A Single-wavelength NIR-triggered Polymer for in situ Generation of Peroxynitrite (ONOO−) to Enhance Phototherapeutic Efficacy

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Abstract Phototherapies including photodynamic therapy (PDT) and photothermal therapy (PTT) are the most promising and non-invasive cancer treatments. However, the efficacy of mono-therapy of PDT or PTT is often limited by the phototherapeutic defects such as low light penetration depth of photosensitizers and insufficiency of photothermal agents. Peroxynitrite (ONOO−) has been proved to be an efficient oxidizing and nitrating agent that involves in various physiological and pathological processes. Therefore, ONOO− produced in tumor site could be an effective treatment in cancer therapy. Herein, a novel cyanine dye-based (Cy7) polymer nanoplatform is developed for enhanced phototherapy by in situ producing ONOO−. The Cy7 units in the nanoparticles can not only be served as the photosensitizer to produce reactive oxygen species (ROS) including singlet oxygen and superoxide anion for PDT, but also be used as a heat source for PTT and the release of NO gas from N-nitrosated napthalimide (NORM) at the same time. Since NO can react quickly with superoxide anion to generate ONOO−, the enhanced phototherapy could be achieved by in situ ONOO− produced by PCy7-NO upon exposure to the near infrared (NIR) light. Therefore, the NIR-triggered Cy7-based nanoplatform for ONOO−-enhanced phototherapy may provide a new perspective in cancer therapy.

Keywords Peroxynitrite (ONOO−); Phototherapy; Cyanine; Napthalimide; Near-infrared

INTRODUCTION

In recent years, phototherapies including photothermal therapy (PTT) and photodynamic therapy (PDT) have been becoming promising treatments for many diseases, such as inflammation and cancer, due to their non-invasive features and controllability.¹⁻⁴ In PTT, the energy of implementing light transforms into thermal energy to realize irreversible death of the tumor cells, while PDT utilizes a specific way to generate highly cytotoxic reactive oxygen species (ROS) to ablate the cancer cells.⁵⁻⁻⁷ However, the efficacy of phototherapies for cancer therapy is often limited by their own defects. In PDT, photosensitizers (PSs) are activated to the excited state by light irradiation, and then react with oxygen from surrounding environment to produce ROS, but the efficacy of PDT treatment often suffers to low light penetration depth of PSs and the hypoxia of the tumors.¹²⁻⁻¹⁵ Similarly, in PTT¹⁶ photothermal agents can be promoted to the excited state with light irradiation, and then produce vibrational energy in the form of thermal energy to increase temperature of tumor tissue to kill tumor cells, but tumor cells could generate heat shock proteins (HSP) to avoid apoptosis, leading to a relatively low efficacy of PTT.¹⁷⁻⁻¹⁹ More recently, the combination of phototherapy with other therapies has been developed including immunotherapy and chemotherapy, and the efficacy of phototherapy could be enhanced in comparison with the single phototherapy.²⁰⁻⁻²⁵ Peroxynitrite (ONOO−) has been considered to be an efficient oxidizing or nitrating agent that has a higher toxicity to cells than most free radicals since ONOO− is almost independent on some counteractive intracellular materials such as oxyhemoglobin and superoxide dismutase.²⁶⁻⁻³⁰ Therefore, the overexpression of ONOO− in normal cells can cause many diseases.³⁰⁻⁻³¹ For example, ONOO− can produce the pathologic effect on the vasculature, leading to the occurrence of diabetes, hypertension and other vascular diseases by the following pathways such as triggering programmed cell apoptosis, incremental regulation of endothelial cell adhesion molecules, decomposition of endothelial cell envelope, and enhancement of neutrophil adhesion.³²⁻⁻³³ But recently, ONOO− has been found to be an efficient tool to improve the efficacy of antibacterial treatment.³⁴⁻⁻³⁶ Therefore, ONOO− seems to
be an enhanced agent for phototherapies.\cite{37-39} Generally, it is the most common and convenient approach to produce ONOO\textsuperscript{−} via the direct reaction of NO and O\textsubscript{2}\textsuperscript{−} in a physiological environment. However, the in situ formation of ONOO\textsuperscript{−} in the therapeutic process is a great challenge, since its precursors of NO and O\textsubscript{2}\textsuperscript{−} both have short half-life time and narrow diffusion distance, and the generation process of ONOO\textsuperscript{−} is spontaneous and uncontrolled.\cite{30} So, it would be an ingenious and promising strategy to combine photosensitizers and NO donors\cite{41-43} for realizing in situ controllable formation of ONOO\textsuperscript{−} during phototherapeutic process, further achieving the ONOO\textsuperscript{−}-enhanced phototherapy.

Herein, we propose a novel cyanine dye-based (Cy7) polymer nanoplatform which could achieve in situ formation of ONOO\textsuperscript{−} to enhance phototherapy (Scheme 1). The nanoplatform is constructed by using Cy7 as the photosensitizer\cite{44,45} and N-nitrosated napthalimide (NORM) as the NO donor,\cite{46-49} where NO can be produced by the photothermal process of Cy7, and superoxide anion can be simultaneously generated during the photodynamic process, thereby the in situ formation of ONOO\textsuperscript{−} is accompanied with phototherapy. The Cy7 molecule was first functionalized to Cy7 monomer, and then utilized to prepare amphiphilic Cy7-based block copolymer via reversible addition-fragmentation chain transfer (RAFT) polymerization by using poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMA) as the macromolecular chain transfer agent (CTA). The Cy7-based block copolymer was self-assembled in aqueous solution with NORM to form the nanoparticles (PCy7-NO). Under 808 nm light irradiation, PCy7-NO nanoparticles could not only produce ROS such as \textsuperscript{1}O\textsubscript{2} and O\textsubscript{2}\textsuperscript{−} and heat effectively, but also generate a large amount of NO gas under the photothermal process. Thus, the nanoplatform could achieve simultaneous production of NO and O\textsubscript{2}\textsuperscript{−} at the tumor sites, thereby realizing the in situ generation of ONOO\textsuperscript{−} for enhanced phototherapy only through one-step NIR light activation. A series of in vitro and in vivo experiments have been performed to verify the above assumptions. Therefore, PCy7-NO nanoparticles would be used as the promising agent for enhanced phototherapy.

**Scheme 1** Schematic illustration for the fabrication of PCy7-NO nanoparticles and the processes of in situ generating ONOO\textsuperscript{−} for enhanced phototherapy.

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**EXPERIMENTAL**

**Synthesis of Hydroxyindole**

Hydroxyindole was prepared by mixing 2,3,3-trimethyl-3H indole (1.59 g, 10 mmol) with 2-iodoethanol (1.72 g, 10 mmol) in acetonitrile at 80 °C for 8 h under nitrogen atmosphere. Then crude product was obtained by evaporating acetonitrile. The product was further purified by precipitating in ice ether and the pink powder was obtained (Yield: 74%).

**Synthesis of Cy7**

Hydroxyindole (1.33 g, 4 mmol) and the condensing agent (0.35 g, 2 mmol) were dissolved in the mixed solvent of 1-butanol and toluene (V\textsubscript{1-butanol} / V\textsubscript{toluene} = 7/3), and then the mixture was heated at 120 °C for 12 h under nitrogen atmosphere. After the reaction, the mixture was cooled to room temperature and the solvent was evaporated. The crude product was precipitated in ice ether. Further purification was performed with column chromatography using dichloromethane/methanol (20/1, V/V) as the eluent. Green solid with metallic luster was obtained (Yield: 67%).

**Synthesis of OH-Cy7-alkenyl (Cy7-based Monomer)**

Cy7 (536 mg, 0.8 mmol) in anhydrous dichloromethane was added into a round-bottom flask and cooled to 0 °C. The mixture was then sealed under vacuum. The degassed solution was immersed into an oil bath at 70 °C and stirred for 24 h. The ice bath was then removed, and the mixture was kept at room temperature for 24 h with stirring. The solution was evaporated under reduced pressure to afford the desired compound Cy7-based monomer as a purple solid with metallic luster (Yield: 43%).

**Synthesis of POEGMA**

OEGMA\textsubscript{475} (1.0 g, 2.1 mmol), CDB (19 mg, 70.2 μmol), AIBN (3.84 mg, 23.4 μmol) and 2 mL of DMF were added into a dry glass tube equipped with a magnetic stirring bar. The mixture was degassed by passing nitrogen into the tube through at least three freeze-pump-thaw cycles, and the polymerization tube was then sealed under vacuum. The degassed solution was immersed into an oil bath at 70 °C and stirred for 24 h. The polymerization was quenched by plunging the reaction flask into liquid nitrogen. The mixture was then precipitated in ice ether for three times, and then the product was dried in vacuum oven at room temperature for 12 h (Yield: 78%).

**Synthesis of Amphiphilic Block Copolymer, PCy7**

The block copolymer was prepared via the subsequent RAFT polymerization of the HO-Cy7-alkenyl monomer using POEGMA as the macro-RAFT agent. Typically, POEGMA (100 mg, 16.65 μmol), Cy7-based monomer (150 mg, 18.8 μmol), AIBN (0.91 mg, 5.55 μmol) and 3 mL of DMF were added into a dry polymerization tube with a magnetic stirring bar. The mixture was degassed through at least three freeze-pump-thaw cycles, and the polymerization tube was then sealed under vacuum. After the polymerization reaction was performed with stirring at 70 °C for 72 h, the polymerization was quenched by plunging the reaction flask into liquid nitrogen. The mixture was dialyzed with deionized water/methanol (4/1, V/V) as dialysate.
at least three times to remove small molecules, and finally, the product was dried by lyophilization (Yield: 64%).

**Synthesis of BNA**
4-Bromo-1,8-naphthalic anhydride (20 g, 72.18 mmol) and butylamine (6.32 g, 86.58 mmol) were added into 500 mL of ethanol. Under refluxing, the mixture was stirred for 8 h, and then filtered and washed by ethanol to afford a crude product, purified by recrystallization in the mixed solvent of ethanol and water to obtain BNA (Yield: 68%).

**Synthesis of MOEANA**
BNA (10 g, 30.12 mmol), butylamine (3.30 g, 45.18 mmol) and NaHCO$_3$ (3.80 g, 45.18 mmol) were added into 300 mL of DMSO. The mixture was stirred and heated to 110 °C for 12 h and then cooled to room temperature. After that, the reaction solvent was evaporated under reduced pressure. The resulting residue was transferred into water, and then extracted by CH$_2$Cl$_2$ for three times. The organic layer was dried with Na$_2$SO$_4$, filtered, and evaporated under reduced pressure to obtain the crude product. The product was further purified with a column chromatography with DCM/PE (1/1.5, V/V) as the eluent to obtain the MOEANA (Yield: 62%).

**Synthesis of NORM**
MOEANA (1.1 g, 3.5 mmol) was dissolved in the mixed solvent of AcOH/CH$_2$Cl$_2$ (10/0.5/7, V/V/V, 40 mL), and the solution was cooled to 0 °C. Sodium nitrite (483 mg, 7 mmol) was added into the mixture under stirring. After the ice bath was removed, the reaction mixture was stirred at room temperature for another 2 h. A saturated NaHCO$_3$ solution was added into the flask until the pH was adjusted to neutral. The mixture was extracted three times with DCM, and then the organic layer was collected, dried with Na$_2$SO$_4$, filtered and evaporated under reduced pressure to produce the crude product. The product was further purified with a column chromatography with DCM/MeOH (100/0.5, V/V) as eluent to obtain the NORM (Yield: 68%).

**Methods**

**Preparation of the PCy7-NO Nanoparticles**
The PCy7-NO nanoparticles were prepared by a typical nano-preparation method. PCy7-NO nanoparticles (10 mg) and NORM (2 mg) were first dissolved in 1 mL of DMF to form the solution. Then the solution was added dropwise into 4 mL of deionized water under magnetic stirring at room temperature in 1 h. The mixed solution was stirred for 4 h and dialyzed in deionized water using a dialysis membrane (MWCO=3500) for 3 days. Finally, the concentration of the assembled solution was adjusted to 200 μg·mL$^{-1}$ (Cy7).

**Characterization**
All 1H-NMR spectra were recorded on a Bruker AV400 Spectrophotometer at 400 MHz in CDCl$_3$ with tetramethylsilane (TMS) as an internal reference. The molecular weight was measured by a Waters 1515 gel permeation chromatography (GPC) system using DMF as the eluent with the flow rate of 1 mL·min$^{-1}$. GPC data were calibrated as polystyrene standards. Absorption spectra were recorded on a Shimadzu UV-2550 UV spectrophotometer using a quartz cuvette with a 1 cm beam path length, and fluorescence spectra measurements were performed on a Varian Cary Eclipse fluorescence spectrophotometer at room temperature with excitation and emission slit widths of 10 nm and 5 nm, respectively.

**Detection of Singlet Oxygen and Superoxide Anion in Vitro**
Typically, 1,3-diphenylisobenzofuran (DPBF, 30 μL, 8 mmol·L$^{-1}$) as a probe of singlet oxygen was added into the solutions of PCy7-NO and PCy7 nanoparticles (5 μg·mL$^{-1}$ for Cy7) in 3 mL of PBS, respectively. The solution was irradiated by the 808 nm laser for different time and the absorption intensity of DPBF at 425 nm was recorded.

Dihydro ethidium (DHE) as a probe of superoxide was first mixed with DNA solution, and the mixed solution was added into PCy7-NO and PCy7 aqueous solutions, respectively. The solution was irradiated by the 808 nm laser for different time and the emission fluorescence intensity at 610 nm was recorded ($A_{	ext{ex}}=480$ nm).

**Detection of Extracellular and Intracellular NO**
Griess assay, a typical method for quantitatively detecting NO, was used to detect the released NO from the PCy7-NO nanoparticles. Griess reagent I (600 μL) was added into the PCy7-NO aqueous solution (irradiated with 808 nm light for different durations) and shaken for 1 min. Then, griess reagent II (600 μL) was added into the mixture solution and shaken for another 2 min at room temperature. At last, the mixture was measured by a UV-Vis spectrophotometer.

Intracellular NO levels were examined by confocal laser scanning microscopy (CLSM) by using the fluorescence of the sample itself. When the PCy7-NO nanoparticles were irradiated with 808 nm light, NO was immediately released and the corresponding molecules of MOEANA were also produced. Thus, the fluorescence was turned on. 4T1 cells were seeded into confocal dishes and treated with PCy7-NO nanoparticles at 37 °C overnight. The cells were then irradiated for different time with 808 nm laser (0.8 W·cm$^{-2}$) and the cells were incubated for another 4 h. A reappeared green fluorescence signal of NORM was obtained by using CLSM ($E_x/E_m=488/525$ nm).

**Detection of Peroxynitrite from PCy7-NO Nanoparticles**
Peroxynitrite (ONOO$^-$) can be detected by L-tyr solution in weak alkaline environment with the presence of CO$_2$. DL-tyr will be generated by the oxidation of peroxynitrite, and then detected by the fluorescence spectrophotometer with the excited wavelength at 313 nm. Typically, 100 mL of PBS (0.10 mol·L$^{-1}$, pH=8.2) with NaHCO$_3$ (0.015 mol·L$^{-1}$) and L-tyr (5.0×10$^{-5}$) was prepared at room temperature. Then, 1 mg of PCy7-NO or PCy7 nanoparticles was added into the solution and irradiated with 808 nm laser for 1 min. The change of the fluorescence intensity at 404 nm was recorded.

**Cell Culture**
Mouse breast cancer (4T1) cells kindly provided by the Institute of Biochemistry and Cell Biology, SIBS, Chinese Academy of Sciences were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics (50 units per mL penicillin and 50 units per mL streptomycin) under a humidified atmosphere with 5% CO$_2$ at 37 °C.

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Cell Manipulation with PCy7-NO and PCy7 Nanoparticles

Dark toxicity

4T1 cells were seeded into a 96-well plate and then cultured at 37 °C for 24 h. The nanoparticles with different concentrations dispersed in culture medium were added into the wells and incubated for another 24 h. Subsequently, the cells were washed with sterilized PBS for three times, and the culture medium was replaced with 200 μL fresh DMEM included 20 μL of MTI (5 mg·mL⁻¹). The cells were incubated for another 4 h. After that, 150 μL of DMSO was added into the wells with gentle shaking to extract the formazan products. Then, the absorbance was measured at 560 nm using a microplate reader. Cell viability (%) was calculated according to the equation: cell viability (%) = (OD_{test} - OD_{background})/(OD_{control} - OD_{background}) × 100%, where OD_{test} is the absorbance in the presence of sample solutions, and OD_{control} is the absorbance of the control group.

Photo-toxicity

4T1 cells were seeded into a 96-well plate and then cultured at 37 °C for 24 h. The nanoparticles with different concentrations dispersed in culture medium were added into the wells and incubated for another 24 h. Subsequently, the cells were washed with sterilized PBS for three times to remove residual nanoparticles, and fresh culture medium was added into the plate. Then the cells were irradiated with 808 nm laser (0.5 W·cm⁻²) for 60 s, and put back into incubator for another 24 h. Finally, cell viability was evaluated using MTT assay.

Detection of the Released ROS from the PCy7-NO and PCy7 Nanoparticles

Intracellular ROS levels were examined by CLSM by using the ROS-sensitive probe, DCFH-DA. 4T1 cells were seeded into confocal dishes (10⁵ cells per well) and treated with PCy7 and PCy7-NO nanoparticles (4 μmol·L⁻¹) at 37 °C overnight. The cells were incubated with 20 μmol·L⁻¹ DCFH-DA for 30 min, and then irradiated with 808 nm laser (500 mW·cm⁻²) for 3 min. After irradiation, the cells were incubated for an additional 4 h. A green fluorescent signal of intracellular ROS was detected by using CLSM (E532/E520=488/520 nm).

Fluorescence and photothermal imaging was accomplished by following the protocol. PCy7-NO and PCy7 nanoparticles (2.0 mg·kg⁻¹) were injected into the tail vein of 4T1-bearing nude mice when the tumor volume reached 100 mm³. After injection, the fluorescence imaging was performed with an in vivo imaging system at 4, 8, 12 and 24 h, respectively. Photothermal imaging was performed at 24 h post-injection. The tumor site was irradiated by 808 nm laser for 3 min, and the change of temperature was recorded.

In Vivo Anti-tumor Efficacy of PCy7-NO Nanoparticles

In vivo treatment was evaluated on 4T1 tumor-bearing mice. The mice were randomly divided into 5 groups (n=4) when the tumor volume reached 100 mm³: (a) control; (b) PCy7; (c) PCy7 nanoparticles with 808 nm laser; (d) PCy7-NO; (e) PCy7-NO nanoparticles with 808 nm laser. Each group was performed on demand at first day. At 24 h post-injection, groups (c) and (e) were irradiated by 808 nm laser for 3 min (0.5 W·cm⁻²). The tumor volumes were measured every two days. After 14-day treatment, mice were sacrificed to collect all the tumors and major organs for H&E staining to evaluate anti-tumor efficiency and the biocompatibility of nanoparticles.

RESULTS AND DISCUSSION

Synthesis and Characterization of the PCy7-NO Nanoparticles

To prepare the nanoparticles with a superior capability of generating ONOO⁻ for enhanced phototherapy, the Cy7-based amphilic block copolymer was prepared. First, Cy7 was synthesized according to previous literature,¹³ and then it was functionalized into Cy7-based monomer by the reaction of acetyl chloride and hydroxyl group of Cy7. The Cy7-based block copolymer was obtained via the RAFT polymerization of Cy7-based monomer with POEGMA as a macromolecular CTA. The successful preparation of Cy7-based monomer and block copolymer was confirmed by ¹H-NMR spectra (Figs. S1–S5 in the electronic supplementary information, ESI). The degree of polymerization (DP) of the block copolymer could be calculated by comparing the integration area of the signal at 3.36 ppm (protons of OEGMA units) to that at 8.16 ppm (protons of Cy7 units)[⁶⁸]. The DP of POEGMA macromolecular CTA was obtained as DP_{POEGMA} = 2I_{1.36}/I_{12.29} = 12 (Fig. S4 in ESI), and the DP of PCy7 block was obtained as DP_{PCy7} = (3I_{1.16}/I_{1.36} × DP_{POEGMA}) = 6 (Fig. S5 in ESI). Additionally, the symmetrical GPC trace of the block copolymer revealed that the RAFT polymerization of Cy7-based monomer was performed in a living manner (Fig. S6 in ESI). Then, NORM, as a NO donor, was synthesized in three steps, where 4-bromo-1,8-naphthalic anhydride (BNA) was condensed with butyramine to produce MOEANA, then MOEANA was nitrosated to prepare NORM, which was verified by ¹H-NMR spectra (Figs. S7–S9 in ESI), and finally the NORM was encapsulated by amphiphilic Cy7-based block copolymer to form the nanoparticles (PCy7-NO) by a nanoprecipitation method. In addition, the amphiphilic copolymer was self-assembled into micelles without NORM as the control, which was denoted as PCy7. As shown in Fig. 1(a), PCy7 presents a broad characteristic absorbance of Cy7 in the near-infrared (NIR) region with a strong shoulder peak, which is attributed to the formation of H-aggregation of Cy7 during the self-assembly process.¹⁹ Similiarly, PCy7-NO has a similar strong characteristic absorbance in NIR region, but it has a new broad absorbance at 365 nm, indicating that NORM was successfully entrapped into the nanoparticles. The average diameters of PCy7 and PCy7-NO nanoparticles measured by dynamic light scattering (DLS) were 156 and 165 nm, respectively (Fig. 1b). The sizes of the PCy7-NO and PCy7 nanoparticles measured by DLS were slightly bigger than those measured by transmission electron microscopy (TEM), since POEGMA chains were solvated and therefore fully extended in aqueous solution (the inset of Fig. 1b and Fig. S10 in ESI).

NIR-responsive Generation of O²⁻, O₂⁻ and NO in Solution

As a typical cyanine dye, Cy7 could produce reactive oxygen species (ROS) such as singlet oxygen (O₂¹) and superoxide anion (O₂⁻) under light irradiation. First, we evaluated the producing ability of O₂⁻ of the two kinds of nanoparticles by detecting the absorbance of 1,3-diphenylisobenzofuran (DPBF), where the absorbance decrease of DPBF corresponds to the production of O₂⁻. As shown in Fig. 1c, although there was slight difference at the point of 30 s between the two kinds of nanoparticles, the
absorbance decrease rate of DPBF in the presence of PCy7-NO nanoparticles was similar to that of PCy7 nanoparticles, indicating that the ability to generate $\cdot{O}_2$ of PCy7-based nanoparticles was not affected by the incorporation of NORM. Further, $O_2^\cdot$ produced by Cy7 was detected by dihydro ethidium (DHE) as an $O_2^\cdot$ probe, where the fluorescence intensity of oxidized DHE at 610 nm ($\lambda_{ex}=480$ nm) was recorded. The result showed that both of the PCy7 and PCy7-NO nanoparticles can generate $O_2^\cdot$ effectively after light irradiation (Fig. S11 in ESI).

Next, the release behavior of NO from PCy7-NO nanoparticles was studied by using a typical Griess assay which is a commercial reagent for detecting NO. The quantity of the released NO was calculated by absorbance at 540 nm from the full wavelength UV-Vis spectrophotometer, and it can be...
observed that the release of NO increased gradually with the irradiation time (Fig. 1d). The final quantity of NO produced from 20 μmol·L⁻¹ PCy7-NO nanoparticles at 3-min light irradiation could be up to around 60 μmol·L⁻¹, which might be enough for synergistic cancer therapy. Moreover, after the NO release from NORM, the fluorescence signal at 528 nm of MOEANA recovered, and the fluorescence intensity increased with irradiation time (Fig. S12 in ESI), which further confirmed that NO could be effectively produced from PCy7-NO nanoparticles.

**Mechanism of the NO Release**

We also explored the NO release mechanism of PCy7-NO nanoparticles. First, the release capability of NORM was directly evaluated under heating. It can be observed that NORM could not generate NO gas at low temperature, while it could produce a large amount of NO with the temperature above 85 °C (Fig. S13 in ESI), indicating that NORM as a NO donor has a superior capability to generate NO gas at a high temperature. Meanwhile, Cy7 has been confirmed to be utilized as an effective photothermal agent to produce heat quickly in the physiological environment. Here, Cy7-based nanoparticles exhibited excellent and similar photothermal behavior, and they could induce the rapid increase of the temperature to 80 °C under light irradiation (Fig. 1e). What’s more, it has been proved that the surface temperature of the nanoparticles is actually much higher than the average temperature of the solution in the previous work.[52]

**Detection of Produced ONOO⁻ from the PCy7-NO Nanoparticles**

Finally, ONOO⁻ produced from PCy7-NO nanoparticles was detected by L-tyrosine (L-tyr) in the presence of CO₂ in a weak alkaline environment, where ONOO⁻ could oxidize L-tyr to the dimerization of L-tyrosine (DL-tyr) by detecting the fluorescence signal of DL-tyr at 400 nm.[37] As shown in Fig. 1(f), no fluorescence signal at 400 nm was detected in L-tyr and L-tyr+PCy7 groups with or without light irradiation, indicating that no ONOO⁻ was generated in these groups. For L-tyr+PCy7-NO group without 808 nm light irradiation, the weak fluorescence signal could be observed at 400 nm, which might be resulted from the slight amount of NO generated at natural light. However, it is exciting to find that a very strong fluorescence signal appeared at 400 nm of DL-tyr for L-tyr+PCy7-NO group with 808 nm light irradiation, which suggests ONOO⁻ could be produced efficiently from PCy7-NO nanoparticles. Therefore, it can be concluded that ONOO⁻ could be in situ formed during Cy7-based phototherapeutic process by the redox reaction between O₂⁻ and heat-released NO.

**Cellular Uptake of the Nanoparticles**

In order to evaluate the intracellular uptake of nanoparticles, 4T1 cells were separately incubated with PCy7 and PCy7-NO nanoparticles. It can be observed that the fluorescence intensity of Cy7 in both PCy7 and PCy7-NO nanoparticles increased with the incubation time by using CLSM, indicating these two kinds of nanoparticles could be internalized by 4T1 cells gradually (Fig. 2a). Furthermore, ROS and NO generation within cells were characterized by CLSM. Intracellular ROS generation was firstly detected by the ROS probe of 2′,7′-dichlorodihydrofluorescein diacetate (DCFHDA). After 808 nm laser irradiation, clear green fluorescence appeared in the samples treated with PCy7 and PCy7-NO nanoparticles, suggesting that a large number of ROS generated within cells as shown in Fig. 2(b). NO gas generation was also examined with NORM itself since it could recover fluo-

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![Fig. 2](https://doi.org/10.1007/s10118-021-2540-0)
cence after releasing NO. As shown in Fig. 2(c), there was no fluorescence detected for the cells treated with PCy7 nanoparticles before and after irradiation, while the cells treated with PCy7-NO nanoparticles could produce intense fluorescence after light irradiation. Therefore, it could be concluded that PCy7-NO nanoparticles could generate large amount of ROS and NO at the same time within cells.

**Cell Cytotoxicity**

Encouraged by the above results, the cell cytotoxicity of PCy7-NO nanoparticles was further studied by MMT assay. First, the cell cytotoxicity of NORM was evaluated with or without 808 nm light irradiation, and it could be found that all the cell viabilities were around 100%, indicating that NORM has no toxicity to the cells (Fig. S15 in ESI). Then, the cell viabilities of PCy7 and PCy7-NO nanoparticles were assessed without light irradiation, and the results showed that more than 90% cell viabilities were remained (Fig. 3a), suggesting these two kinds of PCy7-based nanoparticles have no dark cytotoxicity. The light cytotoxicity of PCy7 and PCy7-NO nanoparticles on 4T1 cells was further investigated by using 808 nm light irradiation (0.5 W·cm⁻²), and it can be found that the cytotoxicity of the two kinds of nanoparticles increased with their concentrations. Moreover, the cytotoxicity of PCy7-NO nanoparticles was much higher than that of PCy7 nanoparticles at the same concentration. Therefore, the results revealed that PCy7-NO nanoparticles could realize the ONOO⁻-enhanced phototherapy by in situ formation of ONOO⁻ during phototherapeutic process.

**In Vivo Biodistribution of PCy7 and PCy7-NO Nanoparticles**

Nude mice bearing 4T1 tumors were used as the xenograft model to verify the distribution of nanoparticles in vivo using fluorescence imaging firstly. The fluorescence intensity of the Cy7 was examined at different time after intravenous injection of PCy7 or PCy7-NO nanoparticles (Cy7, 2 mg·mL⁻¹). As shown in Fig. 3(b) and Fig. S16 (in ESI), mice treated with PCy7-NO and PCy7 nanoparticles both show remarkable fluorescence intensity, which indicated that the loading of NORM would not affect the biodistribution of Cy7. More excitingly, the accumulation of the nanoparticles in the tumor site increased gradually with circulation time, and the fluorescence intensity of Cy7 in these nanoparticles was observed to be maximal at 24 h. These results demonstrated that PCy7-NO nanoparticles could be well accumulated in the tumor sites.

**In Vivo Photothermal Effect of PCy7 and PCy7-NO Nanoparticles**

The in vivo photothermal effect was evaluated using 4T1-bearing mice which were intravenously administered PCy7 and PCy7-NO nanoparticles and exposed to the 808 nm laser (0.5 W·cm⁻²) for 3 min after 24 h post-injection. Obviously, both PCy7 and PCy7-NO groups exhibited similar tendencies for the photothermal behavior. Within 3 min of the irradiation, the temperature of tumor sites in both groups increased gradually and reached around 65 °C for 2 min and remained constant during the rest of the time (Fig. 3c and Fig. S17 in ESI), thus the temperature is high enough for the threshold of photothermal

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**Fig. 3** (a) Cell viability of 4T1 cells treated with PCy7 or PCy7-NO nanoparticles with or without 808 nm laser irradiation. (b) Fluorescence images of 4T1 tumor-bearing mice post i.v. injection of PCy7-NO nanoparticles for different time. (c) Photothermal images of 4T1 tumor-bearing mice post i.v. injection of PCy7-NO and PCy7 nanoparticles for 24 h and exposed to the 808 nm laser for different time.

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therapy (around 40–50 °C) to induce the ablation of the cancer. The results confirmed that these nanoparticles can accumulate in the tumor sites and produce enough heat to realize photothermal therapy.

**In Vivo Anti-tumor Efficacy of PCy7-NO Nanoparticles**

Based on the excellent performance of the nanoparticles in treatment and imaging, their therapeutic potency using 4T1-bearing mice was further evaluated. The mice were divided into five groups randomly: (a) control (saline), (b) PCy7, (c) PCy7+L, (d) PCy7-NO, (e) PCy7-NO+L. Intravenous injection was administrated when the tumor volume reached 100 mm$^3$. Then the 808 nm laser was used to irradiate the tumor site for 3 min at 24 h post-injection under the guidance of in vivo imaging. Tumor volumes and the weights of the mice were recorded every two days over the following 14 days. As shown in Figs. 4(a) and 4(b), the tumor volumes of the mice treated with saline show a fast increase in the control group. The PCy7 and PCy7-NO groups also show lower anti-tumor efficacy. The PCy7+L group has a good antitumor efficacy due to the toxic ROS and quantity of heat generated from Cy7.

However, the PCy7-NO+L group showed the best efficacy of tumor inhabitation, suggesting that NO gas could be effectively generated from the nanoparticles to form ONOO$^-$ with $O_2^-$, leading to ONOO$^-$-enhanced phototherapy. Then the mice were sacrificed and the tumors were collected and weighed. As shown in Fig. 4(c), the tumor weight in the PCy7-NO+L group was the lowest compared with those of other groups. Additionally, the body weights had no significant difference among all the groups (Fig. 4d), confirming that there was almost no biological toxicity of the nanoparticles. All

![Fig. 4](https://doi.org/10.1007/s10118-021-2540-0)
these results indicated that PCy7-NO nanoparticles presented a great potential in tumor inhabitation.

To further examine the efficiency of ONOO−-enhanced phototherapy and systemic toxicity of the PCy7-NO nanoparticles, hematoxylin and eosin (H&E) staining of the major organs and tumors were performed after treatment. As shown in H&E staining of tumor slices (Fig. 4e), tumors of the PCy7-NO+L group showed more significant apoptosis than those of other groups, which confirmed ONOO−-enhanced phototherapy of PCy7-NO nanoparticles. What’s more, the histology of major organs showed there was no obvious damage in major organs in all treated groups, indicating good biological compatibility of these nanoparticles (Fig. S18 in ESI).

CONCLUSIONS

In summary, a NIR-triggered ONOO−-generated nanoplatform has been successfully constructed through the self-assembly of amphiphilic Cy7-based block copolymer with NORM, which can realize the release of NO and O2− simultaneously under single-wavelength 808 nm light irradiation to produce ONOO− for enhanced phototherapy. A series of experiments including UV-Vis absorption spectroscopy, fluorescence spectroscopy and CLSM proved the generation of NO and ROS (O2− and O2•−), and then in situ formation of ONOO− from PCy7-NO nanoparticles during the process of phototherapy. Finally, PCy7-NO nanoparticles exhibited excellent ONOO−-enhanced phototherapy efficacy with a remarkable cytotoxicity of 89% against 4T1 cells in vitro, and a superior tumor inhabitation against 4T1 tumor model with minimal side effects in vivo. Therefore, this work provides a novel strategy for the fabrication of multi-functional nanoplatform to enhance phototherapy of cancer.

Electronic Supplementary Information

Electronic supplementary information (ESI) is available free of charge in the online version of this article at https://doi.org/10.1007/s10118-021-2540-0.

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