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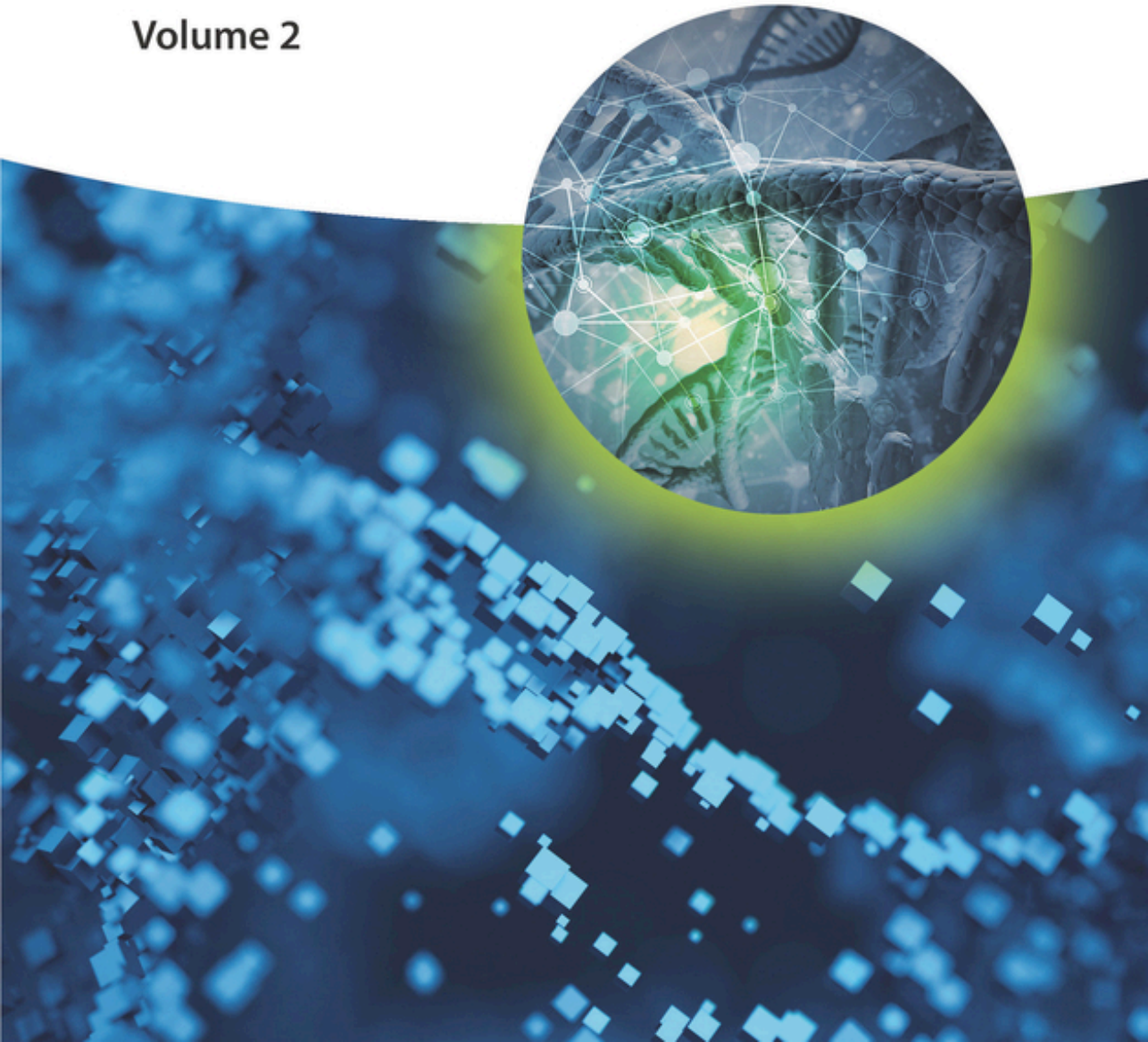
Molecular Technology

Life Innovation

Volume 2



Molecular Technology

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Contents

Foreword by Dr Hamaguchi

Molecular Technology is a newly developed research field supported through Japan Science and Technology Agency (JST) research funding programs. These programs aim to establish an innovative research field that harnesses the characteristics of molecules to enable new scientific and commercial applications. It is our great pleasure to publish this book, with the ambition that it will develop both an understanding of and further support for this new research field within the research and student community.

Molecular Technology as introduced in this book began in 2012 as a research area within JST's Strategic Basic Research Programs. JST is an advanced network-based research institution that promotes state-of-the-art R&D projects and leads the way in the cocreation of future innovation in tandem with wider society. JST develops a wide range of funding programs related to the promotion of scientific and technological innovation, which include strategy planning, target-driven basic research, and promotion of research and development.

Various research projects focused on Molecular Technology are currently underway within JST's Strategic Basic Research Programs:

- The team-based research program "CREST (Core Research for Evolutionary Science and Technology)"
- The individual research program "PRESTO (Precursory Research for Embryonic Science and Technology)."

Dr Yamamoto (CREST) and Dr Kato (PREST) manage the Molecular Technology Research Area as research supervisors.

In addition, JST's Strategic International Collaborative Research Program promotes research projects in the area of Molecular Technology, including ongoing cooperation with *L'Agence nationale de la recherche* (The French National Research Agency, ANR).

A wide range of researchers from young to senior across the fields from green science, life science, and energy are participating in successful research aimed at establishing the new field of Molecular Technology. They are already producing excellent research results, and it is our hope that these will develop into technologies capable of initiating a new era in energy, green, and life sciences.

I encourage you to read not only researchers in related fields but also look more broadly to researchers working in other fields. Inspired by this book, I look forward to emerging new research fields and seeds toward future innovation.

Michinari Hamaguchi
President, Japan Science and Technology Agency

Foreword by Dr Noyori

As an affiliated institution of the Japan Science and Technology Agency (JST), the Center for Research and Development Strategy (CRDS) navigates the latest global trends in science, technology, and innovation to aid the Japanese government in formulating its national strategies. *Molecular Technology* is the outcome of a research project born of a CRDS Strategic Proposal realized under the excellent editorial supervision of Hisashi Yamamoto and Takashi Kato. To them and to the scientists who have made major advances in molecular technology through their uninhibited research, I extend my heartfelt congratulations and respect.

The significance of molecular science in all areas of scientific endeavor is certain to increase. Accurate understanding of molecular assemblies and molecular complexes is essential for comprehending the elaborate workings of natural phenomena and of the genesis and mechanisms of materials and life functions. Now, more than ever, science must be seen as a single entity, a comprehensive whole. Mathematical science and the most advanced technologies of observation and information help us to explore the essence of materials and substances in a way that brings together all fields of science. It is the nature of molecular science to continually advance and expand. Using the metaphor of light, we can say that molecules behave in the manner of both "waves and particles."

The traditional separation of science into physics, chemistry, and biology no longer applies. Neither does it make any sense to maintain those seemingly self-contained subdivisions of organic chemistry, inorganic chemistry, physical chemistry, or polymer chemistry. So long as specialized groups and rigid educational systems cling to outdated perceptions, the more important it is to encourage an "antidisciplinary" type of science in which diverse fields converge rather than conventional interdisciplinary or transdisciplinary attempts to link diverse fields.

Molecular Technology, while firmly grounded in fundamental scientific knowledge, aims for practical applications within contemporary society. Johann Wolfgang von Goethe once said, "Knowing is not enough; we must apply. Willing is not enough; we must do." Technology with no practical application is meaningless to society. Researchers should not hesitate to set their own themes and topics of exploration in academia where self-determination holds strong and creativity wins the highest respect. Researchers must show ingenuity in the pursuit of their chosen mission even as they fulfill their duty to pursue science-based technology

for society. Never forget that it is by no means advisable to function purely as a support for activities that industry should actually undertake on its own.

The creative outcomes of the Molecular Technology Project launched in 2013 in conjunction with new collaborations are certain to lead to a wide range of innovations and to make significant contribution to achieving the Sustainable Development Goals (SDGs) of the United Nations' 2030 Agenda.

Science is one; and the world is one. Those who will follow us have a responsibility to the world after 2030, and it is my hope that new generations will pioneer revolutionary molecular technology that will bring science and humanity ever closer together. Brain circulation and international collaboration are essential to achieve these goals. V. S. Naipaul, winner of the 2001 Nobel Prize in Literature, once noted that knowing what you wanted to write was three-quarters of the task of writing. Humanity's future is to be found in the unbounded imagination of the young and in its ability to support the challenges they undertake.

December 2017

Ryoji Noyori
Tokyo, Japan

Preface

Chemical science enables us to qualitatively change exiting science and technology by purposefully designing and synthesizing molecules and creating the desired physical, chemical, and biological functions of materials and drugs at molecular level. In 2012, we started the big funding project in Japan, "Molecular Technology" (Establishment of Molecular Technology toward the Creation of New Functions (CREST) and Molecular Technology and New Functions (PRESTO)), and numerous research groups in Japan join the project of diverse research areas. All of these are typical transdisciplinary research projects between chemistry and various research areas of science and technology. In other words, Molecular Technology is the brand new scientific discipline. In principle, most of the proposed projects try to create the big bridge between chemistry and other basic science and technology. We thus propose a nice model for this bridge that is able to make valuable contribution for human welfares.

Between Japan Science and Technology Agency (JST) and French National Research Agency (ANR), we initiated a number of international collaboration projects of Molecular Technology in 2014. Since then 12 new collaboration projects started. The project provides quite unique collaboration opportunities between Japan and France, and quite active research groups involved in very close discussions of molecular technology between two countries. We are sure this project gave us close contacts between research groups of Japan and France for numerous discoveries. Overall, this international collaboration will be new entry for even more important discoveries in the future.

In 2016, we started the discussion for making a new and comprehensive book of molecular technology for the benefit of all researchers in the world to provide typical and leading examples of molecular technology. Overall, researchers of 15 CREST, 50 PRESTO, and 12 INTERNATIONAL groups have contributed to this book. Because of the wide areas of molecular technology, this book covers extremely diverse areas of science and technology from material to pharmaceuticals.

Hisashi Yamamoto
Chubu University, Supervisor of CREST

Takashi Kato
The University of Tokyo, Supervisor of PRESTO

1

Control of DNA Packaging by Block Catiomers for Systemic Gene Delivery System

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1.1 Introduction

DNA undergoes large volume transition from extended coil to compact state by polyion complexation with polycations for minimizing the contact surface area of the charge-neutralized polyplex from water [1–3]. The transition called DNA condensation is the essential mechanism of genomic DNA packaging and is the important process in preparing a nonviral gene delivery system [4–8]. The self-assembly formed from pDNA and block catiomers has been gaining attention as a potential systemic gene delivery system, in which the pDNA is condensed into a core by complexed with cationic block and the neutral blocks surround it as a shell to form a 100-nm-sized core–shell-structured polyplex micelle [9–12]. Polyplex micelles, launched from our group [13, 14], had been developed by the encouragement of the precedent development of polymeric micelles for drug delivery, which are currently under investigation of clinical trials [15, 16]. Originated from the firstly prepared polyplex micelles from PEG-*b*-P(Lys) [13, 17, 18], a variety of block catiomers or graft catiomers have been elaborated in order to improve the transfection efficiency by modulating parameters of their degree of polymerization (DP), grafting density for the case of graft catiomers, and varying mixing ratio with pDNA as described elsewhere in details [10, 17, 19, 20]. By these efforts, gene transfection efficiency has been remarkably promoted and a feasible formulation had proceeded to human clinical trial with local application [21, 22].

Nonetheless, development of polyplex micelles for systemic application has yet to be reached the level of clinical trial in spite of the structural analogy with polymeric micelles for drug delivery. This is mainly ascribed to the limited bioavailability of pDNA in active form at the final targeted nucleus; particularly, its instability in bloodstream precludes the secure delivery. To this end, the key issue that should be addressed is the packaging of pDNA into polyplex micelles because it regulates the basic character of polyplex micelles such as

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size, surface potential, stability, shape, and PEG crowding and thereby their biological performances such as blood circulation capacity, protection from nuclease attack, efficiencies of extravasation and migration into tissue, cellular entry efficiency, and transcription efficiency, all of which affect the ultimate gene expression efficiency. For achieving proper packaging, it is imperative to know the character of pDNA as a molecule and the principle mechanism of polyplex micelle formation so as to freely handle the structure. Moreover, it is necessary to know the suitable structure and the required functionalities to accommodate each step of delivery process. These processes should clearly point out the demanding issues for entirely managing the systemic gene delivery, which ultimately lead to a proper molecular design in structure and functionality to prepare polyplex micelles for achieving systemic gene therapy.

In this context, this review first focuses on the packaging of pDNA by block catiomers as the primal subject. Then, the required property and functionality for managing each of the delivery process are focused from intravenous (IV) injection to the last process of transcription. Finally, rational design criteria of block catiomers for systemic gene delivery are outlined.

1.2 Packaging of pDNA by Block Catiomers

It is important to first recognize the molecular character of pDNA for the sake of elucidating the mechanism of pDNA packaging. pDNA is a large molecule comprising typically a few kbp, which correspond to millions in molecular weight and a few micrometers in contour length, and has supercoiled closed circular form. DNA behaves as a semiflexible chain in solution with persistence length of 50 nm. Then, it is complexed with a large number of block catiomers for compensating the negative charges of pDNA, e.g. 200 block catiomers are required to compensate negative charges of pDNA of 5000 bp when block catiomers with 50 positive charges in their cationic segment are used. The formed polyplex micelles consist of single pDNA, wherein the concept of CAC is not defined as opposed to the polymeric micelles prepared from amphiphilic block copolymers, which are formed by association of multimolecules. Note that the single pDNA packaging is ensured as long as conducting the complexation at a diluted condition, which allows the accomplishment of the PEG shell formation before the collision of complexed pDNA to associate with neighboring complexed pDNA molecule due to translational motion. Otherwise, the secondary association occurs when the polyplex collision takes place faster than the formation of PEG shell, which is evidenced in the network-like complex formation by conducting the complexation exceeding the overlapping concentration of pDNA strands [23]. Polyplex micelles are characterized as approximately 100 nm particles by dynamic light scattering (DLS) and neutral zeta-potential value due to the charge-shielding effect by the PEG shell. When considering packaging of pDNA into polyplex micelles with respect to the aforementioned character of pDNA, several fundamental questions should rise: how the long pDNA changes its conformation within the characteristic topology and how

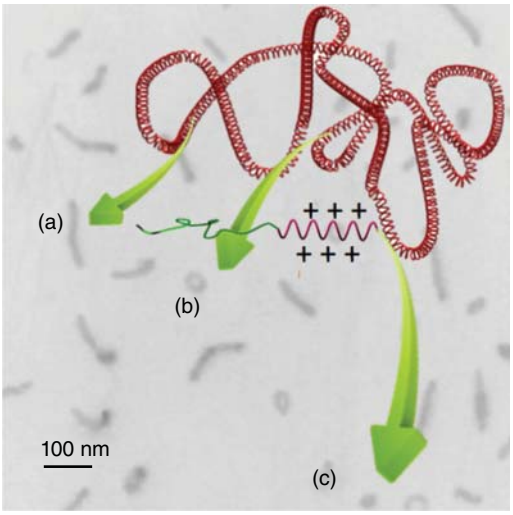

Figure 1.1 Packaging of pDNA within polyplex micelles by PEG_{12k}-$P(Lys)_{70}$ block catiomers observed by TEM. Various shapes are observed: (a) rod shape, (b) toroid shape, and (c) globular shape.

DNA accommodates its stiffness. To these questions, transmission electron microscopic (TEM) or AFM observations revealed that pDNA undergoes a variety of packaging to form structural polymorphism [24–31] such as rod shape, doughnut-like shape (toroid), and globular shape (Figure 1.1). This is actually intriguing with respect to the driving force of the DNA condensation because globular shape is the most expected shape for minimizing the surface area. The next section deals with the subject of pDNA packaging to address this question focusing on the rod shape and globular shape.

1.2.1 Rod-Shaped Packaging of pDNA

The rod shape is the most frequently observed shape among structural polymorphism. A specific folding scheme of pDNA was found from the study based on PEG-*b*-poly(L-lysine) [PEG-*b*-P(Lys)] polyplex micelles, named "quantized folding scheme." pDNA is folded by n-times (f_n) to form a rod shape consisting of $2(n+1)$ numbers of double-stranded DNA packed as a bundle in the orthogonal cross section (Figure 1.2b). Accordingly, the length is regulated to multiple of $1/[2(n+1)]$ of pDNA contour length as found in the discrete rod length distribution measured from TEM images (Figure 1.2a) [32]. This folding scheme has been observed in various polyplex micelles irrespective of the species of hydrophilic block and cationic block [30, 33] as well as the length of pDNAs; thus, it takes place independent of DNA sequences [34]. Another intriguing scheme is the relevancy with DNA rigidity; DNA is folded back at the rod ends, which is actually unacceptable assuming the persistence length of the double-stranded DNA (50 nm). However, this is made possible by local dissociation of double-stranded structure at the rod ends. The flexible nature of single-stranded DNA with persistence length of a few nanometers or less permits DNA to fold back. S1 nuclease, a single-stranded DNA-specific nuclease, could detect the occurrence of the double-stranded DNA dissociation,

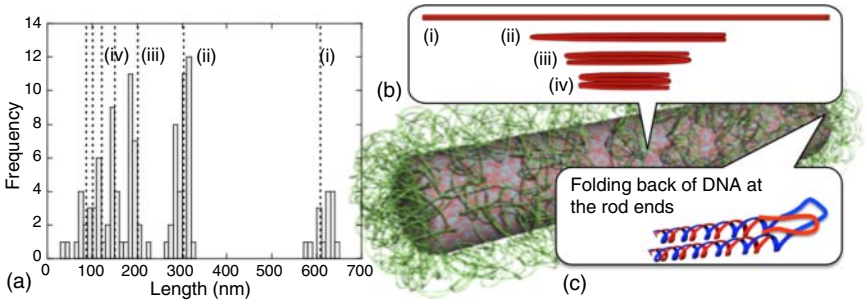

Figure 1.2 Quantized folding scheme of pDNA to form bundled structure within polyplex micelles. (a) Rod length distribution measured from TEM images. (b) DNA is folded to bundled rod within polyplex micelles. Folded pDNA (i)–(iv) in (b) corresponds to the rod lengths in (a). (c) Double-stranded structure of DNA at the rod ends is locally dissociated to single strand for folding back.

presenting a specific fragmentation pattern with lengths exactly corresponded to the multiples of the rod lengths [28, 32, 34].

The rod length, determined by f_n of pDNA, was changed by DP in P(Lys) block. The rod length shifted to short with increasing P(Lys) DP; major f_n was 1–5, 2–8, and 3–9 for polyplex micelles prepared from PEG_{12k}-*b*-P(Lys) with P(Lys) DP 19, 39, and 70, respectively [35]. This P(Lys) DP dependence is mechanistically accounted based on the PEG contribution. A quantitative analysis of the PEG crowding of polyplex micelles allowed for depicting the rod shape by the balances of free energies for DNA compaction ($dF_{\text{compaction,DNA}} = Gl\,dl - dE_{\text{surface}}$) and free energies for PEG repulsion ($dF_{\text{anti-compaction,PEG}} = \Pi(dV_{\text{occ,PEG}}) - T(dS_{\text{conf,PEG}})$). Here, G, l, E_{surface}, Π, $V_{\text{occ,PEG}}$, T, and $S_{\text{conf,PEG}}$ represent modulus of rigidity of bundled DNA core, rod length, surface energy developed on the core, PEG osmotic pressure, number-average occupied volume of PEG, temperature, and conformational entropy of PEG, respectively. Briefly, polyplex micelles prepared from lower P(Lys) DP retain more number of PEG in the shell because the associating number of block catiomer is inversely proportional to P(Lys) DP. Thus, those polyplex micelles are elongated by the increased PEG steric repulsion. The long rod structure costs higher interfacial free energy than the shorter rod; thus, the higher PEG crowding than the shorter rod balances it. Eventually, the long rod shape has the higher PEG crowding for accommodating the energetic balance. The consistent relevancy is observed between the rod length and analyzed PEG crowding. The PEG_{12k}-*b*-$P(Lys)_{70}$ polyplex micelles showing shorter rod length were analyzed to have so-called mushroom conformation from the estimated reduced tethering density (RTD) value of 2.6, whereas the PEG_{12k}-*b*-$P(Lys)_{20}$ showing longer rod length was analyzed to have upward squeezed conformation from the RTD value of 5.2 [35]. The cryo-TEM observation confirmed the consistent PEG height with those analyzed PEG conformation [35].

The energetic description in the rod shape is further examined by the investigation using PEG cleavable polyplex micelles prepared from PEG_{12k}-acetal-$P(Lys)_{19}$ block copolymers [36]. Upon incubating the polyplex micelles in acidic milieu

for releasing PEG blocks from the polyplex core, the originally formed rod shape had changed to globular shape, indicating that the presence of PEG sustained the rod shape from being globular shape. This study concomitantly revealed the contribution of rigidity of the bundled DNA in the rod shape. The rod shape maintained the lengths unchanged even when PEG had been continuously removed out from polyplex micelles representing the contribution of the bundled DNA rigidity to sustain the rod shape. The rod shapes had, however, collapsed into globular shapes when the synergistic contributions of PEG and DNA rigidity could not endure the request of DNA condensation.

1.2.2 Rod Shape or Globular Shape

It is found that pDNA is condensed into a globular shape by complexing with homo-catiomers [23, 37], which is actually the most expected shape following the request of DNA condensation. This fact indicates that the presence of PEG interferes the condensation into the globular shape, suggesting a possibility that the extent of PEG interference may regulate the condensation into rod shape or globular shape. This view is examined by modulating the PEG crowding on polyplex micelles prepared from PEG-*b*-P(Lys) block catiomers through changing the number of associated PEG on complexed pDNA attainable by changing DP of P(Lys) block or changing the PEG volume by changing the molecular weights of PEG block. Consequently, it was found that the PEG crowding covering pDNA in a pre-condensed state before undergoing condensation regulated the packaging pathways to form either structure. The rod shape was preferentially formed when the tethered PEG chains in a pre-condensed state were dense enough for overlapping one another, whereas the globular shape was preferentially formed when they were not overlapped [23]. In the PEG_{12k}-*b*-P(Lys) block catiomers, the globular shape becomes predominant when P(Lys) DP was higher than 100. It should be noted that DNA double-stranded structure is impaired to dissociation in the globular shape as evidenced by the S1 nuclease assay so that DNA can accommodate the rigidity issue [23].

These mechanistic studies have provided a general scheme to understand structures of polyplex micelles prepared from various block or graft catiomers. For example, change in rod length was observed for polyplex micelles prepared form PEG-*g*-cysteine-$P(Lys)_{30}$ with fixed P(Lys) DP and varied PEG molecular weight [29]. This can be interpreted that the increased PEG crowding on polyplex micelles by the increased PEG molecular weight eventually elongated the rod length. The rod-shaped polyplex micelles prepared from poly[2-(methacryloyloxy)ethylphosphorylcholine]$_{30}$-*b*-poly(dimethylaminoethyl methacrylate), $PMPC_{30}$-*b*-PDMAEMA, decreased their rod length by increasing DP of PDMAEMA segment from 10 to 40 [24]. This trend is consistent with the increase in P(Lys) DP in the PEG-P(Lys) polyplex micelles and is understood that the increased DP of PDMAEMA segment decreases the number of associating PMPC chains and results in a decrease in the PMPC crowding, thereby permitting polyplex core to take shorter rod. The polyplex micelles changed the predominant shape from rods to globules by further increasing DP of PDMAEMA up to 60, which is again the same trend with the observations

for PEG-P(Lys) polyplex micelles. Globular shape was also found in the polyplex micelles formed from PEG_{3k}-*b*-$DMAEMA_{100}$ [38] and PEG_{2k}-*b*-$DMAEMA_{37}$ [39]. The formation of these globular shapes are understood by the fact that the decreased number of PMPC chains or lowered PEG molecular weight permitted pDNA to undergo globular collapsing instead of rod folding. The variation of structures was also found by modulating solvent polarity for complexation. Polyplex micelles of PEG_{10k}-*b*-polyphosphoramidate (PPA) formed long rod shapes by preparing in pure water. This was changed to shorter rod shape and ultimately changed to globular shape by increasing DMF fraction in the DMF/water cosolvent [40]. This could be presumed that the change of solvent polarity changed the energetic balance for the condensation and anti-condensation, leading to such structural change.

In this way, these studies have provided potential answers to the fundamental questions concerning the conformational change of DNA strands and the rigidity of DNA upon condensation and also the rod-shaped formation instead of the globular shape.

1.3 Polyplex Micelles as a Systemic Gene Delivery System

After understanding the basic structural characters, it is important to know the suitable structure and the required functionalities to accommodate each step of delivery process so that one could identify demanding issues for entirely managing systemic gene delivery. This section focuses such issues starting from a subject of stable encapsulation as the basic requirements for circulation, cellular entry, endosome escape, nuclear translocation, and ultimate transfection efficiency.

1.3.1 Stable Encapsulation of pDNA Within Polyplex Micelles for Systemic Delivery

Polyplex micelles need to overcome various biological barriers as depicted in Figure 1.3, whereby they confront a variety of biological components that preclude their systemic delivery such as nucleases, negative-charged substances to cause disassembly of polyplex through polyion exchange reaction, and inherent biological defense system. This section describes the required structure and functionality against these obstacles.

Nuclease attack gives fatal impact for gene delivery because pDNA impairs the ability of gene expression even permitting one site cleavage along the long DNA strands. It is acknowledged that the complexed pDNA attains tolerability against nuclease attack and the PEG shell coverage further improves tolerability [12, 14, 18, 34]. However, pDNA was still ultimately digested even within polyplex micelles with elevated PEG crowding during systemic circulation [35]. Thus, a strategy to physically block the access of nucleases was considered. It is thought that such polyplex micelles may be prepared from block copolymers retaining hydrophobic segment serving for blocking layer; however, the

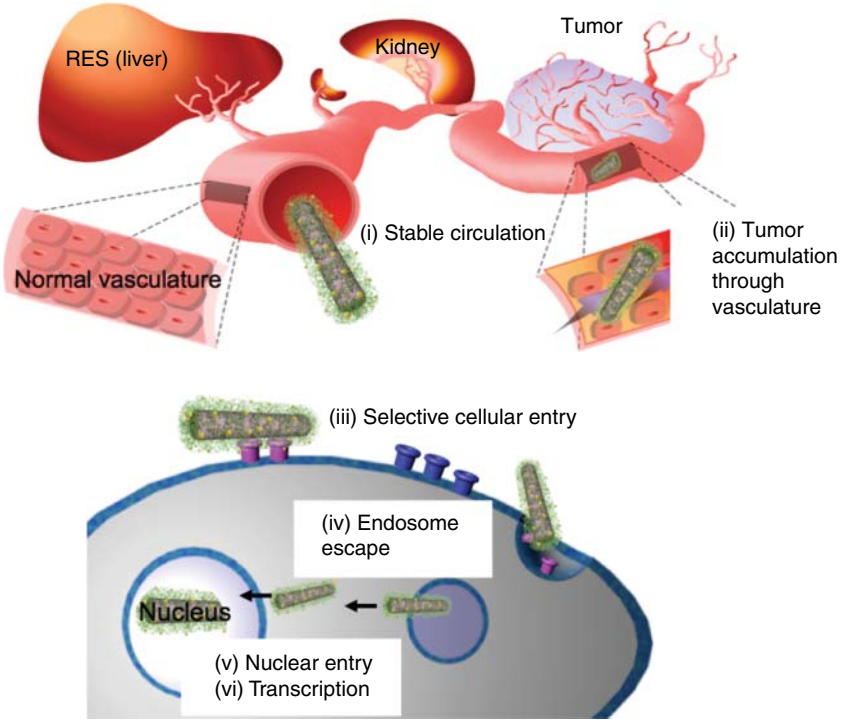

Figure 1.3 Schematic illustration of systemic gene delivery processes.

hydrophobic segment may spontaneously cause interpolymer association before complexation with pDNA, giving rise to interference in the smooth polyion complexation. This problem is smartly solved by use of thermoresponsive polymers, which behave as a hydrophilic chain in the solution below the lower critical solution temperature (LCST) while it behave as a hydrophobic chain in the solution above the LCST. Thus, a triblock copolymer consisting of hydrophilic poly(2-ethyl-2-oxazoline), thermoresponsible poly-(2-*n*-propyl-2-oxazoline) (PnPrOx), and cationic P(Lys) was designed noticing its lower LCST(25 °C) than the body temperature. A two-step procedure, mixing the triblock copolymers with pDNA below the LCST of PnPrOx followed by incubation above the LCST (37 °C), allowed for collapsing of the PnPrOx segment positioning in the middle layer of polyplex micelles [30]. The polyplex micelles exhibited significant tolerance against nuclease attacks over the control polyplex micelles without the protective layer, presenting great utility of this strategy for preventing the nuclease access. Such double-protective-layered polyplex micelles were also prepared from diblock catiomers but from a set of diblock catiomers each having hydrophilic block and thermoresponsive block. These were prepared by complexing pDNA with a mixed block catiomers of PEG-*b*-poly{*N*′-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide} {PEG-*b*-P[Asp(DET)]} and poly(*N*-isopropylacrylamide)-*b*-P(Asp(DET)) (PNIPAM-*b*-P(Asp(DET)) at

room temperature, which is below the LCST of PNIPAM, for the formation of polyplex micelles with hydrophilic shell of PEG and PNIPAM, followed by incubation at 37 °C for collapsing the PNIPAM segment to prepare hydrophobic palisade. The polyplex micelles also exhibited significant improvement in nuclease tolerability compared with those without hydrophobic palisade [31].

Association with negatively charged substances is another critical factor for precluding the bioavailability of polyplex micelles because it causes disintegration of polyplex micelles through polyion exchange reaction. Note that this is a common problem in most of the nucleic acid delivery systems prepared by electrostatic assembly [41, 42]. Typically, glycosaminoglycans (GAGs), negatively charged polysaccharides, including chondroitin sulfate (HS) and heparan sulfate (HS), are addressed as such substances. These molecules are abundantly presenting in the cell membrane, including vascular endothelial cells and blood cells, and in the glomerular basement membrane in kidney; therefore, polyplex micelles inevitably encounter them during the delivery process, particularly during cellular entry and blood circulation. In this view, strategies of stabilization are imperative in addition to the inherent electrostatic interaction, although overstabilization impairs the transcription process. This trade-off can be addressed by core cross-linking with redox-responsive disulfide linkage because it allows for maintaining the linkage in extracellular entity including bloodstream, while it is cleaved in the intracellular reductive environment [25, 43–45]. Photocleavable cross-linkers and pH-responsive cross-linkers are also feasible reversible cross-linkers to manage the trade-off. Phenylboronic acids (PBA) are a recently highlighted cross-linker, worked by dual stimuli of pH and ATP concentration [46]. An installation of hydrophobic group into the core compartment is another effective strategy to prevent disintegration through its coagulation force, although it has no reversibility [47–49]. DNA intercalators, such as acridine, were also considered to prevent the dissociation, although the no reversibility and the inherent carcinogenic property remain as a concern [50]. The aforementioned hydrophobic barrier compartment can also improve the stability due to its blocking capacity against the access of GAGs to the polyplex core [30, 31]. Polyplex micelles modified with these strategies eventually promote longevity in blood circulation as well as later-described cellular uptake efficiency.

A property to prevent adsorption of biological compounds present in blood is important for ensuring prolonged blood circulation aside of the stabilization. Nonspecific interaction causes the formation of aggregates, which readily results in embolization of capillary. Furthermore, adsorption of serum proteins triggers the elimination by mononuclear phagocyte system (MPS) [51]. It is widely acknowledged that PEGylation affords this property onto nanocarriers [11, 12, 52–54]. The significance of the PEG shell is indeed visualized by the *in situ* observation using the intravital real-time CLSM (IVRTCLSM) [55]. Polyplexes prepared from non-PEG catiomers immediately caused the formation of distinct aggregates after intravenous injection, whereas their PEGylated micelle formulation was observed to circulate without such aggregate formation. To prevent the adsorption of serum proteins, it is important to consider PEG crowding of polyplex micelles because it determines the extent of serum

protein adsorption. It is reported that inhibition of the protein adsorption on PEG-modified surface started when PEG chains were crowded for overlapping with neighboring chains and maximized when the overlapped PEG chains were substantially crowded ($\langle L \rangle / 2R_g < 0.48$; L indicates distance between tethering PEG sites) [51]. In this regard, PEG crowding of polyplex micelles prepared from PEG_{12k}-P(Lys) block catiomers was evaluated, giving $\langle L \rangle / 2R_g = 0.39$, 0.47, and 0.55 for polyplex micelles of $P(Lys)_{19}$, $P(Lys)_{39}$, and $P(Lys)_{70}$, respectively [35]. The PEG crowding suggested that polyplex micelles with $P(Lys)_{19}$ and $P(Lys)_{39}$ may have higher chance to escape the elimination mode by MPS, whereas those with $P(Lys)_{70}$ may be captured. The evaluation using IVRTCLSM consistently proved the projection because the blood circulation profiles were improved in the following order: $P(Lys)_{70} < P(Lys)_{39} < P(Lys)_{19}$ [35]. Notably, the profiles showed that a certain fraction had been eliminated from the bloodstream at the early circulating stage for polyplex micelles of $P(Lys)_{70}$. It should be further noted that polyplex micelles prepared from PEG_{20k}-$P(Asp(DET))_{60}$-Chole were analyzed to have much high PEG crowding with scalable brush conformation and exhibited a profile suggesting successful escape from the early elimination mode [48]. These observations demonstrate that the PEGylation can indeed serve for prolonging the blood circulation, but the crowding is essential for escaping the early elimination mode.

In addition, a crucial impact of shear stress was recently indicated in the capacity of blood circulation. It was demonstrated that the shear stress removed block catiomers from polyplex micelles when they were exposed at magnitudes in the bloodstream resulted in structural deterioration, which eventually led to accelerated degradation by nucleases. Instead, installation of the core cross-linking prevented the structural deterioration and remarkably improved the blood circulation profile [56]. Thus, it is reasonable to assume that the stabilization by either means of cross-linking, hydrophobic moieties or intercalators, can improve the tolerability against shear stress and thereby improve the blood circulation [25, 47, 50, 57, 58].

1.3.2 Polyplex Micelles for Efficient Cellular Entry

Polyplex micelles are intrinsically unfavorable for cellular entry because the presence of PEG shell prevents association with cell surface; particularly, an attempt to elevate PEG crowding for improved shielding further impairs their interaction with cells. Moreover, as opposed to positively charged non-PEG polyplexes, polyplex micelles with neutral zeta-potential do not have strong interaction with negatively charged cells. Furthermore, GAGs on cellular membrane cause destabilization of polyplexes. Thus, the installation of stabilizing functionality as described in Section 1.3.1 can promote their cellular uptake efficiency [25, 47–49, 59]. Ligand molecules can also promote the cellular uptake efficiency for which transferrin [11], peptides such as cRGD [52] or peptides for EGFR [60], sugars, aptamers, and antibodies [11, 61–63] are often used. In the choice of ligands, it is important to consider not only the efficiency but also the specificity to distinguish the targeted cells from nontargeted cells in order for avoiding false delivery. Also, the ligand molecules should not compromise the stealth

effect of the carriers. In this sense, cell-penetrating peptides (CPP) [64–68] may not be suitable for systemic application due to their limited cell specificity and their positive charges cause nonspecific interaction with biological molecules, although the strong potency to promote cellular entry is attractive. After choice of appropriate ligands, it is important to consider the ligand density on polyplex micelles for obtaining maximized efficacy. The low ligand density may not suffice to ensure the multivalent binding with their receptors, whereas the high density may impair the stealth character of the carriers. Moreover, the strong multivalent binding with receptors may not allow the captured polyplex micelles release to the other side, resulting in the decreased efficiency of transcytosis [69]. It is also important to consider the mobility of the attached ligand molecules on the polyplex micelles for providing a chance to bind with receptors [70]. PEG cleavage is an interesting strategy to tackle the so-called PEG dilemma. Disulfide bond was installed between PEG and catiomer to expect PEG cleavage at the extracellular space [71–75]. This strategy could be effective in local applications although the nonspecificity may be not suite for systemic application. In this respect, peptides susceptible to matrix metalloproteinase (MMP), presenting rich in tumor, may be useful [76, 77].

In addition, it is addressed that the shape and size are the parameters to determine cellular uptake [78]; e.g. inhibited cellular uptake was observed for filamentous nanoparticles [79–81]. Nonetheless, it is still controversial because different nanoparticles with different surface properties are used in order to change their sizes and shapes. To this issue, polyplex micelles of PEG_{21k}-P(Lys)(SH) revealed a significant effect of rod length on cellular uptake based on their capacity to change the rod length but maintaining their surface properties including PEG crowding and zeta-potential. Note that the polyplex micelles were installed with disulfide cross-linking to address the GAG-mediated destabilization upon cellular entry. This study uncovers the critical rod length of 200 nm for efficient uptake [59]. This was reasonably explained by the upper limit sizes of endocytotic vesicles, which were evaluated as 5 μm [82], 200 nm [83, 84], 80 nm [85], and 90 nm [86] for macropinocytosis, clathrin-dependent, caveolae-dependent, and clathrin/caveolae-independent endocytosis, respectively. Thus, polyplex micelles with rod length fractions below 200 nm can be taken up by any of these four endocytotic pathways, whereas that rod length fraction above 200 nm can be taken up only by macropinocytosis. The rod length limitation was observed in various cell lines including HeLa, BxPC3 cells, and HUVECs in the examined PEG-P(Lys) polyplex micelles as well as in other polyplex micelles prepared from PMPC-*b*-PDMAEMA block catiomers against A549 cells [24] and PEG-*b*-PPA against HEK 293 cells [40]. Importantly, the modifications of cRGD peptide on PEG-*b*-P(Lys) polyplex micelles could remarkably increase the cellular uptake efficiency for the polyplex micelles, which satisfied the rod length limitation, i.e. PEG-*b*-$P(Lys)_{42}$ and PEG-*b*-$P(Lys)_{69}$, but not for polyplex micelles with rod length above the 200 nm, i.e. PEG-*b*-$P(Lys)_{20}$, indicating the significance of rod length limitation.