Efficient Short Interference RNA Delivery to Tumor Cells Using a Combination of Octaarginine, GALA and Tumor-Specific, Cleavable Polyethylene Glycol System

Yu SAKURAI,[a,c] Hiroto HATAKEYAMA,[a,c] Hidetaka AKITA,[a,c] Motoi OISHI,[b,c] Yukio NAGASAKI,[b,c] Shiro FUTAKI,[d] and Hideyoshi HARASHIMA*,[a,c]

[a] *Laboratory for Molecular Design of Pharmaceutics, Faculty of Pharmaceutical Sciences, Hokkaido University; Kita 12, Nishi 6, Kita-ku, Sapporo 060–0812, Japan:* [b] *Tsukuba Research Center for Interdisciplinary Material Science (TIMS), University of Tsukuba; Tsukuba 305–8573, Japan:* [c] *CREST, Japan Science and Technology Agency (JST), Saitama 332–0012, Japan: and* [d] *Institute for Chemical Research, Kyoto University; Uji, Kyoto 611–0011, Japan.*
Received January 15, 2009; accepted February 23, 2009; published online February 27, 2009

We recently developed a multifunctional envelope-type nano device (MEND) for efficient nucleic acid delivery. Here, we report on the development of an octaarigine (R8)-modified MEND encapsulating small interfering RNA (siRNA) with a tumor-specific, cleavable, polyethylene glycol (PEG)-lipid (PPD). We first determined the optimal concentration of R8 and pH-sensitive fusogenic peptide (GALA) on the lipid envelope of MEND (R8/GALA-MEND). Then, we examined the combination of optimized R8/GALA-MEND with a PEG-lipid. When a conventional PEG-lipid was used, the R8/GALA-MEND failed to knockdown expression of the target gene. On the other hand, PPD-modified R8/GALA-MEND exhibited efficient silencing activity to the level of the PEG-unmodified R8/GALA-MEND. In addition, we compared a R8/GALA-MEND with a MEND composed of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) that is a conventional cationic lipid used as a lipoplex component. The knockdown ability of the R8/GALA-MEND was much higher than that of the DOTAP-based MEND at the dose that is commonly employed in *in vitro* siRNA transfection. These results demonstrate that the R8/GALA-MEND is a promising delivery system for the transfer of siRNA to tumor cells.

Key words non-viral delivery system; stearyl octaarginine; cancer gene therapy; multifunctional envelope-type nano device; pH-sensitive furogenic peptide

RNA interference (RNAi) triggered by short interference RNA (siRNA) can suppress expression of a specific gene and is considered a potential therapeutic tool for many conditions, including cancer, infection and hypercholesteremia.[1,2] To obtain efficient knockdown of the target gene, siRNA must be delivered to the cytosol of target cells. Therefore, a successful siRNA delivery system requires a rational strategy that can overcome many biological barriers by controlling both biodistribution and intracellular trafficking. To address these issues, many groups have developed various types of carrier systems for siRNA delivery, such as lipoplexes and polyplexes.[3—5] We recently proposed a novel packaging approach for the assembly of multiple devices in a single delivery system, a multifunctional envelope-type nano device (MEND), in which nucleic acid is condensed using a polycation to form a core particle, followed by encapsulation in a lipid envelope.[6,7] When the MEND was modified with stearylated octaarginine (STR-R8) on the lipid envelope (R8-MEND), it was efficiently taken up by cells *via* a unique pathway called macropinocytosis, which allowed escape from lysosomal degradation and led to transfection activity as high as that of adenovirus.[6,8] The R8-MEND is also applicable to siRNA delivery. Using STR-R8 as a polycation, siRNA can form a complex 60—80 nm and be efficiently encapsulated into the MEND.[9] As a result, the R8-MEND results in a greater silencing effect than does a commercially available reagent, TransIT-TKO.[9] To further improve silencing activity, an additional modification was developed that allows the cytoplasmic delivery of encapsulated siRNA. We recently established that R8-modified liposomes prepared with dioleoylphosphatidyl ethanolamine (DOPE) and phosphatidic acid (PA) (molar ratio＝9 : 2) have high fusogenic activity with the endosome, and efficiently release functional siRNA into the cytosol.[10] Additionally, we used a pH-sensitive fusogenic peptide GALA (WEAALAEALAEALAEHLAEALAEALEALAA) in the form of cholesteryl-GALA (Chol-GALA)[11] to modify the MEND lipid envelope, which improved transfection activity of an encapsulating plasmid DNA due to an enhanced endosomal release.[12] Therefore, we postulated that an R8-MEND, prepared with a lipid composition of DOPE/PA plus Chol-GALA (R8/GALA-MEND) would be ideal for the promotion of the cytoplasmic release of functional siRNA.

For *in vivo* tumor targeting, PEGylation of the gene carriers is useful strategy due to enhancement of the permeability and retention (EPR) effect.[13] However, PEGylation hampers the cellular association of gene carriers with cell membrane and lowers subsequent cellular uptake and endosomal escape.[14,15] To overcome this polyethylene glycol (PEG)-associated dilemma, we previously constructed a novel, cleavable, PEG-peptide-DOPE conjugate named PPD, that is cleaved in response to matrix metalloproteinase (MMP), which is specifically secreted from tumor cells.[16]

In the present study, we developed an R8/GALA-MEND modified with PPD for siRNA delivery. We first examined the optimal concentration of STR-R8 and Chol-GALA on the lipid envelope to exert sufficient gene silencing, as assessed by *in vitro* luciferase activity. Finally, we introduced a non-cleavable PEG-lipid or PPD into the R8/GALA-MEND, and the silencing effect of PEGylated R8/GALA-MEND was evaluated by comparison with a conventional MEND that had been prepared using 1,2-dioleoyl-3-trimethylammonium-

* To whom correspondence should be addressed. e-mail: harasima@pharm.hokudai.ac.jp

propane (DOTAP), DOPE and cholesterol (molar ratio= 3 : 4 : 3).

MATERIALS AND METHODS

Materials Anti-luciferase siRNA (21-mer, 5′-GCGCUGCUGGUGCCAACCCTT-3′, 5′-GGGUUGGCACCAGCAGCAGCGCTT-3′) and anti-green fluorescent protein (GFP) siRNA (5′-GCUGACCCUGAAGUUCAUCTT-3′, GAUGAACUUCAGGGUCAGCTT-3′) were obtained from Thermo Electron GmbH (Ulm, Germany). STR-R8 was synthesized as described previously.[17] DOPE, DOTAP, cholesterol and distearoyl-*sn*-glycero-3-phoshoethanolamine-*N*-[methoxy (polyethylene glycol)-2000] (PEG-DSPE) were purchased from AVANTI Polar Lipids (Alabaster, AL, U.S.A.). PA was purchased from Sigma (St. Louis, MO, U.S.A.). PPD and Chol-GALA were synthesized as described previously.[16,18] Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, U.S.A.). Diethyl pyrocarbonate (DEPC)-treated water and G418 were obtained from Nacalai Tesque (Kyoto, Japan). Luciferase assay reagents and reporter lysis buffer were obtained from Promega (Madison, WI, U.S.A.). HeLa human cervical carcinoma cells were obtained from RIKEN Cell Bank (Tsukuba, Japan). All other chemicals were commercially available reagent-grade products.

Cell Culture HeLa cells stably expressing luciferase (HeLa-luc) were prepared as described previously,[19] and cultured in cell-culture dishes (Coring) containing DMEM supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), G418 (0.4 mg/ml) at 37 °C in an atmosphere of 5% CO_2 and 95% humidity. Matrix metalloproteinase-2 (MMP-2) in the supernatant of HeLa-luc was quantified using a commercially available enzyme-linked immunosorbent assay (ELISA) specific for human MMP-2 (Biotrak ELISA System; Amersham Biosciences, Uppsala, Sweden). Samples were prepared according to the manufacturer's protocol.

Preparation of R8/GALA-MEND To prepare the siRNA/STR-R8 complex, the siRNA solution (0.1 mg/ml in DEPC-treated water) was added to the STR-R8 solution (0.1 mg/ml in DEPC-treated water) under vortexing at room temperature, at a nitrogen/phosphate (N/P) ratio of 3.2. A lipid film was formed by evaporating a chloroform solution (125 nmol total lipids), composed of DOPE, PA, STR-R8 and Chol-GALA (molar ratio of DOPE/PA was fixed at 7 : 2). For modification of the MEND with PEG-DSPE or PPD, the lipid film was prepared by evaporation with a known amount of PEG-DSPE or PPD. The siRNA/STR-R8 complex was applied to the lipid film, followed by incubation for 10 min at room temperature to hydrate the lipids. To coat the siRNA/STR-R8 complex with the lipid, the lipid film was sonicated for approximately 1 min in a bath-type sonicator (AU-25, AIWA Co., Tokyo, Japan).

Preparation of DOTAP-MEND To prepare the siRNA/STR-R8 complex for the DOTAP-MEND, a STR-R8 solution (0.1 mg/ml in *N*-(2-hydoxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes) buffer, pH 7.4) was added to a siRNA solution (0.1 mg/ml in Hepes buffer, pH 7.4) under vortexing at room temperature, at a N/P ratio of 1.2. A lipid film was formed by the evaporation of a chloroform solution (125 nmol total lipids), composed of DOTAP, DOPE and cholesterol (3 : 4 : 3 molar ratio). In the case of PEG-DSPE and PPD modification, the lipid film was prepared by evaporation with a certain amount of PEG-DSPE or PPD. The siRNA/STR-R8 complex was applied to the lipid film, followed by incubation for 10 min at room temperature to hydrate the lipids. To coat the siRNA/STR-R8 complex with the lipid, the hydrated lipid film was then sonicated for approximately 1 min in a bath-type sonicator.

Measurement of Diameter and ζ-Potential for Prepared MENDs The average diameter and the ζ-potential of the condensed siRNA core and the MENDs were determined using a Zetasizer Nano ZS ZEN3600 (MALVERN Instrument, Worchestershire, U.K.).

Gene Silencing Effect of MENDs To examine the silencing effect of the MEND, 4×10^4 HeLa-luc cells were seeded in a 24-well dish 1 d prior to transfection. The MEND, containing the indicated dose of siRNA, was added to 0.25 ml of DMEM containing 10% FBS, followed by incubation at 37 °C for 3 h. Then, 0.75 ml of DMEM containing 10% FBS was added to the cells, followed by incubation for an additional 21 h. The cells were then washed with 0.5 ml of PBS and lysed with reporter lysis buffer. Luciferase activity in the cell lysate was then measured using a luminometer (Luminescencer-PSN, ATTO, Japan). The protein concentration was determined with a BCA Protein Assay Kit (Pierce, Rockford, IL, U.S.A.). Luciferase activity was expressed as relative light units (RLU) per mg of protein. The silencing effect was calculated as a percentage using the following equation:

$$\text{silencing effect (\%)} = \left(1 - \frac{\text{TE}_{\text{anti-luc}}}{\text{TE}_{\text{anti-GFP}}}\right) \times 100$$

where $\text{TE}_{\text{anti-luc}}$ and $\text{TE}_{\text{anti-GFP}}$ represent luciferase gene expression after transfection with either anti-luciferase siRNA or anti-GFP siRNA, respectively.

Statistic Analysis Comparisons between multiple treatments were made using one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls test. Pair-wise comparisons between treatments were made using a two-tail Student *t*-test. A *p*-value of <0.05 was considered significant.

RESULTS AND DISCUSSION

Optimization of Chol-GALA Concentration of R8/GALA-MEND Initially, we optimized the density of Chol-GALA on the R8/GALA-MEND. R8/GALA-MENDs with 10 mol% of STR-R8 were prepared containing various densities of Chol-GALA (0—4 mol%). The average diameter and ζ-potential of prepared R8/GALA-MENDs were comparable at each density, as shown in Table 1. On the other hand, the knockdown activity of the R8/GALA-MEND at a dose of 0.2 μg siRNA was dependent on the Chol-GALA density up to 2 mol%, as shown in Fig. 1, which was presumably due to an increase in endosomal escape by virtue of the function of GALA after the R8/GALA-MEND was taken up *via* endocytosis.[11,12] No further enhancement in activity was observed >2 mol%, because the ability of GALA to accelerate the endosomal escape might reach the limit in this formulation.

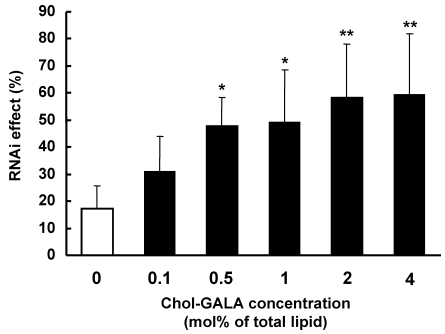

Fig. 1. Effect of Chol-GALA Modification on Silence Activity of an R8-MEND

HeLa-luc (4×10^4 cells/well) were transfected with R8-MEND (0.2 μg siRNA/well) modified with Chol-GALA at various densities (0—4 mol%). Luciferase activity was measured 24 h after transfection. The RNAi effect was calculated by normalization to cells treated with non-specific (anti-GFP) siRNA. The RNAi effect is expressed as the mean±S.D. ($n=3$). $*p<0.05$. $**p<0.01$ *vs.* R8-MEND without Chol-GALA (open bar).

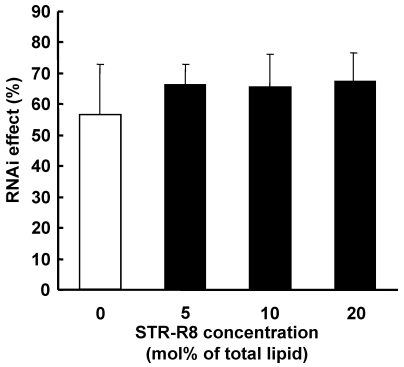

Fig. 2. Comparison of the Silence Activity of an R8/GALA-MEND Containing Various Densities of STR-R8

An R8-MEND (0.2 μg siRNA/well) containing various densities of STR-R8 (0—20 mol%) and modified with 2 mol% Chol-GALA was transfected to HeLa-luc (4×10^4 cells/well), and luciferase activity was measured at 24 h after transfection. The RNAi effect was calculated by normalization to cells treated with non-specific (anti-GFP) siRNA. The RNAi effect is expressed as the mean±S.D. ($n=3$).

Table 1. Characteristics of R8/GALA-MEND

STR-R8 (mol%)	Chol-GALA (mol%)	Diameter (nm)	ζ-Potential (mV)
10	0	186±31	58±7
	0.1	212±19	60±6
	0.5	200±42	60±6
	1	183±23	51±23
	2	196±31	57±11
	4	227±57	62±12
0	2	196±22	−57±6
5		186±22	−3.7±14
10		196±31	52±19
20		204±35	54±34

Each value is represented by the mean±S.D. ($n=3$).

Table 2. Characteristics of PEGylated R8/GALA-MEND and DOTAP-MEND

	PEG-DSPE (mol%)	PPD (mol%)	Diameter (nm)	ζ-Potential (mV)
R8/GALA-MEND	—	—	180±7	−3±20
	5	0	123±6	3±11
	2.5	2.5	115±6	13±6
	0	5	168±29	16±8
DOTAP-MEND	—	—	187±2	51±19
	5	0	122±12	−4±3
	0	5	171±6	6±10

Each value is represented by the mean±S.D. ($n=3$).

Furthermore, in the case of 4 mol% of Chol-GALA, the aggregation occurred occasionally in R8/GALA-MEND formulation, which could have been caused by fusion between the lipid envelope of the MEND due to an excess amount of GALA. Based on these results, it was concluded that the optimal density of Chol-GALA on the R8/GALA-MEND was 2 mol%.

Optimization of STR-R8 Concentration of R8/GALA-MEND We next determined the optimal density of STR-R8 in the R8/GALA-MEND. The density of Chol-GALA was fixed at 2 mol%, and the STR-R8 density was varied between 0 mol% and 20 mol% of total lipid. The average diameter of the R8/GALA-MEND remained unchanged, regardless of the STR-R8 concentration. In contrast, modification of STR-R8 resulted in a gradual increase in ζ-potential by as much as 10 mol% and reached a plateau at 20 mol% as shown in Table 1. At 5 mol% of STR-R8, the ζ-potential was nearly neutral. The knockdown activity of the R8/GALA-MEND was slightly enhanced at 5 mol% of STR-R8 compared with the MEND with no STR-R8 (0 mol%). However, additional modification of STR-R8 had a minor effect on gene knockdown activity (Fig. 2). Since positively or negatively charged particles strongly interact with serum proteins and biomolecules,[20,21] a neutral surface is desirable for *in vivo* application. Collectively, 5 mol% of STR-R8 seemed to be the optimal density.

Effect of Modification of MENDs with PEG Lipid on Gene Silencing Then, we evaluated the effect of PEGylation on silencing activity. PEG-DSPE or PPD was used to modify the R8/GALA-MEND at 5 mol% of total lipid. PEG-DSPE modification reduced the average diameter of R8/GALA-MEND compared with the PEG-unmodified R8/GALA-MEND (Table 2). On the other hand, PPD modified R8/GALA-MEND showed a slightly smaller diameter than the PEG-unmodified R8/GALA-MEND. Because hydrophobic amino acids accounted for most of the peptide sequence of PPD, the PEG layer might not have exhibited an appropriate formation on the surface of the lipid envelope. To the contrary, the ζ-potential of both PEG- and PPD-modified MEND showed no significant difference. The prepared MENDs were transfected at a dose of 0.4 μg siRNA. PEG-DSPE modification inhibited the silencing activity of an R8/GALA-MEND by reducing both cellular uptake and endosomal escape (Fig. 3). This is presumably due to the blockage of the interaction of R8 by the cellular membrane and GALA with the endosomal membrane *via* the steric hindrance of PEG.[12] Then, PEG-DSPE was partially or fully replaced with PPD at 2.5 or 5.0 mol%. The knockdown effect of the R8/GALA-MEND was increased depending on the amount of PPD (Fig. 3). Of note, gene knockdown activity of the R8/GALA-MEND modified with 5 mol% PPD was nearly equal to that of the PEG-unmodified R8/GALA-MEND. The expression of MMP-2 in the supernatant of

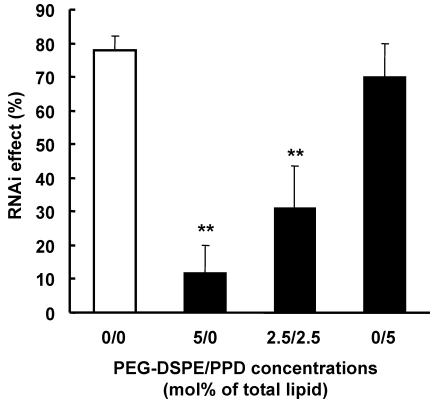

Fig. 3. Effect of Modification of PEG-DSPE and PPD on Silencing Activity

An R8/GALA-MEND (0.4 μg siRNA/well) modified with PEG-DSPE and PPD at indicated concentrations was transfected to HeLa-luc (4×10^4 cells/well), and luciferase activity was measured 24 h after transfection. The RNAi effect was calculated by normalization to cells treated with non-specific (anti-GFP) siRNA. The RNAi effect is expressed as the mean±S.D. ($n=3$). ** $p<0.01$ *vs.* PEG-unmodified R8/GALA-MEND (open bar).

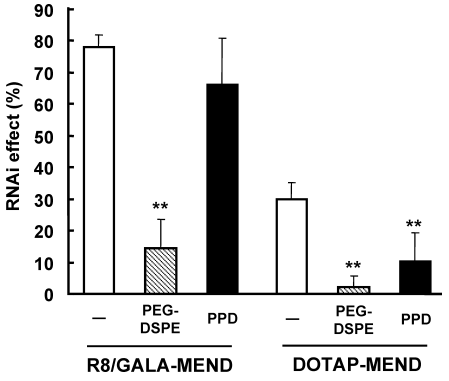

Fig. 4. Comparison of Silencing Activity between an R8/GALA-MEND and a DOTAP-MEND

An R8/GALA-MEND and a DOTAP-MEND (0.4 μg siRNA/well) were transfected to HeLa-luc (4×10^4 cells/well). At 24 h after transfection, luciferase activity was measured. The RNAi effect was calculated by normalization to cells treated with non-specific (anti-GFP) siRNA. The RNAi effect is expressed as the mean±S.D. ($n=3$). ** $p<0.01$ *vs.* PEG-unmodified MEND (open bar).

HeLa-luc was approximately 20 ng/ml, which is sufficient to trigger the cleavage of PEG.[16] Therefore, the PEG moiety was removed from the surface of the PPD-modified R8/GALA-MEND in response to MMP in the culture medium. Then, the R8 of the naked R8/GALA-MEND was able to associate with cell-surface components such as heparane sulfate proteoglycans,[22] which triggered the cellular uptake of R8/GALA-MEND through endocytosis. Subsequently, the interaction of the GALA moiety of the endocytosed R8/GALA-MEND with the endosomal membrane allowed efficient cytosolic release of encapsulating siRNA, which resulted in a higher knockdown activity. Collectively, these results suggest that a high silencing effect can be achieved because of the synergistic functions of GALA and PPD.

Comparison between R8/GALA-MEND and DOTAP-MEND Finally, we compared the silencing activity of R8/GALA-MEND to that of the DOTAP-MEND, which was composed of the conventional cationic lipid DOTAP,[23] DOPE and cholesterol (molar ratio=3 : 4 : 3), as described previously.[16] The average diameters and ζ-potentials of the DOTAP-MEND without and with PEG-lipid are shown in Table 2. In the PEG-unmodified MEND, the gene knockdown activity of the R8/GALA-MEND was double that of the DOTAP-MEND at a dose of 0.4 μg siRNA. In addition, the R8/GALA-MEND demonstrated a gene-knockdown effect even when the PEG was modified, whereas that of the DOTAP-MEND was negligible (Fig. 4). When the PEG was replaced with PPD in the DOTAP-MEND, the gene-knockdown effect was increased; however, it remained less than that of the PEG-unmodified example. By contrast, the R8/GALA-MEND with PPD exhibited significant gene silencing, which was comparable to that of the PEG-unmodified MEND. These data collectively indicate that the R8/GALA-MEND is more compatible with PPD modification, relative to the DOTAP-MEND, and is superior to the DOTAP-MEND from the point of view of gene knockdown efficiency.

In conclusion, we succeeded in developing siRNA-encapsulated R8/GALA-MENDs with optimized STR-R8 and GALA concentration. The R8/GALA-MEND exhibited a higher RNAi effect than the conventional DOTAP-based MEND. Furthermore, even with the PPD modification, the R8/GALA-MEND provided sufficient *in vitro* gene silencing. Collectively, the R8/GALA-MEND combined with PPD is a promising new carrier of siRNA for silencing of specific gene in tumor cells.

Acknowledgements This work was financially supported by Grants-in-Aid for Scientific Research (A) and Grant-in-Aid for Young Scientists (Start-up) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by Grants-in-Aid for Scientific Research on Priority Areas from the Japan Society for the Promotion of Science. We also thank Dr. J. L. McDonald for his helpful advice in editing the manuscript.

REFERENCES

1) Kim D. H., Rossi J. J., *Nat. Rev. Genet.*, **8**, 173—184 (2007).
2) Fougerolles A., Vornlocher H. P., Maraganore J., Liberman J., *Nat. Rev. Drug Discov.*, **6**, 443—453 (2007).
3) Li S. D., Chono S., Hunag L., *J. Controlled Release*, **126**, 77—84 (2008).
4) Tietze N., Pelisek J., Philipp A., Roed W., Merdan T., Tarcha P., Ogris M., Wagner E., *Oligonucleotides*, **18**, 161—174 (2008).
5) Leng Q., Scaria P., Lu P., Woodle M. C., Mixson A. J., *Cancer Gene Ther.*, **15**, 485—495 (2008).
6) Kogure K., Moriguchi R., Sasaki K., Ueno M., Futaki S., Harashima H., *J. Controlled Release*, **98**, 317—323 (2004).
7) Kogure K., Akita H., Yamada Y., Harashima H., *Adv. Drug Deliv. Rev.*, **60**, 559—571 (2008).
8) Khalil I. A., Kogure K., Futaki S., Hama S., Akita H., Ueno M., Kishida H., Kudoh M., Mishina Y., Kataoka K., Yamada M., Harashima H., *Gene Ther.*, **14**, 682—689 (2007).
9) Nakamura Y., Kogure K., Futaki S., Harashima H., *J. Controlled Release*, **119**, 360—367 (2007).
10) El-Sayed A., Khalil I. A., Kogure K., Futaki S., Harashima H., *J. Biol. Chem.*, **283**, 23450—23461 (2008).
11) Kakudo T., Chaki S., Futaki S., Nakase I., Akaji K., Kawakami T., Maruyama K., Kamiya H., Harashima H., *Biochemistry*, **43**, 5618—5628 (2004).
12) Sasaki K., Kogure K., Chaki S., Nakamura Y., Moriguchi R., Hamada H., Danev R., Nagayama K., Futaki S., Harashima H., *Anal. Bioanal. Chem.*, **391**, 2717—2727 (2008).
13) Maeda H., Wu J., Sawa T., Matsumura Y., Hori K., *J. Controlled Re-*

lease, **65**, 271—284 (2000).
14) Mishra S., Webster P., Davis M. E., *Eur. J. Cell Biol.*, **83**, 97—111 (2004).
15) Remaut K., Lucas B., Braeckmans K., Demeester J., De Smedt S. C., *J. Controlled Release*, **117**, 256—266 (2007).
16) Hatakeyama H., Akita H., Kogure K., Oishi M., Nagasaki Y., Kihira Y., Ueno M., Kobayashi H., Kikuchi H., Harashima H., *Gene Ther.*, **14**, 68—77 (2007).
17) Futaki S., Ohashi W., Suzuki T., Niwa M., Tanaka S., Ueda K., Harashima H., Sugiura Y., *Bioconjug. Chem.*, **12**, 1005—1011 (2001).
18) Futaki S., Ishikawa T., Niwa M., Kitagawa K., Yagami T., *Bioorg. Med. Chem.*, **5**, 1883—1891 (1997).
19) Masuda T., Akita H., Nishio T., Niikura K., Kogure K., Ijiro K., Harashima H., *Biomaterials*, **29**, 709—723 (2008).
20) Li S., Tseng W.-C., Stolz D. B., Wu S.-P., Watkins S. C., Huang L., *Gene Ther.*, **6**, 585—594 (1999).
21) Price M. E., Cornelius R. M., Brash J. L., *Biochim. Biophys. Acta*, **1512**, 191—205 (2001).
22) Khalil I. A., Kogure K., Futaki S., Harashima H., *J. Biol. Chem.*, **281**, 3544—3551 (2006).
23) Regelin A. E., Fankhaenel S., Gürtesch L., Prinz C., von Kiedrowski G., Massing U., *Biochim. Biophys. Acta*, **1464**, 151—164 (2000).