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Out-of-Plane Coordinated Porphyrin Nanotubes with Enhanced Singlet Oxygen Generation Efficiency

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A supramolecular porphyrin nanotube displaying J-aggregation feature was constructed by out-of-plane coordinated bismuth-porphyrin. Significantly, compared to traditional J-aggregated porphyrin suffering from fluorescence and singlet oxygen quenching, the nanotube exhibits excellent bio-imaging ability and enhanced production efficiency of singlet oxygen. The out-of-plane structure of bismuth to porphyrin makes the aggregation an appropriate material for theranostics. Furthermore, it is also a potential radio-therapeutic drug owing to the presence of radio-active bismuth. Thus, the self-assembly of out-of-plane coordinated porphyrin can be a facile approach toward effective therapy of tumors and other diseases.

Photodynamic therapy (PDT) is a clinical method to eradicate early-stage cancer and to reduce the size of tumor in the end-stage cancer^{1–3}, which includes the combination of a photosensitizer, light and molecular oxygen ($^3\text{O}_2$)^{4,5}. When exposed to the appropriate light, the photosensitizers can produce cytotoxic oxygen species leading to a selective and effective damage of diseased cells and tissues^{6,7}. Hence, the photosensitizers with high singlet oxygen ($^1\text{O}_2$) quantum yield are desirable⁸. Porphyrins, as one of promising photosensitizers, have received much attention in PDT for their intense absorption and excellent singlet oxygen generation quantum yield⁹. Upon photo irradiation in UV-visible region, porphyrins transfer energy to triplet oxygen ($^3\text{O}_2$) and generate cytotoxic single oxygen ($^1\text{O}_2$) which causes cellular and tissue damage ultimately^{10,11}.

However, the large π -conjugate of planar porphyrin often undergoes obvious aggregation which causes severe quenching of the excited state thus leads to the decrease of $^1\text{O}_2$ generation^{12,13}. It greatly limits the applications in biomedical field, especially in PDT application. To address this important issue, some space-demanding porphyrins have been introduced to segregate porphyrin units and suppress the quenching effect, such as co-assemble porphyrins with bulky molecules¹⁴, load porphyrins to other nanostructures^{15–17}, and introduce steric moieties *via* organic synthesis^{18–20}, *etc.* In these processes, the strategies often involve complicated chemical synthesis, which is time-consuming and rises the risk of cytotoxicity due to the use of organic solvents. Therefore, pursuing a non-covalent method to make functional porphyrin nanostructures still remains challenging.

Metal porphyrins are known to facilitate the energy transfer *via* intersystem crossing^{21,22}, which is of key importance in the generation of singlet oxygen for PDT. Some metals coordinated with porphyrin and formed an out-of-plane structure that break the planar geometry and orderly arrangement of porphyrin^{23–25}. Unfortunately, out-of-plane coordination can hardly drive well-defined porphyrin nanostructures so far. If the out-of-plane coordinated porphyrin can be employed properly, porphyrin nanostructures could be generated without close π - π stacking. It is of significant importance for suppressing the self-quenching in porphyrin aggregations and enhancing the efficiency of singlet oxygen generation.

Herein we report a rare case of J-aggregated porphyrin nanotubes with enhanced singlet oxygen generation efficiency. Tetrasodiummeso-tetra(sulfonatophenyl)porphine (TPPS₄) was employed to react with Bi(NO₃)₃ to generate TPPS₄-Bi nanotube. TPPS₄ is widely used as model porphyrins to study their self-assembling

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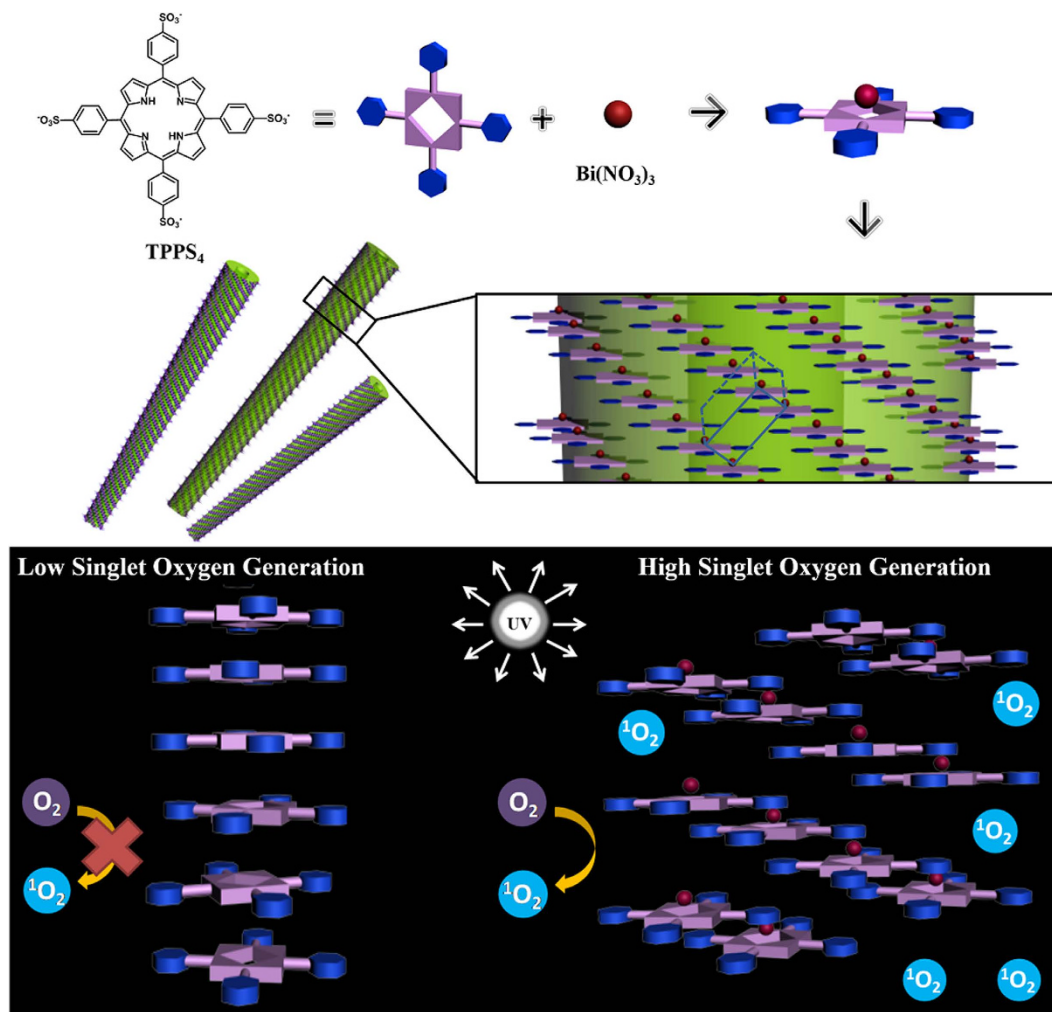


Figure 1. Chemical structures of the TPPS₄ and the mechanism for the enhanced singlet oxygen efficiency of TPPS₄-Bi nanotubes compared with that of TPPS₄ aggregation.

behaviors^{26,27}. In this work, the TPPS₄ was found to self-assemble into nanotubes with Bi³⁺ by out-of-plane coordination. The coordination of Bi³⁺ makes a staggered arrangement of porphyrins so that it could reduce the overlap of porphyrin planar and suppress the self-quenching in aggregation (Fig. 1). Surprisingly, the nanotube exhibited an enhanced production of singlet oxygen similar to the monomeric porphyrins and an excellent fluorescence imaging ability in cells. Moreover, considering that Bi³⁺ also has wide applications in radio-therapy for the treatment of tumors and gastrointestinal disorders^{28,29}, the TPPS₄-Bi nanotube is expected to multiple functions as theranostics. The self-assembly of porphyrin which could suppress self-quenching without other co-assembling molecules or covalent modification is still challenging. Therefore, the present work opens a new vista to construct more porphyrin photosensitizers for the therapy of tumors and other diseases.

Results

The TPPS₄-Bi nanotube is fabricated in aqueous solution at pH 3.6, which is a condition that prohibits the hydrolysis of Bi³⁺. In such acidic solution, TPPS₄ only could self-assemble into flexible and fiber-like J-aggregates which suspended in the solution (Supplementary Fig. S1)^{30–33}. However, upon addition of Bi(NO₃)₃, precipitates were formed. TEM and SEM images demonstrate that the precipitates are composed of nanotubes with lengths and widths around 500 ± 100 nm and 50 ± 5 nm, respectively (Fig. 2). The wall-thickness of these nanotubes is only about 10–20 nm. EDX analysis reveals the presence of Na and Bi, suggesting these nanotubes are formed with TPPS₄ and Bi(NO₃)₃ (Supplementary Fig. S2).

Supramolecular structure of TPPS₄-Bi nanotubes was investigated using UV-Vis, fluorescence and circular dichroism (CD) spectroscopy. The TPPS₄ in aqueous solution displays two UV-Vis absorption bands at 434 and 645 nm. Upon addition of equimolar Bi³⁺, these two bands gradually red shift to 491 and 707 nm, respectively, suggesting an increased extent of J-aggregation (Fig. 3a)^{30–33}. Meanwhile, the fluorescence emission blue shifts from 680 nm to 516 nm, confirming that Bi³⁺ has induced further J-aggregation of TPPS₄ (Supplementary Fig. S3).

Figure 3b shows the X-ray diffraction (XRD) data for the TPPS₄-Bi nanotubes. Compared with J-aggregation of TPPS₄ only^{30–33}, sharp bragg diffractions occur in TPPS₄-Bi nanotubes, suggesting Bi³⁺ has induced orthorhombic TPPS₄ crystal cells with a dimension of a = 1.9 nm, b = 1.4 nm, and c = 0.21 nm. It is noteworthy that

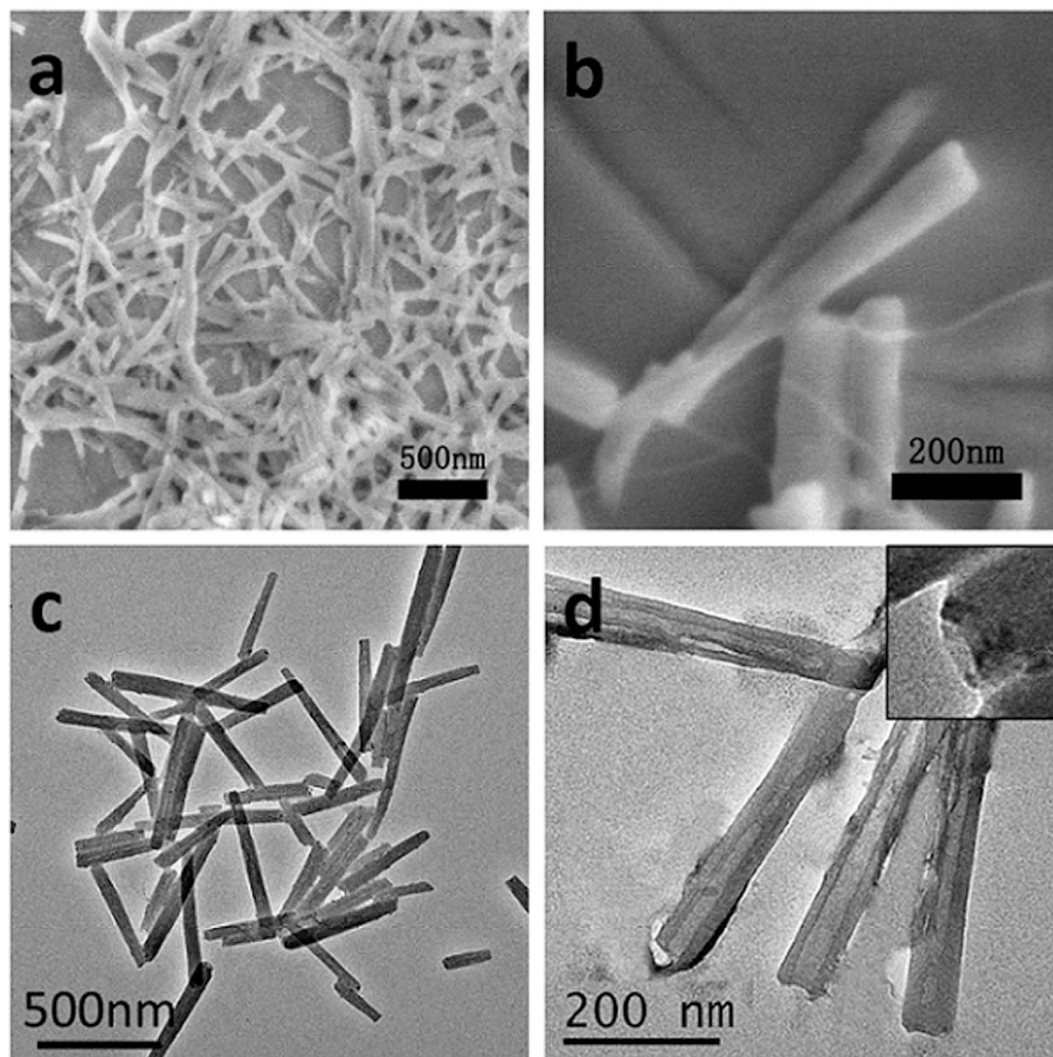


Figure 2. The SEM (a,b) and TEM (c,d) images of TPPS₄-Bi nanotubes. [TPPS₄] = 0.1 mM, [Bi³⁺] = 0.2 mM, pH 3.6.

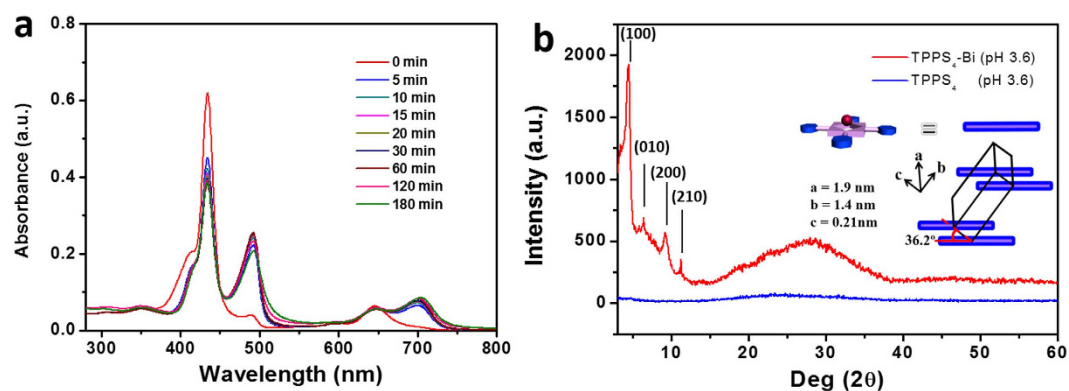


Figure 3. (a) Time dependent UV-Vis absorption of TPPS₄-Bi aqueous solution. [TPPS₄] = 0.025 mM, [Bi³⁺] = 0.05 mM (pH 3.6). (b) XRD measurements for the TPPS₄-Bi nanotubes and TPPS₄ aggregates in acid aqueous solution (pH 3.6). The inset in (b) shows the arrangement of the TPPS₄ molecules in one cubic cell. The dislocation angle between the neighboring top and down TPPS₄ plane was 36.2°.

Bi³⁺ coordinates to TPPS₄ *via* out-of-plane coordination due to its large size with radius about 0.1 nm²³. The distance between two neighboring TPPS₄ face is only 0.21 nm, much smaller than that of the 0.3 nm for the

	pH	τ /ns	Percentage of molecules with each lifetime (%)	Fitting quality χ^2
TPPS ₄	6.8	11.75	84.03	0.9444
TPPS ₄	3.6	3.783	94.13	1.062
TPPS ₄ -Bi	3.6	11.10	65.34	1.295

Table 1. The longest fluorescence lifetime (τ) of TPPS₄ under different conditions. The fraction of molecules with long lifetimes was given as hundred percent (%). The fitting quality was expressed by the parameter χ^2 .

usual J-aggregated TPPS₄²⁵. The electrostatic interactions between the Bi³⁺ of one TPPS₄ and the SO₃⁻ of another one decrease distance between two TPPS₄ and result in the formation of the J-aggregated TPPS₄-Bi nanotubes. Based on the crystal cell parameters, the coordinated Bi³⁺ locates in between two dislocated TPPS₄ planes and makes porphyrins arrange in a dislocation angle at about 36.2°, which is much smaller than the critical dislocation angle of 54.7° featuring J-aggregation³⁴. As a result, the dislocation of porphyrin induces the stair-case type self-assembly of TPPS₄ as illustrated in Fig. 1 and greatly reduces the extent of overlapping of porphyrin rings. In this way, the π - π stacking overlap was significantly reduced. Since the radius of Bi³⁺ is only around 0.1 nm which did not trigger extra steric hindrance for the arrangement of TPPS₄, no induced chiral signal was observed upon formation of the nanotube (Supplementary Fig. S4). It is noticed that NO₃⁻ could exist in nanostructure by coordination interaction between Bi and NO₃⁻³⁵. In addition, the TPPS₄-Bi nanotubes can be viewed as a nanostructure with 100% loading of porphyrins because no additional co-assembling molecules were added into the system.

The suppression of self-quenching in TPPS₄-Bi was confirmed with time-resolved fluorescence studies (Supplementary Fig. S5 and Table 1). In neutral solution, 84% TPPS₄ exhibits a lifetime of 11.75 ns corresponding to the monomeric TPPS₄³⁶. Before addition of Bi³⁺, 94% porphyrin in J-aggregated TPPS₄ at pH 3.6 exhibits a short lifetime of 3.8 ns, which indicates that the π - π stacking overlap of TPPS₄ in J-aggregation have resulted in self-quenching of porphyrin. However, the lifetime is remarkably enhanced in J-aggregated TPPS₄-Bi system at pH 3.6, 65% TPPS₄ has the unprecedented long lifetime of about 11.10 ns, similar to that of monomeric TPPS₄ (Table 1). In addition, the long lifetime luminescence of TPPS₄-Bi corresponds with triplet stated that the emission at 600 nm could be quenched by oxygen³⁷, as shown in Supplementary Fig. S6. It is suggested that TPPS₄-Bi system possesses the potential in generation of singlet oxygen. The tubular structures and presence of heavy atom both promoted the intra-system crossing (ISC) which leads to the enhancement of photophysical properties.

The long lifetimes of porphyrin in TPPS₄-Bi system allow the nanotubes display excellent ability of singlet oxygen generation which was verified by iodide method and electron paramagnetic resonance (EPR) spectroscopy. The principle of this method is that the amount of I₃⁻ produced by oxidation of I⁻ with ¹O₂ is proportional to the concentration of ¹O₂ under continuous irradiation³⁸. The light induced oxidation of iodide is chosen as model reactions for catalysis as Scheme S1. Figure 4a,b show that upon irradiation with UV light, the characteristic absorption of I₃⁻ at $\lambda = 350$ nm and $\lambda = 288$ nm occurred, indicating the generation of ¹O₂ depending on time increasing. EPR spectroscopy was also employed to monitor the ¹O₂ generation ability of TPPS₄-Bi upon light irradiation. Trace amounts of 2,2,6,6-tetramethylpiperidine (TEMP), which is a diamagnetic and water-soluble molecule, was applied to capture ¹O₂ by yielding a paramagnetic product TEMPO^{39,40}. The unpaired electron located on the NO group of TEMPO can lead to the hyperfine splitting of the EPR signal into three narrow lines, arising from the interaction between the unpaired electronic spin and the nitrogen nucleus. When the oxygen-saturated solution of TPPS₄-Bi was irradiated in the presence of TEMP for 10 min, the EPR signal increased as shown in Fig. 4c. The result of EPR test was in accordance with iodide method which confirmed the generation of singlet oxygen in TPPS₄-Bi system.

Moreover, compared with self-quenching J-aggregated TPPS₄ at pH 3.6, the TPPS₄-Bi nanotubes display a significantly enhanced singlet oxygen production (Fig. 4d). It is noteworthy that ability of singlet generation for TPPS₄-Bi nanotubes is comparable to that of monomeric porphyrins, which is probably benefited from the reduced π - π stacking by staggered porphyrin arrangement. Moreover, the nanotubes remain stable when dispersed in neutral water and the singlet oxygen generation ability was not apparently affected by changing pH (Supplementary Fig. S7). When the TPPS₄-Bi nanotubes were dispersed and irradiated by light in neutral solution or in oxygen free solution, there is no obvious generation of ¹O₂ (Figs 4b and S6).

The excellent singlet oxygen generation ability of the TPPS₄-Bi nanotube in neutral solution indicates a potential application in cells. We incubated the TPPS₄-Bi nanotubes with HeLa cell for 24 h. The cells are lighted up by the nanotube (Fig. 5a), and the strength of the green fluorescence is comparable to that of the commercial lysosome imaging dye Hoechst 33258 (Fig. 5b). The green and the blue window overlapped perfectly and resulted in the cyan fluorescence in the cell nucleus (Fig. 5c,d). Since the fluorescence of porphyrins is usually obtained as they exist in the isolated states, the present results indicate that the out-of-plane coordination have retained the monomeric porphyrin, which is in agreement with the detection of long lifetimes in time resolved fluorescence measurements.

The sufficient fluorescence of TPPS₄-Bi in HeLa cells manifests the possibilities of using this nanotube as theranostics, far beyond function solely as the PDT drug. Therefore, the PDT effect of the nanotube was tracked with its own fluorescence, rather than under the help of commercial dyes. Figure 5e,f are the images of the HeLa cells treated with 10 μ M TPPS₄-Bi nanotubes before and after irradiation. Before irradiation, large amount of living cells are lighted up. In contrast, only several cells are observed after 10 min's irradiation followed by washing out the dead cells with water. A qualitative SRB analysis in Fig. 5g shows that half of the HeLa cells have been

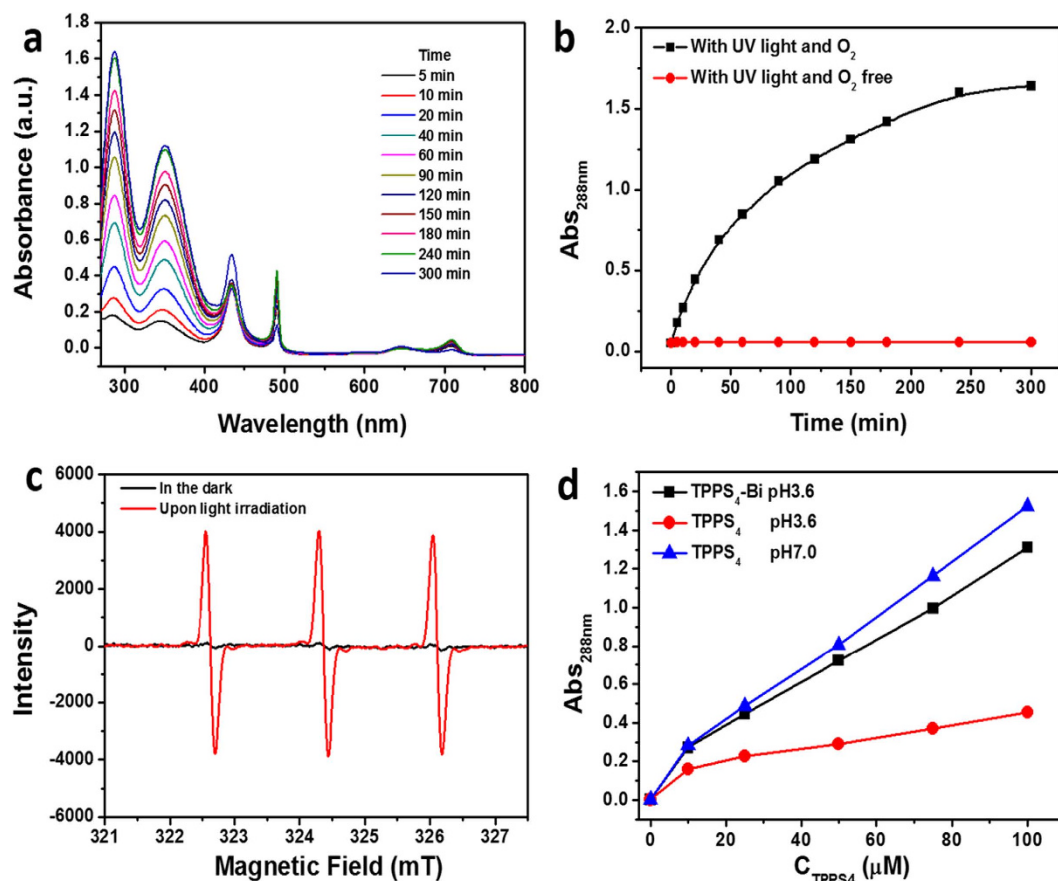


Figure 4. Time depending (a) UV-Vis spectra of the TPPS₄-Bi nanotubes in the presence of KI and (b) the comparison of TPPS₄-Bi nanotubes in the presence of UV light and O₂ (□) and O₂ free (○), respectively. The absorption was at 288 nm. (c) EPR spectra of the TPPS₄-Bi nanotubes solution in which trace TEMP acts to capture the ¹O₂ generated in the dark or upon light irradiation for 10 min. (d) Compared singlet oxygen generation tested by UV-Vis spectra at λ = 288 nm with increasing concentrations at TPPS₄ (pH 7.0), TPPS₄ (pH 3.6) and TPPS₄-Bi (pH 3.6) systems. [TPPS₄] = 0.025 mM, [Bi³⁺] = 0.05 mM, C_{KI} = 1 M.

killed to death under the same condition. However, controlled experiments suggest that no cell death can be observed without irradiation. Meanwhile, blank test manifests that irradiation of untreated cells would not lead to considerable cell death, too (Supplementary Fig. S8), showing that the TPPS₄-Bi nanotubes display excellent phototoxicity.

In summary, we have prepared TPPS₄-Bi nanotubes through the facile out-of-plane coordination assisted self-assembly. The Bi³⁺ locates between two porphyrins at a dislocation angle which induces staggered arrangement of porphyrins and prevents π-π stacking overlap. It allows the TPPS₄-Bi J-aggregation to suppress the self-quenching effect and display an enhanced singlet oxygen generation compared with traditional TPPS₄ J-aggregation. Since the component Bi³⁺ is also a radio-therapy agent for tumors and other diseases, and there are judicious choices of functionally large metal ions and porphyrins, we expect that the out-of-plane coordination approach inspires much exciting design of porphyrin aggregations for multifunctional theranostics.

Methods

Materials. The tetrasodiummeso-tetra(sulfonatophenyl)porphine (TPPS₄, >97.0%) was purchased from Alfa Aesar. Bi(NO₃)₃·5H₂O, Zn(NO₃)₂, Pb(NO₃)₂ and TiCl₄ were purchased from Beijing Chemical Reagents (Beijing, China, >99.0%). All the aqueous solutions were prepared using Milli-Q water (Millipore, 18 MΩ/cm resistivity).

Preparation of TPPS₄-Bi Nanotubes. The porphyrin nanostructure were fabricated by mixing aqueous solutions of TPPS₄ and Bi(NO₃)₃ at pH 3.6. Typically, 0.1 mL of H₄TPPS₄²⁻ solution (1 mM) was mixed with 0.2 mL of Bi(NO₃)₃ solution (1 mM) in 0.7 mL water, and the mixture as left undisturbed in the dark at 25 °C for 24 h. Over time, it provides a high yield of nanostructure.

Scanning Electron Microscopy (SEM). A drop of TPPS₄-Bi suspending solution was placed on clean silicon sheets and dried freely under ambient conditions. The samples were observed by a SEM, Hitachi S4800, 5 kV.

Transmission Electron Micrograph (TEM). Samples were observed by a Jeol JEM 100 CX, 80 kV and JEM-2100, 200 kV, together with energy-dispersive spectroscopy (EDS) measurement. Drops of samples were put

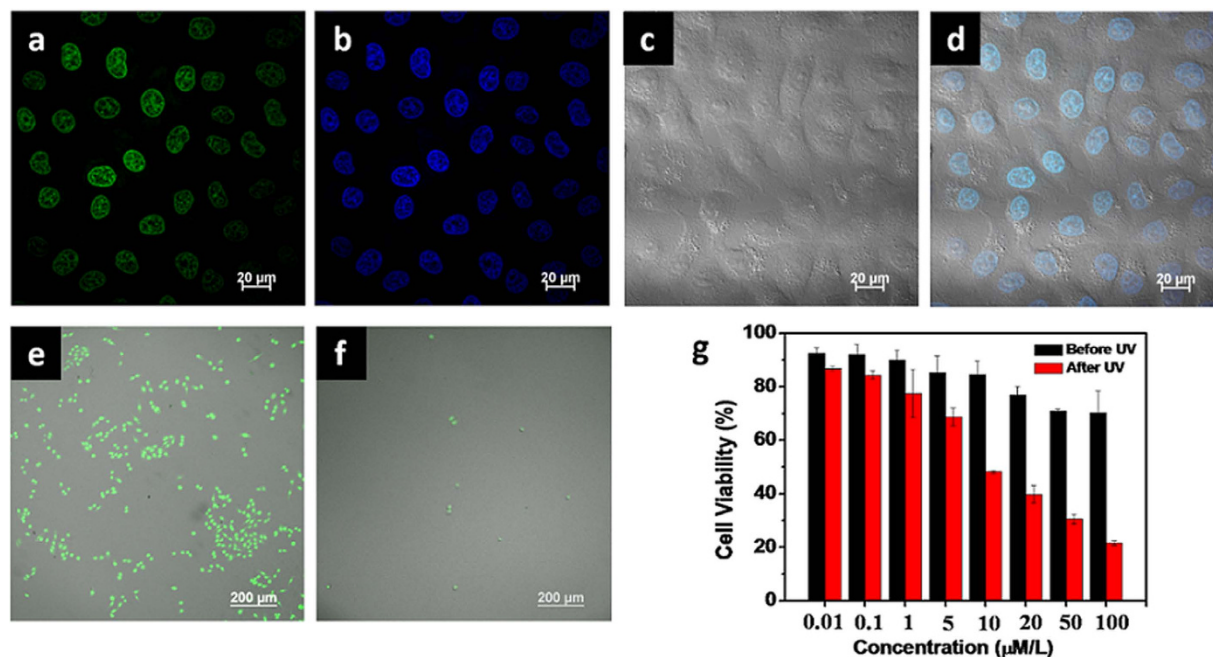


Figure 5. Confocal images of HeLa cells labeled with Hoechst 33258 after incubating with TPPS₄-Bi nanotubes. The fluorescent fields after (a) treated with 10 μM mL⁻¹ TPPS₄-Bi nanotubes and (b) Hoechst 33258 stained. (c) The optical field and (d) the overlap images of bright and fluorescence field in HeLa cells. The overlap images of bright field and fluorescence field (e) before and (f) after light irradiation in HeLa cells which were treated with 10 μM mL⁻¹ TPPS₄-Bi nanotubes. (g) SRB test for the cytotoxicity of TPPS₄-Bi nanotubes to HeLa cells after 48 hours incubation in various concentrations before and after UV irradiation. Data were presented as the mean ± standard deviation (N = 3).

onto 230 mesh copper grids coated with formvar film. Excess water was removed by filter paper and the samples were allowed to dry in ambient air at RT, before TEM observation. It is noticed that all samples were not stained except for the one which was made of TPPS₄ only.

Spectra Measurements. UV-Vis absorbance measurements were carried out on the Beijing Purkinje General Instrument Co. Ltd. TU-1810. The generation of singlet oxygen measured by UV-Vis absorption was carried out at 288 nm. The fluorescent measurements were tested by a fluorescent spectrophotometer, Hitachi F7000. The lifetime measurements of all samples were tested by a lifetime and steady state spectrometer (Edinburgh Instruments Ltd. FLS920). The circular dichroism (CD) spectrum was tested by a circular dichroism chiroptical spectrometer (Jasco Co.). All the spectra measurements were conducted at RT.

The light induced oxidation of iodide is chosen as model reaction for catalysis as previous reports. The reaction was shown in Scheme S1. The solution was deoxygen by nitrogen and kept in dark for 24 h. Before test, the solution was flowed by oxygen and added with KI. Then light was applied to induce the generation of ¹O₂. The ¹O₂ generation efficiency was tested by UV-Vis measurement at 288 nm absorption.

X-Ray Diffraction (XRD). For XRD measurements, several drops of suspension of TPPS₄ and TPPS₄-Bi were dropped on a clean glass slide respectively, followed by drying in the air. All samples were tested by an instrument (Rigaku Dmax-2000, Ni-filtered Cu Kα radiation) under ambient conditions at RT.

Singlet Oxygen Measurement. The EPR spectroscopy was used to monitor the generation of singlet oxygen in aqueous solutions. Singlet oxygen was detected as TEMP-¹O₂ adduct (TEMPO) using TEMP as a singlet oxygen trap. The EPR experiments were performed at room temperature on a JEOL JES-FA200 apparatus. The solution was saturated with oxygen, followed by addition of trace amount of TEMP and then irradiated by a xenon lamp with a sharp-cut filter (the cut off wavelength is 450 nm). The TEMPOL signal was analyzed by EPR.

Confocal Laser Scan Microscopy (CLSM). The samples were dropped on a clean glass slide and covered by a clean cover slip. A TCS-sp inverted confocal laser scanning microscope (Leica, Germany) was used to conduct experiments in fluorescence and differential interference contrast (DIC) modes.

Cell Culture and Cytotoxicity Assay *in Vitro*. In this study, HeLa cell lines were used. The HeLa cells (Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China) were routinely grown in DMEM medium supplemented by 10% heated-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin. Cells were maintained at 37 °C with 5% CO₂. For cytotoxicity tests, HeLa cells were seeded into 96-well culture plates at a density of 5 × 10³ cells/well and grown for 24 h. Then TPPS₄-Bi nanotubes were added into 96-well culture plates. The concentration of TPPS₄-Bi nanotubes

were in the range of $1 \times 10^{-3} - 50 \mu\text{M mL}^{-1}$. Then after 48 h incubation, the cell viability was measured by a microplate reader at 540 nm with the SRB staining assay. The following formula was used: $\text{Survival\%} = (\text{A}_{540\text{nm}} \text{ for the treated cells} / \text{A}_{540\text{nm}} \text{ for the control cells}) \times 100\%$, where the $\text{A}_{540\text{nm}}$ was the absorbance value. Each assay was repeated for 5 times.

Confocal Microscopic Imaging of Cells Using TPPS₄-Bi Nanotubes. The HeLa cells were seeded into 96-well culture plates at a density of 5×10^3 cells/well and grown for 24 h. The cells were taken with $4 \mu\text{g mL}^{-1}$ TPPS₄-Bi nanotubes for 4 h at 37 °C. Afterward, the cells were washed three times with PBS to remove the non-internalized nanotubes and incubated for another 24 h in a 24-well plate. Then the cells were fixed with 4% paraformaldehyde for 10 min and stained with $10 \mu\text{g mL}^{-1}$ Hoechst 33258 at room temperature. Cell images were taken with a confocal laser scanning microscope with the excitation wavelengths of 405 nm and 488 nm.

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Author Contributions

Q.Z. performed all the experiments and involved in writing; Y.W. provided useful help in cell tests; Y.X. provided useful help in XRD tests; J.H. and Y.Y. proposed the study and revised the manuscript. All authors reviewed the manuscript.

Additional Information

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