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# Novel carrier-free nanoparticles composed of 7-ethyl-10-hydroxycamptothecin and chlorin e6: Self-assembly mechanism investigation and *in vitro/in vivo* evaluation

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ABSTRACT

The combination therapy strategy based on both chemotherapy and photodynamic therapy (PDT) exhibits great potential for advanced cancer treatment. Multimodal nanodrug delivery systems based on both chemotherapeutic drug and photodynamic agent have been proven to possess excellent synergistic efficacy. In this study, 7-ethyl-10-hydroxycamptothecin (SN38) and chlorin e6 (Ce6) were co-assembled into novel carrier-free nanoparticles (SN38/Ce6 NPs) *via* simple antisolvent precipitation method. As expected, SN38/Ce6 NPs exhibited uniform morphology with a particle size of around 150 nm and a zeta potential of about −30 mV, good stability in aqueous solution/at lyophilized state and high cellular uptake efficiency against murine mammary carcinoma (4T1) cell lines. Besides, enhanced singlet oxygen generation capacity of the nanoparticles was both observed in test-tube and in 4T1 cell lines in contrast with Ce6 injection. Moreover, a ~85 % inhibition rate of SN38/Ce6 NPs with laser was detected, which was significantly higher ($P < 0.05$) than those without laser (~65 %) and injections (less than 20 %), verified the excellent synergistic antitumor efficacy of the nanoparticles due to combined chemo-photodynamic therapy, enhanced tumor accumulation and higher cellular internalization. Notably, chemical thermodynamic method and molecular dynamics (MD) simulations supplied solid data and visual images to estimate the driving forces for the self-assembly process of the carrier-free nanoparticles as primary hydrophobic interactions (π-π stacking) and subordinate hydrogen bonds. Conclusively, the above self-assembled carrier-free nanoparticles represented a promising synergistic anticancer strategy capable of maximal therapeutic efficacy and minimal systemic toxicity. Moreover, the application of thermodynamic method together with MD simulations in the investigation of NPs self-assembly process also provided new ideas for the assembly mechanism exploration of more complicated nanodrug delivery system.

## 1. Introduction

Cancer is reported to remain as one of the major life-threatening and death-inducing diseases around the world [1]. Apart from surgery and radiotherapy, systemic chemotherapy is still the most preferred treatment for anticancer therapy [2,3]. Therefore, over the past century, tremendous efforts have been made to discover potential chemotherapeutic agents acting through specific targeted pathways. The natural alkaloid camptothecin (CPT) and its derives including 10-hydroxycamptothecin (10−HCPT) and 7-ethyl-10-hydroxycamptothecin (SN38), which exert their antitumor effects as the topoisomerase I inhibitor, have attracted massive attention as prominent and broad spectrum chemotherapeutic agents [4]. Among the CPTs, SN38, one of the most active analogues, has even poorer solubility in comparison with other analogues including HCPT, which significantly limited its application in clinic [5]. As a result, there is still no clinically available SN38 formulations up to now.

Irinotecan hydrochloride (CPT11), a soluble prodrug derived from CPT, is a clinically first-line chemotherapeutic agent for treatments of a wide range of cancer. CPT11 implements its therapeutic effect through converting to the active metabolite SN38 *via* carboxylesterase (CE)-mediated de-esterifcation *in vivo* [6,7]. However, the conversion rate of

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CPT11 to SN38 is mere 5 % in human body due to the inferior carboxylesterase activity in human liver, which leads to the poor performance of CPT11 in clinical application [8]. For instance, the insoluble drug SN38 has been extensively investigated in drug delivery field for the purpose of fully exploiting its therapeutic potential.

Nevertheless, despite the progressive achievement in SN38-related drug delivery systems, the monotherapy relies on barely chemotherapeutic agent is still unsatisfied due to the multilevel complexities and variability of advanced cancer. Consequently, the combination therapy strategy based on both chemotherapy and photodynamic therapy (PDT) is widely applied for cancer treatment [9]. PDT has been reported as a promising treatment modality for a variety of cancer diseases, which exerts its antitumor effect of severe cell hypoxia and apoptosis *via* reactive oxygen species (ROS) produced by photosensitizers (PSs) in the presence of specific near-infrared (NIR) laser [10]. Notably, in contrast with conventional chemotherapy method, PDT reveals multiple advantages such as decreased systemic toxicity, lower invasiveness, reproducible dose, and reversal of the multidrug resistance (MDR) [11,12]. Interestingly, the phenomenon of photochemical internalization (PCI) has also been reported as the as-generated ROS by PDT could oxidize and further disrupt the endosomal membrane and accelerate the intracellular release of the chemotherapeutic agents delivered by the nanoparticle drug delivery system (NDDS), which is typical uptake by the endosome [9,13].

To date, tremendous varieties of NDDS have been developed for combined chemo-photodynamic therapy, such as nanoparticles [14,15], mesoporous silica nanoparticles (MSNs) [16], liposomes [17], polymeric microgel [10], and polymer micelles [9], et al. However, in most cases, the complicated fabrication of the nano-carriers utilized in the above NDDS limited their manufacture at large scale, not to mention the potential toxicity and inflammation of the carriers *in vivo* [18]. Moreover, the low drug loading efficiency (usually less than 20 %) of the NDDS increases their production cost as well [19]. Therefore, carrier-free nanoparticles merely composed of chemo-photodynamic pharmaceutical molecules [20–22] have attracted the attention of many researchers recently. However, the above reported carrier-free nanoparticles either based on water-soluble/amphiphilic drugs (DOX and ICG) as surfactants, or involved alkaline solution (preferable solvent) in the final product (DOX/Ce6 NPs and HCPT/Ce6 NPs), indicating a majority of HCPT probably existed as carboxylic salt form. More importantly, although the above carrier-free nanodrug delivery systems were speculated to merely self-assemble *via* hydrophobic interactions (π-π stacking) and electrostatic interactions from the structures of the molecules, the driving forces for the self-assembly process were still unverified.

Herein, novel carrier-free SN38/Ce6 NPs (Scheme 1) based on the collaborative assembly of both insoluble agents SN38 and Ce6 were developed *via* simple antisolvent precipitation method without the usage of alkaline solution for synergistic chemo-photodynamic combination therapy. Besides, the physiochemical characteristics of the NPs containing size, morphology, and singlet oxygen ($^1O_2$) generation capacity were particularly evaluated. Furthermore, the *in vitro* and *in vivo* combined chemo-photodynamic antitumor efficacy, together with the *in vivo* biodistribution were assessed as well. Mentionablely, the driving forces for the self-assembly process of the carrier-free nanoparticles were corporately disclosed *via* substantial data calculated by chemical thermodynamic method and visual image simulated by molecular dynamics (MD) simulations, which would eventually provide fresh ideas for the exploration of the self-assembly mechanism of the NDDS.

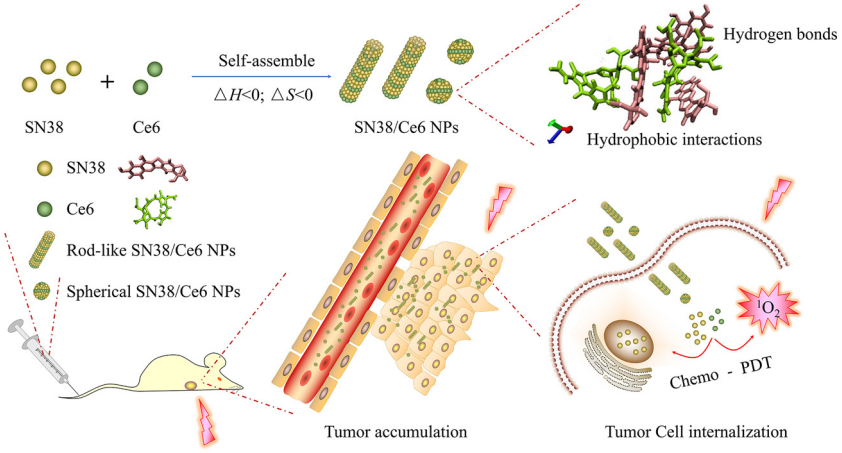
## 2. Experimental section

Previous reported antisolvent precipitation method [23], which was augmented by ultrasonication and ended with high-pressure homogenization, was utilized to fabricate carrier-free 7-ethyl-10-hydroxycamptothecin/chlorin e6 nanoparticles (SN38/Ce6 NPs). Singlet oxygen ($^1O_2$) generation of SN38/Ce6 NPs was detected with commercially available luminescent probe SOSG in test-tube and luminescent probe DCFH-DA in 4T1 cell lines. MTT assay was employed to evaluate the *in vitro* cytotoxicity of SN38/Ce6 NPs against 4T1 cells, while the *in vivo* antitumor activity was evaluated with 4T1-tumor bearing mice models. Spectroscopic measurements and molecular dynamics simulations were utilized to clarify the self-assembly mechanism of the NPs. Data were shown as mean ± standard deviation (SD) for all experiments. Independent-samples t-test, and one-way analysis of variance (ANOVA) (SPSS 19.0, USA) were utilized for the statistical analysis. P-value less than 0.05 was regarded as statistically significant, and p-value less than 0.001 was regarded as highly significant. The detailed experimental procedures could be found in Supporting Information.

## 3. Results and discussion

### 3.1. The particle size, zeta potential and morphology

Antisolvent precipitation method was a generally effective and simple organic solvent-based preparation method for hydrophobic drug-loaded NPs [24]. In this study, the method was employed for fabricating SN38/Ce6 NPs. As shown in Table S1, drug ratio of 3:1 (mol/mol, SN38/Ce6) was verified as the most suitable radio for preparing SN38/Ce6 NPs due to the minimum size ($P < 0.001$), the minimum PDI and the maximum zeta potential ($P < 0.001$). The average particle size of SN38/Ce6 NPs prepared at a drug ratio of 3:1 (mol/mol, SN38/Ce6) was 154.87 ± 1.82 nm with a narrow distribution (PDI = 0.207 ± 0.024), which was close to the particle size of




**Scheme 1.** Illustration of the self-assembled SN38/Ce6 NPs and the NPs guided chemo-PDT combinational therapy through intravenous injection.