博士論文　（要約）

Study on the Design of Glutathione Sensitive Polymeric Micelles for the Delivery of Anticancer Drugs

(制がん作用を有するグルタチオン感受性ポリマーミセルに関する研究)

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Nanocarriers responding to specific stimulus have revealed high potential for achieving on-demand release in a spatiotemporally controlled manner and improved treatment modalities. Particularly, nanocarriers responding to intracellular redox-potential have represented a promising strategy for achieving selective intracellular delivery of therapeutic agents and avoiding detrimental side effects to normal tissues. Owing to the essential role of endosomal escape for disulfide-based nanocarriers due to the insufficient reduction potential of endocytic compartments, endowing such nanocarriers with an additional external stimulus to induce cytosolic translocation may provide further spatial and temporal manipulation at the targeted tissue region. Moreover, the precise operation at the site of triggering can eliminate detrimental issues rising from the off-target distribution of these drug delivery systems.

Polymeric micelles incorporating therapeutic agents in their core have demonstrated outstanding features as nanocarriers in both preclinical and clinical studies, including their adaptable drug release in response to specific stimuli by directly engineering the bond between the drugs and the core-forming segment of the block copolymers. Our group has previously developed polymeric micelles exploiting disulfide bonds in their core for specifically acting under the reductive conditions of the cytosol, which shows 100- to 1000-fold higher glutathione (GSH) concentration than those in blood and extracellular milieu. As polymeric micelles enter cells *via* endocytosis and accumulate in endosomal/lysosomal compartments with insufficient redox-potential for thiol/disulfide exchange reaction, micelle systems sensitive to reductive cytosolic environment could be effectively combined with external stimuli for spatiotemporal controlling the therapy after inducing their escape from endosomes into the cytosol.

Herein, I developed drug-loaded polymeric micelles designed to release their content under the reductive condition of the cytosol and exploit an external trigger, namely, reactive oxygen species induced by light-irradiated photosensitizers capable of permeabilizing endosomal membranes, so called photochemical internalization (PCI), for allowing these micelles to escape from endosomes into the cytosol and controlling their drug release *in situ*. In **Chapter 2** of this thesis, these reduction-sensitive micelles were self-assembled in water after conjugating the anticancer drug camptothecin (CPT) to the poly(L-lysine) block of poly(ethylene glycol)-poly(L-lysine) (PEG-P(Lys)) block copolymers *via* a reduction-sensitive disulfide linker and their physicochemical properties were evaluated. The drug-conjugated ratio can be effectively controlled from 5 to 16% (w/w) by modulating the feeding amount of CPT (1, 2, and 5 eq.). According to the dynamic light scattering (DLS) and transmission electron microscopy (TEM) analysis, the sub-100 nm micellar size and condensed PEG shell can confer improved tumor accumulation through the enhanced permeability and retention (EPR) effect, that is, the enhanced accumulation of macromolecules in tumor tissues due to the augmented permeability and reduced lymphatic drainage, and prolonged retention time in bloodstream for further *in vivo* application. The drug release study of CPT/m using GSH or dithiothreitol (DTT) as the reducing agents showed preferential release at reductive conditions simulating the cytosol, and the negligible release of these micelles at extracellular conditions. As the hydrophobic drug was covalently conjugated with the thiolated block copolymer, the preferential intracellular release and excellent extracellular stability suggested the reduction-sensitive micelles could be a promising platform for the delivery of hydrophobic agents.

The *in vitro* cell viability of various cancer cells after incubation with free CPT and CPT/m was studied in **Chapter 3** of this thesis to determine the cytotoxic effect of these micelles. CPT/m

showed usually 100-fold lower cytotoxicity than the free drug, indicating the safety of these micelles and suggesting the limited intracellular drug release without the activation. Both drugs were further combined with 0.1 μg/ml the clinically used photosensitizer Photofrin in **Chapter 4**, which was found to be non-cytotoxic after 10 min irradiation with white light at 1.8 J/cm^2, for inducing endosomal escape of the micelles. While without light irradiation, free CPT plus Photofrin showed approximately 100-fold lower 50% inhibitory concentration (IC50) than micelles alone, 10 min irradiation of CPT/m plus Photofrin enhanced the cytotoxicity of the micelles, showing more than 3-fold decrease in the IC50 compared to the non-irradiated sample. Even 0.01 μg/ml Photofrin and 10 min irradiation induced the enhancement of *in vitro* cytotoxicity of CPT/m, suggesting the capability of Photofrin for improving the efficacy of these reduction-sensitive micelles. This enhancement of the cytotoxicity of CPT/m could be attributed to the photo-induced permeabilization of endocytic membranes by the generated ROS species from Photofrin after light exposure, followed by the endosomal escape of micelles and the accelerated release of CPT.

The capability of Photofrin to induced endosomal escape of CPT/m by PCI was evaluated *in vitro* by following the time-dependent intracellular distribution of CPT/m labeled with Alexa 555 in AY27 cancer cells *via* confocal laser scanning microscopy (CLSM). The late endosomes of AY27 cells were marked by using CellLight Late Endosome-GFP. These cells were incubated with Alexa 555-labeled CPT/m and Photofrin for 24 h. At this time-point, the signal of micelles was colocalized with that of late endosomes. Then, the cells were irradiated with a light dose similar to that of the *in vitro* cytotoxicity experiment, that is, 1.8 J/cm^2, and the escape of the micelles from the late endosomes was followed by assessing the CPT/m/endosomes colocalization ratio. After 2 h, the endosomal escape of Alexa 555-labeled CPT/m in irradiated cells was evident, whereas the cells without light irradiation maintained the level of colocalization of micelles with late endosomes. Thus, this PCI-mediated translocation of CPT/m from endosomal compartments into the reductive cytosol may result in the enhanced intracellular release of CPT, which could be a substantial advantage for developing safe and tumor-specific cytotoxic therapies.

In **Chapter 5**, the in vivo performance of CPT/m and the feasibility of the therapeutic strategy were evaluated in detail. For effectively exploiting the selective tumor accumulation through EPR effect, CPT/m should stably circulate in the bloodstream for prolonged time. Therefore, in **Chapter 5**, we assessed the *in vivo* circulation time of CPT/m in the bloodstream by using *in vivo* confocal laser scanning microscopy, which allows real-time non-invasive and quantitative evaluation of *in vivo* dynamic processes. Thus, the fluorescence intensities of Fluorescently-labeled CPT/m in the blood vessels of the ear-lobe, as well as in the interstitial space, were followed for more than 15 h. Snap-shots at 1 min and 1, 6 and 15 h post i.v. injection of the micelles and quantification of the signal in the blood vessels revealed that the Fluorescently-labeled CPT/m have prolonged circulation in the bloodstream for more than 15 h, with a blood half-life of 12.8 h, which is probably associated with the surface, size and controlled release rate of the micelles. Moreover, CPT/m did not extravasate into the interstitial space of the ear lobe, indicating that the micelles did not dissociate into the forming block copolymers during blood circulation. Moreover, the blood circulation of Photofrin after i.v. injection was also evaluated. The half-life of Photofrin in blood was calculated to be 7.9 h. Contrary to CPT/m, Photofrin gradually extravasated into the interstitial space of healthy skin tissue, which is associated with its skin phototoxicity reported in the clinic. It is worth mentioning that, even though the PS molecules accumulate in healthy tissue, the strategy proposed in this study can overcome such

drawback as the extravasation and accumulation of CTP/m in healthy tissue is minimal and the PS doses required for PCI are nontoxic, which may result in safe light-activated chemotherapies.

Considering the significant role of Photofrin in PDT against bladder cancer, as well as the requirements for avoiding typical symptoms of PDT to healthy bladder, the *in vivo* antitumor effects resulting from the PCI-mediated activation of CPT/m were studied in **Chapter 5** against a xenograft tumor model of AY27 rat bladder cancer. Accordingly, 24 h after i.v. administration, the amount of CPT delivered by CPT/m in subcutaneous AY27 xenografts was determined to be 11% of the injected dose per gram of tumor. The effect of Photofrin-induced PCI on the *in vivo* antitumor activity of CPT/m was then evaluated in nude mice bearing subcutaneous AY27 xenografts. Mice treated with 3 i.v. injections every 2nd day, *i.e.* on days 0, 2 and 4, of Photofrin at 2 mg/kg followed by irradiation with a light-dose of 10 J/cm^2 (fluence rate: 100 mW/cm^2) on days 1, 3 and 5 showed comparable tumor growth to mice receiving PBS injections, indicating that Photofrin at this dosage does not afford significant antitumor effect. Thus, this Photofrin dosing was used in combination with free CPT at its maximum tolerated dose, that is, 5 mg/kg, or CPT/m at 50 mg/kg on a CPT base, which were i.v. injected following the same schedule as Photofrin, for determining the enhancement of the antitumor efficacy after PCI. The activity of free CPT at 5 mg/kg was not affected by the induction of PCI by Photofrin, as free CPT enters cells by diffusion. Conversely, while CPT/m at 50 mg/kg showed moderate antitumor effect, the application of PCI significantly enhanced the activity of the micelles compared to PBS ($P < 0.01$) and CPT/m at 50 mg/kg groups ($P < 0.05$). Also, it is worth noting that, while free CPT at 5 mg/kg caused considerable loss of the body weight of mice ($P < 0.001$), and 1 out of 5 mice died at day 8 in the free CPT plus PCI group due to toxicity, the weight of mice treated with CPT/m at 50 mg/kg was not affected during the antitumor activity experiment, most likely due to the site-specific control of the micelles by PCI, and the controlled release of CPT from the micelles under cytosolic conditions.

To confirm that Photofrin induced PCI of CPT/m *in vivo*, the intracellular distribution of Fluorescently-labeled CPT/m in AY27 xenografts in a living mouse was analyzed by *in vivo* CLSM. Initially, a subcutaneous AY27 tumor was surgically exposed, and the late endosomes in the cells of the tumor were marked by dropping 10 µl of CellLight Late Endosome-GFP on the xenograft. Twenty-four hours later, Photofrin at 2 mg/kg and the fluorescent micelles were i.v. injected. After 24 h, the micelles were visualized in the late endosomes of the cells in the tumor. In addition, Hoechst 33342 was used to stain the nucleus of AY27 cancer cells to distinguish the position of single cells. After irradiating with a light-dose of 2.0 J/cm^2 (fluence rate: 100 mW/cm^2), the signal from CPT/m gradually separated from that of late endosomes, indicating the endosomal escape of the micelles. Conversely, in a non-irradiated tumor, the colocalization of the micelles and late endosomes was observed throughout the experiment. Moreover, the colocalization ratio of micelles and late endosomes was reduced from 70% to 59% 5 h after irradiation. The facilitated access of CPT/m to the cytosol after PCI, observed in these intravital microscopies, may promote the drug release from the micelles within the tumors, improving their therapeutic efficiency.

Twenty-four hour after co-injection of CPT/m and Photofrin, the tumors were irradiated at a light dose of 10 J/cm^2 to induce the PCI effect. Then, the PCI-induced release of CPT from CPT/m within AY27 tumors was assessed by calculating the amount of free CPT at 0 and 24 h after irradiation obtained by reverse phase liquid chromatography of homogenized tumor tissues. Thus, while the released of CPT from the micelles within tumors without irradiation was less than 4%, the drug release was more than 70% 24 h after irradiation. This increment of CPT release was further demonstrated by

studying the fluorescence of CPT in tumor sections by using CLSM. Accordingly, after PCI, the fluorescence signal from CPT in tumor tissues augmented due to the release and dequenching of the CPT molecules that were loaded in the core of CPT/m, whereas in non-irradiated tumor tissues CPT molecules remained quenched in the core of micelles. These results support the enhancement of the *in vivo* antitumor activity of CPT/m after the application of PCI, and denote the ability of reduction-sensitive drug-loaded polymeric micelles for developing safe and site-specific chemotherapies activated by PCI. Moreover, other external stimuli capable of permeabilizing endosomal membranes could be potentially combined with polymeric micelles releasing their payloads in response to cytosolic signals for designing highly selective antitumor treatments.

In this study, we designed and developed reduction-sensitive polymeric micelles for selectively release of CPT under cytosolic conditions, and demonstrated their practicality as safe nanocarriers for effective light-activated chemotherapy in combination with Photofrin. To our knowledge, this is the first study demonstrating in real-time the controlled operation of nanocarriers from endosomal compartments after application of light *in vivo*, which not only allowed us to confirm the design of our strategy by direct visualization of the process, but could also serve for expanding the insights of the intracellular processes for other nanocarriers and therapeutics after the application of triggers permeabilizing endosomal membranes *in situ*. The specific features of these micelles can be remotely controlled after promoting their escape from endosomal compartments in cells within tumors by PCI, to exert strong inhibition of the tumor growth without toxicity. This first demonstration of PCI-induced endosomal escape indicated the capability of spatiotemporal control of the cytosolic activation of reduction-sensitive micelles for developing safe and effective therapies, and suggested the potential of modulating biological events within diseased tissues by using exogenous triggers for amplifying the activity nanocarriers. Moreover, since a broad variety of therapeutic agents can be incorporated inside polymeric micelles through disulfide coupling, the design of reduction-sensitive polymeric micelles can be readily tailored to fulfill the therapeutic requirements and facilitate the development of PCI-activated therapies with clinical potential.