Accepted Article

Title: Size-Reducible Nanodrug with Aggregation-Enhanced Photodynamic Effect for Deep Chemo-Photodynamic Therapy

Authors: Chendong Ji, Qin Gao, Xinghua Dong, Wenyan Yin, Zhanjun Gu, Zhihua Gan, Yuliang Zhao, and Meizhen Yin

This manuscript has been accepted after peer review and appears as an Accepted Article online prior to editing, proofing, and formal publication of the final Version of Record (VoR). This work is currently citable by using the Digital Object Identifier (DOI) given below. The VoR will be published online in Early View as soon as possible and may be different to this Accepted Article as a result of editing. Readers should obtain the VoR from the journal website shown below when it is published to ensure accuracy of information. The authors are responsible for the content of this Accepted Article.

To be cited as: Angew. Chem. Int. Ed. 10.1002/anie.201807602
Angew. Chem. 10.1002/ange.201807602

Link to VoR: http://dx.doi.org/10.1002/anie.201807602
http://dx.doi.org/10.1002/ange.201807602
A Size-Reducible Nanodrug with Aggregation-Enhanced Photodynamic Effect for Deep Chemo-Photodynamic Therapy


Abstract: Fluorescent dyes with multi-functionality are of great interest for photo-based cancer theranostics. However, their low singlet oxygen quantum yield impedes their potential applications for photodynamic therapy (PDT). Herein, we report a molecular self-assembly strategy for the nanodrug with a remarkably enhanced photodynamic effect based on a dye-chemodrug conjugate. The self-assembled nanodrug possesses an increased interystem crossing rate due to the aggregation of dye, leading to a distinct singlet oxygen quantum yield ($\Phi(SO_2)$). Subsequently, upon red light irradiation, the generated singlet oxygen reduces the size of the nanodrug from 90 to 10 nm, which facilitates deep tumor penetration of the nanodrug and release of chemodrug. The nanodrug achieved in situ tumor imaging and potent tumor inhibition via deep photodynamic therapy. Our work verifies a facile and effective self-assembly strategy to construct nanodrugs with enhanced performance for cancer theranostics.

Photodynamic therapy (PDT) is an important therapeutic protocol with minimal invasiveness, high selectivity and low toxicity to bio-system, showing great potential for clinical cancer treatment.[7] The efficiency of PDT relies on high singlet oxygen quantum yields ($\Phi(SO_2)$) of photosensitizer (PS) to generate cytotoxic $O_2$. Traditional PSs such as methyl blue, rose bengal and porphyrin derivatives have been widely applied for PDT.[5] The $\Phi(SO_2)$ of PS can be effectively enhanced by introducing heavy atoms such as iodine and metal to the chromophore.[8] However, these PSs possess intrinsically weak fluorescence and they are lack of functionality. Functional fluorescent dyes conjugated with targeting groups, peptides, antibodies or chemodrugs are powerful for photo-based cancer diagnosis and treatment (i.e. phototheranostics).[9] Unfortunately, the low $\Phi(SO_2)$ of these dyes impede their anti-cancer efficiency in PDT.[10] Therefore, it is urgently needed to design novel multi-functional dyes with enhanced photodynamic ability for improving current PDT.

Self-assembly of fluorescent dyes is a feasible and versatile method to construct nanodrugs for photo-based theranostics of cancer.[9] During the self-assembly process, molecular aggregation undergoing different mechanisms (i.e. phototheranostics).[10] Recently, it has been confirmed that molecular aggregation of fluorescent dye is effective in minimizing energy gap ($\Delta_E_{ST}$) between the lowest excited singlet state (S1) and lowest triplet state (T1) of the dye.[8] With a lower $\Delta_E_{ST}$, the fluorescent dye is likely to exhibit a higher interystem crossing rate ($k_{ISC}$) and longer lifetime in the triplet state, both of which are essential for improving the energy transfer from excited dye molecules to oxygen during the photodynamic process.[9]

To maximize the efficiency of PDT, we should rationally consider the self-assembly manner, integrated functionality, and delivery strategy of photosensitizers to tumor cells. Herein, we design a self-assembled phototheranostic nanodrug (PTN) based on multi-functional pentamethine indocyanine (ICy5) dye, with both aggregation-enhanced photodynamic efficiency and reducible size for deep chemo-PDT. The precursor (ICy5-CPT-RGD) of nanodrug is synthesized by covalently attaching ICy5 with cyclic Arg-Gly-Asp (RGD) peptide, a targeting group of $\alpha_5\beta_3$ integrin,[10] and camptothecin (CPT), an anti-cancer drug[11] (Figure 1A). ICy5-CPT-RGD is amphiphilic and hence readily self-assembles in water to form PTN (I). Due to the aggregation of ICy5, PTN undergoes improved singlet to triplet transition (with higher $k_{ISC}$) and exhibits enhanced photodynamic efficiency (Figure 1B, II). Upon red light irradiation, the generated $O_2$ triggers the cascaded size reduction from 90 to 10 nm of PTN, which leads to deep tumor penetration and accelerates the release of CPT into cancer cells (Figure 1B, III, IV and V). On account of these features, PTN represents an “all in one” nanodrug for tumor-targeting, drug delivery, in vivo imaging and deep chemo-PDT.

The synthesis route of ICy5-CPT-RGD is outlined in Scheme S1. ICy5 bearing two carboxyl groups was prepared according to our previous work.[12] To functionalize ICy5, maleimide was firstly introduced by amidation. Then, effective and catalyst-free thiol-maleimide “click” reactions[13] were performed stepwise to conjugate ICy5 with RGD and CPT-thiol (compound 3) to yield ICy5-CPT-RGD. Detailed synthesis procedures and characterizations are provided in Figures S1-S5. The hydrolysable ester bond linker[14] between CPT and ICy5 moiety was designed for the intracellular release of CPT.

Owing to its amphiphilic nature, ICy5-CPT-RGD could readily self-assemble in water into PTN with RGD-rich hydrophilic surface and hydrophobic inner part.[14] As shown in scanning electron microscopy (SEM) image (Figure 2A), sphere-like nanoparticles with a narrow size distribution and an average diameter of 90 nm are formed in water. Both hard and hollow spheres are observed in transmission electron microscopy (TEM) images (Figures 2A and S8). The hydration radius of PTN is about 105 nm in different medium and remains stable after storage for 4 weeks (Figures S9 and S10). PTN has a negative charged surface (Figure S11) and high CPT content (16.9%, calculated from Figure S12).

The optical and photodynamic properties of PTN were next investigated. PTN forms a clear blue solution and emits bright violet light (Figure 2B). The fluorescence quantum yields (FQY) of PTN in DMSO and 10% fetal bovine serum (FBS) are 59.2±0.2% and 16.5±0.3%, respectively. PTN has two main

[a] C. Ji, Prof. Z. Gan, Prof. M. Yin
State Key Laboratory of Chemical Resource Engineering, Beijing Laboratory of Biomedical Materials, Beijing University of Chemical Technology. E-mail: yinwy@mail.buct.edu.cn

[b] Q. Gao, X. Dong, Prof. W. Yin, Prof. Z. Gu, Prof. Y. Zhao
Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety Institute of High Energy Physics, Chinese Academy of Sciences. E-mail: yinwy@ihep.ac.cn

Supporting information for this article is given via a link at the end of the document.
absorption/emission peaks at 360/416 nm and 654/665 nm, which are attributed to the CPT and ICy5 moieties, respectively (Figure 2C). The shoulder peak at 610 nm is properly clarified by the aggregation of ICy5. After chemical conjugation of CPT and ICy5 moieties, neither spectral overlap of the two moieties nor obvious peak shift is observed (Figure S13), suggesting no charge or energy transfer between the two fluorophores. With emission in the first window (650-900 nm) for bioimaging, PTN is a potential agent for diagnosis of solid tumors.

Next, we examined the photodynamic ability i.e. \( \Phi(s) \) generation efficiency of PTN using 1,3-diphenylisobenzofuran (DPBF) as a probe. \(^{[15]}\) When the PTN and DPBF mixed solution was irradiated with light (660 nm, 50 mW/cm\(^2\)), the absorption peak of DPBF at 418 nm decreases in intensity rapidly, indicating rapid \( ^{1}O_2 \) generation of PTN in FBS (Figure S14). As expected, PTN exhibits stronger photodynamic ability than its single molecular state of ICy5-CPT-RGD in DMSO (Figures 2D and S15). The \( \Phi(s) \) of PTN is 26.2\%, which is about 10 folds higher than that (2.7\%) of ICy5-CPT-RGD molecule (calculated from Figure S15).

This aggregation-enhanced photodynamic effect of ICy5-CPT-RGD was further confirmed by tuning the aggregation degree in DMSO/water co-solvents (Figures S16 and S17). To elucidate the mechanism of such effect, we compared the photo-physical properties of PTN and ICy5-CPT-RGD in Table S1. PTN has a lower FOY but a dramatically higher non-radiative decay rate \( (k_{isc}) \), indicating that the absorbed energy of PTN is mainly dissipated through non-radiative transition, which includes internal conversion, vibrational relaxation and intersystem crossing (ISC). \(^{[8a]}\) Meanwhile, under light irradiation, negligible photothermal effect of PTN caused by internal conversion and vibrational relaxation is observed (Figure S18). \(^{[6a]}\) Thus, the strong photodynamic effect is speculated to be dominated by the ISC process. \(^{[10]}\) Subsequently, time-dependent density functional theory was applied to calculate the \( \Delta E_{ST} \) of ICy dyes (Table S2 and Figure S19). A higher aggregation degree of the ICy dye leads to a lower \( \Delta E_{ST} \), which results in higher \( k_{isc} \). Thus, more excited electrons of the fluorophores in triplet states are activated for photodynamic reactions. \(^{[13a]}\) Although higher \( \Phi(s) \) leads to higher photobleaching ratio of PSs, the absorption of PTN still remains above 90\% under irradiation for 10 min, indicating good photostability of PTN (Figure S20).

The generated \( ^{1}O_2 \) of PTN led to the degradation of ICy5, which could simultaneously induce disassembly of nanoparticles. Thus, we explored the size reduction of PTN and its impact on chemodrug CPT release. Under light irradiation of PTN, nanoparticles with smaller sizes (down to 10 nm) and a broader size distribution (Figures 2E, S21-S23) are observed, revealing the size-reduced process benefit hydrolysis of the ester linkage between CPT and ICy5 under acidic conditions. As confirmed by dialysis experiment, the amount of free CPT increases under light irradiation at pH 5.0 (simulative lysosome condition) (Figure

---

**Figure 1.** (A) Chemical structure and (l) self-assembly of ICy5-CPT-RGD to form PTN. (B) Characteristics and delivery process of PTN. (II) Aggregation-enhanced photodynamic effect of PTN (Abs. absorption; FL, fluorescence; \( k_{isc} \), intersystem crossing rate). (III) \( ^{1}O_2 \)-triggered size reduction and drug release. Thus, we explored the size reduction of PTN and its impact on chemodrug CPT release. Under light irradiation of PTN, nanoparticles with smaller sizes (down to 10 nm) and a broader size distribution (Figures 2E, S21-S23) are observed, revealing the size-reduced process benefit hydrolysis of the ester linkage between CPT and ICy5 under acidic conditions. As confirmed by dialysis experiment, the amount of free CPT increases under light irradiation at pH 5.0 (simulative lysosome condition) (Figure

**Figure 2.** (A) SEM and TEM (inset) images of PTN in water (concentration, \( 1 \times 10^{-3} M \)). (B) Photographs of PTN under natural light and ultraviolet (UV) light (FBS, 10\% fetal bovine serum). (C) Absorption and emission spectra of PTN in FBS. (D) Optical density change (\( \Delta (OD) \)) of DPBF at 418 nm mixed with PTN upon light irradiation in FBS and DMSO; DPBF in DMSO was set as control. (E) SEM and TEM (inset) images of PTN in water after light irradiation for 10 min (concentration, \( 1 \times 10^{-3} M \)). (F) Cumulative drug release profiles of PTN under different conditions by dialysis. (Light source: 660 nm, 50 mW/cm\(^2\)).
2F). We supposed that the size reduction of PTN was caused by the degradation of ICy5 through the C-C cleavage of polyene chains (Figure S24).\textsuperscript{[11]} As a confirmation, the degradation products of ICy5-CPT-RGD were detected by high performance liquid chromatography-mass spectrometry (Figures S25-S26). Although acidic conditions could cause degradation of nanoparticles,\textsuperscript{[18]} 1O2 triggered a noticeable acceleration of size reduction and chemodrug release of PTN. The photodynamic efficiency of size-reduced nanoparticles was further investigated. During light irradiation, the Φ(O2) of PTN decreased gradually (Figure S27). The calculated average Φ(O2) of PTN upon light irradiation for 10 min was 20.7% (Figure S28). The decreased Φ(O2) of PTN may be attributed to combined effects of dense molecular packing, degradation of ICy5 chromophore and deaggregation of PTN (The detailed discussion is presented in Supporting Information). Both 1O2 generation and CPT release ability of PTN were verified in cancer cells (Figures S29 and S30). Thus, PTN is promising for potential chemo-PDT combined therapy. Moreover, the generated size-reduced nanoparticles would facilitate their deep penetration in tumors\textsuperscript{[19]}.

**Figure 3** (A) Co-localization of PTN and Lysotracker Green. Scale bars = 10 μm. Co-localization experiment of HeLa cells incubated with PTN and SYTO (B) with and (C) without light irradiation. Scale bars = 20 μm. Excitation: 640 nm, and the red emission was from ICy5 chromophore. (D) Cell viabilities of HeLa, BEL-7402, and HUVEC cells co-incubated with ICy5 for 24 h in the dark. E: Cell viability of BEL-7402 cells co-incubated with culture medium (set as control), ICy5 or PTN with or without light treatment.

To gain insight into its cellular uptake and localization, PTN was incubated with human cervical carcinoma cell lines (HeLa). First, the positive targeting ability of PTN was confirmed by comparing with a non-RGD molecule ICy5-CPT-COOH (Scheme S2, Figures S6 and S7). Because of the RGD motif in ICy5-CPT-RGD, PTN exhibited enhanced cellular uptake efficiency (Figure S31). Besides, the addition of free RGD peptide can competitively inhibit the cellular uptake of PTN (Figure S32), thus PTN is probably uptaken by HeLa cells through αvβ3 receptor-mediated endocytosis.\textsuperscript{[10a]} Next, co-localization experiments were performed and lysosome marker Lysotracker Green was used to visually evaluate the cell internalization of PTN. The red fluorescence of ICy5 matches the green fluorescence of Lysotracker Green, indicating that PTN localize in lysosome (Figure 3A). It is noteworthy that PSs accumulated in lysosome would cause cell death via the lysosomal pathway.\textsuperscript{[22]} The acidic condition of lysosome would also enable hydrolysis of the ester bond within PTN and trigger CPT release. After light irradiation, the fluorescence of PTN increases significantly (Figure S33), implying the intracellular disassembly of PTN. In contrast with non-irradiated HeLa cells, light also accelerates the release and transfer of CPT drug to the cell nucleus (Figures 3B and 3C). Based on these results, the in vitro therapeutic effect of PTN was further evaluated by the Cell Counting Kit-8 assay.\textsuperscript{[21]} In the dark, ICy5 exhibits low dark toxicity in both cancer cells and normal cells (Figures 3D and 3E). Also, PTN shows a comparable cell toxicity to that of free CPT (Figure S35). However, after light treatment, PTN causes death to over 85% of human hepatoma (BEL-7402) cells (Figure 3E). Compared with chemotherapy or PDT alone, PTN with light treatment achieved combined chemo-PDT and exhibited significant cytotoxicity to cancer cells. Taken together, these results suggest that PTN can deliver ICy5 and the CPT into cancer cells and achieves high therapeutic efficiency in vivo.

**Figure 4.** (A) In vivo fluorescence imaging of PTN at different times after intravenously injection. (B) Ex vivo fluorescence imaging of PTN at the tumor, heart, liver, spleen, lung, and kidney. (C) Immuno-fluorescence staining images of the intratumor distribution of PTN with and without light irradiation. Cell nuclei were stained with DAPI (blue), the tumor vessels were stained with FITC-tagged CD31 antibody (green), and ICy5 was represented by red. (D) Tumor volumes of BEL-7402 xenograft tumor-bearing mice treated with PBS (control), PBS + light, CPT, PTN, Cy-PEG + light, and PTN + light within 20 days. (*p < 0.05). (E) Photographs of tumors on the 20th day after different treatments.

Pharmacokinetics assay of PTN was next investigated. A water soluble ICy5 (Cy-PEG) was synthesized (Scheme S3) and used as control. The pharmacokinetics result demonstrated that...
the self-assembled PTN has an elongated retention time in the bloodstream of mice compared with Cy-PEG (Figure S36). The in vivo fluorescence imaging of PTN showed strong signal at tumor sites after 24 h treatment, demonstrating the excellent tumor-targeting ability and long retention time at the tumor sites (Figures 4A and S38). The in vivo fluorescence images at 24 h show that most of the PTN accumulates at the tumor sites, whereas limited fluorescence is observed in major organs (Figures 4E and S40). It is worth noting that, PTN exhibited very low accumulation at the liver and spleen, thus had a higher bioavailability and low toxicity.22] By contrast, Cy-PEG accumulates in the tumor at only 1 h post-injection and then undergoes rapid clearance through blood circulation (Figure S39). Thus, PTN has shown great potential for imaging-guided cancer therapy.

To validate that the size-reduced PTN can enhance its penetration depth, we compared the immunofluorescence staining of tumor section. As shown in Figure 4C, without light treatment, the fluorescence of PTN is relatively weak and is mainly located within the tumor vessels. In contrast, upon light treatment, the bright fluorescence of PTN can be observed both inside and outside of the tumor vessels. Remarkably, the size-reduced PTN leads to extravasation from the tumor vessels and penetration into the tumor parenchyma, demonstrating deeper tumor penetration ability than that of PTN without light irradiation.

The in vivo tumor inhibition efficiency of PTN was evaluated using BEL-7402 tumor-bearing mice with different treatment (Figure 4D). The mice treated with PTN plus light irradiation receiving the combined therapy, exhibits the highest tumor inhibition efficiency (Figure 4D). The inhibition rate of tumor growth for the combined therapy is 77.5%, which is higher than that for PDT (33.1%) or chemotherapy (lower than 20%) alone. After treatment for 20 days, the photographs, dissected tumor tissues, and tumor weights of the mice under each treatment (Figures 4E, S40 and S41) also lead to the same conclusion. Qualitative histological examinations of the tumor slices showed that the group undergoing combined therapy clearly has the highest necrotic rate among all test groups. Notably, both PTN and Cy-PEG showed negligible in vivo side effects (Figures S42-46). These results confirmed the high treatment efficiency of PTN with deep chemo-PDT for tumor therapy.

In summary, by using a dye-chemodrug conjugate (ICy5-CPT-RGD), we have developed a multi-functional nanodrug PTN with enhanced photodynamic efficiency and reducible size, thus realizing in vivo tumor imaging and deep chemo-PDT. PTN was sphere-like with an average diameter of 90 nm, showing good physiological stability and long wavelength emission. Importantly, the photodynamic efficiency of PTN improved significantly (up to 10 folds) due to the aggregation-enhanced photodynamic effect. Upon light irradiation, the generated 1O2 of PTN triggered its size reduction (down to 10 nm), leading to accelerated chemodrug release and deep tumor penetration of the nanodrug. In vivo experiments confirmed the targeting and diagnosis ability and high tumor inhibition efficiency of PTN through deep chemo-PDT. For the first time, we have revealed the aggregation-enhanced photodynamic effect of cyanine dye. Considering the facile preparation of the multi-functional nanodrug, our strategy holds a promising future for dye-based functional nanomaterial engineering and cancer theranostics.

Keywords: aggregation-enhanced photodynamic effect • size-reducible • pentamethine indocyanine • multi-functionality • nanodrugs

Acknowledgements
This work was financially supported by the National Natural Science Foundation of China (21774007, 21574009, 51521062, and 51772293).

References
A size-reducible nanodrug with enhanced photodynamic effect is constructed by the self-assembly of a multi-functional indocyanine dye. Due to the aggregation-enhanced photodynamic effect of the dye, the nanodrug exhibits a distinct singlet oxygen quantum yield. Especially, the generated singlet oxygen self-triggers the size reduction of the nanodrug, which facilitates the deep tumor penetration of the nanodrug and accelerates chemodrug CPT release.