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Review

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Multifunctional Envelope-Type Nano Device: Evolution from Nonselective to Active Targeting System

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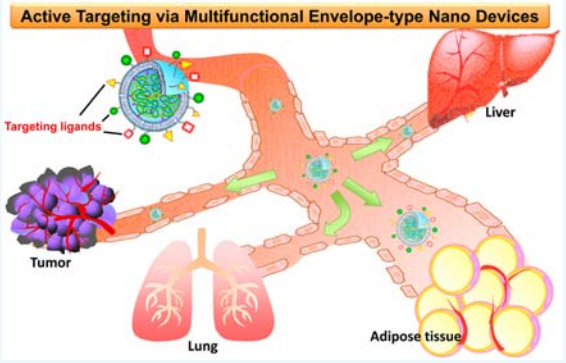
ABSTRACT: A paradigm shift has occurred in the field of drug delivery systems (DDS), one being intracellular targeting, and the other, active targeting. An important aspect of intracellular targeting involves delivering nucleic acids such as siRNA/pDNA rather than small molecular compounds, since the mechanism responsible for their entering a target cell is usually via endocytosis, and the efficiency of endosomal escape is a critical factor in determining the functional activities of siRNA/pDNA. A multifunctional envelope-type nano device (MEND) was developed to control the intracellular trafficking of nano carriers containing siRNA/pDNA. An octaarginine (R8) modified MEND was developed to achieve this. Considerable progress has been made in active targeting to selective tissue vasculature such as tumor, adipose tissue, and the lung where endothelial barrier is tight against nanoparticles with diameters larger than 50 nm. A dual-ligand system is proposed to enhance active targeting ability by virtue of a synergistic interaction between a selective ligand and a cell penetrating ligand. Prohibitin targeted nanoparticles (PTNP) were developed to target endothelial cells in adipose tissue, which deliver apoptotic peptides/proteins to the adipose vasculature. Lung endothelial cells can be targeted by means of the GALA peptide, which is usually used to enhance endosomal escape. These active targeting systems can induce pharmacological effects in in vivo conditions. Finally, a novel strategy for producing an original ligand has been developed, especially for the tumor vasculature. This progress in DDS promises to extend the area of nanomedicine as a breakthrough technology.



1. NEW PACKAGING CONCEPT

A paradigm shift has occurred in the field of drug discovery and development, the molecular size of drugs expanded from small size to macromolecules including proteins and nucleic acids. This change necessitates the introduction of innovative drug delivery systems (DDS) to achieve successful new therapy for unmet medical needs. In this process, a breakthrough technology which can control intracellular trafficking as well as biodistribution will become an essential tool for successful nanomedicine. Recently, we developed a new delivery system, a multifunctional envelope-type nano device (MEND) based on a new packaging concept called "Programmed Packaging". In this delivery system multifunctional nano devices are integrated into a nano carrier system using a strategy designed to overcome all barriers during biodistribution, intracellular trafficking, and bioactivity.[1]

The main requirement for successful nanomedicine, especially gene therapy, is that the therapeutic nucleic acids must reach their intracellular target sites such as nucleus or cytosol after internalization via endocytosis, because the DNA (or RNA) molecules are hydrophilic, negatively charged, and high molecular weight; therefore, it is difficult for them to diffuse through the plasma membrane. We recently developed a multifunctional envelope-type nano device (MEND) based on a new packaging concept called "Programmed Packaging". Using MEND, various functional devices are packaged into a single nanoparticle permitting them to function at the appropriate location and time. The new packaging concept consists of three components: (1) a program to overcome all barriers; (2) the design of functional devices and their three-dimensional arrangement; and (3) a nanotechnology approach that permits all of the devices to be assembled into a nanosize structure. The ultimate MEND consists of a condensed plasmid DNA (pDNA) core and a lipid envelope structure equipped with the various functional devices. To date, we have been successful in efficiently packaging not only pDNA, but also oligo nucleic acids and proteins into a MEND.[2] In this chapter, we will discuss how to control the intracellular trafficking of functional nucleic acids such as pDNA as well as siRNA using octaarginine (R8) as a nonselective but highly efficient ligand. Then, we will introduce new active targeting strategies such as the dual ligand system for tumor endothelial cells as well as active targeting system for lung or adipose tissue.

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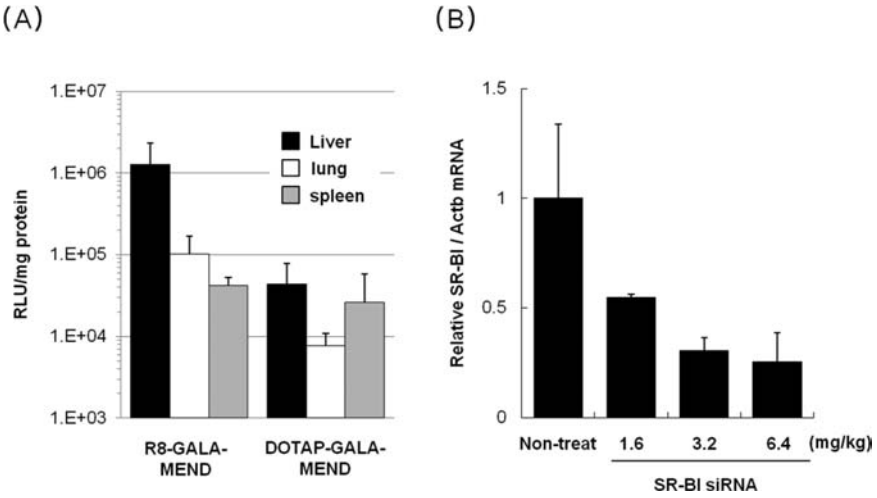

Figure 1. Hepatic pDNA and siRNA delivery with R8-GALA-MEND. (A) Luciferase expression activities in liver, lungs, and spleen in balb/c mice. 50 μg of luciferase encoded pDNA loaded with R8-GALA-MEND or DOTAP-GALA-MEND were treated via the tail vein and assayed 6 h after injection. Values are the mean ± SD (n = 3). (B) Gene silencing activities of target gene in C57BL/6 mice at different doses. SR-BI mRNA levels in the liver was measured 24 h after the intravenous injection. Values are the mean ± SD (n = 3). Reprinted with permission from (A) *J. Controlled Release* **20**, *156* (3), 374−80 (2011) and (B) *Int. J. Pharm.* **419**, *1*−*2*, 308−13 (2011). Copyright 2015 Elsevier.

These strategies for subcellular and cell type-specific targeting combined with a respiratory route of application tools can be used to specifically deliver drugs into various cells present in the alveolar region (or lungs), making it possible to devise a highly specific and effective treatment for respiratory illnesses.

2. R8-MEND FOR IN VIVO DELIVERY

2.1. Properties of the Octaarginine (R8) Peptide. Cell penetrating peptides (CPPs) are short peptides that transport many different types of cargo, such as small compounds, nucleic acids, and mid- and large-sized molecules across plasma membrane, which have great potential for in vitro and in vivo delivery vectors. CPPs typically have an amino acid composition that either contains positively charged amino acids, such as lysine or arginine, or has some sequences that change pattern of polar/charged amino acids in response to pH. In particular, octaarginine (R8), a synthetic peptide, is one of the well-known peptides for achieving efficient intracellular delivery.[3] The peptide plays an important role in the cellular uptake mechanism as well as in intracellular trafficking. A high density of stearylated R8 modified liposomes (R8-liposomes) are taken up mainly through macropinocytosis, where they are less susceptible to lysosomal degradation, and leads to high gene expression in vitro.[4] Another interesting property of the R8-liposomes is the ability to escape endocytic vesicles at both neutral and acidic pH,[5] suggesting that the R8 peptide has promise for intracellular delivery.

Table 1. Relative Value of Nonlinearity between in Vitro and in Vivo in Each Category[a]

	in vitro (serum−)	in vitro (serum+)	in vivo
PK	1	1.4	88
Intracellular PK	1	1.5	1.2
PD	1	1	1.2

[a]Relative nonlinearities were estimated by values such as siRNA molecules per cell, input dose, and gene silencing activity. Reprinted with permission from *J. Controlled Release* **161**, 3, 757−762 (2012). Copyright 2015 Elsevier.

2.2. R8-MEND for pDNA and siRNA Delivery to the Liver. In our initial studies, we focused on the design of a gene delivery system. Contrary to our expectations, the gene expression activity of the R8-MEND loaded with pDNA encoding luciferase in the liver was very low (2×10^2 RLU/mg protein) via tail injection. This can be explained by inadequate intracellular trafficking. Next, GALA, a pH-sensitive fusogenic peptide,[6] was modified on the surface of R8-MEND to increase gene expression activity in the mouse liver. When they are administered via tail vein, its activity was dramatically increased (1640-fold), but we found that property of pDNA condensed particles inside GALA modified R8-liposome is a critical factor. We used PEI (polyethylenimine) as a condenser of pDNA, which is a positively charged polymer with endosome-escaping function. The gene expression activity was maximum when the R8-MEND was loaded with negatively charged pDNA/PEI condensed particles (R8-MEND$_{NCP}$) (Particle size, 147 ± 13 nm; and Lipid composition, STR-R8:DOPE = 2:9), but not with positively charged pDNA/PEI condensed particles (R8-MEND$_{PCP}$) (Particle size, 210 ± 17 nm; and Lipid composition, STR-R8:DOPE:CHEMS = 2:7:3). The number of pDNA molecules delivered to liver cells and their nuclei for the R8-MEND$_{NCP}$ and R8-GALA-MEND$_{PCP}$ was comparable indicating that the substantial improvement in gene expression can be explained by an improved gene expression efficiency per pDNA in the presence of GALA. This result suggests that GALA could render more efficient assistance in decoating the lipid envelope or the decondensation from polycations, even though the general belief is that the GALA's effect is mainly related to facilitating endosomal escape and subsequent nuclear delivery. The use of the optimized R8-GALA-MEND$_{NCP}$ (Particle size, 181 ± 5 nm; and Lipid composition, STR-R8:DOPE:Cho-GALA = 2:8.5:0.5) resulted in a 3350-fold increase in liver luciferase gene expression (1.3×10^6 RLU/mg protein) compared with that of the R8-MEND$_{NCP}$. In addition, the liver gene expression of the R8-GALA-MEND$_{NCP}$ was 29 times higher than that of DOTAP-GALA-MEND, in which a cationic lipid is commonly used in the drug delivery research

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field. Furthermore, their gene expression activity in the liver was higher than that of the lungs (13-fold) and spleen (31-fold) (Figure 1A).[7] We next examined the pharmacological effect against lipopolysaccharide/D-galactosamine (LPS/D-GalN) induced acute liver injury using the R8-GALA-MEND$_{NCP}$ loaded with hepatocyte growth factor (HGF) pDNA. HGF was originally found in the serum, and promotes hepatocyte growth and DNA synthesis, which improves liver injury in this model. Pretreatment with R8-GALA-MEND$_{NCP}$ resulted in a reduction in elevated ALT and AST levels, which are indicators of liver toxicity. A similar tendency was observed in the case of in vivo-jetPEI-Gal, a commercially available in vivo liver transfection reagent; however, the survival rate of the mice was increased significantly in the case of R8-GALA-MEND$_{NCP}$, but not in the case of the in vivo-jetPEI-Gal.[8] These results demonstrate that the R8-GALA-MEND$_{NCP}$ is a useful gene delivery system for the treatment of acute liver injury.

Second, we applied the optimized in vivo gene delivery system of the R8-GALA-MEND$_{NCP}$ to systemic siRNA delivery. The R8-GALA-MEND loaded with target siRNA (SR-BI; a scavenger receptor class B, member 1) (particle size, 162 ± 8 nm; and lipid composition, STR-R8:DOPE:Cho-GALA = 18:77.5:4.5) induced a significant reduction in SR-BI mRNA in a dose dependent manner in primary hepatocytes. Confocal microscopic analyses showed that only a small fraction of the siRNA was colocalized with the lysosomal compartment, indicating that the R8-GALA-MEND can be used as a delivery system of siRNA to the cytoplasm. When R8-GALA-MENDs loaded with SR-BI siRNA were injected via the tail vein, a dose-dependent reduction in SR-BI mRNA in the liver was observed (Figure 1B). In contrast, no significantly detectable reduction was observed with buffer or R8-GALA-MENDs loaded with luc siRNA, indicating that the target mRNA had been selectively degraded. ALT and AST and inflammatory cytokines were all in the normal range 1 day after the single injection of a maximum dose of R8-GALA-MENDs loaded with siRNA (6.4 mg/kg), compared with HEPES buffer injection controls, even though there was slight increase in both ALT and AST values.[9]

2.3. Pharmacokinetics (PK) and Pharmacodynamics (PD) Analyses with R8-MEND. Last, we analyzed the intracellular pharmacokinetics (PK) and pharmacodynamics (PD) properties of the R8-GALA-MEND in vitro and in vivo from the viewpoint of dose-dependency in order to rationally develop an efficient siRNA delivery system for the purposes of clinical testing. We divided them into three categories: PK, intracellular PK, and PD. The PK defines a biodistribution of nanoparticles before reaching cells or tissues. The intracellular PK, including cellular uptake, endosomal escape, and decoating and decondensation from the lipid envelope, can have a substantial effect on the efficiency of siRNA delivery. On the other hand, the PD, a relationship between the amount of siRNA in cytoplasm and its silencing effect, shows how the siRNAs delivered to the cytoplasm were effectively used. A quantitative study of siRNA molecules revealed that 5.0×10^4 and 4.4×10^4 siRNA molecules per cell were required to achieve an ED_{50} for SR-BI gene silencing in both cultured cells and the mouse liver, demonstrating that there was no significant difference in the efficiency in PD. Furthermore, siRNA degradation profile was similar in both cultured cells and the mouse liver. However, a remarkable nonlinearity was observed in PK as shown in Table 1,[10] indicating that the percentage of siRNA amount detected in the mouse liver was drastically decreased as the treatment dose was decreased. These results demonstrated that the PK is a causative factor in the huge gap between the in vitro and in vivo situations, and these findings provide a promising clue to achieving a more efficient CPP-mediated in vivo siRNA delivery at a lower dose.

3. DUAL-LIGAND BASED LIPOSOMES FOR TARGETING TUMOR ENDOTHELIAL CELLS (TECS)

3.1. The Concept of a Dual-Ligand for Active Targeting. When we develop a system to target tumor tissue

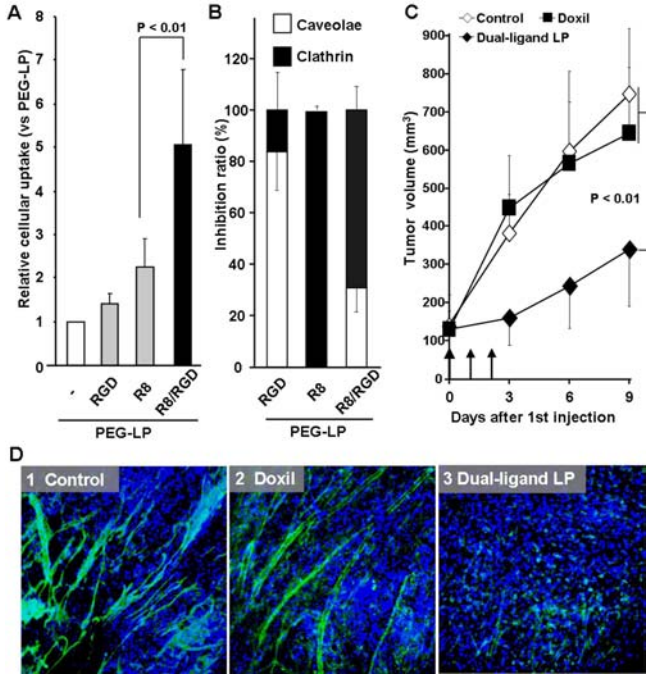

Figure 2. In vitro and in vivo evaluation of a dual-ligand LP. (A) Cellular uptake of LPs. Different formulations of PEG-LPs were incubated with HUVEC cells for 3 h. Cellular uptake is expressed as the mean ± SD. (B) Percent inhibition of cellular uptake of PEG-LPs in the presence of specific inhibitors, sucrose (clathrin), and fillipin (caveolae) in HUVEC cells. The relative cellular uptake is expressed as the percentage uptake in the presence of the inhibitors. (C) Comparison of tumor growth by a dual-ligand LP with Doxil. PBS or LPs containing 1.5 mg/kg of DXR were i.v.-injected on days 0, 1, and 2. Tumor volume was monitored at indicated times. (D) Effect of cytotoxicity on blood vessels. After treatment of PBS or LPs, TECs and nucleus in unfixed tissues were stained and are shown as green and blue. Images were captured by a confocal microscopy. . Reprinted with permission from *J. Controlled Release* **153**, 2,141−148 (2011) and *J. Controlled Release* **162**, *1*, 225−232 (2012). Copyright 2015 Elsevier.

based on passive targeting,[11] the long circulating property is very important by modification with poly(ethylene glycol) (PEG). In the case of active targeting, selective and efficient targeting is more important than long circulating property. Tumor targeting PEGylated liposomes have been developed by attaching specific targeting ligands to the distal end of the PEG chain.[12] Although it is possible to achieve active targeting via the use of specific ligands, receptor mediated endocytosis is a saturable pathway, which restricts the amount of liposomes available for cellular uptake. On the other hand, CPPs are widely utilized for the delivery of drugs and genes.[13] CPPs are capable of entering cells efficiently, either alone or linked to not only small molecules, but also bulky cargos such as peptides,

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proteins, oligonucleotides, pDNA, and liposomes.[13,14] With both specific ligand-mediated active targeting and CCP-mediated efficient intracellular delivery taken into consideration, a rational strategy designed to take advantage of a combination of both specific ligands and CPPs could allow PEGylated liposomes to be used as a more selective and efficient in vivo systemic application. To accomplish this, we developed a dual-ligand system, in which specific ligand-modified PEGylated liposomes are combined with cationic ligands, such as CPPs.[15−17] In the dual-ligand system, CPPs are masked by PEG and, as a result, are unfunctional and opsonin-free in the systemic circulation. After their arrival at the target site, cellular association via specific ligands permits the CPPs to exert their powerful ability to internalize the liposomes into target cells due to the close proximity of the liposomes to the surface of the target cells. In this section, we describe the application of dual-ligand liposomes for active targeting to tumor endothelial cells (TECs), both in vitro and in vivo.

3.2. Dual-Ligand Liposomes for Targeting TECs in Vitro. It has been reported that Arg-Gly-Asp (RGD) or Asn-Gly-Arg (NGR) motif peptides are able to recognize Integrin $\alpha v\beta 3$ and amiopeptidase N (CD13), which are overexpressed in angiogenic blood vessels.[18] Therefore, we employed the NGR and RGD motif peptides as specific ligands for the neovasculature, and oligoarginine (R4 and R8) as a CPP ligand.[15,16] PEGylated liposomes were prepared by hydration method and diameter of the liposome was controlled around 100 nm by extrusion. The single modification of PEGylated liposome (PEG-LP) with either a RGD motif on the surface of the PEG (RGD-PEG-LP) or R8 on the surface of a liposome (R8/PEG-LP) resulted in a 1.4-fold or 2.2-fold enhancement in cellular uptake compared to PEG-LP in Integrin $\alpha v\beta 3$ expressing HUVEC (Figure 2A). On the other hand, an approximately 5.1-fold cellular uptake was observed for a PEG-LP that was dually modified with RGD and R8 (R8/RGD-PEG-LP). However, in integrin $\alpha v\beta 3$ negative cells, no synergistic effect was observed on cellular uptake.[16] To determine the mechanism for the uptake of dual-ligand liposomes, cellular uptake was evaluated in the presence of specific inhibitors. As shown in Figure 2B, R8/PEG-LP was taken up via the clathrin-mediated endocytosis pathway which was induced by R8, and RGD-PEG-LP was taken up mainly by caveolae-mediated endocytosis triggered by RGD motif. Interestingly, it was observed that the internalization of R8/RGD-PEG-LP was predominantly governed by the clathrin-mediated endocytosis pathway, which allows it to be efficiently internalized by cells by R8.

3.3. Large-Sized Liposomes (300 nm in Diameter) For a Dual-Ligand System Targeting in Vivo TECs. Chemotherapeutic resistance in tumor cells, including renal cell carcinomas (RCCs), is a serious obstacle in cancer therapy. Although RCC patients suffer from severe side effects such as gastrointestinal perforation, new classes of drugs, such as sunitinib, sorafenib, or bevacizumab, designed to target specific molecules that are related to the angiogenesis process, such as vascular endothelial growth factor (VEGF) and VEGF receptors (VEGFRs), have been indicated for the treatment of RCC.[19] Further increases in therapeutic activity and fewer side effects could be achieved by specifically targeting the neovasculature with nanomedicines.

We hypothesized that a large size would be more advantageous for targeting TECs than a small size by preventing the liposomes from extravasation to the tumor through permeable tumor blood vessels and would allow the liposomes to efficiently interact with the blood vessels. To test this hypothesis, we employed a liposome with a diameter of 300 nm in designing a dual-ligand formulation, not a 100-nm-diameter liposome. PEGylated liposomes with 300 nm were prepared by hydration method, followed by extrusion. As we expected, PEGylated liposomes with diameters of 300 nm were detected mainly along the blood vessels because 300 nm in diameter would prevent PEGylated liposomes from the extravasation, while PEGylated liposomes with a diameter of 100 nm were distributed both inside via the EPR effect and outside the blood vessels in RCC tumors.[17] In addition, it is possible that large liposomes represent an advantage in terms of targeting vascular walls rather than small ones. It was reported that nontargeted particle diameters of >200 nm appear to be more effective in adhering firmly to the margins of vascular walls under flow than particles with diameters of <200 nm.[20] When the in vitro cellular binding affinity of a large dual-ligand LP modified with NGR and R4 was evaluated in comparison with the small one, the relative K_d value of the large dual-ligand LP was around 10 times lower than that of the small one.[17]

3.4. Size Controlled Dual-Ligand Liposomes for Treatment of Drug Resistant Cancer. Doxil (Caelyx), PEGylated liposomal doxorubicin (DXR) with 100 nm in diameter, accumulates at high levels in solid tumors via the EPR effect and has fewer side effects compared with free DXR, and is clinically used in the treatment of AIDS-related Kaposi's sarcoma and ovarian carcinomas. Antitumor activity in RCC tumor-bearing mice was evaluated by the systemic administration of a dual-ligand LP with 300 nm in diameter encapsulating DXR and Doxil. Biodistribution study showed that the amount of Doxil in the tumor was 3−4-fold higher than that of a dual-ligand LP, because Doxil accumulated in tumor via the EPR effect.[17] Despite an enhanced accumulation in tumors, no antitumor effect was observed in the case of the systemic treatment of Doxil (Figure 2C). By contrast, tumor growth suppression was clearly observed for the case of treatment with a dual-ligand LP. Furthermore, blood vessel density was clearly diminished as the result of treatment with the dual-ligand LP, while Doxil had no effect, similar to the controls (Figure 2D). Even though the liver and spleen are major clearance organs for the prepared formulations, neither damage nor an abnormal morphology of blood vessels was observed in the liver and spleen.[17] These results suggest that the dual-ligand LP specifically disrupted the neovasculature in OS-RC-2 tumors, but had no effect on normal endothelial cells in normal tissues such as the liver and spleen.

If 1 g of tumor tissue contains 10^8 cells, we estimate that the availability of DXR in tumor cells would be 5% injected dose (ID)/10^8 cells, since the amount of liposome in a tumor via the EPR effect was approximately 5% ID/g tumor. On the other hand, TECs constitute only approximately 2% of the total tumor tissue (2×10^6 cells/g tumor).[21] Because 1.5%ID/g tumor of liposomes was found in the case of the dual-ligand LP, the availability of DXR in TEC is calculated to be 0.75%ID/10^6 cells, which means the concentration of DXR in TEC would be expected to be at least 10-fold higher than that in OS-RC-2 cells. Furthermore, cytotoxicity analyses indicated that TECs derived from RCC tissue are approximately 2 orders of magnitude more sensitive to DXR than RCC tumor cells.[17] Taking these facts into consideration, targeting TECs should be around 3 orders of magnitude more efficient in terms of exerting cytotoxicity by DXR than targeting RCC tumor cells.

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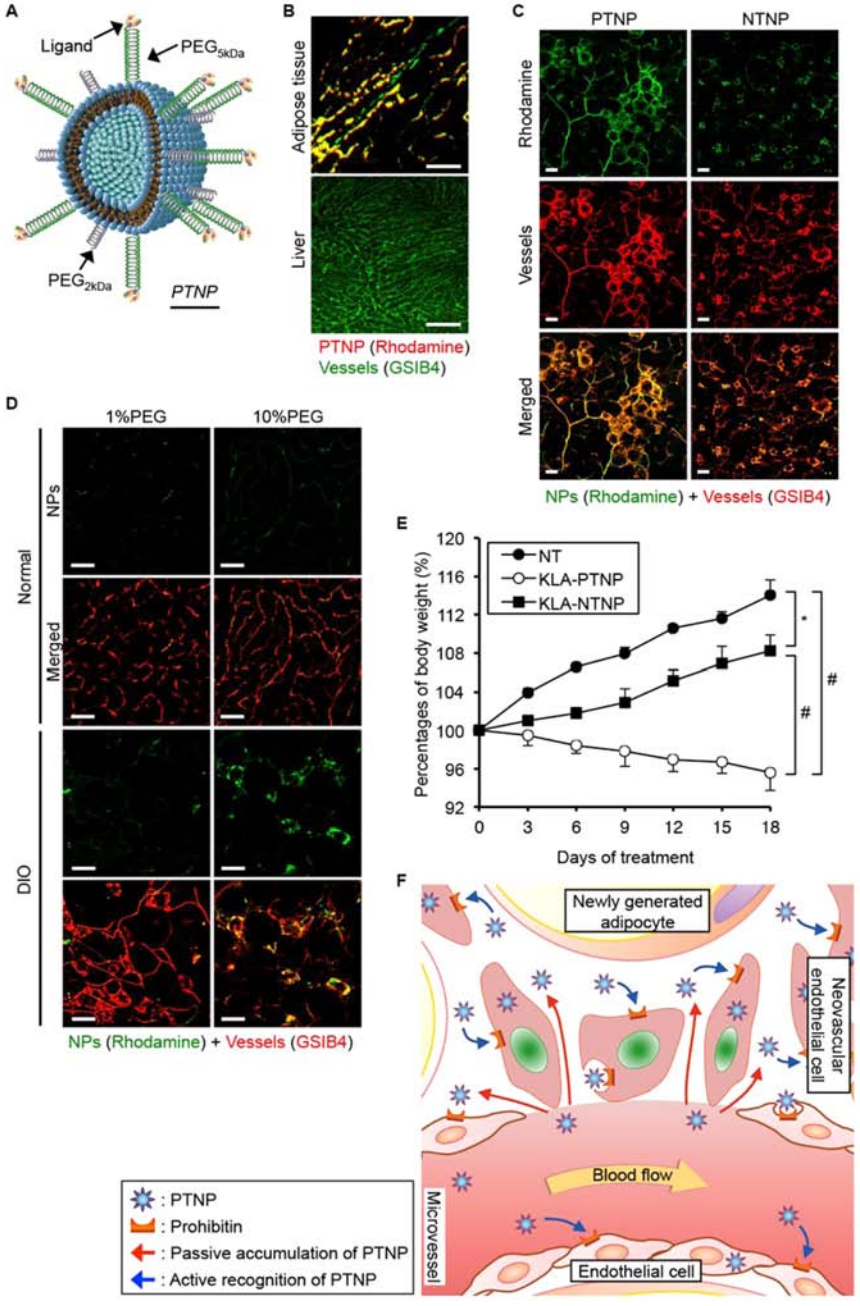

Figure 3. Vascular-targeted nanoparticulate carrier system for obesity nanotherapy. (A) Schematic illustration of prohibitin-targeted nanoparticle (PTNP). (B) Confocal evaluation of targeted delivery of rhodamine-labeled PTNP into adipose vessels and off-targeted accumulation into liver. (C) In vivo accumulation of PTNP and NTNP into adipose vessels in DIO mice. (D) Passive accumulation of PEGylated NPs into the angiogenic clusters in obese fat. (E) Body weight gain in response to the treatment of KLA-PTNP and KLA-NTNP in DIO mice. Data represents the mean ± SD; #$P < 0.0001$ and *$P < 0.05$, one-way ANOVA followed by Tukey-Kramer's HSD test. (F) Graphical illustration of dual mechanisms for passive and active targeting by which intravenously administered PTNP accumulates into endothelial cells of obese adipose tissue. Bars represent 100 μm (B–D). Adapted and/or reprinted in part with permission from *J. Controlled Release* **163**, *2*, 101–110 (2012). Copyright 2015 Elsevier.

Collectively, the dual-ligand LP can be considered a promising carrier for the treatment of drug resistant RCC via the disruption of TECs.

4. TARGETED DRUG DELIVERY TO WHITE FAT VESSELS WITH NANOPARTICLES VIA A DUAL-TARGETING MECHANISM

4.1. Antiobesity Therapy and Angiogenesis in Adipose Tissue. A growing demand exists in developed societies worldwide for an effective and safe therapy for the management of obesity. The only remaining FDA-approved antiobesity drug is Orlistat, a lipase inhibitor that acts in the gastrointestinal tract (GIT), but it is also associated with numerous side effects, including severe diarrhea and nephrotoxicity. One potential alternative approach to obesity control is the targeting of endothelial cells in white fat vessels (WFV). While endothelial cells that form the walls of WFV provide oxygen and nutrients needed for the survival and growth of resident cells including parenchymal adipocytes, these cells therefore represent an attractive target for obesity therapy.

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Moreover, new blood vessel growth in adipose tissue may simultaneously be associated with the growth of adipocytes and subsequently result in an increase in fat mass through the process of angiogenesis-dependent adipogenesis.[22] Several studies have indicated that the use of a nonspecific angiogenic inhibitor (TNP-470) that functions via the apoptosis/necrosis of endothelial cells in adipose tissue may be useful for controlling obesity.[23,24] However, the angiogenesis inhibitor has an undesirable toxicity caused by nonspecific action. Thus, an unmet need exists for an effective, safe, and better-tolerated obesity therapy.

4.2. Development of a Prohibitin-Targeted Nanoparticle for the in Vivo Targeted Drug Delivery to Adipose Vessels. We recently reported on the development of an in vitro adipose endothelial cell targeted nanoparticle (NP)[25] utilizing a WFV-homing peptide motif (KGGRAKD) that can specifically bind to prohibitin.[26] The preparation was a liposomal NP composed of biocompatible lipids with its surface modified with a linear peptide containing a WFV-targeting motif, attached via a poly(ethylene glycol) (PEG) spacer. In a previous study, we reported that the targeted NP specifically delivered encapsulated compounds to the cytoplasm of primary cultured endothelial cells derived from murine adipose tissue[27] through their uptake by prohibitin-mediated endocytosis. For in vivo application of the targeted NP, we attempted to optimize the preparation of NPs in normal healthy mice with regard to the density of ligand modification, the length of the PEG linker, and comodification of the PEG polymer to increase the steric stability of NPs for maximizing the targeted accumulation of NPs in WFV and minimizing the undesired accumulation of NPs to other organs, such as the liver. As a result, we found that the NPs that were co-modified with the targeting ligand via a longer PEG linker ($PEG_{5\ kDa}$) and the short PEG polymer (Figure 3A) (particle size: 116 ± 9 nm) were substantially and widely distributed in WFV after systemic injection, while there was no evidence for their accumulation in liver (Figure 3B).[28] Thus, we denoted the in vivo optimized NPs as prohibitin-targeted NPs (PTNP).[28] In addition, we found that PTNP was specifically internalized by vascular endothelial cells in adipose tissue after systemic injection and can deliver the encapsulated rhodamine to WFV.[28] From these results, we concluded that the development of an in vivo targeted delivery system to adipose vasculature was successful.

4.3. Unexpected Enhancement of Targeted Delivery of PTNP to Obese Adipose Tissue through the Passive Accumulation of NPs into Angiogenic Regions. As mentioned above, we succeeded with in vivo optimization of PTNP in normal healthy mice. However, in order to apply the PTNP system to obesity therapy, verification that PTNP targets WFV in obese animals is a critical issue. Thus, we next compared the accumulation of PTNP in adipose tissue in diet-induced obese (DIO) mice. As a result, PTNP accumulated in WFV at high levels within 24 h after i.v.-injection (Figure 3C).[28] Surprisingly, nontargeted NP (NTNP), which is essentially a preparation of PTNP, but with the targeting ligand absent, used as a negative control of PTNP, was also substantially accumulated in obese adipose tissue (Figure 3C).[28] The fluorescence of PTNP was detected in both cluster-like regions and capillary vessels, whereas that of NTNP appeared to be limited to the clusters. It is known that the sprouting of new blood vessels from the preexisting vasculature is coupled to new adipocyte formation in the adipogenic/angiogenic cell clusters in obese adipose tissue.[29] In addition, it has also been reported that an increase in vascular permeability in obese visceral fat is a hallmark of inflammation. The mechanisms that cause adipose tissue to be inflamed have not been fully elucidated. However, increase in vascular permeability due to chronic inflammation in obese fat tissue is widely accepted. We would like to discuss the putative mechanism of NTNP accumulation.[30] Therefore, we hypothesized that the physical properties (enhanced angiogenesis and vascular permeability) of obese adipose vessels might enhance the passive accumulation of sterically stabilized NPs such as PEGylated NPs. To assess this hypothesis, we examined the accumulation of PEGylated NPs with different PEG densities (1 or 10 mol % of total lipids) in adipose tissue of normal and DIO mice. The findings showed that the low density PEGylated NPs accumulated poorly in both adipose tissues of normal and DIO mice. In contrast, the high density PEGylated NPs accumulated at substantial levels around the clusters in DIO fat depots at 24 h post injection, whereas in normal adipose tissue, the extent of accumulation was much lower (particle size: 101 ± 2 nm) (Figure 3D).[28] These results strongly support our hypothesis and also suggest that the accumulation of PTNP, which is equipped with active targeting machinery, into WFV of obese mice could be accelerated by the passive targeting mechanism (EPR effect).[28]

4.4. Vascular-Targeted Nanotherapy of Diet-Induced Obesity. For a pharmacological assessment, we selected the pro-apoptotic peptide ($_D(KLAKLAK)_2$) as a model compound and prepared KLA-loaded PTNP (KLA-PTNP) and NTNP (KLA-NTNP). DIO mice were treated with KLA-PTNP or -NTNP (1 mg/kg) at 3 day intervals by i.v.-injection. The weight gain of the nontreated (NT) mice was accelerated by feeding a high-fat diet. The weight gain for the KLA-NTNP treated mice was significantly less than that of the NT group, but the body mass was not significantly decreased during the treatment period. On the contrary, the body weight of KLA-PTNP treated mice was significantly less than their initial weight (Figure 3E).[28] In addition, vascular density in adipose tissue was significantly lower in KLA-PTNP treated mice, compared to KLA-NTNP treated mice.[28] These results may be reasonable, since it was known that the $_D(KLAKLAK)_2$ peptide itself was not internalized by cells and required an appropriate intracellular delivery strategy to induce apoptosis.[31] Furthermore, we recently showed that the KLA-PTNP has higher therapeutic ability to treat obesity, compared to the peptidomimetic (CKGGRAKDC-GG-$_D(KLAKLAK)_2$), which is composed of the prohibitin-targeting peptide and the proapoptotic peptide.[32] This may also be reasonable, since the peptidomimetic would not passively accumulate to adipose tissue due to the relatively low molecular mass. Collectively, our data clearly demonstrate that the higher therapeutic effects of vascular-targeted nanotherapy are mediated by the enhanced delivery of drugs to the target site via dual mechanisms, i.e., both passive and active targeting (Figure 3F).[32]

5. TARGETED DRUG DELIVERY TO THE LUNG EPITHELIUM BY GALA PEPTIDE

5.1. GALA as a Targeting Ligand for the Lung. A drug delivery system that specifically targets the lung would be extraordinarily useful in terms of curing a number of lung-related diseases. Previous attempts to accomplish this by using cationic materials involved risks, since temporal trapping in microvessels with the simultaneous formation of large aggregates is a main driving force for the accumulation of

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such materials in the lung. Thus, the nanoparticle that can accumulate to the lung endothelium via active targeting is highly desired. As a candidate ligand, we reported on an IRQ peptide motif (IRQRRRR: PEP_{IRQ}) as a targeting ligand for the lung endothelium.[33] With the objective of improving the endosomal escape, the surface of the PEP_{IRQ}-modified particle was further modified with Chol-GALA, an α-helical peptide as described in section 2.2. However, during these investigations, we found it to be incredible that GALA, originally invented as a pH-dependent fusion inducer, also has a novel function beyond our assumption: to target a ligand to the lung endothelium.[34] In this study, modification of the Chol-GALA onto the conventional liposomes composed of EPC and cholesterol (GALA-LPs) sized approximately 100 nm drastically improved the lung by approximately 24-fold in comparison with that of ordinary liposomes at 360 min after administration. Since the GALA was originally designed to mimic the function of hemagglutinin (HA), a key protein that functions in a membrane fusion in influenza, it might recognize the sialic acid-terminated sugar chain. In fact, the cellular uptake of the rhodamine-labeled liposomes to lung epithelium-derived cell line was blocked in the presence of the lectins that recognize the sialic acid-terminated sugar chains.

Furthermore, the dynamic behavior of the GALA/MEND was visualized by intrapulmonary intravital real-time confocal laser scanning microscopy (IVRTCLSM) in living animals, an optical methodology. This imaging technology permitted the active lung-targeting process to be verified and provided evidence to show that large aggregates are not formed.

5.2. GALA-Modified MEND as a siRNA Carrier for the Lung Epithelium. As to the functional achievement, the GALA was modified on the siRNA-encapsulating MEND (GALA-MEND) using CD31 as an endothelium specific gene.[34] As a result, it is revealed that it confers pulmonary endothelium-specific gene knockdown more efficiently compared with AtuPLEX (Silence Therapeutics), a prior technology that is currently undergoing Phase I clinical studies. Moreover, the pharmacological effect of GALA/MEND-mediated siRNA delivery was evidenced by the prevention of pulmonary metastasis of melanomas with the smallest known dose. Thus, this GALA/MEND opens a new-generation therapeutic approach to satisfy unmet medical needs in curing various lung diseases.

Collectively, we successfully developed a GALA-MEND that mounts dual function analogous to the influenza virus: (1) It efficiently delivers a nanoparticle to the lung endothelium by recognizing sugar chains that are terminated with sialic acid residues, and (2) it further triggers the cytoplasmic release of encapsulated RNA (siRNA) via membrane fusion to the endosome.

6. SELECTION OF DNA APTAMER LIGANDS FOR TUMOR ENDOTHELIAL CELLS TO ACHIEVE AN ACTIVE TARGETING DDS

6.1. Aptamer and SELEX Strategies. Active targeting is one of the promising approaches for achieving efficient DDS, a process that can send drugs to specific target tissues/cells by virtue of specific interactions between receptors and ligands such as antibodies and peptides.[35] It is essential to identify an ideal ligand for the use in DDS to accomplish active targeting. Aptamers are new classes of molecules that bind to many types of target molecules such as small molecules, proteins, nucleic acids, viruses, microbes, and live cells.[36] Aptamers are DNAs or

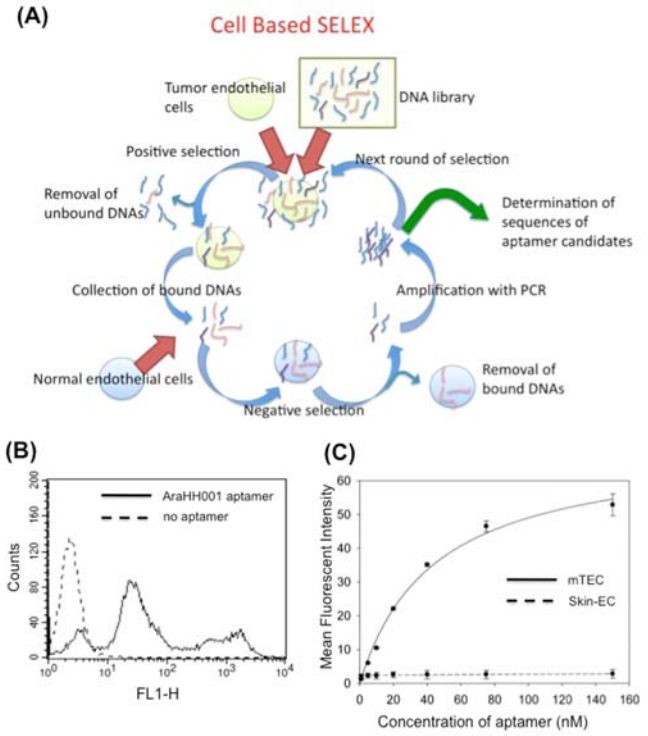

Figure 4. Investigation of DNA aptamer via the cell-based SELEX method. (A) Schematic representation of cell-based SELEX method. (B) Flow cytometric binding assay of FITC labeled aptamer with mTEC. 200 pmol of aptamer was treated with mTEC and applied for FACS analysis. (C) Determination of dissociation constant (K_d) of this aptamer toward mTEC and Skin-EC. The experiment was repeated three times and error bars represent standard deviations of each mean. Averages for each concentration were calculated and plotted to determine the dissociation constant. The regression curve was fitted using *SigmaPlot 12*. Adapted with permission from *J. Controlled Release* **193**, 316−324 (2014). Copyright 2015 Elsevier.

RNAs comparable to antibodies, but aptamers have advantages over antibodies. It can be easily prepared and readily modified with chemical synthesis and have a low toxicity and low immunogenicity. Aptamers are developed by the SELEX (Systemic Evolution of Ligands by EXponential enrichment) method. The SELEX method for selecting aptamers from combinatorial libraries by an iterative in vitro selection procedure was first reported by two research groups, Ellington and Tuerk, in the early 1990s, independently.[37,38] Extensive studies of aptamers have indicated that they have great potential for use in a variety of areas, including diagnosis, therapy, biomarker identification, a targeting ligand for the DDS, in vivo imaging, and as biosensors. These advantages and recent applications of aptamers in different biomedical areas and their potential power have resulted in their having a high priority for development. Cell-based SELEX is a recently invented selection method that can be used in exploring aptamers. The cell-based SELEX method can use live cells as the target. This method has beneficial aspects compared to conventional selection when the specific target protein of interest is unknown. The new target receptor can be identified with a newly isolated aptamer found with cell-based SELEX through aptamer facilitated biomarker discovery.[39] Moreover, aptamers can be identified as the suitable sequence for binding, to the corresponding domain expressed on the cell surface.

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Table 2. Characteristics of Various Types of MEND

	MEND				
characteristics	R8	Dual-ligand	PTNP	GALA	Aptamer
Approximate particle size	150−200 nm	300 nm	100 nm	150 nm	100 nm
Approximate ζ-potential	40−50mV	Neutral to slightly negative (∼ −10 mV)	Neutral	30 mV	−40 mV
Lipid composition	STR-R8/DOPE/Chol-GALA = 20/85/5	HSPC/Chol/Ligand-PEG_{2000}-DSPE/STR-R4 = 70/30/10/2.5	EPC/Chol/PEG_{2000}-DSPE/Ligand-PEG_{5000}-DSPE = 67/33/1/1.25	DOTMA/Chol/EPC/STR-PEG/Chol-GALA = 30/40/30/2/2	EPC/Chol/Aptamer-PEG_{2000}-DSPE = 70/30/5
Ligand	R8	Peptide (NGR or RGD)	Peptide (KGGRAKD)	GALA	DNA (ACGTACCGACTTCGTATGCCAACAGCCCTTTATC-CACCTC)
Encapsulated drug	pDNA or siRNA	Doxorubicin	Proapoptotic peptide ($_D$(KLAKLAK)$_2$) or protein (Cytochrome c)	siRNA	under investigation
Target site (binding receptor)	Liver (no-selective receptors)	Tumor endothelial cells (CD13, Integrin $\alpha v\beta 3$)	Vascular endothelial cells in white adipose tissue (Prohibitin)	Sugar chains that are terminated with sialic acid	Tumor endothelial cell (Tropoinin T)
Target diseases	Not tested	Tumor	Obesity	Metastasis	Cancer
Mechanisms for accumulation into target site	Largely accumulated into liver tissue through electrostatic interactions between cationic natures of R8-GALA-MEND and negatively charged cellular membrane.	The active targeting to tumor vasculature by ligands and avoidance of the EPR effect by the size of liposome (300 nm)	The active targeting of PTNP to adipose vasculature is passively enhanced by the EPR effect in obese animals.	The active targeting to lung vasculature	Active targeting manner, this aptamer could bind to Troponin T which is expressing some kinds of tumor endothelial cells.
Internalization into target cells	Mainly taken up via macropinocytosis	Mainly taken up via clathrin mediated endocytosis	Multivalent interaction between the ligands on PTNP and the receptors on adipose vascular ECs significantly enhances the cellular uptake of PTNP via the prohibitin-mediated endocytosis	Unknown	It was proved that this aptamer could internalize into tumor endothelial cells (in vitro experiment)
Drug release in the target cells	Dissociate nucleic acids (drug) from R8-GALA-MENDs in the process of membrane fusion with endosome	Spontaneous leakage of doxorubicin	PTNP might have the ability to escape from endosomes/lysosomes through the unknown mechanisms.	Triggered by the membrane fusion with endosome with an aid of GALA	under investigation
References	4, 5, 7, 9	15−17	23, 26, 30	34	44−46

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6.2. Tumor Endothelial Cells as the Target of Aptamer for Cancer Therapy. Tumor growth that is dependent on angiogenesis was first reported by Folkman in 1971.[40] Preventing or inhibiting angiogenesis, which is associated with the increased vascularity necessary for tumor progression and metastasis, is a challenging issue in combating cancer. Tumor blood vessels provide nutrients and oxygen, and remove waste from tumor tissue, resulting in tumor progression. Tumor blood vessels have been shown to differ from their normal counterparts in that they are more likely to leak, and the thickness of the basement membrane is uneven. This suggests that tumor endothelial cells (TECs) may well express surface markers that are different from those found in normal cells. Tumor blood vessels contain tumor endothelial cells that might be genetically normal and stable, even though these endothelial cells are structurally and functionally abnormal. Since tumor growth is somewhat dependent on the development of a neovascular supply, inhibiting angiogenesis by targeting tumor endothelial cells represents the ultimate goal in cancer therapy. We decided to use tumor endothelial cells as a target to explore an efficient ligand using the cell-based SELEX method to achieve antiangiogenesis therapy through the active targeting DDS (Figure 4A).

6.3. Aptamer Selection for Tumor Endothelial Cells as the Target via the Cell-SELEX Method. A library for the selection was first chemically synthesized by the phosphoramidite method.[41] It had a 40mer random sequence flanked by 21mer nucleotide sequences for forward and reverse primers to amplify with PCR to regenerate the recovered library. The sequence was (5′-CGTAGAATTCATGAGGACGTT-N40-AGCTAAGCTTACCAGTGCGAT-3′). This ssDNA library was mixed with primary cultured mouse TECs, removed unbound ssDNAs, and collected bound ssDNAs that had been heated to denature the secondary structure. Trypsin is usually used to detach cells, but it digested some of the surface proteins on the TECs. To overcome this problem, we used RepCell, temperature-sensitive cell culture dishes. Using this technique, it is possible to detach cells by lowering the temperature of the dish, while keeping the surface proteins intact. This method has improved the diversity of recovered DNA libraries. The library was purified by phenol-chloroform extraction and ethanol precipitation and amplified by conventional PCR. Amplifying the random sequence with PCR was a problem sometimes, because the random sequence acts as the primer and an unexpected sequence is also produced.[42] It is known that too much template causes this unexpected amplification so that the amount of library produced in each PCR cycle must be monitored. The PCR amplified library was a double-stranded DNA, but single-stranded DNA is needed for selection so that the asymmetric PCR method was employed to generate ssDNA. Asymmetric PCR (also called Linear After The Exponential PCR (LATE PCR)) was conceived by Gyllensten.[43] This method contains two sequential PCR procedures. The first PCR was regular PCR and the second was done without a reverse primer to produce only the desired strand. The counter selection was also applied to Skin-EC as the normal endothelial cell model and OS-RC-II as the tumor parenchymal cell model. The affinity of each cycle of the library was checked via a FACS experiment and the entire SELEX procedure was finished when a sufficient binding affinity of the library was observed.

6.4. Identification and Physicochemical Properties of DNA Aptamers. After 12 rounds of selection, we found a great shift in the fluorescent intensity on FACS. This indicates that the enrichment of the DNA library was sufficient. The DNA library was cloned and sequenced to determine the DNA sequence, and 48 sequences were identified as candidate aptamers. The binding affinities of these aptamers were investigated by flow cytometry and one aptamer was found that bound strongly to mTEC (Figure 4B) but not Skin-EC or OS-RC-II cells. We denoted the aptamer as AraHH001. The mean fluorescent intensities were measured as a function of aptamer concentration and the dissociation constant of this aptamer was determined to be 43.8 ± 13.7 nM (Figure 4C). These findings indicate that this aptamer has a strong binding affinity and good selectivity.[44] Next, the target protein which this aptamer could bind has been identified. Biotin-modified aptamer was mixed with TEC cell lysate and the target protein was isolated with streptavidin beads, separated with SDS-PAGE, and identified with peptide mass fingerprinting. It was Troponin T, known as the contraction of muscles, and this approach was proven to find new class of ligand on the surface of cells.[45]

Next, this aptamer was applied to the ligand of drug delivery system. At first, this aptamer was conjugated with PEG-lipid and aptamer-bound liposome was constructed via conventional hydration method. The targeting abilities of this aptamer-bound liposome were examined in vitro and in vivo, and it was proven that this aptamer-bound liposome could bind and was taken up to tumor endothelial cells. From these results, we could show that the aptamer is one of the most promising targeting devices on DDS research.[46]

A new cell-based SELEX method with RepCell has been used to investigate primary cultured mTECs as the target. Using this method, cell surface proteins can be kept intact, while this is not necessarily the case when the trypsin method is used. Additionally, when cell lines are used, important markers can be lost during passages. On the other hand, the use of primary cultured mTECs provides the opportunity to identify many kinds of aptamer candidates that can target proteins expressed by live animals. The Cell-based SELEX method also has an advantage in identifying an aptamer that can recognize the protein expressed on cell surface. We also have investigated other aptamers toward tumor endothelial cells,[47] protein,[48] and mitochondria[49] and are trying to develop a new aptamer ligand drug delivery system which could explore the new horizon of active targeting drug delivery system.

7. PERSPECTIVES

The characteristics of our nanodevices described in this Review are summarized in Table 2. Principle of passive targeting was introduced at the end of the 20th century and Doxil has been used clinically in the world. A concept of active targeting has also been introduced at the same time; however, there is no clinically successful DDS based therapy. Recent progress described herein encourages us to move breakthrough technologies into clinical testing for innovative nanomedicine in the near future.

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Notes

The authors declare no competing financial interest.

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