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Hypoxia-Responsive Polymeric Nanoprodrugs for Combo Photodynamic and Chemotherapy

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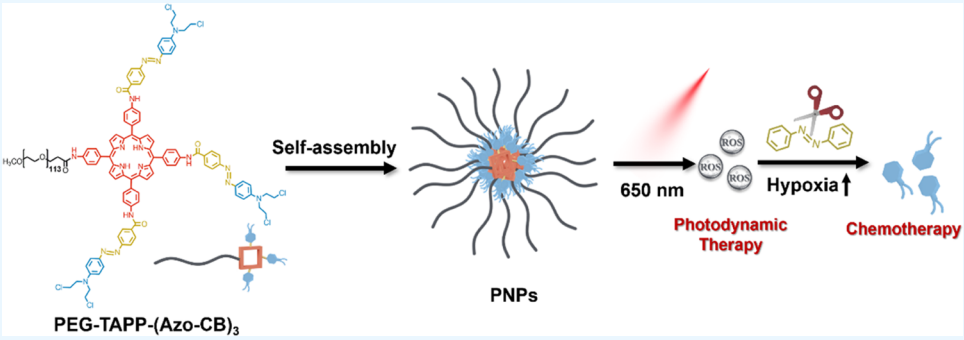

ABSTRACT: Hypoxia in most solid tumors is a major challenge for photodynamic therapy (PDT), and the combination of hypoxia-activated chemotherapy and PDT is a promising approach for enhanced anticancer activity. Herein, we designed hypoxia-responsive polymeric nanoprodrug PNPs to co-deliver photosensitizer 5,10,5,20-tetrakis(4-aminophenyl)-porphine (TAPP) and chlorambucil (CB) to improve the overall therapeutic efficacy. Upon laser irradiation, the central TAPP converted oxygen to produce single oxygen ($^1O_2$) for PDT and induced PDT-reduced hypoxia environment, which accelerated the release of activated CB for synergetic cancer cell killing. Consequently, these hypoxia-responsive polymeric nanoprodrugs with a considerable drug-loading content and synergistic therapeutic effect of PDT-CT had great potential for tumor therapy.

1. INTRODUCTION

As a characteristic feature of most solid tumors, hypoxia results from the imbalance between aggressive oxygen consumption by cancer cells and inadequate oxygen supply.[1−5] The hypoxia-responsive drug delivery system was established by utilizing hypoxia-responsive linkers, e.g., nitroaromatic groups and azobenzene.[6−9] Among them, azobenzene compounds and their derivatives could be reduced to amino derivatives by various azoreductases, which were easier to generate in hypoxic cells due to the decreased oxygen content.[9] However, the antitumor effect of the hypoxia-responsive drug delivery system is generally inhibited in oxygen-rich tumor cells near tumor vasculatures, which may cause tumor recurrence.[10−14] Thus, the approach to reducing hypoxia in tumor tissues is an urgent need to exploit enhanced anticancer therapy.

The hypoxia-responsive drug delivery system combined with photodynamic therapy (PDT) seems to be promising. As an effective and promising cancer treatment, PDT has presented a series of advantages in contrast to traditional chemotherapy, including low systemic side effects, minimal invasiveness, and almost no drug immunosuppression.[15−19] The photosensitizers (PSs) can convert endogenous oxygen into cytotoxic reactive oxygen species (ROS) under light irradiation for the apoptosis of tumor cells.[15,20−22] As a consequence, PDT can effectively deplete endogenous oxygen in and around the tumor site to aggravate the underlying hypoxia.[9] Sequentially, the hypoxia-responsive drug delivery system releases activated drugs to exhibit enhanced combined treatment effects by synergizing cancer cell elimination. Additionally, the poor water solubility of most PSs can cause poor circulation performance and tumor-selective accumulation. Prodrugs formed by chemical conjugation possess some advantages including high drug loading, low systemic toxicity, and high stability.[23−27]

Herein, we report the design and synthesis of hypoxia-responsive polymeric nanoprodrug PNPs for combo photodynamic and chemotherapy (PDT-CT) (Scheme 1). These PNPs are constituted by a tetrameric polymer prodrug PEG-TAPP-$(Azo\text{-}CB)_3$ with a 5,10,5,20-tetrakis(4-aminophenyl)-porphine (TAPP) center via the synergy of hydrophobic and $\pi-\pi$ stacking interactions, which was synthesized by

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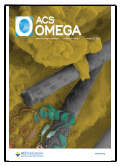
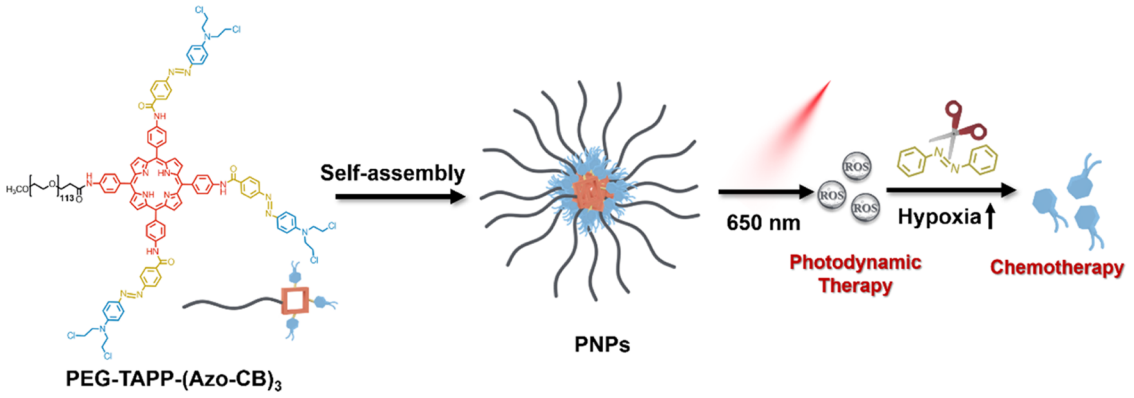


Scheme 1. Schematic Illustration for the Fabrication of Hypoxia-Responsive Polymeric Nanoprodrug PNPs and the Mechanism of PDT-CT



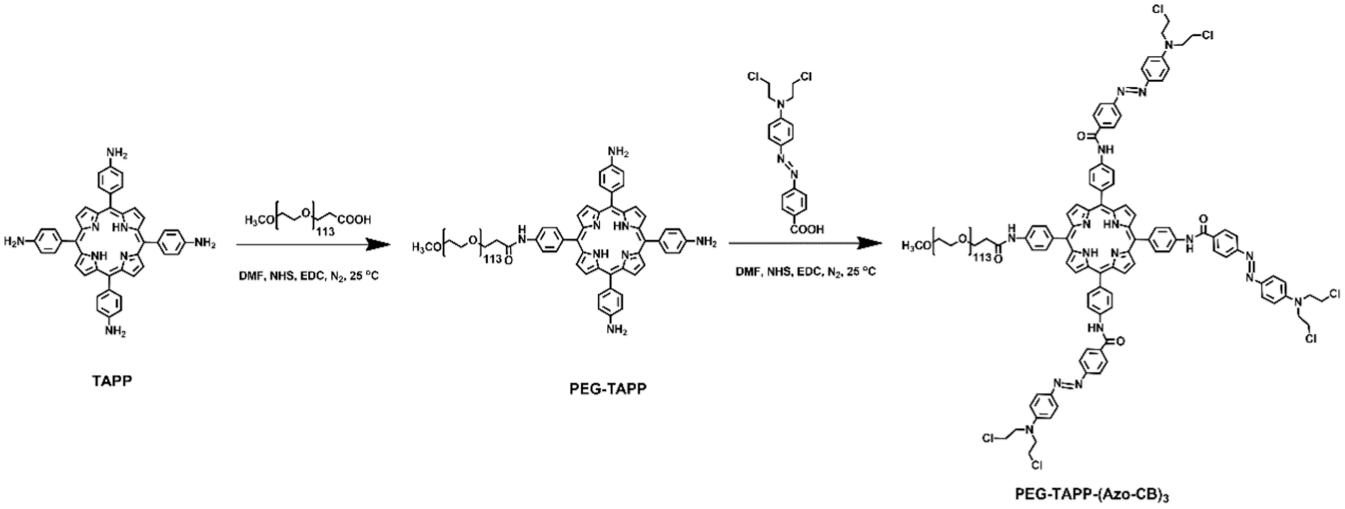


Figure 1. Synthetic route of PEG-TAPP-$(Azo\text{-}CB)_3$.

successively conjugating one poly(ethylene glycol) (PEG) and three azobenzene-modified chlorambucil molecules (Azo-CB) with TAPP using amido linkages. Upon laser irradiation at the tumor site, the central TAPP generated a large amount of single oxygen ($^1O_2$), which not only killed cancer cells for TAPP-mediated PDT but also consumed oxygen to induce a PDT-reduced hypoxia environment. This severe hypoxia caused the disassembly of PNPs to release activated CB for enhanced synergistic anticancer activity. As a result, the PNPs can be a promising candidate for cancer theranostics.

2. MATERIALS AND METHODS

2.1. Reagents and Materials. 5,10,5,20-Tetrakis(4-aminophenyl)-porphine (TAPP), 4-aminobenzoic acid, sodium dithionite ($Na_2S_2O_4$), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) were purchased from Adamas (China). PEG-COOH (M_w = 5000) was obtained from Ponsure Co., Ltd.

2.2. Synthesis of PEG-TAPP. The EDC (81.6 mg, 0.42 mmol) and NHS (49.2 mg, 0.43 mmol) were added to anhydrous DMF (100 mL) of PEG-COOH (1.38 g, 0.28 mmol) under N_2 conditions and stirred for 3 h. Thereafter, the solution of TAPP (250 mg, 0.38 mmol) in anhydrous DMF (15 mL) was added and further reacted for 24 h. The mixed solution was precipitated into diethyl ether to obtain the PEG-TAPP product.

2.3. Synthesis of PEG-TAPP-$(Azo\text{-}CB)_3$. The EDC (81.6 mg, 0.42 mmol) and NHS (49.2 mg, 0.43 mmol) were both added into anhydrous DMF (20 mL) of Azo-CB (102.2 mg, 0.28 mmol) under nitrogen conditions and stirred for 3 h. Thereafter, anhydrous DMF (30 mL) of PEG-TAPP (342.1 mg, 0.06 mmol) was added and further reacted for 24 h. The mixed resulting solution was precipitated into diethyl ether to obtain the PEG-TAPP-$(Azo\text{-}CB)_3$ product.

2.4. Fabrication of Polymeric Nanoprodrug PNPs. Generally, PEG-TAPP-$(Azo\text{-}CB)_3$ (10 mg) was dissolved in DMF (1 mL). Then, deionized water (10 mL) was added into the above solution under stirring overnight. Thereafter, the mixture was put into a dialysis tube (MWCO 3500 Da) to dialyze against distilled water to obtain PNPs.

2.5. Detection of $^1O_2$ Generation. ABDA (0.05 mg/mL) was added into the PBS (20 mL) of PNPs with an equivalent TAPP concentration of 40 μg/mL. For the NIR-triggered $^1O_2$ release, the mixed solution was irradiated with laser irradiation (650 nm, 0.5 W/cm^2, 5 min). Finally, the absorption intensity of ABDA at 380 nm was analyzed by UV−vis spectroscopy.

2.6. In Vitro Drug Release. The PNP solution (1 mg/mL) was blended with $Na_2S_2O_4$ at a concentration of 6.4 mM. Then, the mixture was transferred into a dialysis bag after stirring for 2 h and immersed in methanol (15 mL) with 24 h stirring. Finally, the above solution outside of the dialysis bag was dried and measured by HPLC.

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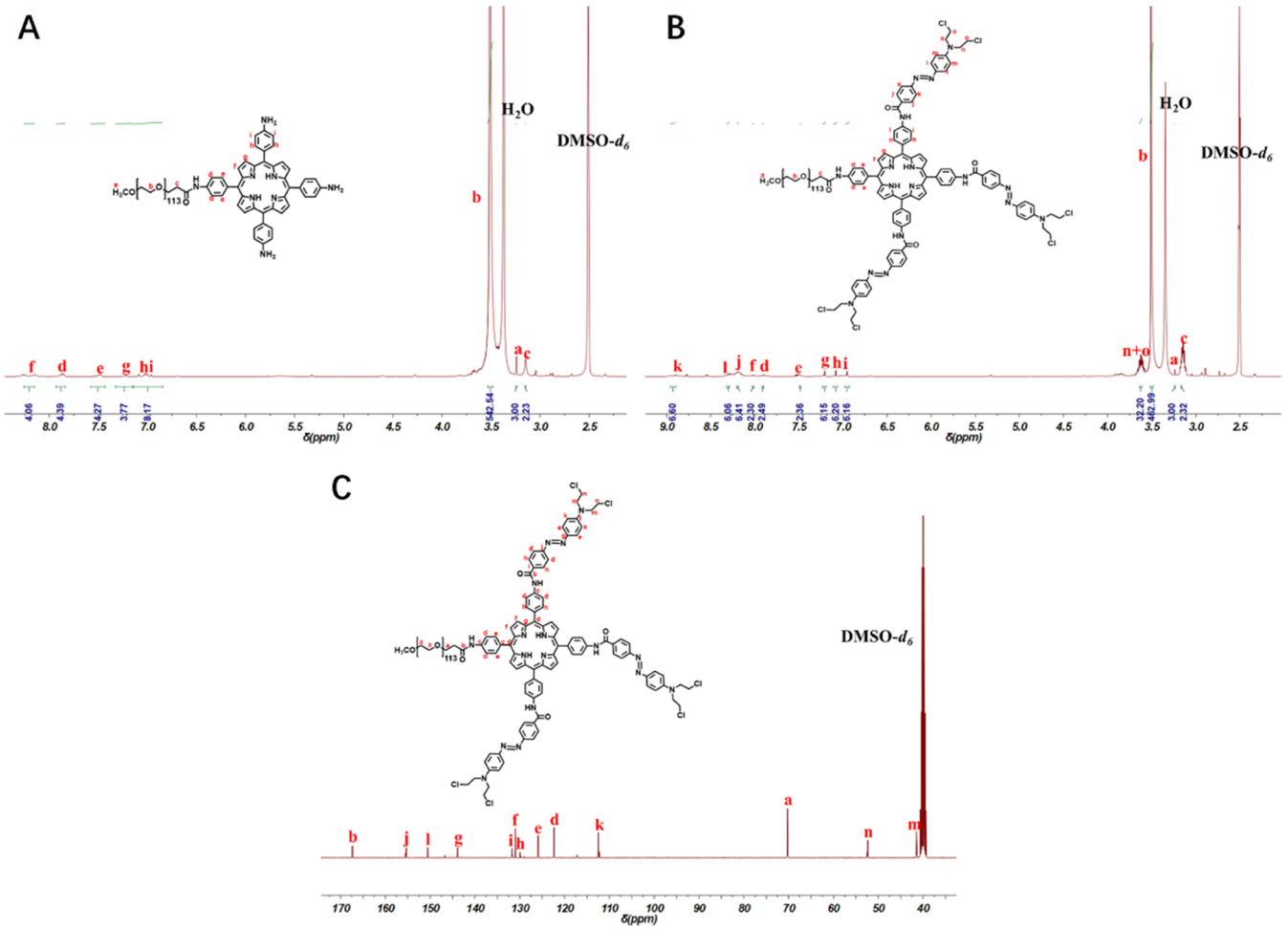

Figure 2. ^{1}H NMR spectra of PEG-TAPP (A) and PEG-TAPP-(Azo-CB)$_3$ (B) (DMSO-d_6). (C) ^{13}C NMR spectra of PEG-TAPP-(Azo-CB)$_3$.

2.7. In Vitro Cytotoxicity. HeLa cells were put in a 96-well plate at a density of 1×10^4 cells/well (200 μL), and the cells were incubated for 12 h. Then, the medium was substituted with fresh DMEM containing PNPs with different concentrations and incubated for 6 h under hypoxia or normoxia conditions. For irradiation groups, the cells were irradiated with NIR irradiation (650 nm, 0.5 W/cm^2, 5 min), followed by further incubation for 18 h. Subsequently, the cytotoxicity was analyzed by using MTT assays.

3. RESULTS AND DISCUSSION

As shown in Figure 1, PEG-TAPP-(Azo-CB)$_3$ was synthesized by successively conjugating one poly(ethylene glycol) (PEG) and three azobenzene-modified chlorambucil molecules (Azo-CB) with tetrameric TAPP using amido linkages. The relevant ^{1}H NMR spectra and ^{13}C NMR spectra are illustrated in Figure 2. Furthermore, the characteristic absorption peaks of TAPP and CB distinctly appeared in the UV−vis spectrophotometry of PEG-TAPP-(Azo-CB)$_3$, demonstrating that CB molecules were successfully grafted to PEG-TAPP (Figure 3).

The polymeric nanoprodrug PNPs were prepared by classical dialysis method using PEG-TAPP-(Azo-CB)$_3$ through the synergy of hydrophobic interactions and $\pi-\pi$ stacking between TAPP groups. Sequentially, dynamic light scattering (DLS) and transmission electron microscopy (TEM) were applied to investigate the diameter and morphology of nanoprodrug PNPs. As illustrated in Figure 4A, the hydrodynamic diameter (D_h) was 179.8 ± 9.8 nm (PDI = 0.38 ± 0.01), which was suitable for the transportation to the tumor site by enhanced permeation and retention (EPR) effect.[28−32] Meanwhile, the morphology of PNPs was a spherical micelle with a diameter of 140.1 ± 7 nm (Figure 4B), which was smaller than the D_h due to a shrunken size in the dry state. Sequentially, the D_h of PNPs under different conditions, including 5, 10, or 15% fetal bovine serum (FBS) and PBS with pH 7.4 or 5.5, was monitored by DLS and exhibited negligible changes, indicating good biostability.

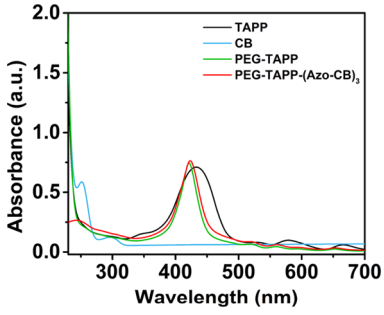

Figure 3. UV−vis absorption spectra of TAPP, CB, PEG-TAPP, and PEG-TAPP-(Azo-CB)$_3$.

The ROS production capability of polymeric nanoprodrug PNPs upon NIR irradiation (650 nm, 0.5 W/cm^2) was assessed by detecting the UV−vis spectrum change of 9,10-

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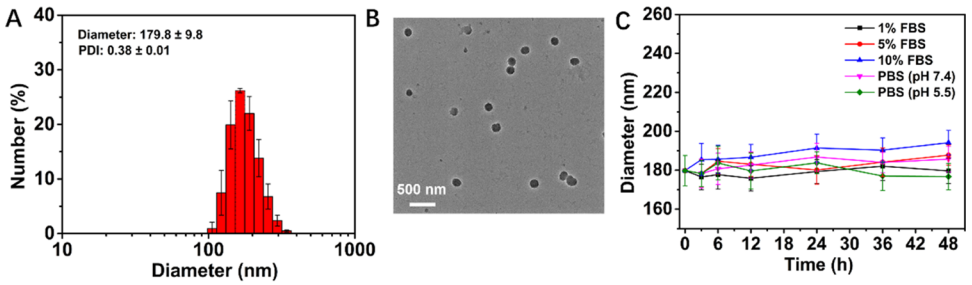

Figure 4. DLS data (A) and TEM image (B) of polymeric nanoprodrug PNPs. (C) Hydrodynamic diameter distribution of PNPs under different conditions.

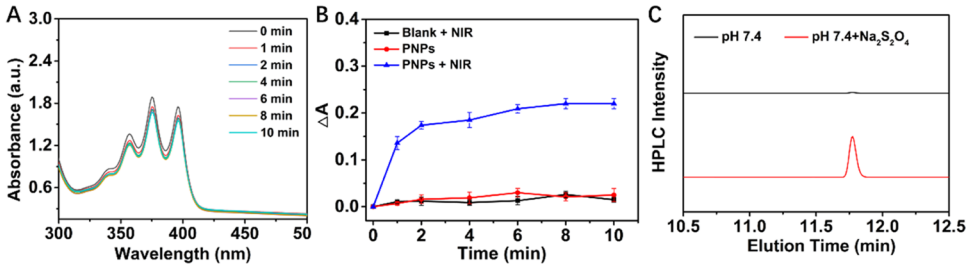

Figure 5. (A) Absorption spectra of ABDA after irradiation by $^1O_2$ generated from PNPs. (B) $^1O_2$ generation at different conditions. (C) HPLC analysis of CB release with or without $Na_2S_2O_4$.

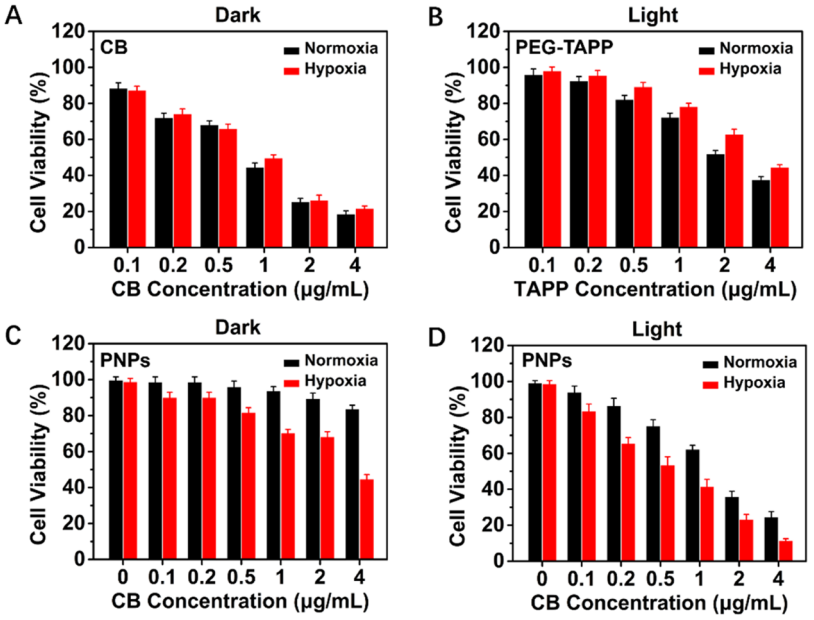

Figure 6. In vitro cell cytotoxicity of free CB in the dark (A), PEG-TAPP under light irradiation (B), and PNPs under normoxia or hypoxia in the dark (C) or under light irradiation (D) against HeLa cells after incubation for 48 h.

anthracenediyl-bis(methylene) dimalonic acid (ABDA, a $^1O_2$ probe), which could irreversibly react with $^1O_2$ to show reduced absorption intensity.[33] As seen in Figure 5A,B, the absorption intensity of ABDA at 375 nm in the PNPs + NIR group was reduced sharply, suggesting that $^1O_2$ could be efficiently generated with the extension of irradiation time. Thus, the polymeric nanoprodrug PNPs can act as a promising candidate for PDT under 650 nm laser irradiation.

The in vitro drug release from PNPs was studied by high-performance liquid chromatography (HPLC) at pH 7.4 in the presence or absence of sodium dithionite ($Na_2S_2O_4$), which was generally used to mimic hypoxic conditions for 2 h.[3] In contrast to the no CB release from PNPs under the normoxic condition without $Na_2S_2O_4$, the peak representing CB at 11.9 min distinctly appeared under the hypoxic condition in $Na_2S_2O_4$ (Figure 5C). The above results demonstrated that the activated CB could be effectively released for hypoxia-triggered breakage of azobenzene linkers between the CB and TAPP.

Finally, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed against HeLa cells to estimate the synergetic anticancer activity of PDT-CT. The HeLa cells were incubated with different concentrations of PNPs under normoxia (21% O_2) or hypoxia (1% O_2) with or without light irradiation. As depicted in Figure 6, the PNPs exhibited dose-dependent cytotoxicity against HeLa cells both

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ACS Omega 2024, 9, 1821−1826

under normoxia with light irradiation and under hypoxia without light irradiation, indicating the hypoxia-triggered CB release for CT and NIR-triggered $^1O_2$ generation for PDT. In sharp contrast, the highest cytotoxicity of PNPs was obviously displayed under the hypoxic condition with light irradiation owing to the synergistic effect on anticancer activities of PDT-CT. Therefore, the above results adequately demonstrated the excellent biocompatibility and therapeutic potential of PNPs in vitro.

In addition, flow cytometry was further carried out to evaluate the cellular uptake capacity of PNPs at the predetermined time intervals (i.e., 30 min, 1, 2, and 4 h) (Figure 7). As a result, the TAPP fluorescence intensity

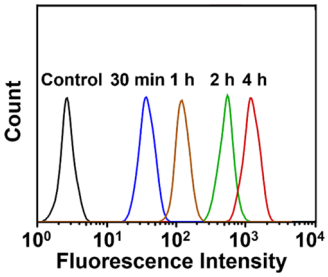

Figure 7. Flow cytometry profiles of HeLa cells incubated with PNPs at predetermined time intervals (i.e., 30 min, 1, 2, and 4 h).

increased obviously in a time-dependent manner within 4 h and reached a maximum at 4 h, demonstrating that the PNPs could be effectively internalized by HeLa cells in an endocytosis manner.

4. CONCLUSIONS

In summary, we have prepared a tetrameric polymer prodrug PEG-TAPP-$(Azo\text{-}CB)_3$ with a 5,10,5,20-tetrakis(4-aminophenyl)-porphine (TAPP) center, which was self-assembled into hypoxia-responsive polymeric nanoprodrug PNPs based on the synergy of hydrophobic and $\pi-\pi$ stacking interactions for synergistic PDT-CT. Upon laser irradiation, the central TAPP moieties consumed oxygen and produced $^1O_2$, inducing an aggravated hypoxia environment. Thereafter, the activated CB was released by the hypoxic-responsive cleavage of the azobenzene linkages. The experimental results demonstrated the synergistic antitumor efficiency of PNPs. Consequently, it is expected that his study provided a promising strategy for the development of hypoxia-activated combinational therapy.

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Notes

The authors declare no competing financial interest.

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