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NIR Light Activated Combination Therapy with Precisely Integrated Photosensitizer and Prodrug by a Host-Guest Strategy

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Abstract: Co-delivery of photosensitizers with reactive oxygen species (ROS) sensitive prodrugs for light triggered ROS generation and cascaded prodrug activation has drawn tremendous attention. However, the absence of a feasible method to deliver the two components with a precise ratio has impaired the application potential. Herein, we report an efficient method to fabricate a nanosized delivery platform with an optimized ratio of the two components by the means of host-guest strategy for maximizing the combination therapy efficacy in the cancer treatment. The key features of this host-guest strategy for the combination therapy lie in (i) the ratio between photosensitizer and ROS sensitive prodrug could be tuned easily, (ii) near-infrared (NIR) irradiation as single external stimulus could sensitize the photosensitizer and activate paclitaxel prodrug for its release, and (iii) the accumulation process could be tracked by NIR imaging for maximizing the efficacy of photodynamic and chemotherapy.

Photodynamic therapy (PDT), as an important cancer treatment modality, has been intensively investigated and widely utilized in clinic.^[1,2] Upon irradiation with light of specific wavelength, photosensitizers (PS) can absorb the energy from the light then transfer the energy to endogenous oxygen to produce highly reactive oxygen species (ROS) for causing cellular damage.^[3] Compared with other conventional therapy methods, PDT exhibits numerous advantages including low systemic toxicity, minimal invasiveness, and repeatable dose.^[4] Since limited water solubility of most available PS results in poor circulation performance and low ability of tumor tissue-selective accumulation, the development of novel nanocarriers for the PS delivery shows significant scientific and clinical values.^[5,6]

Although chemotherapy, as a major cancer treatment approach, has greatly improved the survival rate, its clinical performance is still restricted due to systemic toxicity and lack of specificity.^[7,8] The design and preparation of prodrugs have been a popular strategy to overcome these drawbacks of chemotherapeutic drugs.^[9] Prodrugs are derivatives of chemotherapeutic drugs with the blockage of their active site, which exhibit low toxicity in normal tissues and could be converted to original forms with high toxicity in tumor tissues

triggered by tumor microenvironment or external stimuli.^[10–12] Thus, side effects of chemotherapy are greatly suppressed by selective activation.^[13–18] Many ROS responsive prodrugs based on different ROS sensitive linkers such as aminoacrylate,^[19–22] diselenide,^[23] thioketal,^[24] arylboronic ester^[25] and thioether^[26] have been reported to show the application potential in clinical cancer treatment.

Scheme 1. Schematic illustration for the structures of the polymer, PS, and prodrug (detailed chemical structures are shown in the Supporting Information), the self-assembly process of the nanoparticles with the optimized ratio, and its light activated combination therapy process.

On account of these disadvantages in each therapeutic practice, the co-delivery of two or more therapeutic agents has been developed to exhibit synergistic effects.^[27,28] The construction of co-delivery systems renders greater therapeutic effect as compared to a single treatment modality. Even though numerous studies have been reported in this field,^[29–31] the unavailability of an effective method to fine-tune the ratio of co-loaded therapeutic agents has obstructed successful cancer treatment. There are three popular strategies employed for the co-delivery of PS with prodrugs, *i.e.*, (i) covalent conjugation of PS to prodrugs,^[24] (ii) grafting PS or drugs with carriers, meanwhile loading the other component as the cargo,^[32] (iii) co-loading the two components within carriers such as polymers and inorganic porous nanoparticles.^[33–36] Each of these

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strategies has its own disadvantages. The main challenge is the difficulty in fine-tuning the ratio between PS and drugs, because the ratio is largely related to chemical structures, functionalities, and performance. For the fabrication of covalently conjugated prodrugs, there are some drawbacks including complicated organic synthesis and purification process. For the other two strategies, achieving a precise ratio between the two components is also difficult due to potential premature leakage during the preparation and transportation processes. The uncertainty arising from imprecise loading of therapeutic components may lead to low or unpredictable therapeutic efficacy in the cancer treatment.

On account of intrinsic cavity and easy functionalization, macrocycles including crown ethers,^[37] cyclodextrins (CDs),^[38–40] cucurbiturils,^[41,42] calixarenes,^[43] and pillararenes^[44] have been widely employed as drug carriers by the encapsulation of chemotherapeutic drugs as guests. Through the formation of supramolecular complexes, the solubility of the drugs is enhanced, leading to longer circulation time and better therapeutic performance. CDs are the most extensively investigated macrocycles in pharmaceutical filed for clinical usage.^[45] Herein, we developed a supramolecular system with the optimized loading ratio between the PS and prodrug (Scheme 1). A diblock copolymer (PEG-PGA- β -CD) consisting of polyethylene glycol (PEG) and poly-L-glutamic acid (PGA) moieties was synthesized, followed by the conjugation with β -CD. Adamantane modified aza-BODIPY (Ada-BODIPY) and paclitaxel (Ada-PTX) were prepared as PS and prodrug guest molecules, respectively. Through strong interaction between β -CD and adamantane unit to form stable host-guest inclusion complex, the introduction of PS and prodrug components with an optimized ratio to afford highly therapeutic efficacy could be easily realized. Having the same host-guest complexation motif and similar binding interaction, the precise loading ratio between PS and prodrug components would be maintained *in vivo* even if premature leakage occurs. Low cytotoxicity and high accumulation in tumor could be further achieved with the surface PEG segment. Upon near-infrared (NIR) light irradiation, ROS was generated for PDT. The ROS sensitive aminoacrylate group in Ada-PTX was simultaneously cleaved to free PTX for chemotherapy. Thus, PDT and cascaded Ada-PTX activation for combination therapy were obtained.

The host-guest interaction between PEG-PGA- β -CD and the two guests were investigated by two-dimensional rotating-frame overhauser spectroscopy (2D ROSEY) NMR spectroscopy, where β -CD was employed as a model host. Obvious nuclear overhauser effect was observed between β -CD and the adamantane moiety of the two guests, indicating similar host-guest binding motif (Figures S1 and S2). Computational simulation results also confirmed the host-guest interaction (Figure S3). While there were about 14 β -CD rings conjugated on each polymer, only 10 of them were employed to load the two components in order to ensure sufficient loading. To optimize the loading ratio of the PS and prodrug, the mixtures of Ada-BODIPY and Ada-PTX with different ratios ranging from 0:10 to 10:0 were slowly injected into the solution of PEG-PGA- β -CD under sonication (Figure S4). After introducing Ada-BODIPY and Ada-PTX to PEG-PGA- β -CD, self-assembled polymer nanoparticles (PNs) with narrow size distributions were obtained

(Figure S5). The PEG unit acts as the hydrophilic domain, while the polypeptide segment becomes the hydrophobic domain in PNs due to the formation of host-guest inclusion complexes.

HeLa cells were then incubated with these PNs with different loading ratios for 48 h followed by the irradiation with 660 nm light (100 mW) for 20 min. The cell viability was investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Figure 1a). 100% Ada-PTX PNs (Ada-PTX_(100%)-PNs) and 100% Ada-BODIPY PNs (Ada-BODIPY_(100%)-PNs) exhibited lower cytotoxicity than other samples under the light irradiation, while 60% Ada-PTX : 40% Ada-BODIPY PNs (Ada-PTX_(60%)-BODIPY_(40%)-PNs) showed the highest cytotoxicity. Based on the MTT assay screening on HeLa cells, Ada-BODIPY and Ada-PTX were found to achieve the optimized synergistic efficacy at the loading ratio of 3:2. Thus, PNs with Ada-PTX : Ada-BODIPY ratios of 10:0, 3:2 and 0:10 were chosen for further investigations. Transmission electron microscopy (TEM) images of Ada-PTX_(100%)-PNs, Ada-BODIPY_(100%)-PNs, and Ada-PTX_(60%)-BODIPY_(40%)-PNs presented uniform polymersome-like morphology with the diameters of about 105 nm, 100 nm and 90 nm, respectively (Figures 1b and S6). Their dynamic light scattering (DLS) measurements (about 110 nm, 109 nm and 92 nm from Figure S5) were in accordance with the TEM results. By monitoring the change of hydrodynamic diameter upon diluting the nanoparticle concentration from 20 μ M to 0.001 μ M, the critical aggregation concentration (0.01 μ M) of Ada-PTX_(60%)-BODIPY_(40%)-PNs was determined. This relatively low critical aggregation concentration means that the obtained Ada-PTX_(60%)-BODIPY_(40%)-PNs are suitable for the *in vivo* application with certain resistance to the dilution effect.

Figure 1. a) Cell viability of Ada-BODIPY and Ada-PTX with different ratios. b) TEM images of i) Ada-PTX_(100%)-PNs, ii) Ada-PTX_(60%)-BODIPY_(40%)-PNs, and iii) Ada-BODIPY_(100%)-PNs. c) HPLC retention time of PTX and Ada-PTX_(60%)-BODIPY_(40%)-PNs detected upon NIR light irradiation for different time. The PTX release profile from Ada-PTX_(60%)-BODIPY_(40%)-PNs in the presence of light was obtained. d) UV-vis spectrum of (I) Ada-BODIPY_(100%)-PNs and (II) Ada-PTX_(60%)-BODIPY_(40%)-PNs. e) TEM image of Ada-PTX_(60%)-BODIPY_(40%)-PNs after NIR light irradiation for 30 min.

UV-vis spectrum of Ada-BODIPY_(100%)-PNs in aqueous solution showed a broad absorption band from 600 nm to 740 nm with a maximum peak at 678 nm. Ada-PTX_(60%)-BODIPY_(40%)-PNs also exhibited a similar absorption band (Figure 1c). The

Figure 2. a) Detection of cellular ROS level using carboxy-DCFDA enzyme: control group i) in the dark and ii) under light, and Ada-BODIPY_(100%)-PNs iii) without light and iv) under light irradiation. Live/dead assays on HeLa cells at different conditions b) in the dark and c) under the light irradiation: i) control group, ii) Ada-PTX_(100%)-PNs, iii) Ada-BODIPY_(100%)-PNs, iv) Ada-PTX_(60%)-BODIPY_(40%)-PNs. Concentration: 500 nM. Scale bar: 100 μ m. DAPI: 4',6-diamidino-2-phenylindole, PI: propidium iodide.

generation of singlet oxygen ($^1\text{O}_2$) from Ada-BODIPY_(100%)-PNs in the presence of NIR light irradiation (660 nm, 20 mW) was studied by using 1,3-diphenylisobenzofuran (DPBF) as a probe. The DPBF absorption band at 423 nm was rapidly quenched, since the generated $^1\text{O}_2$ by Ada-BODIPY_(100%)-PNs could react with DPBF (Figure S7a). The singlet oxygen quantum yield of Ada-BODIPY_(100%)-PNs was also measured by using methylene blue as a reference, and the quantum yield was about 66% (Figure S7). The high singlet oxygen quantum yield of Ada-BODIPY_(100%)-PNs indicates its excellent ROS generation capacity suitable for PDT.

Although Ada-PTX_(60%)-BODIPY_(40%)-PNs could maintain its polymersome-like morphology after NIR irradiation (660 nm, 100 mW) for 30 min, smaller size around 68 nm was observed by DLS and TEM, suggesting the PTX release (Figures 1d and S8). In contrast, Ada-BODIPY_(100%)-PNs and Ada-PTX_(100%)-PNs kept their morphology unchanged after the irradiation. The aminoacrylate bond in Ada-PTX could be cleaved through the oxidation of generated ROS to release the active PTX drug (Figure S9). The prodrug activation process was also monitored with high performance liquid chromatography (HPLC). Free PTX and Ada-PTX exhibited peaks at retention time of 28.03 min and 27.35 min, respectively. When Ada-PTX_(60%)-BODIPY_(40%)-PNs were irradiated, the emergence of the PTX peak was observed at 28.03 min. The intensity of the peak at 27.38 min decreased upon the irradiation time, while the intensity of peak at 28.03 min enhanced. A fast light triggered release was observed, and more than 62% prodrug was activated with the irradiation over 5 min. After the irradiation for 20 min, the peak at 27.38 min totally disappeared, indicating that the Ada-PTX prodrug was fully converted to PTX (Figure 1e). As a control, no peak at 28.03 min was detected in the dark for overnight, indicating the stability of Ada-PTX_(60%)-BODIPY_(40%)-PNs without light. Meanwhile, Ada-PTX_(100%)-PNs were also irradiated for 40 min, and no change in

the retention time was observed due to lack of the ROS source (Figures S10 and S11). These results proved that the prodrug in Ada-PTX_(60%)-BODIPY_(40%)-PNs could be efficiently activated by NIR light.

The internalization of these nanoparticles in cells was evaluated by confocal laser scanning microscope (CLSM). HeLa cells were incubated with Ada-BODIPY_(100%)-PNs (1 μ M) for 4 h. When the cells were treated under the excitation of 640 nm laser, obvious red fluorescence belonging to BODIPY indicates successful uptake of Ada-BODIPY_(100%)-PNs (Figure S12). The MTT assay was employed to assess the cytotoxicity of PEG-PGA- β -CD and Ada-PTX. HeLa cells exhibited high viability (over 90%) after the treatment with PEG-PGA- β -CD for 24 h and 48 h, demonstrating no obvious cytotoxicity of the polymer (Figure S13). The Ada-PTX prodrug showed lower cytotoxicity (IC_{50} 529 nM) than that of free PTX (IC_{50} 204 nM) especially at low concentration, indicating efficient deactivation of PTX after the modification. Ada-PTX_(60%)-BODIPY_(40%)-PNs exhibited the highest cytotoxicity under NIR light irradiation, while Ada-PTX_(100%)-PNs and Ada-BODIPY_(100%)-PNs presented lower cytotoxicity under the same conditions. The cell viability of Ada-PTX_(60%)-BODIPY_(40%)-PNs in the dark showed much lower cytotoxicity. To confirm the combination therapy effect, the combination index (CI) was calculated for Ada-PTX_(60%)-BODIPY_(40%)-PNs. The CI values were all below 1 at the range from IC_{30} to IC_{60} , indicating synergistic effect from PS and prodrug within Ada-PTX_(60%)-BODIPY_(40%)-PNs (Figure S13).

To investigate the ROS generation ability of Ada-BODIPY *in vitro*, 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-DCFDA) was employed to detect the ROS level in HeLa cells. Negligible green fluorescence from the cells was observed in the absence of Ada-BODIPY_(100%)-PNs whenever in the dark or upon light irradiation. Weak green fluorescence was detected after treated with Ada-BODIPY_(100%)-PNs in the dark

(Figure 2a). Enhanced green fluorescence after light irradiation indicates successfully production of ROS by Ada-BODIPY_(100%)-PNs. The enhanced ROS level in cells was a key factor to activate the prodrug for responsive drug release.

In vitro anticancer efficacy of these samples was investigated by CLSM (Figure 2b). For Ada-PTX_(100%)-PNs, strong green fluorescence (indicative of live cells) was observed, while weak red fluorescence (indicative of dead cells) was observed in the dark, indicating the cytotoxicity alleviation by blocking active site of PTX. For Ada-BODIPY_(100%)-PNs in the absence of light, negligible red fluorescence detected indicates good bio-safety of the PS. For Ada-PTX_(60%)-BODIPY_(40%)-PNs in the dark, a similar green/red fluorescence intensity ratio was observed. Upon NIR light irradiation for 20 min, green/red fluorescence intensity ratio for Ada-PTX_(100%)-PNs had no obvious change, since Ada-PTX_(100%)-PNs cannot release PTX without ROS. Strong red fluorescence observed for Ada-BODIPY_(100%)-PNs indicates its phototoxicity. Significantly, only red fluorescence was observed for Ada-PTX_(60%)-BODIPY_(40%)-PNs in the presence of the light, proving its high cytotoxicity with the optimized ratio (PS:prodrug = 3:2). The live/dead assays are well consistent with the MTT results, demonstrating that Ada-PTX_(60%)-BODIPY_(40%)-PNs present the highest cytotoxicity to HeLa cells. These results also indicate that Ada-PTX_(60%)-BODIPY_(40%)-PNs are mainly activated by the generated ROS, but not hydrolyzed by esterases.

Figure 3. a) Biodistribution of I) Ada-BODIPY and II) Ada-BODIPY_(100%)-PNs in mice after injection for 3 h and 24 h. The tumors are circled. b) Biodistribution comparison of I and II in tumor tissues determined from a). c) Ex vivo fluorescence image of major organs and tumor excised from mice injected with I and II. d) Biodistribution comparison of I and II in various organs determined from c). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

To study the *in vivo* biodistribution, we performed animal experiments using xenografted model of HeLa tumor in nude mice. Fluorescent Ada-BODIPY and Ada-BODIPY_(100%)-PNs were chosen for the investigation. After intravenous injection into tumor-bearing mice, the *in vivo* imaging system (IVIS) was employed to monitor the biodistribution at different time (Figure 3a,b). Ada-BODIPY and Ada-BODIPY_(100%)-PNs exhibited similar distribution in nude mice after 3 h of injection. After 24 h of injection, obvious tumor accumulation was observed for Ada-BODIPY_(100%)-PNs, while negligible fluorescence was detected in the tumor site for Ada-BODIPY. The mice were sacrificed after 24 h, and the tumor and major organs were resected and visualized. No significant fluorescence intensity difference in

major organs except for liver was observed for Ada-BODIPY, while higher fluorescence signal was observed from the tumor tissue for Ada-BODIPY_(100%)-PNs (Figure 3c,d). The fluorescence intensity of the tumor tissue for Ada-BODIPY_(100%)-PNs was 6.5-folds higher than that for Ada-BODIPY. To understand the circulation time of commercial Taxol®, Ada-PTX_(100%)-PNs and Ada-PTX_(60%)-BODIPY_(40%)-PNs, the *in vivo* pharmacokinetics were performed. Compared to Taxol® ($t_{1/2} < 0.25$ h), Ada-PTX_(100%)-PNs and Ada-PTX_(60%)-BODIPY_(40%)-PNs showed longer circulation time with $t_{1/2}$ about 5 h (Figure 4a). These results suggest that these nanoparticles could accumulate selectively at the tumor sites with long circulation time in blood stream, which are highly suitable for antitumor applications *in vivo*.

Figure 4. a) *In vivo* pharmacokinetics of PTX in blood after the groups III), IV) and V) were intravenously injected into the tumor-bearing mice through the tail vein. b) Time dependent tumor sizes from different groups of mice with different treatments over a period of 20 days. c) Time dependent tumor weights from different groups of mice with different treatments over a period of 20 days. d) Body weights of the mice in different groups after the treatments at different time intervals. e) H&E staining of tumor tissues (magnification 100x) from different samples of euthanized mice. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. I: saline, II: Ada-BODIPY_(100%)-PNs, III: free Taxol®, IV: Ada-PTX_(100%)-PNs, V: Ada-PTX_(60%)-BODIPY_(40%)-PNs, VI: Ada-BODIPY_(100%)-PNs + light, VII: Ada-PTX_(60%)-BODIPY_(40%)-PNs + light.

The *in vivo* antitumor efficacy was conducted on the nude mice bearing HeLa tumor (Figure 4b-e). The mice were randomly divided into seven groups ($n=5$), followed by monitoring and treatment for 20 days. Compared with the saline group and the Ada-BODIPY_(100%)-PNs group in the dark, other groups exhibited the tumor growth inhibition. The group of Ada-PTX_(60%)-BODIPY_(40%)-PNs under the light irradiation exhibited the best tumor growth inhibition performance on account of PDT along with cascaded prodrug activation. Moreover, the tumor from one of the mice was disappeared completely (Figure S14). Ada-PTX_(60%)-BODIPY_(40%)-PNs achieved much higher therapeutic outcome with lower dose of chemotherapeutic drug than that of Ada-PTX_(100%)-PNs. The average tumor size of the

Ada-PTX_(60%)-BODIPY_(40%)-PNs group under the light irradiation was about $22.8 \pm 10.0 \text{ mm}^3$, while the saline group showed the average size of about $622.4 \pm 165.1 \text{ mm}^3$ (Figure 4b). The average tumor weights of all groups were also measured. The average tumor weight in the group of Ada-PTX_(60%)-BODIPY_(40%)-PNs under light irradiation was about $11.4 \pm 4.1 \text{ mg}$, which was about 6 times smaller than the best result from other therapeutic groups and about 35 times smaller than the saline group (Figure 4c). Furthermore, the tumor growth inhibition rate for the Ada-PTX_(60%)-BODIPY_(40%)-PNs group under light irradiation was 97.2%. Except for the Taxol® group, minimal differences in the average body weights of the mice were observed for all other groups as compared with the saline group, indicating that the prodrug and nanoparticle formation strategy could significantly reduce the systemic toxicity of the commercial Taxol® (Figure 4d). Hematoxylin and eosin (H&E) staining of tumors was performed on all seven groups (Figure 4e). Significantly damaged regions of the tumor could be observed from the Ada-PTX_(60%)-BODIPY_(40%)-PNs group under light irradiation, while negligible damage from the saline and Ada-BODIPY_(100%)-PNs groups was observed, and minor damages were detected from the rest groups. No serious inflammation or lesion was observed from various major organs in these treated mice (Figure S15). These results clearly demonstrate light activated combination therapy provided by Ada-PTX_(60%)-BODIPY_(40%)-PNs with high antitumor efficacy and low systemic toxicity *in vivo*.

In summary, we have successfully developed a new strategy to optimize the ratio of PS and prodrug based on the host-guest interaction. The obtained nanoparticles with the optimized ratio have exhibited excellent NIR activated therapeutic performance both *in vitro* and *in vivo*. The nanoparticles with different ratios of PS and prodrug present polymersome like morphology and suitable size for biomedical applications. NIR light triggered ROS generation by PS and cascaded prodrug activation within self-assembled nanosystems have been confirmed. Cellular uptake and MTT assay have been employed to optimize the ratio and investigate the *in vitro* anticancer efficacy. Excellent tumor-selective accumulation of the nanoparticles has been proven using subcutaneous murine xenograft models. Their *in vivo* tumor growth inhibition ability has been examined on the mice models, and significant tumor growth inhibition has been achieved from the Ada-PTX_(60%)-BODIPY_(40%)-PNs group under the light irradiation with low dose of chemotherapeutic drug. The combined PDT and chemotherapy provided by Ada-PTX_(60%)-BODIPY_(40%)-PNs have been efficiently maximized, enabling its further applications for practical cancer treatment. In our follow-up research, we will improve this nanosystem in order to track real-time distributions of the key components *in vitro* and *in vivo*.

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COMMUNICATION

A host-guest strategy is developed to co-load photosensitizer and prodrug with the optimized ratio onto a polymeric system, achieving excellent NIR activated therapeutic performance *in vitro* and *in vivo*.

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Page No. – Page No.

NIR Light Activated Combination Therapy with Precisely Integrated Photosensitizer and Prodrug by a Host-Guest Strategy