Review

Study on Development of Polymeric Micellar Gene Carrier and Evaluation of Its Functionality

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Received April 11, 2013

Polymeric micelles, which are formed by the self-assembly of block copolymers, can load various substances and have received attention as drug carriers. Negatively charged nucleic acids such as DNA or RNA are some of the drugs delivered by polyion complex-based polymeric micelles (polyplex micelles). Polyplex micelles are expected to be nonviral gene carriers instead of viral vectors, which have several problems including safety, the limited size of the loading gene, and productivity. In this review, recent studies by our group on smart polyplex micelles delivering genes will be introduced.

Key words gene carrier; block copolymer; polyplex micelle; polymeric micelle; gene delivery system; gene therapy

INTRODUCTION

Nucleic acid-based drugs for gene therapy have been expected as new types of drugs. Plasmid DNA (pDNA) has been utilized to introduce deficient genes into disease cells and express pharmaceutical proteins, while antisense DNA and short interfering RNA (siRNA) have been utilized to suppress gene functions causing diseases. Many diseases have been targeted by gene therapy, and the potential of gene therapy is versatile. However, these nucleic acid-based drugs are easily degraded by nucleases in the body and have exhibited low cellular uptake efficiency because of negatively charged biomacromolecules. A major key to success in gene therapy is the development of gene carriers that have high delivery efficiency without any toxicity. Viral vectors such as adenoviruses, retroviruses, and adeno-associated viruses have played a pivotal role in gene therapy because of their excellent gene transfection efficiency. However, the clinical use of viral vectors has considerable limitations related to their safety such as antigenicity and oncogenicity, the limited size of the loaded nucleic acid, and the difficulty of formulating them with good quality control.[1–3] These limitations have led to the recent trend in developing nonviral vectors with efficient transfection ability, safety, and high productivity as alternative carriers to viral vectors.[4,5] Lipoplexes[6,7] and polyplexes[8,9] based on cationic lipids and polymers, respectively, have been extensively studied as nonviral gene carriers. Nevertheless, these carriers are still insufficient for *in vivo* applications, particularly those administered systemically, because they generally contain excessive cationic lipids or polymers to increase solubility in an aqueous solution. Shifting their surface charge to a positive value induces nonspecific interactions with anionic components in the body such as plasma proteins and blood cells. To achieve sufficient *in vivo* systemic transfection, nonviral vectors need to satisfy several properties.

Polyplex micelles,[10] composed of poly(ethylene glycol)-polycation (PEG-polycation) block copolymers and nucleic acids including DNA and RNA, are characterized by the unique core–shell architecture of the hydrophilic shell layer surrounding their polyplex core, with a suitable size for systemic administration being approximately 100 nm (Fig. 1). A biocompatible PEG shell layer minimizes nonspecific interactions with biocomponents, achieves higher stability in a medium containing serum than that of conventional lypoplexes and polyplexes,[11] and has an increased retention time in the bloodstream,[12] which suggests that polyplex micelles may be a promising candidate for carriers that can be used in systemic gene delivery. Nevertheless, in order to enhance transfection efficiency into the targeted tissue after systemic administration and achieve successful gene therapy, further appropriate

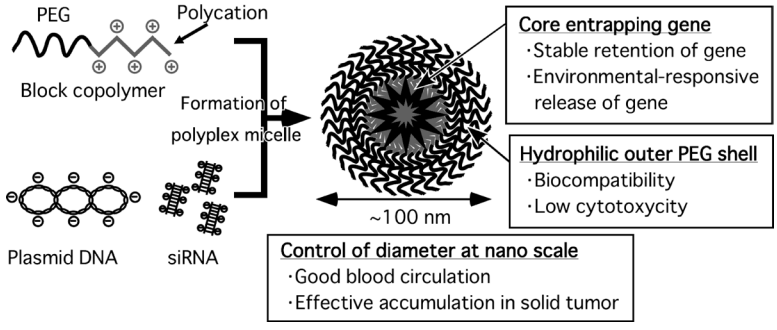

Fig. 1. Polymeric Micellar Gene Carrier

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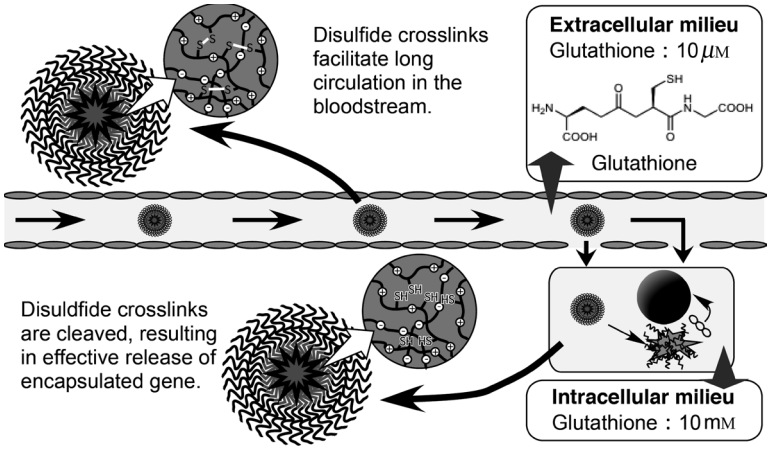

Fig. 2. Disulfide Crosslinked Polyplex Micelle in the Extra- and Intracellular Milieu

functions need to be introduced in polyplex micelles. This review describes advanced systems of polyplex micelles carrying pDNA and siRNA that have recently been developed.

1. DISULFIDE CROSSLINKED POLYPLEX MICELLES

PEG-poly(L-lysine) (PEG-PLys)/pDNA polyplex micelles are relatively more stable than other nonviral gene carriers such as polyplexes and lipoplexes; however, they are destabilized under harsh *in vivo* conditions by exchanging reactions with negatively charged biomacromolecules. The incorporated gene in the polyplex micelle needs to be strongly protected from nucleases and serum proteins in the extracellular milieu such as in the blood, while it must be released from the micelle only under the appropriate environment. In order to satisfy these requirements, disulfide crosslinks were introduced into the core of polyplex micelles[13,14] (Fig. 2). Disulfide bonds are stable covalent bonds under a non-reductive milieu such as the bloodstream, while they are cleaved under a reductive milieu such as an intracellular compartment. This is derived from the different concentrations of glutathione, which is an intracellular reductive compound. The concentration of glutathione is approximately 10 μM in the extracellular milieu and 10 mM in the intracellular milieu.[15] Therefore, the dissociation of polyplex micelles with disulfide crosslinks into the core may be suppressed in the blood after an intravenous injection and the incorporated gene can be released with the cleavage of disulfide bonds after its uptake into the cells. Crosslinked polyplex micelles made by thiolated PEG-PLys efficiently released encapsulated pDNA in response to reductive conditions mimicking the intracellular milieu against exchanging reactions with counter-polyanions, while they exhibited intensive stability under non-reductive conditions mimicking the extracellular milieu. Consequently, crosslinked polyplex micelles with optimal crosslinking densities were approximately 10-fold more efficient for *in vitro* gene transfer than non-crosslinked micelles. Moreover, crosslinked polyplex micelles could be freeze-dried without any excipients, and the original cumulant diameters, shapes, and transfection efficiencies were maintained even after freeze-drying.[16] This achievement is very important for pharmaceutical applications and shows many advantages such as the easy adjustment of drug concentrations, long-term storage stability, and large-scale production reproducibility.

In the *in vivo* applications, disulfide crosslinked polyplex micelles with pDNA encoding the soluble form of vascular endothelial growth factor (VEGF) receptor-1 (sFlt-1) were applied to the anti-angiogenic gene therapy of a subcutaneous pancreatic tumor model.[17] The antitumor activity of polyplex micelles with various crosslinking densities was evaluated in mice bearing subcutaneously xenografted BxPC3 pancreatic adenocarcinoma tumor cells, and polyplex micelles with optimal crosslinking density achieved the effective suppression of tumor growth. Interestingly, the concentration of pDNA in the blood 60 min after an intravenous injection with crosslinked polyplex micelles was approximately 10 times higher than that with micelles without core crosslinking. Significant gene expression of crosslinked micelles was detected selectively in tumor tissue, and their anti-angiogenic effect was confirmed by decreased vascular density inside the tumor tissue. Therefore, disulfide crosslinked polyplex micelles loading sFlt-1 pDNA have a greater potential for anti-angiogenic therapy against the subcutaneous pancreatic tumor model by systemic application.

Disulfide crosslinked polyplex micelles were also applied to siRNA delivery.[18,19] The micellar structure was maintained at physiological ionic strength, but was disrupted under reductive conditions, which is desirable for siRNA release in the intracellular reductive environment. Crosslinked polyplex micelles achieved a 100-fold higher siRNA transfection efficiency than that with non-crosslinked polyplex micelles. Blood circulation was improved for crosslinked polyplex micelles with a circulation half-life that was 3-fold longer than that of naked siRNA. The disulfide crosslinking strategy is effective for both *in vitro* and *in vivo* siRNA delivery.

2. TARGETABLE POLYPLEX MICELLES

The polyplex micelles indicated in the previous chapters accumulate in tumors or some disease regions in a passive manner such as the enhanced permeability and retention effect[20] and enter the cells through adsorptive or fluid-phase endocytosis. In order to prolong the duration of polyplex micelles in the target regions and increase selective uptake into the specific cells, appropriate ligands should be preferably introduced onto the surface of polyplex micelles.[21] In this way,

surface-installed ligands are expected to enhance the uptake rate of polyplex micelles into target cells by receptor-mediated endocytosis, which may lead to higher gene transfection efficiency than that of ligand-free polyplex micelles taken up by cells through adsorptive or fluid-phase endocytosis. For this purpose, a cyclic RGD peptide-conjugated block copolymer was designed and applied to the *in vitro* and *in vivo* experiments.[22–24] Note that the cyclic RGD peptide (cRGD) recognizes $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors,[25] which play a pivotal role in angiogenesis, vascular intima thickening, and the proliferation of malignant tumors. The transfection efficiency of cRGD (+) micelles against the cells possessing no $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins was not significantly different from that of cRGD (−) micelles. On the other hand, transfection efficiency was markedly higher in cRGD (+) micelles than in cRGD (−) micelles for cultured cells possessing $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. Interestingly, this enhancement was not due to an increase in the uptake amount of polyplex micelles, but to a change in their intracellular trafficking route. Detailed confocal laser scanning microscopic (CLSM) observations revealed that cRGD (+) micelles were distributed in the perinuclear region in the early stages preferentially through caveolae-mediated endocytosis, which may be a desirable pathway to avoid the lysosomal degradation of delivered genes. This approach is promising for the construction of nonviral gene carriers that enhance transfection by controlling intracellular distribution (Fig. 3).

Furthermore, cRGD (+) micelles with pDNA encoding sFlt-1 were also tested for their therapeutic effect in mice bearing subcutaneous BxPC3 pancreatic adenocarcinoma tumors by a systemic injection.[26] These micelles significantly inhibited tumor growth as a result of the anti-angiogenic effect confirmed by vascular density measurements. CLSM observations revealed that both cRGD (+) and (−) micelles localized in tumor endothelial cells; however, cRGD (+) micelles accumulated in tumor tissue more effectively than cRGD (−) micelles. These results indicate that cRGD peptide ligands on the surface of polyplex micelles contribute to their targeting of tumor endothelial cells, and that cRGD (+) micelles with sFlt-1 pDNA are promising gene carriers for the treatment of solid tumors. cRGD (+) micelles were also tested for the delivery of siRNA.[27] cRGD peptide installation resulted in increased gene silencing ability, improved cellular uptake, and broader subcellular distribution in *in vitro* experiments and also improved accumulation in both the tumor mass and tumor-associated blood vessels following an intravenous injection into mice. Furthermore, cRGD (+) micelles with anti-angiogenic siRNAs inhibited the growth of subcutaneous HeLa tumor models, which demonstrated that cRGD (+) micelles can be used for siRNA tumor therapies administered by intravenous injections.

3. INTRACELLULAR TRAFFICKING CONTROLLABLE POLYPLEX MICELLES

The major obstacle for nonviral gene carriers internalized into cells through endocytosis is considered to be speedy transport from the endosome, which later fuses with the lysosome. Since DNA and RNA are mostly degraded by nucleases under low pH conditions such as lysosomes, it is important for gene carriers to escape from the early endosome to the

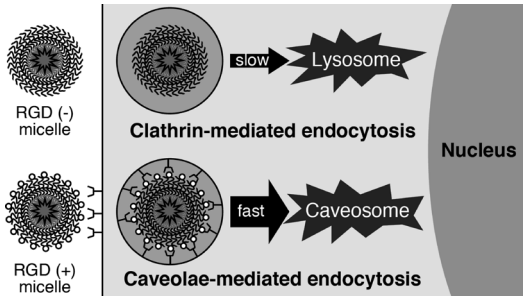

Fig. 3. Intracellular Internalization Mechanism of cRGD (+) and (−) Micelles

cytoplasm. Several approaches for the endosomal escape of nonviral gene carriers have been studied so far. Fusiogenic peptides derived from viruses and artificial peptides were synthesized to enhance transfection efficiency due to their endosomal membrane disruptive activity.[28] Another approach for endosomal escape is the use of polycations such as polyethyleneimine with relatively low pK_a.[29] This approach utilizes the so-called proton sponge effect, and polycations with low pK_a under endosomal acidic conditions (pH 5.5–6.0) caused an influx of chloride ions and protons, leading to osmotic swelling and the disruption of the endosome. Consequently, polyplexes prepared from fusiogenic peptides or polycations with low pK_a can escape from the endosome to the cytoplasm, and high transfection efficiency is achieved. However, it is necessary to add many peptides or polycations for the formation of stable polyplexes under physiological conditions and high transfection efficiency, which results in strong cytotoxicity. The application of such peptides or polycations to *in vivo* gene delivery is therefore limited.

Recently, we investigated a facile and quantitative aminolysis of poly(*β*-benzyl L-aspartate) (PBLA) to obtain an *N*-substituted poly(aspartamide) (PAsp) derivatives library possessing various cationic side chains from a single platform PBLA.[30] In the screening of obtained polycations, we found that polyplexes composed of poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide} [PAsp(DET)] and pDNA exhibited high transfection efficiency with negligible cytotoxicity (Fig. 4).[31,32] Interestingly, PAsp(DET) revealed minimal membrane destabilization at physiological pH (pH *ca.* 7), although there was a significant enhancement in membrane destabilization at acidic pHs mimicking the late endosomal compartment (pH *ca.* 5). The pH-selective membrane destabilization profile of PAsp(DET) apparently corresponded to a protonation change in the flanking diamine unit, from the single-protonated gauche form at neutral pH to the double-protonated anti form at acidic pH. Furthermore, pharmacogenomic analysis demonstrated that PAsp(DET) provided long-term security after transfection because of its biodegradability.[33,34] This PAsp(DET) polycation was applied to the polyplex micellar system[35] described in the following sections.

3.1. PEG-PAsp(DET) Polyplex Micelles PAsp(DET) polyplexes had excellent transfection efficiency without marked cytotoxicity against monolayer cultured cells. However, the surface cationic charge of polyplexes should be avoided for *in vivo* applications, which has been demonstrated by *in vivo* gene transfection for vascular lesions.[36] Naked pDNA, PEI and PAsp(DET) polyplexes, and PEG-PAsp(DET) micelles were installed into a rabbit carotid artery with

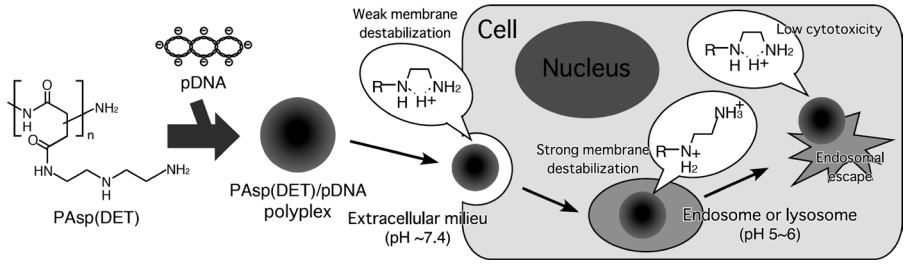

Fig. 4. Proposed Endosomal Escaping Mechanism of PAsp(DET) Polyplex

neointima by an intravascular method, and the expression of the reporter gene in vascular lesions was assessed. Only PEG-PAsp(DET) polyplex micelles showed appreciable gene transfer into vascular lesions without any vessel occlusion by a thrombus, which was in strong contrast to PEI and PAsp(DET) polyplexes which frequently showed occlusion with a thrombus. Recently, a novel method based on intravital real-time confocal laser scanning microscopy (IVRTCLSM) was applied to quantify the dynamic states of polyplexes [PEI, PLys, and PAsp(DET)] and polyplex micelles [PEG-PLys and PEG-PAsp(DET)].[37] Polyplexes formed distinct aggregates immediately after an intravenous injection, followed by an interaction with platelets. In contrast, polyplex micelles had dense PEG palisades, revealing no formulation of aggregates without a visible interaction with platelets during circulation. These findings suggest that the PEGylated strategy on the surface of polyplexes is essential for gene carriers through a systemic injection.

The effects of the chemical structures of polycations and PEG-polycations on transfection and cytotoxicity were elucidated using a multicellular tumor spheroid (MCTS) model,[38] which is known to be a very useful three-dimensional *in vitro* model representing the morphological and functional features of *in vivo* avascular solid tumors. Various features of transfection with polyplex micelles, which have been difficult to observe in conventional monolayer cultures, were revealed by the MCTS model in terms of cytotoxicity and the time-dependent behavior of transfected gene expression under three-dimensional microenvironments. Using this model, PEG-PAsp(DET) micelles were shown to achieve high transfection efficiency as well as low cytotoxicity, both of which are critical properties for successful *in vivo* gene delivery. Furthermore, PEG-PAsp(DET) micelles showed facilitated percolation inside the tumor tissue after incubation with the MCTS model and an intratumoral injection into subcutaneous tumors, whereas PEI and PAsp(DET) polyplexes exhibited limited percolation and localized transfection.[39] Solid tumors are known to possess heterogeneous structures and it is difficult to deliver therapeutic genes to tumor cells distant from the vasculature. This was the first time that polyplex micelles may show improved tumor penetrability over cationic polyplexes, thereby achieving transfection into the inside of tumor tissue.

3.2. PEG Detachable Polyplex Micelles PAsp(DET) polyplexes show higher transfection efficiency than that of PEG-PAsp(DET) micelles, especially at low charge ratios, which suggests that the PEG palisade surrounding micelles may hamper transfection (the PEG dilemma). In order to overcome the PEG dilemma, PEG detachable polyplex micelles sensitive to the intracellular environment were designed as

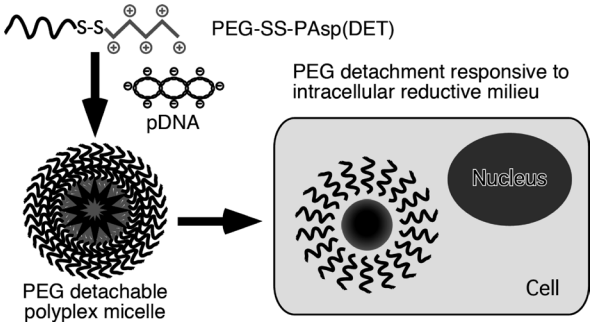

Fig. 5. PEG Detachable Polyplex Micelle Responsive to Intracellular Reductive Milieu

a smart gene carrier. Block copolymers containing disulfide linkages between PEG and PAsp(DET), PEG-SS-PAsp(DET), were synthesized to obtain this design goal[40] (Fig. 5). Disulfide linkage is a biocleavable bond as shown in Section 1. Gene transfection efficiency was several orders of magnitude higher in PEG-SS-PAsp(DET) micelles than in PEG-PAsp(DET) micelles without disulfide linkages in spite of similar levels of cellular uptake. CLSM observations showed the effective endosomal escape of PEG-SS-PAsp(DET) polyplex micelles, which may have been due to the PEG detachment in the endosome.

PEG-SS-PAsp(DET) polyplex micelles were evaluated in a peritoneally disseminated tumor model.[41] Transfection efficiency in tumors was higher with the intraperitoneal administration of PEG-SS-PAsp(DET) micelles than with PEG-PAsp(DET) micelles in spite of the same distribution of micelles. PEG-SS-PAsp(DET) micelles with pDNA encoding human tumor necrosis factor α exhibited a higher antitumor activity than that of saline controls and PEG-PAsp(DET) micelles without hepatic and renal toxicities. PEG detachable polyplex micelles may represent an advantage in gene transfection over PEG undetachable polyplex micelles both *in vitro* and *in vivo*.

3.3. Three-Layered Polyplex Micelles PLys-based polyplex systems have been widely studied due to their strong condensing power against DNA, but have encountered the issue of inefficient transfection activity because of the lack of an endosomal escaping function. On the other hand, PAsp(DET) polycations with an endosomal escaping ability have relatively weak affinity to DNA, which may lead to the detachment of the PAsp(DET) segment from pDNA in the polyplex micelles through the interaction with biological components during circulation in the bloodstream, resulting in a loss of transfection activity. Therefore, PEG-PAsp(DET)-PLys triblock copolymers were prepared to integrate three

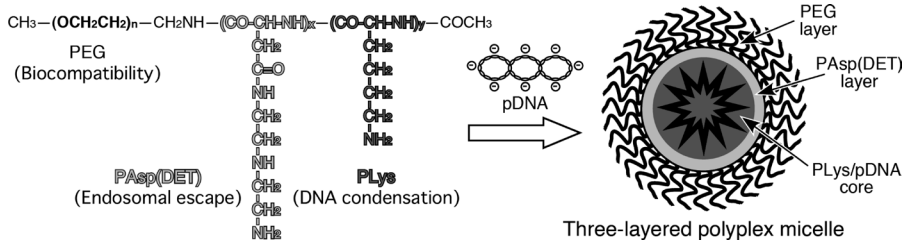

Fig. 6. Three-Layered Polyplex Micelle

functional segments engendering biocompatibility (PEG), efficient endosomal escape [PAsp(DET)], and effective pDNA condensation (PLys), respectively.[42] PEG-PAsp(DET)-PLys three-layered micelles showed one order of magnitude higher transfection efficiency against *in vitro* cultured cells than that of PEG-PLys micelles, which may have been due to the facilitated endosomal escape of pDNA. Reporter gene expression in a BxPC3 pancreatic adenocarcinoma tumor model demonstrated that intravenously injected three-layered polyplex micelles effectively penetrated the tumor vasculature in combination with the transforming growth factor-β type I receptor inhibitor, which enhanced the accumulation of macromolecular drugs in tumor tissues.[43] This was the first example of effective gene expression in BxPC3 tumors with thick fibrotic and hypovascular characteristics *via* the systemic injection of nonviral gene carriers.

3.4. Polyplex Micelles Assisted by Hydrophobic Interactions As shown in Section 3.3, the affinity of PAsp(DET) polycations to pDNA is comparatively weak. Excess PEG-PAsp(DET) block copolymers relative to pDNA were required to achieve high transfection efficiency, which suggested the existence of free polymers. If free polymers play a significant role in gene transfer with PEG-PAsp(DET) micelles, transfection efficiency under highly diluted conditions, such as systemic application, may be markedly decreased. Hence, PEG-PAsp(DET) micelles were further improved as *in vivo* systemic gene carriers by the introduction of cholesterol (Chol) into the ω-terminus of PEG-PAsp(DET) block copolymers to obtain PEG-PAsp(DET)-Chol[44] (Fig. 7). The introduction of Chol resulted in an enhanced association of block copolymers with pDNA, which led to higher stability in the proteinous medium and also in the bloodstream after a systemic injection than that of PEG-PAsp(DET) micelles. The synergistic effect between the enhanced polymer association and increased micellar stability led to high *in vitro* gene transfer even at relatively low charge ratios and low concentrations, due to efficient cellular uptake and the effective endosomal escape of block copolymers and pDNA. In the *in vivo* experiments, PEG-PAsp(DET)-Chol micelles significantly suppressed tumor growth following an intravenous injection into mice bearing a subcutaneous pancreatic adenocarcinoma tumor using sFlt-1 pDNA, which suggests that PEG-PAsp(DET)-Chol micelles can be effective systemic gene carriers for the treatment of solid tumors.

3.5. Polyplex Micelles with Combined Functions Several types of polyplex micelles were developed with a combination of the aforementioned functions. cRGD-PEG-PAsp(DET) block copolymers were designed to obtain both targetability to $\alpha_v\beta_3$ integrin receptors and endosomal escape ability.[45] cRGD-PEG-PAsp(DET) micelles achieved significantly more efficient cellular uptake and gene expression than that of PEG-PAsp(DET) micelles in endothelial cells and vascular smooth muscle cells. Furthermore, *in vivo* evaluations in a rat carotid artery with a neointimal lesion revealed that cRGD-PEG-PAsp(DET) micelles realized sustained gene expression, whereas PEG-PAsp(DET) micelles facilitated rapid, but transient gene expression. These findings suggest that this system may create novel and useful functions for gene transfer and contribute to the establishment of efficient gene therapy for vascular diseases.

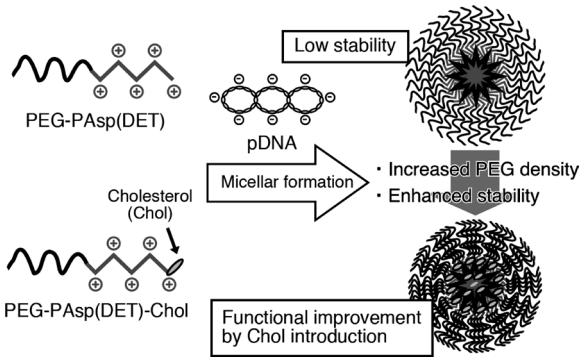

Fig. 7. Chol Introduction of PEG-PAsp(DET) for Functional Improvement

PAsp(DET) polycations were attempted to integrate into PEG-PAsp(DET) micelles with the aim of enhancing cell transfection efficiency for PEGylated polyplex micelles.[46] *In vitro* evaluations verified the PAsp(DET) integration of potent stimulation in enhancing the transfection activity of PEGylated polyplex micelles *via* prompted cellular uptake and facilitated endosomal escape. *In vivo* anti-angiogenic tumor suppression evaluations validated the feasibility of PAsp(DET) integration in promoting gene transfection to the affected cells *via* systemic administration, in which the loaded anti-angiogenic gene was markedly expressed in the tumor site, thereby imparting a significant inhibitory effect on the growth of vascular endothelial cells, ultimately leading to potent tumor growth suppression. PEG-PAsp(DET)/PAsp(DET) micelles with pDNA were also applied to a mice choroidal neovascularization (CNV) model.[47] Fluorescent microscopic observations and Western blotting analysis revealed that the expression of YFP was confirmed in the CNV area after an intravenous injection of PEG-PAsp(DET)/PAsp(DET) micelles; however, expression was not detected in mice that received naked pDNA or in those without CNV. Furthermore, the CNV area in mice that received an intravenous injection of sFlt-1 pDNA encapsulated polyplex micelles was significantly lower than that in control mice, which revealed the potential in gene therapy for regulating CNV.

Regarding systemic siRNA delivery into tumor tissues, stearoyl PEG-SS-PAsp(DET) block copolymers, in which disulfide linkage was cleaved responsive to the intracellular reductive milieu, and a flanking stearoyl moiety assisted stable encapsulation of siRNA.[48] The siRNA-incorporated polyplex micelles allowed significant silencing against cultured pancreatic cancer cells without considerable cytotoxicity, whereas such significant gene silencing was not observed in siRNA polyplex micelles without the disulfide linkage. This enhanced gene silencing activity may be because of the enhanced cellular uptake and subsequent translocation of siRNA into the cytoplasm facilitated by PEG detachment around and/or in the cancer cells. Furthermore, IVRTCLSM observations revealed the effect of hydrophobic stearoyl modification on the stabilization of the siRNA complex for longevity in the bloodstream. Significant *in vivo* gene silencing of the siRNA complex was achieved by the systemic injection of VEGF siRNA in mice bearing a subcutaneous pancreatic tumor, leading to a 40% regression in tumor growth. These results demonstrate the strong potential of stearoyl PEG-SS-PAsp(DET) as a vehicle for the systemic delivery of siRNA in cancer therapy.

4. CONCLUSION

Recent advances in polymeric micellar gene carriers by our groups were described in this review. Studies on polyplex micelles have been conducted for over ten years with significant progress. However, some problems still exist for *in vivo* applications and the development of systems available for clinical use has not yet been achieved. To satisfy this demand, the functions described here should become more sophisticated. In the near future, polymeric micellar gene carriers can be expected with further safety and functionality that will lead to successes in human gene therapy.

Acknowledgments I sincerely appreciate Professor Kazunori Kataoka at Tokyo University for his helpful guidance and suggestions. I also express my sincere thanks to my colleagues and collaborators for their support of my research. This research was supported by a Grant-in-Aid for Young Scientists (A) and Challenging Exploratory Research from the Japan Society for the Promotion of Science.

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