Biocompatible Two-Photon Absorbing Dipyridyldiketopyrrolopyrroles for Metal-Ion-Mediated Self-Assembly Modulation and Fluorescence Imaging

He, Tingchao; Gao, Yang; Sreejith, Sivaramapanicker; Tian, Xiaoqing; Liu, Lin; Wang, Yue; Joshi, Hrishikesh; Phua, Soo Zeng Fiona; Yao, Song; Lin, Xiaodong; Zhao, Yanli; Sun, Handong; Grimsdale, Andrew Clive

2016

He, T., Gao, Y., Sreejith, S., Tian, X., Liu, L., Wang, Y., et al. (2016). Biocompatible Two-Photon Absorbing Dipyridyldiketopyrrolopyrroles for Metal-Ion-Mediated Self-Assembly Modulation and Fluorescence Imaging. *Advanced Optical Materials*, 4(5), 746-755.

<https://hdl.handle.net/10356/83448>

<https://doi.org/10.1002/adom.201500755>

© 2016 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. This is the author created version of a work that has been peer reviewed and accepted for publication by *Advanced Optical Materials*, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. It incorporates referee's comments but changes resulting from the publishing process, such as copyediting, structural formatting, may not be reflected in this document. The published version is available at: [<http://dx.doi.org/10.1002/adom.201500755>].

DOI: 10.1002/ ((please add manuscript number))

Article type: **(Full Paper)**

Biocompatible Two-photon Absorbing Dipyriddyketopyrrolopyrroles for Metal Ions Mediated Self-assembly Modulation and Fluorescence Imaging

Tingchao He, Yang Gao, Sivaramapanicker Sreejith, Xiaoqing Tian, Lin Liu, Yue Wang, Hrishikesh Joshi, Soo Zeng Fiona Phua, Song Yao, Xiaodong Lin, Yanli Zhao, Andrew C. Grimsdale, and Handong Sun**

Prof. T. He, Dr. X. Tian, L. Liu, S. Yao, Prof. X. Lin
College of Physics Science & Technology, Shenzhen University, Shenzhen, 518060, China
Y. Gao, Prof. A. C. Grimsdale
School of Materials Science and Engineering, Nanyang Technological University, 50 Nanyang Avenue, 639798, Singapore
E-mail: acgrimsdale@ntu.edu.sg
Dr. S. Sreejith, H. Joshi, S. Z. F. Phua, Prof. Y. Zhao
Division of Chemistry and Biological Chemistry, School of Physical and Mathematical Sciences, Nanyang Technological University, 21 Nanyang Link, 637371, Singapore
Y. Wang, Prof. H. Sun
Division of Physics and Applied Physics, and Centre for Disruptive Photonic Technologies (CDPT), School of Physical and Mathematical Sciences, Nanyang Technological University, 21 Nanyang Link, 637371, Singapore
E-mail: hdsun@ntu.edu.sg

Keywords: dipyriddyketopyrrolopyrroles, two-photon absorption, cell imaging, self-assembly

Abstract: Diketopyrrolopyrrole (DPP) derivatives are very promising nonlinear optical dyes with excellent thermal resistance and light stability. However, to the best of our knowledge, the fabrication of their organic micro- and/or nanostructures through covalent and/or noncovalent interactions to further adjust their corresponding two-photon absorption (TPA) is rarely reported. In this work, based on previous synthesis method of pyridine-containing ketopyrrolopyrroles, three dipyridyldiketopyrrolopyrroles (dipyridylDPPs) **1**, **2** and **3** containing 2-, 3- and 4-pyridyl substituents respectively have been prepared, which exhibit strong orange fluorescence and large TPA action cross-sections in both organic (tetrahydrofuran) and aqueous media. Importantly, fluorescent organic nanoparticles (FONs) of the di(2-pyridyl)DPP **1** prepared by the reprecipitation method displays strong two-photon emission. Meanwhile, the FONs of **1** not only exhibit Ag⁺ ions mediated self-assembly modulation, but also display low cytotoxicity and allow two-photon bioimaging in HeLa cells.

1. Introduction

Small organic molecules with large two-photon absorption (TPA) cross-sections (δ) have recently attracted increasing attention due to their relative simplicity of preparation and unique optical and optoelectronic properties.^[1-3] Moreover, compared with large molecules, they can be easily loaded into cells and rapidly detect biological targets, which are important requirements for cell-imaging reagents. To date, many kinds of small molecular probes have been designed to target various biomolecules and organelles. Among them, common fluorophores, such as fluorescein, coumarin, BODIPY, and rhodamine, are the most widely used.^[4-6] However, such organic dyes are only useful for one-photon microscopy, which is not suitable for deep-tissue imaging because of the shallow depth of tissue penetration of the short wavelength of excitation light, due to the cellular absorption of the photons, and autofluorescence induced by exciting intrinsic fluorophores.

To fulfill the promise of increased temporal and spatial resolutions, two-photon excitation has emerged as a new, indispensable imaging tool for biomedical research, which provides key advantages over one-photon excitation, such as the capability of imaging deep inside a tissue, higher spatial resolution, and longer observation time.^[7,8] Unfortunately, most of the existing fluorescent probes have small TPA cross-sections, so high excitation intensity should be used for the application of bioimaging to obtain bright images. Therefore, there is a strong need for the development of efficient small two-photon probes in biomedical research. To this end, strategies for the design of molecules with large TPA cross-sections have been investigated both theoretically and experimentally. These designs include symmetric and asymmetric arrangements of electron donating (D) and accepting (A) units linked by π -spacers, with the generic structures D- π -D, A- π -A, and D- π -A.^[9-12] Based on these concepts, a variety of small two-photon probes have been developed, and their utility in bioimaging applications has been demonstrated. Although great progress has been achieved, many two-photon probes in previous reports are only usable in organic solvents due to their negligible

water solubility or the serious fluorescence quenching of their fluorescent organic nanoparticles (FONs) in aqueous media.^[13-15] This limits the applicability of two-photon probes in biomedical application.

One of the most recently discovered groups of small organic dyes, diketopyrrolopyrroles (DPPs) derivatives have distinctive advantages relative to other organic dyes, including high fluorescence quantum yields (QYs), and good light and thermal stability.^[16,17] Significant advances have been made in the development of various optoelectronic devices based on DPPs in recent years. However, their nonlinear optical properties in fluorescent probes are still largely unexplored. Only a few reports describing their nonlinear optical behaviors and related applications have appeared since 2009.^[18-23] Especially, there are only a few reports on the self-assembly of DPPs, although organizing organic dyes by self-assembly is an efficient approach to create defined molecular architectures with appealing topology and properties in the applications of biomedicine, optics, and other technologies.

There are two previous reports on dipyrroldiketopyrrolopyrroles (dipyrroldDPPs) which inspired our work. Ftouni et al designed a dipyrroldDPP for its application in H₂ gas sensor,^[24] while Mizuguchi et al synthesized water-soluble dipyrroldDPPs for two-photon excited fluorescence microscopy.^[25] In seeking DPP derivatives with TPA active self-assembly, in this contribution, inspired by these previously reported dipyrroldDPPs, we have made three dipyrroldDPPs **1**, **2** and **3**, respectively containing 2-, 3-, and 4-pyridyl substituents, and investigated their one- and two-photon optical properties. Among the three chromophores, the di(2-pyridyl)DPP **1** exhibits the largest TPA values in organic solvent, due to the more rigid planar structure induced by having nitrogens atom at the 2-position *ortho* to the ketone. Subsequently, FONs of all the dipyrroldDPPs are prepared by the reprecipitation method. Importantly, the FONs of **1** display self-assembly controllable by the presence or absence of Ag⁺ ions. Moreover, due to the strongly red-shifted two-photon emission (540-660 nm

window) of FONs of **1** and uniformly aggregated morphology, these FONs can be used for cell imaging.

2. Results and discussion

2.1. Linear Optical Properties of the DipyriddyDPPs

To enrich research into the TPA of small molecules and broaden their practical applications, we synthesized three new dipyriddyDPP luminogens, whose chemical structures are presented in **Figure 1**. The linear optical properties of the dipyriddyDPPs in tetrahydrofuran (THF) and aqueous solutions are shown in **Figure 2**. It was found that the absorption maxima of **1**, **2** and **3** in THF were located at 524, 480 and 473nm respectively, while the corresponding emission spectra exhibited two major local maxima. The absorption and emission spectra of **1** were bathochromically shifted relative to those of **2** and **3**, which can be ascribed to the smaller torsional angle between the DPP core and the 2-pyridyl due to the lack of a hydrogen on the N atom at 2-position. For the suspension of FONs of dipyriddyDPP **1**, the entire absorption and fluorescence spectra were red shifted compared to those seen in THF solution. This is a commonly observed phenomenon due to molecular aggregation. The onset wavelengths of absorption spectra of suspensions of FONs of **2** and **3** were also shifted towards longer wavelength relative to their THF solution spectra due to molecular tight π - π stacking. However, new absorption bands at 455 and 453 nm for the suspensions of FONs of dipyriddyDPPs **2** and **3** were observed, accompanied by the blue shift of their fluorescence spectra. This is typically observed for H-aggregates, in which only the transition to a higher energy exciton state is optically allowed as the electric dipole transition moments are aligned parallel to the axis of stacking.^[26-28] Therefore, there existed multiple interactions within the FONs, probably including specific slip-stacking and dimer formation.^[29] Meanwhile, it was found that chromophores **1**, **2** and **3** could be well dissolved in organic solvents with low to high polarity, such as toluene, chloroform (CHCl₃), THF and dimethylsulphoxide (DMSO). The linear absorption and fluorescence spectra of the chromophores in different solvents were

also investigated (Supporting Information, **Figure S1**). Both linear absorption and fluorescence spectra were insensitive to the changes in the polarity of the environment, indicating the excellent stabilization of both the ground and excited states in the organic solvents with various polarity. The QYs of **1**, **2** and **3** were measured to be respectively 81, 87 and 73% in THF, and 4.8, 6.4, and 7.3% in water. Organic dyes are typically poorly soluble in water (with some notable exceptions). Sufficiently soluble organic dyes generally display low emission efficiency in water due to higher nonradiative decay as compared to organic media. H-aggregates are usually weakly or non-fluorescent, since nonradiative decay is the dominant pathway back to the ground state.^[26-28] It was interesting to note that the QY of FONs of **3** was moderate compared to other conjugated molecules in organic solvents, owing to existing intra- and intermolecular interactions.^[29] These effectively slowed exciton motion, turned on a radiative decay channel and inhibited strong H-type coupling to some extent.^[29]

To make further investigations at the molecular level, density functional theory (DFT) calculations were carried out using the Gaussian 03 program. The nonlocal density function of B3LYP with 6-31G basis sets was used for the calculations. The calculated optimal molecular orbitals of the ground state for dipyrindylDPPs **1**, **2** and **3** are shown in **Figure 3**. The calculated energies of the HOMO/LUMO energy gaps for **1**, **2** and **3** were 1.481, 1.569 and 1.519 eV, respectively. Although the calculated energies were not exactly the same as those obtained from absorption spectra, the trend in their magnitude was consistent (**Table 1**).

The fluorescence lifetime is an important parameter for organic emitting molecules, so we also measured time-resolved fluorescence decay curves for the three chromophores (**Figure 4**). Lifetime measurements revealed that **1**, **2**, and **3** in THF displayed almost the same fluorescence decay, consistent with their similar QYs in organic solvent. Compounds **1**, **2**, and **3** displayed single-exponential fluorescence decay behavior with lifetime values of 9.78, 8.14, and 9.38 ns, respectively. The long excited-state lifetime values of the compounds provide a major advantage for fluorescence related applications, such as diagnostics and optical

amplification. By contrast, the overall decay in water for **3** was slower than those for **1** and **2**. This result suggested that the H-type coupling of **3** in aqueous medium was overpowered by the other radiative decay channels. Meanwhile, the FONs exhibited double-exponential fluorescence decay behaviors, with average lifetime values of 0.22, 0.78, 1.78 ns, respectively. The shorter lifetime values of FONs can be attributed to a facile nonradiative decay pathway in aqueous media. The photophysical properties of the three compounds in THF and water are summarized in Table 1.

2.2. Nonlinear Optical Properties of the DipyriddyDPPs

From Figure 2, one can also see that there is a spectral window from 600 to 1200 nm, in which the chromophore solution shows negligible one-photon absorption, which is thus suitable for two-photon excitation. TPA spectra of the dipyriddyDPPs **1**, **2** and **3** were measured by using two-photon excited fluorescence method and excited by femtosecond laser pulses from 720 to 880 nm (**Figure 5**). The TPA mechanism was confirmed by the measurements of input power intensity dependent fluorescence intensity of the dyes under excitation at 800 nm. The linear dependence of their emission intensities on the square of excitation power confirmed that the observed emission indeed arose from two-photon excitation (Supporting Information, **Figure S2**). For THF solutions, the maximum TPA cross-sections, as shown in **Figure 5a**, are 1248, 298 and 170 GM for dipyriddyDPPs **1**, **2**, and **3**, respectively. It was interesting to note that the dipyriddyDPP with electron-withdrawing pyridine rings still had large TPA cross-section, which was attributed to the efficient electronic communication between the DPP cores and the pyridine substituents. A similar phenomenon has also been observed in DPP-imidazolium salts with five-membered heterocyclic substituents.^[19] Our chromophore **3** in THF exhibited smaller TPA cross-sections than those of the thiophene or furan substituted DPP-imidazolium salt, but its maximum TPA cross-section was still larger than that of a benzene substituted DPP-imidazolium salt. Meanwhile, the dipyriddyDPPs exhibited shorter absorption/fluorescence

wavelengths and smaller TPA cross-sections compared to the DPP derivatives tethered with a propeller-like starburst triphenylamine as a strong donor, due to the stronger intramolecular charge transfer of the latter.^[22] Obviously, the TPA cross-section of dipyritylDPP **1** was enhanced greatly by the presence of nitrogen atom at the *ortho*-(2-)position of the pyridine rings. It is well known that a rigid planar structure in chromophores is beneficial for the amplification of TPA.^[30] The presence of nitrogen atoms at different positions could result in different torsional hindrance to the free rotation of the pyridine substituents. From the experimental results, it can be concluded that the dipyritylDPP **1** with nitrogen atom at the 2-position had a more rigid planar structure than the other molecules in which hydrogen atoms at the 2-positions would lead to out of plane twisting. From the comparison of the linear absorption and TPA spectra, it was shown that the transition energies of the TPA bands were greatly blue-shifted compared to their absorption peaks. These experimental results were consistent with the centrosymmetric nature of the compounds.^[21] In such cases, the linear absorption and TPA induced transitions to different excited states.

Inspired by the high TPA cross-section values of the compounds, we were motivated to examine the TPA behaviors of their FONs for their potential applications. The FONs were prepared by using a solvent-exchange process, as described in experimental section in details. The TPA spectra of FONs for chromophores **1–3** are presented in Figure 5b, in which the error bars (ca. 10%) arose from the uncertainties due to scattering influence of FONs. The maximum TPA cross-section values of FONs of **1**, **2** and **3** were 1381 ± 138 , 903 ± 90 and 1588 ± 159 GM, respectively. The FONs of our dipyritylDPPs displayed smaller TPA action cross-sections compared to those prepared from triphenylamine-based DPP derivatives.^[22] However, our FONs still showed comparable two-photon brightness compared to those of red-emitting DPSB-based conjugated polymer.^[31] Although their QYs decreased, the maximum TPA cross-sections of the dipyritylDPPs became larger. Owing to intermolecular π – π interactions as well as dipole–dipole interactions between the chromophores in the FONs,

the TPA cross-sections of the molecules could be greatly modified.^[15] For bioimaging application, molecules with large TPA cross-section value per molecular weight (δ/MW) are needed. The maximum values of δ/MW were calculated as 2.7, 1.8 and 3.1 for FONs of dipyritylDPPs **1**, **2**, and **3**, respectively. These values are larger than 1, which indicates that FONs of dipyritylDPPs are potentially useful for such applications.^[32,33] The maximum TPA action cross-sections ($\phi\delta/\text{MW}$) per molecular weight of chromophores **1–3** in aqueous media were calculated to be 66 ± 7 , 58 ± 6 , and 116 ± 12 GM, respectively. Obviously, the FONs of dipyritylDPP **3** in water displayed a relatively larger TPA action cross-section. When the nitrogen atoms are located at the *para*-(4-)position, the pyridine substituent may tend to twist out-of-plane which could effectively prevent close chromophore-chromophore stacking when the molecules aggregated in aqueous medium. A DFT calculation was carried out to simulate the molecular geometry of the chromophore **3** (Supporting Information, Figure S3). This confirmed the planar structure of the molecular center making the chromophore **3** suitable to form FONs and that the pyridine substituent tended to twist out-of-plane which could effectively prevent close chromophore-chromophore stacking when the molecules aggregated in aqueous medium. Therefore, it was concluded that in the aggregated state the free motion of **3** was hampered and the energy dissipation pathway by the nonradiative decay was inhibited, leading to a relatively higher QY in water. However, dipyritylDPPs **1** and **2**, which have more rigid planar structures, exhibited lower QYs due to higher nonradiative decay. Overall, we concluded that FONs of **1** and **2** underwent aggregation in aqueous conditions via chromophore-chromophore stacking due to their rigid planar structure whereas **3**, owing to its unique three dimensional geometry, did not aggregate uniformly.

2.3. Two-photon active self-assembly

As it is known from literatures that binding of ions to a multiphoton absorbing chromophore produces marked changes in the multiphoton emission spectra,^[34] we suspected

that similar changes would be observed when Ag^+ ions were bound to **1**, **2** and **3** which could lead to major advances in optoelectronic and biomaterials science by providing potential applications. The selection of Ag^+ ions versus other metals was due to the d10 nature of Ag^+ ions, leading to an absence of electronic interactions with the organic ligand. Thus, the selection of Ag^+ ions can simplify the photophysical process and the interpretation of results of investigations therein. In these experiments, a suitable amount of Ag^+ ions was introduced to the suspension of FONs of **1**, **2** and **3**. Subsequently the photophysical properties and morphological changes were monitored by the measurement of fluorescence spectra, and by transmission electron microscopy (TEM) and dynamic light scattering (DLS).

The changes in two-photon emission features of FONs upon addition of Ag^+ ions are shown in **Figure 6**. The figure indicates the evolution of two-photon emission spectra ($\lambda_{\text{ex}} = 800 \text{ nm}$) for the FONs of **1**, **2** and **3** in aqueous dispersion upon adding Ag^+ ions. Upon excitation at 800 nm, all the molecular aqueous dispersions emitted strong two-photon fluorescence. However, the aqueous dispersions displayed distinctively different responses to the addition of small aliquots of Ag^+ ions. For di(2-pyridyl)DPP **1**, upon the addition of Ag^+ solution (0.2 μM), the emission intensity of its FONs decreased immediately (response time <3 s). The fluorescence continually decreased upon further incremental addition of Ag^+ ions and was completely quenched for a Ag^+ concentration of 3 μM . As for dipyridylDPP **2**, with the addition of dilute Ag^+ solution (0.2 to 0.97 μM) its two-photon emission intensity also decreased. However, no further decrease in fluorescence intensity was observed upon increasing Ag^+ concentration above 1.25 μM , indicating a saturated response of the suspension to Ag^+ ions. Although the two-photon emission intensity of dipyridylDPPs **1** and **2** decreased to different extents upon the addition of Ag^+ ions, no decrease was observed in the fluorescence emission intensity of di(4-pyridyl)DPP **3**-based FONs. It is well known that the coordination angle of an Ag^+ ion is 180 degree.^[35] The location of the pyridine nitrogen atom at 2-, 3-, or 4- positions resulted in different torsional hindrance of the pyridine substituent.

As a result, the nitrogen atom located at the 4-positions in dipyridylDPP **3** should more easily coordinate with Ag^+ ions compared to the nitrogen atoms in the other two molecules. However, di(2-pyridyl)DPP **1** displayed the most sensitive two-photon emission towards Ag^+ ions in aqueous medium, while no sensitivity in the two-photon emission was observed for the FONs of dipyridylDPP **3**. This discrepancy may be caused by the different self-assembly behaviors of the chromophores in their aggregates. Due to its three dimensional morphology, the molecular self-assembly in FONs of dipyridylDPP **3** may change the coordination angle of the nitrogen atoms leading to no complexation with Ag^+ ions thus causing no change in morphology. However, for FONs of dipyridylDPP **1**, the coordination angle of the nitrogen atom may suffer subtle changes in the aggregated state and could effectively coordinate with Ag^+ ions leading to disintegration of the self-assembly. In the case of FONs of dipyridylDPP **2**, the molecular arrangement consisted of chromophore stacking may lead to different three dimensional self-assembled structures, with results between **1** and **3**. Obviously, for the dipyridylDPP **1**, strong interactions existed between the nitrogen atom and Ag^+ ions, while for the other two chromophores two-photon emission was relatively less sensitive to the addition of Ag^+ ions, especially for dipyridylDPP **3**. The quenching of two-photon fluorescence of FONs of dipyridylDPP **1** could be attributed to the internal charge transfer between the metal ions and the dye molecules as well as the reduction of the electron accepting strength of the pyridine nitrogen atom(s) upon metal ion binding.^[36-38]

To further corroborate the theory of aggregation in these three FONs, TEM analysis was conducted. The TEM micrographs of FONs suspension of **1**, **2** and **3** before and after addition of Ag^+ ions (**Figure 7**) were obtained to visualize the morphological changes due to the addition of Ag^+ . It is clearly visible that FONs of **1** and **2** (Figure 7a and c) form aggregates with relatively small average sizes in absence of Ag^+ ions. However, in similar conditions, FONs of **3** (Figure 7e) formed large and disoriented self-assembled structures due to out-of plane distortion of the three dimensional structure and inadequate chromophore assembly.

Subsequently, the TEM images after addition of Ag^+ ions showed a disintegration for FONs of **1** (Figure 7b), a slight disintegration for **2** (Figure 7d) and no change in morphology for FONs of **3** (Figure 7f). From these images, the inactive nature of FONs of **3** could be attributed to the disoriented out-of plane structural distortion (Figure 7e) that did not permit the formation of complex with Ag^+ ions. Moreover, the decrease in fluorescence for FONs of **1** caused by complexation with Ag^+ ions arose from disintegration of the aggregates which could be verified from the images (Figure 7a and b). From the TEM images, it was known that the average sizes of prepared FONs for chromophores **1**, **2** and **3** were 290 nm, 325 nm and 1820 nm, respectively. After addition of Ag^+ ions the average sizes of FONs for chromophores **1**, **2** and **3** were 69 nm, 120 nm and 1800 nm, respectively. DLS measurements were performed to further study the hydrodynamic diameter and size distribution of FONs suspensions of **1**, **2** and **3** before and after addition of Ag^+ ions. Again, the experimental results presented in **Figure 8** confirmed the Ag^+ ions induced disintegration for FONs of **1**, a slight disintegration for FONs of **2** and no disintegration for FONs of **3**. Meanwhile, the TEM observation showed smaller average sizes for FONs compared to those observed by DLS measurements, due to the drying process during the TEM sample preparation and the difference between their measurement principles. Therefore, this analysis confirmed the inactive nature of **3** and the susceptibility of **1** to Ag^+ ions and thereby supported the proposed relationship between the morphology of FONs and their photophysical properties.

2.4. Two-photon bioimaging of the dipyriddyDPPs

Next, we tested the efficiency of our FONs for bioimaging in an *in vitro* environment. Although the FONs of dipyriddyDPP **3** exhibited the largest TPA action cross-section in aqueous medium, the aggregation was disoriented and resulted in non-uniform as well as quite large aggregates. However, FONs of dipyriddyDPP **1** showed uniform aggregation and the emission spectrum was red-shifted which would give a better contrast in the bioimaging.

Therefore, as a model system, we investigated the two-photon bioimaging of dipyriddyDPP **1** with the expectation of expanding its potential application.

Good biocompatibility is a prerequisite for any bioimaging probes. Therefore, the cytotoxicity/cell viability of dipyriddyDPP **1** was evaluated by monitoring the metabolic viability of HeLa cells after incubation with its FONs of different concentration. **Figure 9** shows that the cell viabilities remain nearly 100% after incubation with the FONs at the concentration lower than 10 μ M for 24 h, demonstrating their excellent biocompatibility.

The low toxicity and efficient two-photon excited fluorescence from FONs of dipyriddyDPP **1**, are of advantage for imaging in biological specimens. HeLa cancer cells were selected as the model cell lines to study the two-photon *in vitro* imaging capability of FONs of **1**. HeLa cells were incubated with FONs of **1** (10 μ M) along with nuclear counterstain 4',6-diamidino-2-phenylindole (DAPI) following standard imaging protocols. **Figure 10** shows two-photon microscopy images of FONs of dipyriddyDPP **1** upon excited at 800 nm and emission was collected in a 540-640 nm window. Images were collected by excitation of DAPI at 405 nm separately and overlaid. The orange fluorescence from FONs of **1** revealed cellular uptake with good intercellular distribution. It was easily discriminated from the nuclear zone with blue fluorescence, and it meant that dipyriddyDPP **1** had very good counterstain compatibility with DAPI. The two-photon excited fluorescence from FONs of clearly demonstrated the potential of such probes for *in vitro* bioimaging.

3. Conclusion

In summary, we have synthesized three dipyriddyDPPs, containing respectively 2-, 3- and 4-pyridyl substituents. It has been found that dipyriddyDPP containing 2-pyridyl substituent displays the largest TPA cross-section among them in THF solutions, while for their water suspensions the highest TPA cross-section has been observed in the FONs of the dipyriddyDPP containing 4-pyridyl substituent. However, FONs of the dipyriddyDPP containing 2-pyridyl substituent are promising for Ag^+ ions mediated self-assembly

modulation and cell imaging. These studies suggest that these dipyridylDPPs are promising materials for optoelectronic and bioimaging applications.

4. Experimental Section

Synthesis of dipyridylDPPs 1, 2 and 3. All chemicals and reagents used in this project were purchased from commercial sources, e.g. Sigma Aldrich, Alfa Aesar *et al.*, and were used as obtained. All reactions were carried out under an inert N₂ atmosphere. NMR spectra were collected from a Bruker 400 MHz machine, and mass spectra were recorded using Shimadzu Axima Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight mass spectrometer (MALDI-TOF-MS) without matrix. DipyridylDPPs with 2-, 3- and 4-pyridyl substituents were prepared according to literature methods, modified by using potassium *tert*-butoxide as base instead of sodium/FeCl₃.^[24,25,39, 40]

2,5-Bis(2-ethylhexyl)-3,6-di(pyridin-2-yl)pyrrolo[3,4-c]pyrrole-1,4(2H,5H)-dione (1). 3,6-Di(pyridin-2-yl)pyrrolo[3,4-c]pyrrole-1,4(2H,5H)-dione (0.36 g, 1.2 mmol, 1 eq), potassium carbonate (0.65 g, 4.7 mmol, 3.8 eq), and DMF (15 mL) were mixed under N₂ atmosphere in ice-water bath, and stirred for 20 minutes in darkness by wrapping with aluminium foil. 2-ethylhexyl iodide (1.7 mL, 97%, 10.23 mmol, 8.2 eq) was slowly added in three portions, and the mixture was stirred at room temperature for 48 hr before pouring into water. The precipitate was washed with CHCl₃/acetone, and the organic part was condensed. The residue was purified by column chromatography (silica, DCM:Acetone 4:1) to obtain a red powder (0.14 g, Yield: 22.2%). ¹H NMR (400 MHz, CDCl₃, δ) 8.89 (d, *J* = 4 Hz, 2H, Ar H), 8.70 (d, *J* = 8 Hz, 2H, Ar H), 7.91 (t, *J* = 8 Hz, 2H, Ar H), 7.36 (dd, *J* = 4, 2 Hz, 2H, Ar H), 4.34 (m, 4H), 1.53 (m, 2H), 1.19 (m, 16H), 0.82 (dd, *J* = 8, 6.8 Hz, 12H) ppm; ¹³C NMR (100 MHz, CDCl₃, δ) 162.90, 162.84, 149.06, 147.99, 146.03, 137.11, 127.38, 124.92, 111.19, 45.84, 39.70, 30.63, 28.47, 23.99, 23.11, 14.14, 10.79 ppm. MS (MALDI-TOF) *m/z*: calcd for C₃₂H₄₂N₄O₂, 514.70; found: 513.68 ([M-H]⁻), 553.50([M+K]⁺).

2,5-Bis(2-ethylhexyl)-3,6-di(pyridin-3-yl)pyrrolo[3,4-c]pyrrole-1,4(2H,5H)-dione (**2**). 3,6-Di(pyridin-3-yl)pyrrolo[3,4-c]pyrrole-1,4(2H,5H)-dione (0.39 mg, 1.3 mmol, 1 eq), potassium carbonate (0.50 g, 3.6 mmol, 2.7 eq), and DMF (15 mL) were mixed under N₂ atmosphere, and stirred at 140 °C for 20 minutes in darkness by wrapping with aluminium foil. 2-ethylhexyl iodide (1.5 mL, 97%, 9.0 mmol, 6.7 eq) was slowly added in two portions, and the mixture was stirred for 1 hr before cooling down and pouring into water. The precipitate was washed with CHCl₃/acetone, and the organic part was condensed. The residue was purified by column chromatography (silica, *n*-hexane:Acetone 2:1) to obtain a yellow powder (0.13 g, Yield: 18.8%). ¹H NMR (400 MHz, CDCl₃, δ) 8.95 (d, *J* = 1.6 Hz, 2H, Ar H), 8.75 (d, *J* = 1.6 Hz, 1H, Ar H), 8.74 (d, *J* = 1.6 Hz, 1H, Ar H), 8.21 (t, *J* = 2 Hz, 1H, Ar H), 8.19 (t, *J* = 2 Hz, 1H, Ar H), 7.52 (dd, *J* = 4.8, 2.4 Hz, 2H, Ar H), 3.75 (d, *J* = 6.8 Hz, 2H), 1.12 (m, 18H), 0.79 (t, *J* = 6.8 Hz, 6H), 0.71 (t, *J* = 6.8 Hz, 6H) ppm; ¹³C NMR (100 MHz, CDCl₃) δ 162.50, 151.71, 148.75, 146.14, 136.49, 125.00, 123.92, 110.81, 43.26, 38.94, 31.42, 28.37, 23.80, 22.95, 14.08, 10.49 ppm. MS (MALDI-TOF) *m/z*: calcd for C₃₂H₄₂N₄O₂, 514.70; found: 515.79 ([M+H]⁺), 537.83([M+Na]⁺), 553.87([M+K]⁺).

2,5-Bis(2-ethylhexyl)-3,6-di(pyridin-4-yl)pyrrolo[3,4-c]pyrrole-1,4(2H,5H)-dione (**3**). The compound was obtained using the similar procedure to that used for **1** starting with 3,6-di(pyridin-4-yl)pyrrolo[3,4-c]pyrrole-1,4(2H,5H)-dione (0.29 g, 1.0 mmol), potassium carbonate (0.63 g, 4.6 mmol, 4.6 eq), DMF (15 mL), and 2-ethylhexyl iodide (1.2 mL, 97%, 7.2 mmol, 7.2 eq). The desired product was obtained as a yellow powder (50 mg, yield: 10.0%). ¹H NMR (400 MHz, CDCl₃, δ) 8.84 (d, *J* = 5.6 Hz, 2H, Ar H), 7.67 (d, *J* = 5.6 Hz, 2H, Ar H), 3.74 (d, *J* = 6 Hz, 2H), 1.43 (m, 2H), 1.13 (br, 16H), 0.80 (t, *J* = 7.2 Hz, 6H), 0.72(t, *J* = 7.2 Hz, 6H) ppm; δ ¹³C NMR (100 MHz, CDCl₃) δ 161.95, 150.64, 146.72, 135.53, 122.08, 111.01, 45.08, 38.79, 30.26, 28.24, 23.66, 22.80, 13.79, 10.32 ppm. MS (MALDI-TOF) *m/z*: calcd for C₃₂H₄₂N₄O₂, 514.70; found: 514.37 ([M]⁺).

Preparation of FONs. Stock solutions of the compounds in THF with a concentration of 10^{-4} M were prepared. Aliquots of the stock solution were transferred to 10 mL volumetric flasks. After that, appropriate amounts of water were added dropwise under vigorous stirring to furnish 10^{-5} M solution with 98% water content (vol%). The obtained suspension was stirred at room temperature overnight to evaporate the organic solvent to yield aqueous media of FONs of dipyridylDPPs **1**, **2** and **3**. The measurements of the resultant solutions were then performed.

Linear absorption and one-photon fluorescence spectra. UV-Vis absorption and fluorescence spectra of the samples were measured by using a Shimadzu UV-Vis spectrophotometer and Fluorolog-3 (Horiba JOBIN YVON), respectively. Fluorescence QYs were measured by using quinine sulfate in 0.1 M sulphuric acid as the standard.

Time-resolved fluorescence measurements. For the measurements of lifetime, the solutions of three dipyridylDPPs in water and THF were filled in a 1cm thick quartz cuvette and were excited at 360 nm (pulse width: 100 fs; repetition rate: 1000 Hz) with a wavelength-tunable femtosecond amplified laser system. The fluorescence signals were recorded by an Optronics streak camera with a temporal resolution of ~50 ps.

The measurements of TPA cross-sections. The measurements were performed by using femtosecond pulses from a Mai-Tai laser (80 MHz, 100 fs, Spectra-Physics, Inc.), with tunable wavelength range from 720 to 920 nm, as the excitation source. The laser beam was focused onto the sample that was contained in a quartz cuvette with a path length of 1 cm. The emission of the samples was collected from the edge and the signals were dispersed by a 750 mm monochromator combined with suitable filters, and detected by a photomultiplier tube (Hamamatsu R928) using a standard lock-in amplifier technique. A short pass filter with cut-off wavelength at 700 nm was placed before the spectrometer to minimize the scattering from the pump beam. TPA cross-sections were determined from TPA induced fluorescence method

using rhodamine B as a reference with a known value. The TPA cross-section of samples can be calculated at each wavelength according to

$$\delta = \delta_{ref} \frac{n_{ref} \cdot \Phi_{ref} \cdot C_{ref} \cdot F}{n \cdot \Phi \cdot C \cdot F_{ref}} \quad (1)$$

In the equation, the subscript ref stands for the reference molecule, δ is the TPA cross-section value, n is the refractive index of the solution, ϕ is the fluorescence quantum yield, C is the concentration of solution and F is the two-photon emission intensity at each wavelength.^[41]

Two-photon bioimaging. The HeLa cancer cells were first cultured in confocal microscope dishes at 5×10^5 /mL in complete Dulbecco's modified Eagle's medium (DMEM, Gibco, America) for 24 h at 37 °C. After that, the cultured cells were washed with phosphate buffered saline (PBS) three times and then incubated for 4 h with the medium containing FONs of dipyridylDPP **1**. Finally, the cells were washed twice with PBS. Two-photon cell imaging was acquired using a microscope (product model: Olympus BX41) under 800 nm excitation.

Ag⁺ analysis. A stock solution of Ag⁺ in water (1 mM) was mixed with the aqueous suspension of dipyridylDPPs. After that, the mixture was stirred for 1 minute and their two-photon emission spectra were measured immediately. Different molar concentration of Ag⁺ ions were achieved by continually adding Ag⁺ solution into the aqueous suspensions of the FONs. Subsequently the emission spectra, TEM images and DLS results were obtained before and after addition of Ag⁺ ions.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

T. H., Y. G. and S. S. contributed equally to this work. T. H., X. T. and X. L. thank the supports from Natural Science Foundation of China (NSFC Grant nos. 11404219 and 21304046), Natural Science Foundation of Guangdong Province (Grant no.

2014A030313552), Youth Innovation Talent Project (Nature Science) of the Universities of Guangdong Province (Grant no. 2014KQNCX127), Shenzhen Basic Research Project of Science and Technology under Grant (No. JCYJ20150324141711613), State Key Laboratory of Advanced Optical Communication Systems and Networks, Shanghai Jiao Tong University, China (Grant no. 2015GZKF03007). Y. G. thanks NTU for the award of a Postgraduate Research Scholarship.

Received: ((will be filled in by the editorial staff))

Revised: ((will be filled in by the editorial staff))

Published online: ((will be filled in by the editorial staff))

- [1] H. W. Kim, B. R. Cho, *Chem. Rev.* **2015**, *115*, 5014.
- [2] T. D. Ashton, K. A. Jolliffe, F. M. Pfeffer, *Chem. Soc. Rev.* **2015**, *44*, 4547.
- [3] D. Kim, H. G. Ryu, K. H. Ahn, *Org. Biomol. Chem.* **2014**, *12*, 4550.
- [4] C. Xu, and W. W. Webb, *Opt. Soc. Am. B* **1996**, *13*, 481.
- [5] H. Yin, B. Zhang, H. Yu, L. Zhu, Y. Feng, M. Zhu, Q. Guo, X. Meng, *J. Org. Chem.* **2015**, *80*, 4306.
- [6] T. Zhang, X. Zhu, W. K. Wong, H. L. Tam, W.Y. Wong, *Chem. Eur. J.* **2013**, *19*, 739.
- [7] D. Kim, H. Moon, S. H. Baik, S. Singha, Y.W. Jun, T. Wang, K. H. Kim, B. S. Park, J. Jung, I. Mook-Jung, K. H. Ahn, *J. Am. Chem. Soc.* **2015**, *137*, 6781.
- [8] J. Qian, D. Wang, F. -H. Cai, W. Xi, L. Peng, Z. -F. Zhu, H. He, M. -L. Hu, S. He, *Angew. Chem. Int. Ed.* **2012**, *51*, 10570.
- [9] G. S. He L. Tan, Q. Zheng, P. N. Prasad, *Chem. Rev.* **2008**, *108*, 1245.
- [10] M. Albota, D. Beljonne, J. L. Bredas, J. E. Ehrlich, J.Y. Fu, A. A. Heikal, S. E. Hess, T. Kogej, M.D. Levin, S. R. Marder, *Science* **1997**, *281*, 1653.
- [11] M. Pawlicki, H. A. Collins, R. G. Denning, H. L. Anderson, *Angew. Chem. Int. Ed.* **2009**, *48*, 3244.

- [12] T. He, Z. B. Lim, L. Ma, H. Li, , D. Rajwar, Y. Ying, Z. Di, A. C. Grimsdale, H. Sun, *Chem. Asian J.* **2013**, 8, 564.
- [13] K. Amro, J. Daniel, G. Clermont, T. Bsaibess, M. Pucheault, E. Genin, M. Vaultier, M. Blanchard-Desce, *Tetrahedron* **2014**, 70, 1903.
- [14] E. Genin, Z. Gao, J. A. Varela, J. Daniel, T. Bsaibess, I. Gosse, L. Groc, L. Cognet, M. Blanchard-Desce, *Adv. Mater.* **2014**, 26, 2258.
- [15] V. Parthasarathy, S. Fery-Forgues, E. Campioli, G. Recher, F. Terenziani, M. Blanchard-Desce, *Small* **2011**, 7, 3219.
- [16] M. Grzybowski, D. T. Gryko, *Adv. Opt. Mater.* **2015**, 3, 280.
- [17] M. Kaur, D. H. Choi, *Chem. Soc. Rev.* **2015**, 44, 58.
- [18] E. Q. Guo, P. H. Ren, Y. L. Zhang, H. C. Zhang, W. J. Yang, *Chem. Commun.* **2009**, 45, 5859.
- [19] M. Grzybowski, E. Glodkowska-Mrowka, V. Hugues, W. Brutkowski, M. Blanchard-Desce, D. T. Gryko, *Chem. Eur. J.* **2015**, 21, 9101.
- [20] J. Schmitt, V. Heitz, A. Sour, F. Bolze, H. Ftouni, J. -F. Nicoud, L. Flamigni, B. Ventura, *Angew. Chem. Int. Ed.* **2015**, 54, 169.
- [21] A. Purc, K. Sobczyk, Y. Sakagami, A. Ando, K. Kamada, D.T. Gryko, *J. Mater. Chem. C*, **2015**, 3, 742.
- [22] Y. Gao, G. Feng, T. Jiang, C. Goh, L. Ng, B. Liu, B. Li, L. Yang, J. Hua, H. Tian, *Adv. Funct. Mater.* **2015**, 25, 2857.
- [23] H. M. Kimw, B. R. Cho, *Chem. Commun.* **2009**, 45, 153.
- [24] H. Ftouni, F. Bolze, J. Nicoud, *Dyes Pigments* **2013**, 97, 77-83.
- [25] J. Mizuguchi, T. Imoda, H. Takahashi, H. Yamakami, *Dyes Pigments* **2006**, 68, 47.
- [26] F. C. Spano, *Acc. Chem. Res.* **2010**, 43, 429.
- [27] L. Wang, Y. Shen, M. Yang, X. Zhang, W. Xu, Q. Zhu, J. Wu, Y. Tian, H. Zhou, *Chem. Commun.* **2014**, 50, 8723.

- [28] C. B. Winiger, S. M. Langenegger, G. Calzaferri, R. Häner, *Angew. Chem. Int. Ed.* **2015**, *54*, 3643.
- [29] Y. Tian, C. Chen, Y. Cheng, A.C. Young, N. M. Tucker, A. K. -Y. Jen, *Adv. Funct. Mater.* **2007**, *17*, 1691.
- [30] T. C. He, P. C. Too, R. Chen, S. Chiba, H. Sun, *Chem. Asian J.* **2012**, *7*, 2090.
- [31] P. Liu, S. Li, Y. Jin, L. Qian, N. Gao, S. Q. Yao, F. Huang, Q. Xu, Y. Cao, *ACS Appl. Mater. Interfaces* **2015**, *7*, 6754.
- [32] M. Pawlicki, H. A. Collins, R. G. Denning, H. L. Anderson, *Angew. Chem. Int. Ed.* **2009**, *48*, 3244.
- [33] E. H. G. Zadeh, M. V. Bondar, I. A. Mikhailov, K. D. Belfield, *J. Phys. Chem. C* **2015**, *119*, 8864.
- [34] A. K. Mandal, T. He, S. K. Maji, H. Sun, Y. Zhao, *Chem. Commun.* **2014**, *50*, 14378.
- [35] M. Hong, L. Chen, in *Design and Construction of Coordination Polymers*, John Wiley & Sons, Inc. Germany 2009, Ch. 5.
- [36] S. J. K. Pond, O. Tsutsumi, M. Rumi, O. Kwon, E. Zojer, J. Brédas, S. R. Marder, J. W. Perry, *J. Am. Chem. Soc.* **2004**, *126*, 9291.
- [37] X. Dong, Y. Yang, J. Sun, Z. Liu, B. Liu, *Chem. Commun.* **2009**, *26*, 3883.
- [38] H. M. Kim, M. Jeong, H. C. Ahn, S. Jeon, B. R. Cho, *J. Org. Chem.* **2004**, *69*, 5749.
- [39] J. S. Lunák, J. Vynuchal, P. Horácková, B. Frumarová, Z. Zák, J. Kucerík, *J. Mol. Struct.* **2010**, *983*, 39.
- [40] J. W. Jung, F. Liu, T. P. Russell, W. H. Jo, *Chem. Commun.* **2013**, *49*, 8495.
- [41] L. Guo, M. S. Wong, *Adv. Mater.* **2014**, *26*, 5400.

Figure 1. Chemical structures of three dipyrrolylDPPs with 2- (1), 3- (2), and 4-pyridyl (3) units.

Figure 2. Normalized absorption and fluorescence spectra of dipyrrolylDPPs **1**, **2** and **3** in THF (a) and water (b).

Figure 3. HOMO/ LUMO localization and bandgap values in dipyritylDPPs **1**, **2** and **3**

Figure 4. Fluorescence decay curves of dipyritylDPPs **1**, **2** and **3** in THF and water, excited at 360 nm.

Figure 5. TPA spectra of dipyriddyldPPs **1**, **2** and **3** in THF (a) and in water (b).

Figure 6. Two-photon emission spectra evolution of **1** (a), **2** (b), **3** (c) in aqueous dispersion (1.0×10^{-5} M) upon changing Ag^+ ions concentration, excited at 800 nm. (d) A plot of the ratio of two-photon emission intensity as a function of Ag^+ ions concentration.

Figure 7. TEM images of 1×10^{-4} M dipyrrolylDPPs (a) **1**, (c) **2** and (e) **3**. TEM images of 1×10^{-4} M dipyrrolylDPPs (b) **1**, (d) **2** and (f) **3** with equivalent Ag^+ ions

Figure 8. DLS results of 1×10^{-4} M dipyrrolylDPPs (a) **1**, (b) **2** and (c) **3**. TEM images of 1×10^{-4} M dipyrrolylDPPs (d) **1**, (e) **2** and (f) **3** with equivalent Ag^+ ions.

Figure 9. Metabolic viability of HeLa cells after incubation with FONs of dipyriddyDPP **1** at different concentration.

Figure 10. Confocal images of HeLa cells after coincubation of dipyriddyDPP **1**'s FONs and DAPI. (a) Bright field image. (b) Two-photon excited blue fluorescence of DAPI from the nuclear zone. (c) Two-photon excited orange fluorescence of FONs from nucleoli and cytoplasm.