博士論文（要約）

論文題目　**Optical Imaging and Spatiotemporal Control of Drug Delivery Systems**

(ドラッグデリバリーシステムの光学的生体内追跡と時空間制御)

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Nanoscale carriers (nanocarriers) have attracted much attention to achieve pinpoint therapy of intractable tumors, because nanocarriers can deliver therapeutic payloads selectively to the tumor through promoted vascular permeability and immature lymphatic drainage, thereby enhancing therapeutic efficacy with reduced cytotoxicity. To improve the selectivity of therapeutic efficacy, many researchers have further modified the nanocarrier with endogenous stimuli-responsive functionality, with which the nanocarrier can exert its therapeutic functionality selectively in the tumor-associated microenvironment. However, in systemic application, the therapeutic reagents are delivered to normal organs as well as the target tumor, and some normal tissue/cells have similar characteristics with the tumor tissue/cells, which may lead to unfavorable side effects. Thus, in order to improve the specificity of the therapeutic efficacy, it would be a promising approach to spatiotemporally control the function of the nanocarriers in exogenous-stimuli responsive manner, since the effective area can be easily confined by controlling the irradiation area of exogenous stimuli. The combination of endo-/exogenous-stimuli responsive functionality will drastically improve the specificity of therapeutic efficacy by the nanocarriers. In this context, light offers a great potential as the handy exogenous-stimulus triggering the functions of nanocarriers because reasonable compact light sources can be available owing to recent advances in the laser technology and permit the easy confinement of the irradiation area.

Here, this study focused on the utility of the light and was aimed at achieving pinpoint therapy utilizing endo-/exogenous stimuli (pH and light); this thesis proposes a light-responsive nanocarrier for delivering DNA and exerting gene expression selectively in the target tumor and an organic-inorganic hybrid nanocarrier for killing tumor cells in endo-/exogenous stimuli-responsive manner.

For this purpose, this study firstly developed an intravital microscopic system (IVM) to examine what sort of nanoarchitecture offers promise in systemic application (chapter 2). IVM is composed of four components: confocal laser scanning microscopy with high spatiotemporal resolution, holders to remove the undesirable movement from a living animal (such as heartbeat and respiration), equipment to maintain the animal condition, and an image analyzing computer. IVM permits the direct observation of the dynamic behavior of systemically administered nanocarriers with a high spatiotemporal resolution. Here, IVM was applied to the quantitative monitoring of DNA-loaded nanocarriers (polyplexes) in the bloodstream, which are constructed from plasmid DNA (pDNA) and poly(ethylene glycol)-poly(L-lysine) (PEG-PLys) or poly(ethylene glycol)-poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide} [PEG-PAsp(DET)]. IVM consequently demonstrated that the polyplex constructed from these PEG-polycation block copolymers and pDNA (polyplex micelle) avoided aggregate formation and unfavorable interaction with platelets in

the blood owing to the PEG shielding shell, indicating that PEG-PLys/pDNA and PEG-PAsp(DET)/pDNA polyplex micelles are promising nanocarriers for systemic gene delivery.

However, in the term of successful transfection selectively in the targeting tumor, these polyplex micelles still have room to be improved. First, to achieve the efficient transfection efficiency, polyplex micelles need the function to promote the translocation from the endo-/lysosomes into the cytosol (endosomal escape), which is the bottleneck for successful transfection. Second, these polyplex micelles inevitably accumulate to normal organs as well as targeted tumor tissue. Hence, to selectively induce gene expression in the tumor tissue, it is important to utilize physical energy triggering the function of nanocarriers.

In this regard, enhanced cytoplasmic delivery of macromolecular compounds by photochemical disruption of the endo-/lysosomal membrane, termed photochemical internalization (PCI), has recently attracted much attention. PCI is a technique combining photoirradiation and photosensitizer (PS) targeting endo-/lysosomal membranes. Photoactivated PS generates reactive oxygen species (ROSs), which damage the endo-/lysosomal membrane and destabilize the endo-/lysosomes, thereby promoting the endosomal escape of macromolecular compounds entrapped in the endo-/lysosomes. PCI could be applied to anticancer drugs, proteins, and nucleic acids, demonstrating its promising therapeutic efficacy.

However, none of study accomplished PCI-mediated gene transfer in systemic application, because it is prerequisite to construct a nanocarrier delivering gene to the target site. Besides, systemic application of PCI-mediated transfection requires the simultaneous delivery of gene and photosensitizer. For this purpose, polyplex micelles offer a great potential in systemic delivery of these functional molecules, since many studies revealed that the polyplex micelle attained gene transfer in the target tumor after the systemic administration owing to the PEG shielding shell and stably packaged core encapsulating pDNA. PCI function can be mounted to polyplex micelles by incorporating PS into the core compartment; however, the simultaneous encapsulation of pDNA and PS may lead to unfavorable photochemical damage to pDNA. Thus, PS and pDNA should be compartmentalized in a single nanoarchitecture of the polyplex micelle.

In chapter 3, to construct one more compartment in the core-shell polyplex micellar structure, ABC-type triblock copolymer, PEG-PAsp(DET)-PLys was synthesized. PLys has higher affinity to pDNA than PAsp(DET); thus PLys in PEG-PAsp(DET)-PLys preferentially interacts with pDNA and form the polyion complex, forming three-layered polyplex micelle having PEG shielding shell, PAsp(DET) intermediate layer, and pDNA/PLys core compartment. The newly developed cationic PAsp(DET) intermediate layer can encapsulate anionic PS [dendrimeric photosensitizer, dendrimer phthalocyanine (DPc) was used here]. Thus prepared three-layered

nanocarrier has a core compartment for stable packaging of pDNA, an intermediate compartment for incorporation of DPc to induce PCI and an outer hydrophilic compartment of poly(ethylene glycol) (PEG) for the shielding surface. The three-layered nanocarrier, termed as DPc-TPM (a DPc-loaded ternary polyplex micelle), accommodates DPc and pDNA in the segregated compartments, thereby preventing photochemical damage to pDNA. Furthermore, integrated carboxylate groups settling on the periphery of DPc contribute to its stable disposition in the intermediate cationic compartment. In turn, the acidic compartment of the endo-/lysosome neutralizes these carboxylate groups to facilitate the translocation of amphiphilic DPc from the micellar compartment into the endo-/lysosomal membrane, inducing a strong PCI effect. Consequently, successful PCI-mediated gene transduction through systemic route was demonstrated in xenografted tumor models in mice using this DPc-TPM.

In chapter 4, this study considered more about clinical use of light-responsive nanocarriers. DPc, which was used in chapter 3, is useful to construct polyion complex nanocarriers because of its unique characteristics such as polyanionic property and responsiveness to low pH. However, clinically approved photosensitizer is generally an amphiphilic anionic molecule with low molecular weight; it is difficult to encapsulate such molecules into polyion complex nanocarriers. Thus, to endow more versatility to the nanocarrier platform, constituent materials should be more elaborated.

For this purpose, this study focused on inorganic material, calcium phosphate (CaP), which can encapsulate anionic molecules. As bone is comprised of CaP, CaP has been used in the research field of biomaterials, demonstrating its biocompatibility in the body. In the research of drug delivery system (DDS), CaP has been conventionally used for in vitro gene transfection. CaP can encapsulates nucleic acids (anionic molecules) and deliver them into cells in an inexpensive and easy manner. In addition, previous studies reported that CaP increases its solubility with the decrease of pH, and calcium/phosphate ions; therefore this material can be applied to construct pH-responsive nanocarriers. Utilizing these properties of CaP, this study developed an organic-inorganic hybrid nanocarrier encapsulating low molecular photosensitizer for light-selective killing of cancer cells [termed CaPCe6 (calcium phosphate nanocarrier encapsulating chlorin e6)] based on mechanism of photodynamic therapy (PDT).

PDT is a clinically approved therapeutic modality for malignant tumors such as lung, esophageal, bladder, brain, ovarian, and skin cancers. PDT consists of two procedures: accumulation of PSs into the target tissue and their photoactivation to produce cytotoxic singlet oxygen. Because of short lifetime and moderate diffusion of singlet oxygen, the cytotoxic region can be limited in the photoirradiated sites, allowing for less invasiveness than conventional

systemic chemotherapeutics. However, the distribution of PS in the body is dependent on their inherent physicochemical properties; PS sometimes accumulates to normal tissues such as skin as well as the target tumor sites, causing undesirable side-effects including photosensitivity. Hence, successful PDT requires the controlled PS distribution: ideally tumor-selective accumulation. In addition, it is preferable to suppress the PDT efficacy in normal tissue and recover it in the target site in order to improve the therapeutic selectivity.

In this context, CaP offers a great potential as a constituent material for such nanocarriers because CaP may function as an on/off switch of activating photosensitizer in a pH-responsive manner. In physiological condition, CaP stably encapsulates PS and inactivates its photoactivity by blocking the access of oxygen molecules to PS. Under low pH condition inside the lysosome, in turn, the solubility of CaP is increased and the encapsulated PS will be released from CaP, thereby recovering the photoactivity.

To examine the potential of CaP as a pH-responsive material encapsulating low molecular PS, in this study, CaPCe6 was prepared by mixing of calcium/phosphate ions, Ce6 (PS), and PEG-poly(aspartic acid) (PEG-PAsp), followed by hydrothermal synthesis. CaPCe6 possesses the CaP core encapsulating the PSs, which is surrounded by PEG shielding layer. In the physiological condition (pH 7.4), CaPCe6 suppressed photochemical activity of PS by lowering the access of oxygen molecules to the incorporated PS, while PDT efficacy was restored in the acidic condition (pH 5.5) because of the dissolution of CaP and eventual recovery of accession between the oxygen and the PS. Owing to this switch, the nanocarrier reduced the photochemical damage in the bloodstream in the skin, while it induced effective PDT efficacy inside the tumor cell in response to acidic condition of the endo-/lysosomes, indicating that CaP is a promising versatile material to construct the systemically injectable light-responsive nanocarrier.

In conclusion, this study developed IVM to elucidate the dynamic behavior of DDS that could not be clarified by conventional methodology. The newly obtained knowledge was then extended to design the systemically injectable three-layered polyplex micelle for light-induced gene transfer. The polyplex micelle accomplished PCI-mediated gene transduction after the systemic administration. Further, to improve the versatility of the platform for the light-responsive nanocarrier, CaP-based hybrid nanocarrier was developed, demonstrating its potential as the versatile platform. IVM in chapter 2 will be an essential methodology in the DDS research, and the design of the nanocarriers developed in chapter 3 and chapter 4 will provide new approaches to construct multi-functional nanocarriers to ideally achieve the pinpoint therapy.