Recent Advances in Endogenous and Exogenous Stimuli-Responsive Nanocarriers for Drug Delivery and Therapeutics

Hiroto Hatakeyama
Laboratory of Clinical Pharmacology and Pharmacometrics, Graduate School of Pharmaceutical Sciences, Chiba University; 1–8–1 Inohana, Chuo-ku, Chiba 260–8675, Japan.
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Significant progress has been achieved in the development of stimuli-responsive nanocarriers for drug delivery, diagnosis, and therapy. Various types of triggers are utilized in the development of nanocarrier delivery. Endogenous factors such as changes in pH, redox, gradient, and enzyme concentration which are linked to disease progression have been utilized for controlling biodistribution and releasing drugs from nanocarriers, as well as increasing subsequent pharmacological activity at the disease site. Nanocarriers which respond to artificially-induced exogenous factors (such as temperature, light, magnetic field, and ultrasound) have also been developed. This review aims to discuss recent advances in the design of stimuli-responsive nanocarriers which appear to have a promising future in medicine.

Key words drug delivery system; liposome; hyperthermia; stimuli-triggered release; cancer therapy

1. Introduction
To enhance therapeutic effect, and to reduce related side effects, active drugs and molecules are ideally selectively delivered to diseases areas using targeted drug delivery systems (DDSs). Nanocarriers, including nanoparticles and nanostructures, have been developed for imaging, diagnosis and therapeutics based on advances in nanotechnology and insights into the pathology of diseases at the cellular and molecular level. To improve efficacy and reduce side effects, nanocarriers are designed to behave dynamically in response to internal factors in the microenvironment of a diseased area, or to external stimuli, or both; “smart” DDSs require the development of functional devices and materials. Endogenous stimuli characteristic in the pathological areas of disease, include changes in pH, redox gradient, and enzyme concentration. Exogenous stimuli are artificially applied from outside the body, and include temperature, light, magnetic field, and ultrasound (US). The efficacy of nanocarrier-based therapies could be improved by taking into consideration fluctuating biological processes in their design. Recently, we demonstrated that the inhibition of a key molecule for hyperthermia sensitivity enhanced the therapeutic effect of nanoparticle-based hyperthermia treatment on the growth and metastasis of hyperthermia-resistant cancers. Smart nanocarriers offer promise in the application of personalized medicine. This review highlights recent advances in stimuli-responsive nanocarriers responding to endogenous and exogenous factors relative to drug delivery, imaging, and therapeutics.

2. Activation in Response to Endogenous/Internal Stimuli
Intracellular environments with a low pH in the endosomes/lysosomes are employed as triggers for the activation of nanocarriers. A decrease in pH is found in intracellular compartments, such as endosomes and lysosomes where pH values are 5.5 to 5.0. This decrease can be utilized for selectively triggering the activation and drug release of nanoparticles.

Since the 1980s, pH-sensitive liposomes have been developed. pH-Sensitive liposomes composed of phosphatidylethanolamine (DOPE) were protonated in endosomes/lysosomes, which stimulated the release of drug cargos into the cytoplasm.1,2) Later, a series of ionizable aminolipids were synthesized and utilized for nucleic acid delivery to the liver and to tumors as a formulation of stable nucleic acid-lipid particles (SNALPs) or ionizable lipid nanoparticles (iLNPs). 3–6) Recently, we designed a new pH-sensitive cationic lipid, YSK05 (1-methyl-4,4-bis[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]piperidine).7) Liposomes containing YSK derivatives with apparent pKa 6.5 were neutral in the blood and positively charged at the early endosome stage, which triggered the release of a cargo into cytosol via membrane fusion, resulting in efficient knockdown in the liver or tumors after systemic administration8–12) (Fig. 1a).

pH-Labile linkages have been incorporated into pH-responsive compounds. pH-Sensitive polyethylene glycol (PEG) lipids containing an acid cleavable linkage have been developed, which increased the activity of cargos of liposomes in comparison with stable PEGylated lipids13–15) Polymer based nanoparticles composed of pH-sensitive linkages have been also reported.16–18) Walker et al. demonstrated successful gene delivery using pH-sensitive polyplexes with pH-labile hydrazone linkages between the PEG and polycation for in vitro and in vivo use.19) Endosomal disruption by a “proton sponge” mechanism...
has been extensively utilized to accelerate endosomal escape in gene delivery using polyethylenimine (PEI)\(^{19,20}\). The high transfection efficiency of PEI can be attributed to its buffering effect or the “proton sponge effect,” based on its secondary and tertiary amines. Wagner and colleagues conjugated a pH-responsive endosomolytic peptide, melittin, with PEGylated PEI \(\text{via}\) an acid-labile dimethylmaleic anhydride (DMMAn) linker. The resulting conjugate led to the enhanced activity of its cargos. This can be attributed to the enhanced lytic activity at acidic pH, which triggered the destabilization of endosomal membranes.\(^{21,22}\) Other polymers that are protonated in an intracellular acidic compartment have been reported.\(^{23}\)

A pH-sensitive fusogenic membrane peptide GALA (WEAALAEALAEALAEHLAEALAEALEALAA) was developed based on the endosomal escape mechanism of an influenza virus, an envelope-type RNA virus.\(^{24}\) A 30-amino acid GALA contains a glutamic acid-alanine-leucine-alanine sequence that is repeated four times. GALA undergoes structural change to the \(\alpha\)-helix under acidic conditions, which triggers membrane fusion between liposomes and endosomes. The introduction of GALA and its derivative 22 amino acid short version GALA (WEAALAEALAEALAEHLAEALAEALAA: shGALA) to liposomes enhanced the silencing activity of their cargos\(^{25-29}\) (Fig. 1b). Kono and colleagues developed pH-sensitive fusogenic peptide GALA or shGALA. GALAs undergo structural change to the \(\alpha\)-helix under acidic conditions, which triggers membrane fusion between liposomes and endosomes. (b) A liposome modified with pH-sensitive fusogenic peptide GALA or shGALA. GALAs undergo structural change to the \(\alpha\)-helix under acidic conditions, which triggers membrane fusion between liposomes and endosomes. (c) A liposome modified with cleavable PEG-lipid (PPD) in response to an extracellular tumor specific environment such as MMPs.

### Fig. 1. Strategies for Activation in Response to Endogenous Stimuli

(a) A liposome containing pH-sensitive lipids represents a neutral surface in the physiological environment and is positively charged in an acidic environment such as in endosomes and lysosomes, which triggers membrane fusion between liposome and endosome. (b) A liposome modified with pH-sensitive fusogenic peptide GALA or shGALA. GALAs undergo structural change to the \(\alpha\)-helix under acidic conditions, which triggers membrane fusion between liposomes and endosomes. (c) A liposome modified with cleavable PEG-lipid (PPD) in response to an extracellular tumor specific environment such as MMPs.

In addition to the pH gradient, the intracellular reducing environment is considered an alternative source for stimulating triggers. Intracellular glutathione (GSH) concentrations usually ranges from 0.5 to 10 mm, whereas extracellular values are one to three orders of magnitude lower. Since this significant difference in GSH level is an attractive trigger, nanoparticles are designed in response to the difference in redox potential between the intracellular and extracellular environment. PEG-lipid containing disulfide bonds developed by Zalipsky et al., accelerated the rate of release of cargoes from liposomes compared with non-cleavable PEG-lipid modified liposomes.\(^{32,33}\) Reduction-responsive micelles have been reported by several groups.\(^{34}\) A block copolymer containing PEG and thiol-introduced poly(l-lysine) (PLL) was constructed to form polyion complex micelles.\(^{35,36}\) We developed an SS-cleavable proton-activated lipid-like material (ssPalm), which contains two tertiary amines that are positively charged at acidic pH, and with a disulfide bond that can be cleaved in a reducing environment.\(^{37-40}\) Liposomes containing ssPalm were positively charged, with an apparent \(pK_a\) value of 6.2. Furthermore, their envelope structure was designed to be degraded in response to the reductive environment in the cytosol. As a result, these liposomes containing ssPalm exhibited higher transgene expression compared with those developed with conventionally used cationic lipids.

Since enzymes and proteases are related to a disease’s pathological characteristics, and are known to selectively cleave specific substrates, e.g. an amino acid or peptide sequence, enzymes are widely used as triggers in targeting disease sites. A PEG-lipid conjugated \(\text{via}\) a short peptide, which is intracellularly cleaved by cathepsin B, was developed by Allen and colleagues.\(^{41}\) We focused on matrix metalloproteinases (MMPs), since MMPs are overexpressed in numerous cancers. When an MMP substrate peptide was inserted between PEG and DOPE as a linker, the resulting conjugated PEG–peptide–DOPE ternary conjugate is referred to as PPD.\(^{42}\) PPD-modified liposomes represented stability in the systemic circulation, and accumulated in tumors \(\text{via}\) the enhanced permeability and retention (EPR) effect.\(^{43}\) These PPD-modified liposomes deliver cargo, such as pDNA and small interfering RNA (siRNA), to tumor cells more efficiently than conventional PEG modified liposomes, due to the response to MMP in the tumor microenvironment. Hashida et al., and other groups have also reported on MMP based...
delivery systems. Other proteases and enzymes, such as phospholipases and glucose-oxidase, have been utilized as stimulus triggers in drug delivery systems, too.

3. Activation in Response to External Stimuli

Exogenous triggers, including temperature, magnetic field, ultrasound, light, and high energy radiation, can be used to enhance a carrier’s activity, as well as to trigger drug release to achieve diagnosis and/or therapeutic effect at disease sites. Functionalized nanoparticles that can be triggered to enhance their activity by such triggers as temperature, light, magnetic field, and US have been developed.

Temperature is one of the most convenient and effective triggers. In 1978, Yatvin et al. reported the first temperature-sensitive liposome composed of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) at molar ratio of 3 : 1 that was able to release a hydrophilic drug at 44°C due to a gel-to-liquid transition phase transition at a temperature (Tm) of around 44°C. A thermosensitive liposome encapsulating doxorubicin (ThermoDox®), developed by Needham and colleagues, is composed of DPPC monostearoylphosphatidylcholine (MSPC) and polyethylene glycol 2000–distearoylphosphatidylethanolamine (PEG 2000–DSPE) in a molar ratio of 90 : 10 : 4, which allows it to trigger the promotion of thermal drug release at 41–42°C. ThermoDox is currently under evaluation in clinical trials. Tagami et al. developed a heat-activated cytoToxic (HaT) liposome containing DPPC and Brij78, which displayed increased drug release rate constants for many drugs at 40–41°C. Thermosensitive polymeric nanocarriers composed of poly(N-isopropylacrylamide) (PNIPAM) and a variety of polymers have also demonstrated marked transition temperatures, allowing for improved drug release at 40°C.

Basically, light, which is further classified as UV light, visible light, or near infrared (NIR) light, is used as an excitation source. Light-induced activation can be applied as photothermal therapy (PTT), photodynamic therapy (PDT), or image-guided therapy. Gold based materials, including gold nanoparticles, gold nanorods, and gold shells, are widely used in PTT. Niidome et al. developed PEGylated gold nanorods with a peak absorbance at 900nm. The gold nanorods were heated in tumors by irradiation using NIR pulsed laser light, which resulted in the suppression of tumor growth. Because photo sensitive carriers can achieve an on-off drug release by repeatable light irradiation, light-triggered delivery systems have also been developed. Light penetration depth currently remains the limiting factor for practical light wavelength therapy in deep tissues.

Since magnetic nanocarriers, such as iron oxide nanoparticles, can be manipulated under the influence of an external alternating magnetic field (AMF), they have been utilized in applications related to the magnetic targeting of magnetic nanocarriers for drug and gene delivery. By this means, siRNA, electrostatically associated with magnetic nanocarriers could be transferred directly into cancers via AMF therapy. Such magnetofection has improved effectiveness in the transfection of nucleic acids in vitro and in vivo. The feature by which magnetic nanocarriers generate heat under AMF allows magnet nanocarriers to be applied for magnetic hyperthermia as well. Magnetic nanocarriers with unique magnetic properties are able to improve the signal-to-noise ratio in magnetic resonance imaging (MRI), and have been widely applied as MRI contrast agents, useful both in MRI-guided diagnostics and therapeutics.

US has been used extensively in clinics for diagnosis and therapy, due to its intrinsic tissue penetration and high safety. US waves can trigger the release of a drug from a variety of nanocarriers through thermal or mechanical effects generated by cavitation phenomena or radiation forces. US sensitive nanocarriers, such as microbubbles, which are gas-filled microspheres, improve the efficiency of drug delivery and imaging. Maruyama and colleagues have developed liposomal nanobubbles encapsulating perfluropropane which oscillate and collapse in an US field, generating heat and shock waves, thus enhancing the permeability of a cell membrane. Kim et al. reported that a systemically administrated CaCO3-filled

Fig. 2. Strategy for Efficient Hyperthermia Treatment

Tumors were treated with local hyperthermia using CuS NPs activated by NIR laser or iron oxide NPs activated by magnetic field. The knockdown of key molecules of hyperthermia sensitivity using DOPC liposomes sensitized tumors to the hyperthermia treatment.
nanogel enhances the US mediated imaging of tumors.\textsuperscript{85,86}

4. Improved Efficacy of Hyperthermia by Inhibiting a Hyperthermia-Resistant Regulator

As significant progress is made in developing strategies to use heat via exogenous stimuli-responsive nanocarriers, as mentioned above, hyperthermia has been widely attempted in the treatment of disease, especially cancers. However, despite progress in hyperthermia technologies, as mentioned above, the relationship between specific temperatures with the clinical benefits and predictors of sensitivity of cancers to hyperthermia remain poorly understood.\textsuperscript{87} We discovered variable hyperthermia sensitivity of ovarian and uterine cancer cells. Through integrative analyses of static gene signatures and dynamic changes in protein expression between hyperthermia-sensitive and -resistant cells, we identified that connective tissue growth factor (CTGF) regulates hyperthermia resistance by manipulating glucose metabolism under heat stress in ovarian and uterine cancer cells.\textsuperscript{87} Therefore, for efficient hyperthermia therapy of ovarian cancers, we examined the combination of inhibiting CTGF and hyperthermia. The silencing of CTGF was achieved by treatment with 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) liposomal siRNA, which is currently undergoing testing in clinical trials.\textsuperscript{88-90} We carried out localized hyperthermia using copper sulfate nanoparticles (CuS NPs) combined with NIR laser ablation\textsuperscript{89,90} to heat hyperthermia-resistant ovarian tumors. CuS NPs modified with PEG were about 10 nm in diameter and circulated in the blood for long periods after systemic administration, passively accumulating in tumors via the EPR effect. CuS NPs represented the peak of absorption at 980 nm and could convert the optical energy of an NIR laser to thermal energy. As temperatures of 46–60°C are associated with reversible cellular damage in proportion to the exposure time,\textsuperscript{91,92} we maintained the tumor temperature at about 50°C for 5 min. The silencing of CTGF sensitized tumors to hyperthermia, which resulted in inhibiting the growth and metastasis of tumors as compared with tumors treated via hyperthermia alone (Fig. 2). We also demonstrated that the effect of hyperthermia in orthotopic ovarian models with iron oxide nanoparticles irradiated by AMF on tumor suppression was enhanced when used in combination with knockdown of the heat shock protein (HSP) 70 gene by treatment with DOPC liposomal siRNA.\textsuperscript{79} Collectively, we demonstrated that the efficacy of hyperthermia in cancer therapy could be improved by manipulating the biological response under heat stress.

5. Conclusion

As shown in this review, significant progress has been achieved in the development of stimuli-responsive nanocarriers for delivery, diagnosis, and therapy. As we demonstrated, the combination of manipulating stimuli-responsive nanocarriers and regulating biological processes can achieve a better therapeutic effect. Therefore, a key for success in the development of nanocarrier-based therapy would be an understanding of a variety of aspects in nanotechnology as well as basic biology. The translation of the effectiveness of stimuli-responsive nanocarriers is not straightforward: their sophisticated design, makes pharmaceutical development more complex in terms of manufacturing process, reproducibility, and quality control. Enormous challenges and possibilities remain in this new era of smart-nanocarrier-based delivery, diagnosis, and therapy for various diseases that are currently incurable.

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