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Antiviral Efficacy of a Short PNA Targeting microRNA-122 Using Galactosylated Cationic Liposome as a Carrier for the Delivery of the PNA-DNA Hybrid to Hepatocytes

Hyoseon Kim,[a] Kwang Hyun Lee,[a] Kyung Bo Kim, Yong Serk Park,[†] Keun-Sik Kim,[‡,*] and Dong-Eun Kim[*]

Department of Bioscience and Biotechnology, Konkuk University, Seoul 143-701, Korea. [*]E-mail: kimde@konkuk.ac.kr
[†]Department of Biomedical Laboratory Science, Yonsei University, Wonju 220-710, Korea
[‡]Department of Biomedical Laboratory Science, Konyang University, Daejeon 302-718, Korea. [*]E-mail: kskim11@konyang.ac.kr
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Peptide nucleic acids (PNAs) that bind to complementary nucleic acid sequences with extraordinarily high affinity and sequence specificity can be used as antisense oligonucleotides against microRNAs, namely antagomir PNAs. However, methods for efficient cellular delivery must be developed for effective use of PNAs as therapeutic agents. Here, we demonstrate that antagomir PNAs can be delivered to hepatic cells by complementary DNA oligonucleotide and cationic liposomes containing galactosylated ceramide and a novel cationic lipid, DMKE (*O,O'*-dimyristyl-*N*-lysyl glutamate), through glycoprotein-mediated endocytosis. An antagomir PNA was designed to target miR-122, which is required for translation of the hepatitis C virus (HCV) genome in hepatocytes, and was hybridized to a DNA oligonucleotide for complexation with cationic liposome. The PNA-DNA hybrid molecules were efficiently internalized into hepatic cells by complexing with the galactosylated cationic liposome *in vitro*. Galactosylation of liposome significantly enhanced both lipoplex cell binding and PNA delivery to the hepatic cells. After 4-h incubation with galactosylated lipoplexes, PNAs were efficiently delivered into hepatic cells and HCV genome translation was suppressed more than 70% through sequestration of miR-122 in cytoplasm. PNAs were readily released from the PNA-DNA hybrid in the low pH environment of the endosome. The present study indicates that transfection of PNA-DNA hybrid molecules using galactosylated cationic liposomes can be used as an efficient non-viral carrier for antagomir PNAs targeted to hepatocytes.

Key Words : Antagomir, Cationic liposome, Hepatitis C virus, microRNA-122, Peptide nucleic acid (PNA)

Introduction

MicroRNAs (miRNAs) are noncoding RNAs, approximately 22 nucleotides (nts) in length, that mediate post-transcriptional gene regulation usually by repressing the translation of mRNAs through mismatched binding to their 3'-untranslated regions (UTRs).[1] Because of the essential roles played by miRNAs in regulating cellular processes, miRNA malfunction has been attributed to a variety of pathologies, including inflammatory and autoimmune diseases, neurological disorders,[2] myocardial disease,[3] and cancers.[4] In addition, miRNAs are known to play a role in viral infections by invoking antiviral responses or regulating endogenous host miRNAs to aid in infection.[5,6] Hepatitis C virus (HCV) RNA synthesis and translation was previously reported to be stimulated by microRNA-122 (miR-122), a miRNA present primarily in liver cells and the liver cell line Huh-7.[7]

As a therapeutic option, chemically modified anti-miRNA oligonucleotides have been implicated as miRNA inhibitors (also called "antagomirs"[8]) that selectively prevent miRNAs from binding to the 3'-UTR of their target mRNAs.[9] A number of chemically-modified miRNA inhibitors, including peptide nucleic acids (PNAs), locked nucleic acids (LNAs), and 2'-*O*-methyl- and 2'-*O*-methoxyethyl-modified oligonucleotides, have been designed and used to improve their specificity and stability against degradation by nucleases.[10-13] Remarkably, a LNA-modified oligonucleotide complementary to miR-122, which is essential for the translation of HCV RNA in cultured liver cells, leads to long-lasting suppression of HCV in non-human primates.[5,14]

PNAs are synthetic DNA mimics consisting of a polyamide backbone that has a neutral charge and is resistant to degradation by nucleases.[15,16] PNAs bind to complementary DNA or RNA sequences with high affinity and high specificity, and the thermal stability of a PNA-RNA duplex is even higher than that of a PNA-DNA duplex or corresponding oligonucleotide duplex.[17] Due to this high stability when bound to its target oligonucleotide, numerous antisense PNAs have been developed to suppress protein or gene expression.[18] Although PNAs have great potential as antisense and gene-targeting agents, *in vitro* and *in vivo* applications of PNAs are limited because of their poor cell permeability.[19] To enhance cell permeability, several cell-penetrating peptides (CPPs) have been conjugated to PNA.[20,21] However, it has been previously shown that when PNA-oligopeptide conjugates were systematically administered, they were transported to the kidneys due to their small size.[22] Alternative delivery methods that increase the association of PNAs with cationic lipid complexes through conjugation of PNA with

[a]These authors contributed equally to this work.

lipophilic ligands have been suggested.[23] Use of oligonucleotide hybrids or PNA-DNA hybrids have been also attempted,[24] which introduces a negative charge to the uncharged backbone.

Cationic liposomes have been established as facile non-viral gene delivery systems due to the simple, rapid preparation methods for liposome-DNA complexes (lipoplexes) and relative safety, as demonstrated in a number of clinical and preclinical tests.[25,26] Among the several cationic lipids used to form lipoplexes, the cationic lipid called DKME (*O,O'*-dimyristyl-*N*-lysyl glutamate), which consists of a core lysine residue, two of 14 carbon-fatty acyl chains, and glutamic acid, has been shown to possess greater bilayer integrity and a stronger cationic surface charge than DOTAP (*N*-[1-(2,3-dioleoyloxy)]-*N,N,N*-trimethylammoniumpropane-methylsulfate) lipoplexes.[27] In particular, DMKE/cholesterol liposomes have been shown to be very efficient in delivering genes to hepatic cells both *in vitro* and *in vivo.*[27]

In the present study, we designed a PNA-DNA hybrid containing an antisense PNA targeting miR-122 and a 20-mer DNA complementary to the PNA. The PNA-DNA hybrid was complexed with cationic liposome prepared with DMKE/cholesterol and β-D-galactosyl ceramide to facilitate hepatocyte targeting. The cationic lipoplex containing the antagomir PNA-DNA hybrid was evaluated for cell specificity, intracellular distribution, and antiviral activity to reduce HCV translation in HCV-infected Huh-7 cells.

Experimental Section

Lipids. The cationic lipid *O,O'*-dimyristyl-*N*-lysyl glutamate (DMKE) was synthesized as previously reported.[28] C12 β-D-galactosyl ceramide (D-galactosyl- β-1,1 *N*-dodecanoyl-D-*erythro*-sphingosine), POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), DSPE-PEG_{2000} (1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-[methoxy (polyethylene glycerol)-2000], rhodamine-DOPE (rhodamine-[1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine]), and cholesterol were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL).

Preparation of Liposomes. Cationic liposomes were prepared as described previously with a slight modification.[29] Briefly, to prepare the galactosylated cationic liposome, DMKE, cholesterol, β-D-galactosyl ceramide, and rhodamine-DOPE were mixed in a molar ratio of 8:7:5:0.5 in chloroform:methanol (2:1, v/v). Alternatively, to prepare plain cationic liposome without galactosylated lipid, DMKE, cholesterol, POPC, and rhodamine-DOPE were mixed in a molar ratio of 8:7:5:0.5 in chloroform:methanol (2:1, v/v). To prepare neutral liposome, POPC, cholesterol, DSPE-PEG_{2000}, and rhodamine-DOPE were mixed in a molar ratio of 2:1:0.1:0.03 in chloroform:methanol (2:1, v/v). The chloroform and methanol were evaporated under a stream of N_2 gas, and vacuum desiccated for a minimum of 2 h to ensure removal of residual organic solvent. The dried lipid films (0.5 mg total lipids) were hydrated with 500 μL of HEPES buffer (20 mM HEPES, pH 7.4, and 150 mM NaCl) with a vortex mixer for 5 min. The hydrated lipids (1.0 mg/mL) were sonicated with a bath-type sonicator (Branson Inc. Danbury, CT) 3 times for 1 min with a 10-s interval, which produced small unilamellar liposomal vesicles. The liposome solution was then extruded through 800-, 400-, 200- and 100-nm pore size polycarbonate membranes (Millipore) to serially decrease extrusion size.

Preparation of PNA-DNA Hybrids and PNA-DNA Hybrid/Cationic Liposome Complex. The sequences of the anti-miRNA-122 PNA (synthesized and purchased from Panagene, Inc. Daejeon, Korea) and annealing DNA (Cosmo Genetech Co., Seoul, Korea) used in this study were *N*-CCATTGTCACACTCC-C and 5'-GGAGTGTGACAATGG TTTTT-3', respectively. Fluorescently labeled anti-miRNA-122 PNA (F-PNA) was also prepared by linking 6-carboxy-fluorescine (FAM) to the N-terminus with ethylene glycol (*O*-linker; Panagene, Inc.). Various amounts of the F-PNA and the annealing DNA (3:1, 2:1, 1:1, 1:2, and 1:3 molar ratios of F-PNA-DNA with a fixed concentration of 2 μM DNA) were mixed in 10 μL HEPES buffer for 1 h at room temperature. The mixture solutions were loaded onto a 2% agarose gel and visualized by UV illumination. The PNA-DNA hybrid (1:1 molar ratio) was gently mixed with the cationic liposomal solution at various liposome:PNA-DNA hybrid mass ratios (from 3:1 to 15:1) in appropriate volumes depending on experimental needs. For stable PNA-DNA/lipoplex formation, the PNA-DNA/lipoplexes were incubated at room temperature for 1 h prior to transfection.

Electrophoresis Mobility Shift Analysis of Lipoplexes. The differential ability of cationic liposomes to bind PNA-DNA molecules was determined by electrophoresis mobility shift assay. F-PNA-DNA hybrid molecules (0.1 μg) were mixed with rhodamine-labeled cationic liposome at various mass ratios, which were freshly prepared by gentle vortexing of a mixture containing F-PNA-DNA hybrid and liposome suspensions. The complexes were incubated for 1 h at room temperature. The mixture solution was loaded onto a 2% agarose gel and fluorescence images were obtained by UV illumination.

For radioactive detection of PNA-DNA (or RNA) hybrids, annealing DNA and microRNA-122 (sequence shown in Figure 1(a), purchased from ST Pharm Co. Ltd. Seoul, Korea) was end-labeled with ^{32}P using T4 polynucleotide kinase and [γ-^{32}P]ATP (GE Healthcare, Piscataway, NJ). Increasing amounts of liposome were incubated for 30 min in HEPES buffer with PNA-DNA hybrid that was prepared by mixing PNA (0.5 μM) and cold DNA spiked with ^{32}P-5'-end labeled DNA (at a final concentration of 0.5 μM). The PNA-DNA/liposome complexes were analyzed by electrophoresis mobility shift assay on a 2% agarose gel. The gels were scanned for radioactivity using a Cyclone PhosphorImager (PerkinElmer).

Fluorescence Resonance Energy Transfer (FRET). FRET was determined by monitoring the decrease in fluorescence of F-PNA (FAM, fluorescence donor) in the presence of rhodamine-conjugated liposome (rhodamine and fluorescence acceptor) using a spectrofluorophotometer (model RF-5301PC;

Shimadzu Inc., Kyoto, Japan). Fluorescent cationic liposome (1.65 μg) was mixed with F-PNA-DNA hybrid (0.11 μg, 125 nM) to form lipoplexes in 800 μL of HEPES buffer at room temperature for 1 h. After the lipoplex suspension was prepared, emission spectra between 500 and 700 nm were recorded with excitation at 485 nm at 25 °C.

Measurement of Lipoplex Size and Zeta-Potential. The size and zeta-potentials of the lipoplexes were measured using a Zetasizer (Nano ZS, Malvern, UK). The lipoplexes were prepared by mixing different ratios of cationic liposome and the PNA-DNA hybrid. Lipoplex suspensions in HEPES buffer were loaded into the capillary cell mounted on the Zetasizer, and their size and zeta-potentials were measured 5 times per sample at 25 °C.

Cell Culture and Assay for Cell Binding of Cationic Lipoplexes. Huh-7 cells, a human hepatoma cell line, and A549 cells, a human lung cancer epithelial cell line, were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 100 U of penicillin, 100 mg of streptomycin, and 10% fetal bovine serum (FBS). HCV replicon Huh-7 cells carrying HCV subgenomic replicon RNA were prepared and cultured as previously reported.[30] To monitor the cell binding of lipoplexes and cellular uptake of FAM-labeled PNA under confocal microscopy, HCV-replicon Huh-7 cells and A549 cells (1×10^5 cells, each) were seeded in a 35-mm cover glass-bottom dish (SPL Life Sciences, Seoul, Korea) in DMEM supplemented with 10% FBS at 37 °C under 5% CO_2 for 24 h. The fluorescent PNA-DNA hybrid/lipoplexes were prepared prior to addition to the cells by mixing F-PNA-DNA hybrid (1.1 μg) and rhodamine-labeled cationic liposome (16.5 μg). After the cells were rinsed twice with PBS, they were treated with fresh media (200 μL) containing the prepared lipoplex suspension (23.4 μL) and further incubated for 4 or 8 h at 37 °C. After the lipoplex-treated cells were washed 3 times with PBS, fresh media was added. The cells were visualized with a confocal laser-scanning microscope (FV-1000; Olympus, Tokyo, Japan).

Dual-Luciferase Assay. The inhibition of HCV replicon RNA translation by PNA in Huh7 cells was analyzed by measuring luciferase activity from the HCV replicon using the dual-luciferase assay. Huh7 cells harboring HCV subgenomic replicon RNA adapted with the luciferase reporter system (Huh-7 Luc-Neo cells, kindly provided by Dr. Bartenschlager, University of Heidelberg, Germany) were seeded at 8×10^4 cells/well in a 24-well plate and incubated in serum-containing medium at 37 °C for 24 h. The PNA-DNA hybrid/lipoplexes were prepared by mixing PNA-DNA hybrid (1.0 μg) or PNA alone (0.4 μg) with the galactosylated or ungalactosylated cationic liposome (15.0 μg), and then added to the cells. After the cells were washed twice with PBS, they were treated with the serum-free media (200 μL) containing the prepared lipoplex suspension (23.4 μL) or Lipofectamine 2000 (Invitrogen) containing PNA or PNA-DNA hybrid (prepared as suggested by the manufacturer) and further incubated at 37 °C for 4 or 24 h. For experiments with a 4 h liposome treatment, the liposome-treated cells were washed twice with PBS 4 h after transfection. The cells were further incubated in serum-containing medium at 37 °C for 20 h. After incubation, cells in each well were lysed with 100 μL of 1× Passive Lysis Buffer (PLB) (Promega, Madison, WI). Cell lysate (70 μL per condition) was transferred to a 96-well plate, and 70 μL of firefly luciferase assay reagent were added, and then 70 μL of Stop and Glo reagent were added following the instructions for Dual-Luciferase Reporter Assay system (Promega). The relative light units were measured with multilabel plate reader (VICTOR X3; PerkinElmer), and the ratios of firefly luciferase activity was normalized to Renilla luciferase activity. All experiments were performed in duplicate, and values were normalized to the total protein content of each sample.

Dissociation of PNA from PNA-DNA Hybrid. PNA (100 nM) and ^{32}P-DNA (45 nM) were mixed in 5 μL HEPES buffer (pH 7.4) for 30 min at room temperature to generate the ^{32}P-DNA-PNA hybrid. To obtain various pHs (pH 4, 5, 6), different amounts of citrate buffer (pH 2) were added to the ^{32}P-DNA-PNA hybrid solution. After incubation at room temperature for 1 h, excess amounts of the trap DNA with the same sequence as the annealing DNA without the ^{32}P-label was added to the reaction mixture (at a final concentration of 2.5 μM), and the 20-μL solution was incubated at room temperature for 30 min. The solutions were loaded onto and separated on a 10% non-denaturing PAGE. Radioactivity was quantified using a Cyclone PhosphorImager (PerkinElmer) and analyzed by OptiQuant/Cyclone software (Packard Instrument Co. Meriden, CT).

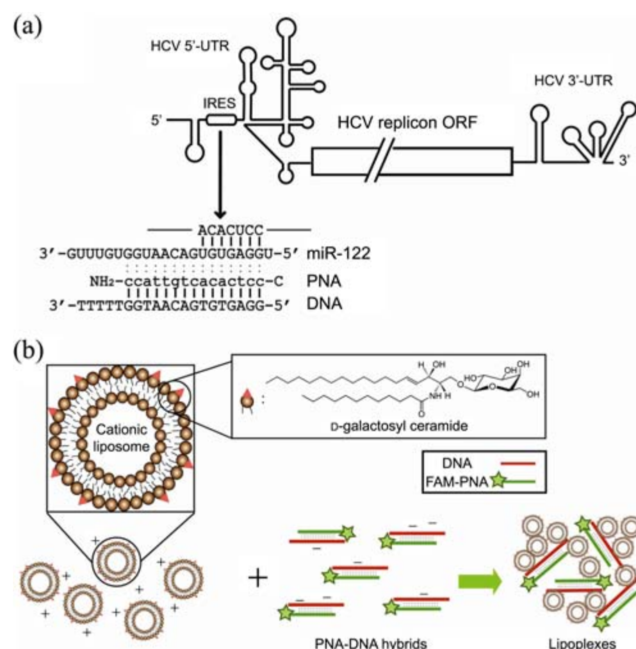

Figure 1. Schematic illustration of antagomir PNA and cationic lipoplex formation. (a) Sequestration of miR-122 with antagomir PNA in Huh-7 cells decreases HCV translation. MiR-122 and its target sequences (a portion of IRES) are shown. Antagomir PNA and its annealing DNA sequences are shown. (b) Preparation of galactosylated cationic lipoplex complexed with PNA-DNA hybrid molecules.

Results and Discussion

Formation of Cationic Lipoplex with PNA-DNA Hybrid. MicroRNA-122 is complementary to the IRES target sequences located in 5'-UTR of the HCV RNA genome (Fig. 1(a)). An antagomir 15-mer PNA was designed to sequester miR-122 in Huh-7 cells carrying the HCV genome. To track the PNA in the cell, the N-terminus was tagged with fluorescein (FAM). DNA (20-mer) with a sequence complementary to the PNA was constructed (Fig. 1(a)). To increase PNA uptake by the target cells, PNA was annealed to the DNA to form a negatively charged PNA-DNA hybrid that was expected to form a complex with cationic liposome (Fig. 1(b)). Formation of the PNA-DNA complex was analyzed by electrophoretic mobility shift assay on a 2% agarose gel, and FAM-labeled PNA and the PNA-DNA hybrid were visualized by UV irradiation. An increase in the annealing DNA relative to PNA caused disappearance of free PNA and appearance of PNA-DNA hybrid as a fast migrating fluorescent band due to its negative charge (Fig. 2(a) left). Equimolar amounts of PNA and DNA (at a 1:1 molar ratio) readily formed a PNA-DNA hybrid, as judged by the disappearance of free PNA. Then, we prepared the novel cationic liposome consisting of galactosylated ceramide, DOPE, cholesterol, and DKME. Since galactose has been shown to selectively bind to the asialoglycoprotein receptor found on the surface of hepatocytes and be internalized *via* receptor-mediated endocytosis,[31] galactosylated ceramide is expected to target the liposome complex to the hepatocyte surface. We used a novel cationic lipid, DMKE, which is composed of lysine (K), glutamate (E), and 14 carbon-fatty acyl chains, all of which are biocompatible and biodegradable.[27] The DNA-binding affinity of DMKE/cholesterol liposomes has been previously shown.[27] Therefore, we confirmed formation of the complex containing F-PNA-DNA hybrid and cationic liposome by electrophoretic mobility shift assay. The amount of F-PNA-DNA hybrid was fixed and the amount of cationic liposome with rhodamine dye was 3 to 15 fold (w/w) higher than that of the F-PNA-DNA hybrid. As the amount of the rhodamine-conjugated cationic liposome increased, the F-PNA-DNA hybrid disappeared and the intensity of the rhodamine fluorescence from the liposome increased (Fig. 2(a) right). Complex formation between the green-colored F-PNA-DNA hybrid and the purple-colored rhodamine liposome resulted in observation of pale yellow-colored mixed fluorescence representing liposome complex in the well. The shift of PNA in the gel was not observed when PNA was mixed with cationic liposome in the absence of the anneal-

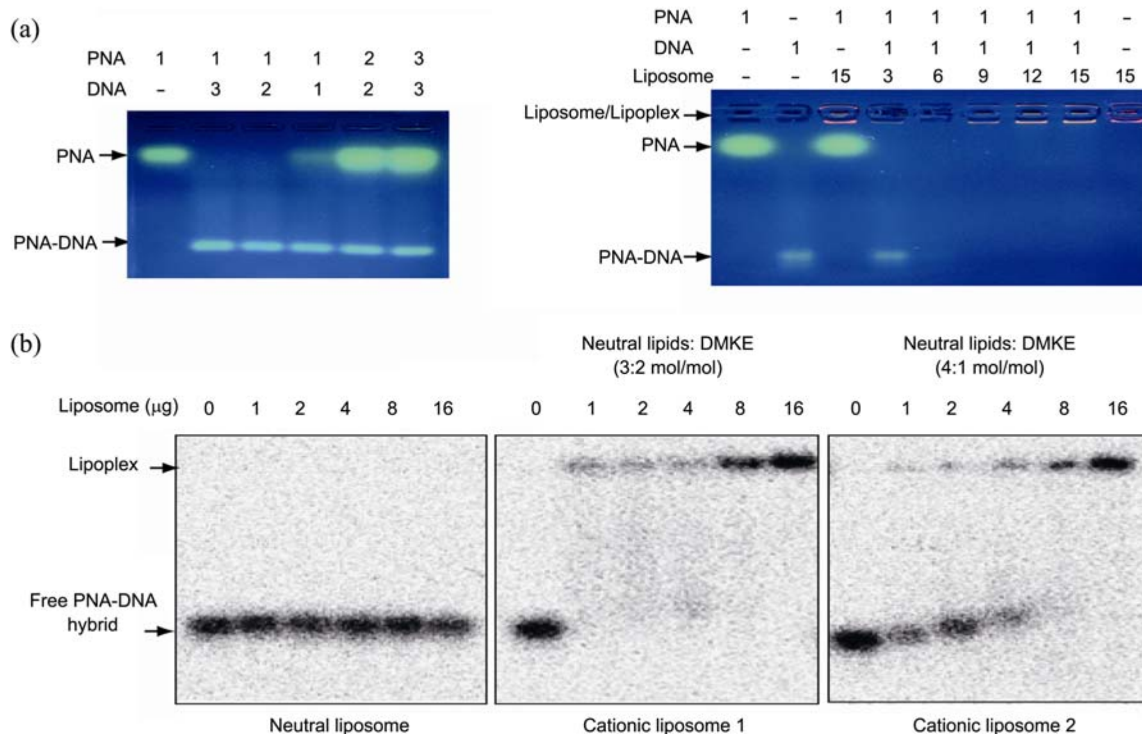

Figure 2. Formation of cationic liposome harboring PNA-DNA hybrid. An electrophoretic mobility shift assay was performed to determine optimal ratios for PNA-DNA hybrid and lipoplex formation. (a) Various amounts of the FAM-labeled PNA and annealing DNA were mixed for 1 h; (at 3:1, 2:1, 1:1, 1:2 and 1:3 molar ratios of F-PNA-DNA at a fixed concentration of 2 mM DNA). Green fluorescence images were obtained by UV radiation. PNA-DNA hybrid (1:1 molar ratio) was mixed with cationic liposomes at increasing ratios (w/w), and then run on a 2% agarose gel. To detect FAM fluorescence (green) and rhodamine fluorescence (purple), gel images were obtained by UV illumination. (b) Comparison of complexation efficiencies of DNA-PNA hybrids with different liposomal formulations. The indicated amounts of liposome were incubated in the presence of cold or ^{32}P-labeled DNA-PNA hybrids, and formed complexes were resolved on an agarose gel and detected by autoradiography. Complexation of PNA-DNA hybrids with liposomes containing different molar ratios of DMKE and neutral lipids was analyzed. Neutral liposomes do not contain any positively-charged lipids. Upper bands corresponding to lipoplexes remained in the wells.

ing DNA. This result indicates that hybridization of the DNA with the PNA in a sequence-specific manner imparts a negative charge to the PNA, resulting in formation of the liposome complex (lipoplex). Hereafter, we used lipoplexes formulated in a PNA:DNA:liposome ratio of 1:1:15 (w/w) to ensure that the PNA-DNA hybrid was fully complexed with the cationic liposomes.

To better understand the properties of the cationic lipid (DMKE) in lipoplex formation, the capability of neutral liposomes and cationic liposomes prepared with DMKE at different molar ratios to bind PNA-DNA hybrids was determined by gel shift experiments using agarose gels (Fig. 2(b)). ^{32}P-labeled DNA was hybridized to PNA in a 1:1 molar ratio, and the PNA-DNA hybrid was then mixed with neutral liposomes and cationic liposomes prepared at 2 different DMKE:neutral lipids ratios (2:3 and 1:4). As expected, only cationic liposomes were able to form complexes with the PNA-DNA hybrid. As the amount of cationic liposome increased, complexation of PNA-DNA hybrids with cationic liposomes were detected, with a concomitant decrease and increase in the intensity of the radioactive bands corresponding to free PNA-DNA hybrids and liposome complexes, respectively. Due to the high stability of PNA-DNA hybrids during electrophoresis,[32] radioactivity trapped in the wells of the agarose gel represents the PNA-DNA/liposome complexes. As judged by the accumulation of the radioactivity in the wells, corresponding to lipoplexes, the efficiency of cationic liposomes binding to PNA-DNA hybrids was higher for the liposomes prepared at a ratio of 2:3 (DMKE:neutral lipids) than those prepared at a ratio of 1:4. This result evidently represents that the positive charge imparted by DMKE is the major determinant of liposome complex formation with negatively-charged PNA-DNA hybrid molecules.

Characterization of Cationic Lipoplexes Containing PNA-DNA Hybrid. Observation of mixed color fluorescence due to complex formation between FAM-labeled PNA and rhodamine-conjugated liposome prompted us to investigate whether fluorescence resonance energy transfer (FRET) would occur due to the proximity of the 2 fluorescent dyes in the lipoplex. In addition to conventional gel retardation assay, detection of FRET between the nucleic acid and liposome confirms lipoplex formation. As shown in Figure 3(a), when excited at 485 nm, FAM-labeled PNA and rhodamine-conjugated liposome have characteristic emission peaks at 525 nm and 585 nm, respectively. When the F-PNA-DNA hybrid and the liposome were mixed at a 1:15 weight ratio, an increase in rhodamine fluorescence (585 nm) and a decrease in FAM fluorescence (525 nm) were observed with excitation at 485 nm (Fig. 3(a)). This result indicates that the FRET donor (*i.e.*, the FAM-PNA) was close to the FRET acceptor (*i.e.*, rhodamine), confirming formation of the lipoplex between the PNA-DNA hybrid and the cationic liposome.

Particle size and the zeta-potential of the lipoplex, major parameters governing nucleotide transfection efficacy, were measured and compared at different mixing ratios of PNA-DNA hybrid and cationic liposomes (Fig. 3(b)). Plain cationic

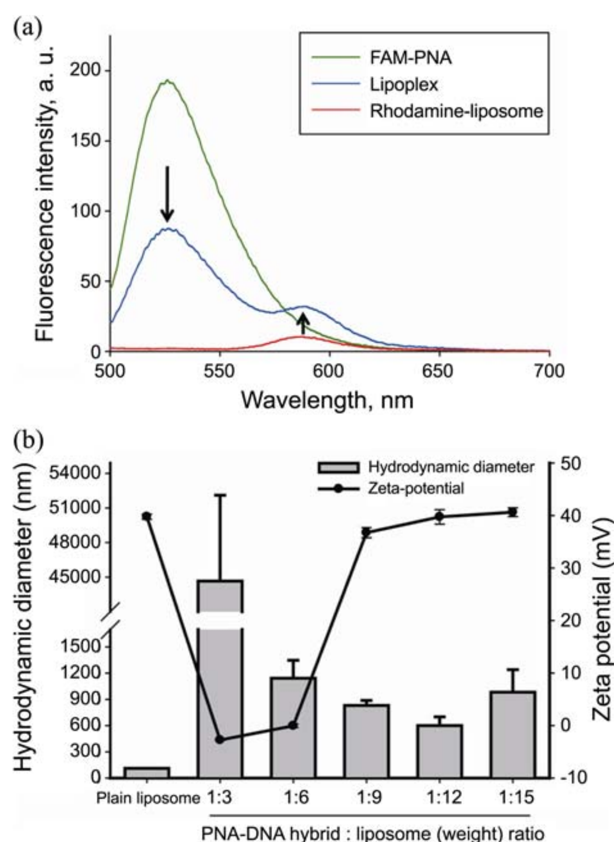

Figure 3. FRET, size, and zeta-potential analysis of cationic lipoplexes. (a) FRET detection in lipoplex formation. Rhodamine-conjugated cationic liposome was mixed with F-PNA-DNA hybrid to form lipoplexes. Decreased F-PNA fluorescence (FAM, FRET donor; 525 nm) and increased rhodamine fluorescence (rhodamine, FRET acceptor; 585 nm) was detected by excitation at 485 nm (indicated by arrows). (b) Particle size and zeta-potential of the lipoplexes. PNA-DNA/liposome lipoplexes (1:3, 1:6, 1:9, 1:12, and 1:15, weight ratios of PNA-DNA:liposome) were loaded into a capillary cell mounted on the Zetasizer. The numbers shown were calculated from 3 measurements at 25 °C.

liposomes were measured as 110 nm in size and 40 mV in surface charge prior to lipoplex formation with the PNA-DNA hybrid. An increase in the amount of cationic liposome at a constant amount of PNA-DNA hybrid led to an increase in surface charge, reaching a saturated zeta-potential of approximately 40 mV after lipoplex formation at a 1:9 weight ratio of PNA-DNA hybrid to liposome. This result suggests that the positively charged liposomes completely bind to PNA-DNA hybrid at a mixing ratio of 1:9. As the amount of liposome increased, lipoplex size decreased, with a minimum of approximately 550 nm at a 1:12 weight ratio of PNA-DNA hybrid to liposome (Fig. 3(b)). The increase in average lipoplex size at a 1:15 ratio can be explained by aggregation of the lipoplexes due to excess liposome.

Cellular Uptake of PNA *via* Selective Binding of Galactosylated Lipoplex to Hepatocytes. The antagomir PNA-transferring capabilities of cationic lipoplexes with or without galactosyl residues were examined in 2 cell lines, HCV-replicon Huh-7 cells harboring asialoglycoprotein receptors for galactose on the cell surface[33] and A549 cells that

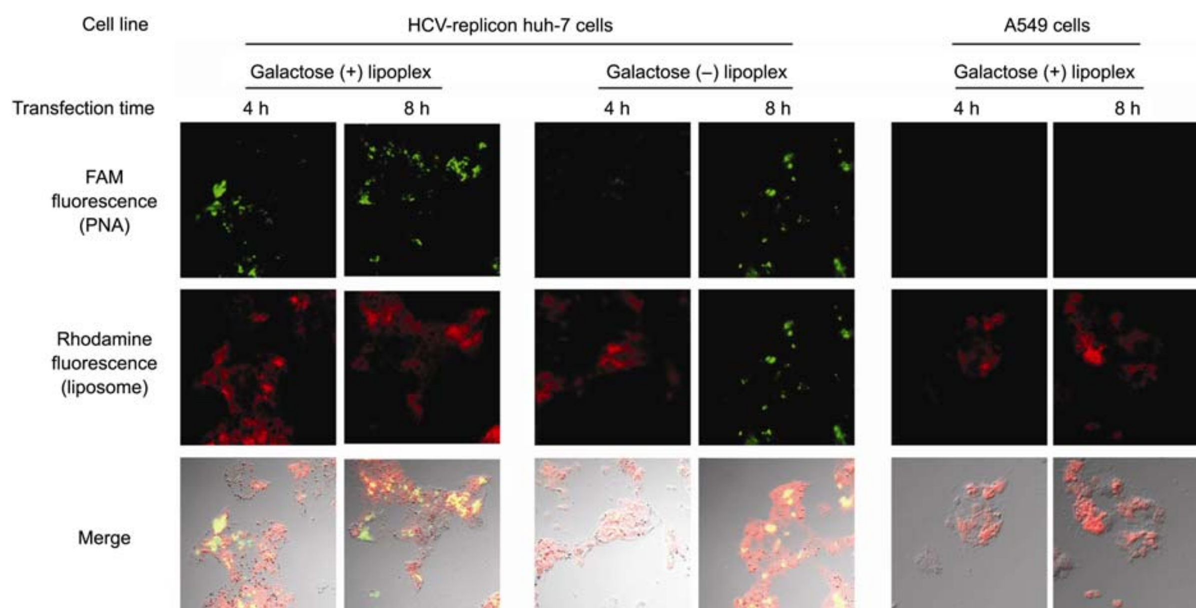

Figure 4. Cell-binding and PNA-transferring activities of cationic lipoplexes in hepatocytes. *In vitro* cell-specific binding was assayed using confocal microscopy. F-PNA-DNA hybrid (1.1 μg) and rhodamine-labeled cationic liposomes (16.5 μg) were incubated for 1 h prior to addition to cells. Transfection of fluorescent lipoplexes into 2 different cell lines (Huh-7 and A549) was performed for 4 and 8 h. Fluorescence confocal images were taken after incubation. FAM-labeled PNA is shown in green, while rhodamine-conjugated liposomes are shown in red inside of the cells. Merged fluorescence in the phase-contrast images of cells are shown at the bottom.

originated from lung cancer epithelium and are devoid of the galactose receptor. The 2 cell lines were incubated with rhodamine-labeled lipoplexes containing FAM-labeled PNA-DNA hybrid molecules for 4 and 8 h. Fluorescently labeled PNAs complexed with DNA and cationic liposome were internalized into hepatocytes, whereas PNA alone was not delivered into the A549 cells (Fig. 4). After a 4-h transfection, FAM fluorescence from PNA was only observed in hepatic cells transfected with the galactosylated lipoplexes. At 8 h post-transfection, internalization of PNA was observed in hepatocytes transfected with non-galactosylated lipoplexes; however, but internalization was lower than that in cells transfected with galactosylated lipoplexes. Therefore, the hepatocyte surface-binding affinities of galactosylated lipoplexes were superior to that of non-galactosylated lipoplexes, suggesting that the galactosylated lipoplexes facilitate targeted delivery of the PNA-DNA hybrid to the hepatocytes. Pale yellow color was observed when FAM and rhodamine fluorescence were merged in confocal images (Fig. 4), indicating that a portion of the PNA delivered into the cells remained as lipoplexes inside the cells. At 12 h post-transfection, slight PNA fluorescence was also observed in A549 cells (data not shown). These results indicate that the cationic lipoplex formulated in our study more efficiently delivered PNA into hepatocytes than into lung epithelial cells. Previous studies suggested that efficient transfection by the DMKE-containing lipoplexes is due to the membrane fluidity of cationic lipid bilayers and the enhanced binding to the cell surface.[27,34]

Suppression of HCV Genome Translation by Antagomir PNA Delivered with Lipoplexes. Sequestration of miRNA-122 has been previously shown to reduce HCV IRES-dependent translation of the HCV genome in cytoplasm.[5,35] We next investigated whether antagomir PNA delivered *via* galactosylated liposomes suppressed HCV genome expression in cells. HCV RNA genome expression was evaluated with an *in vitro* translational system using the luciferase reporter assay in HCV-replicon Huh-7 Luc-Neo cells (Fig. 5(a)). Cells were incubated with PNA-DNA hybrids that were complexed with galactosylated or plain (non-galactosylated) cationic liposomes as well as other conventional transfection reagents, like Lipofectamine 2000 as a control. Luminescence, which reflects HCV genome translation, was measured in each transfected cell at 2 different times of post-transfection (4 and 24 h). At 4 h post-transfection, HCV genome translation was strongly suppressed in cells transfected with the galactosylated lipoplexes, whereas little suppression was observed in cells transfected with PNA-DNA hybrid delivered with plain cationic liposome or Lipofectamine (Fig. 5(b)). These results are consistent with the cellular uptake of PNA observed under confocal microscopy, in which PNA was seen in cells transfected with the galactosylated lipoplexes 4 h post-transfection (Fig. 4). After a 24-h transfection and an additional 24-h incubation, HCV genome translation was suppressed to a similar extent with both galactosylated and plain lipoplexes. However, the cells transfected with Lipofectamine did not exhibit antagomir PNA activity. No cytotoxicity was observed with PNA alone or with the lipoplexes in these culture conditions (data not shown). These results indicate that the galactose moieties in the cationic liposomes efficiently enhanced the cell-binding rate of the lipoplexes and subsequent delivery of PNA into cells. In addition, cationic liposomes prepared in our study were readily capable of delivering PNA-DNA hybrid mole-

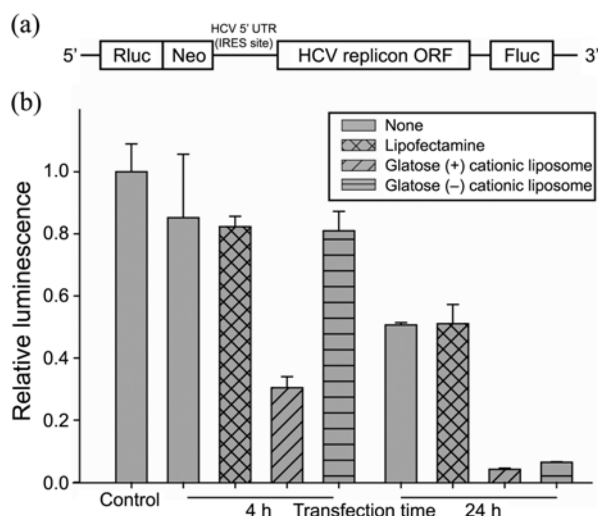

Figure 5. Suppression of HCV translation by PNA delivered into hepatic cells. (a) A luciferase reporter system based on expression of the HCV subgenome in Huh-7 Luc-Neo cells. The construct consists of firefly luciferase (Fluc), Renilla luciferase (Rluc), and a neomycin antibiotic resistance marker (Neo). (b) PNA-DNA hybrids were transfected into HCV-replicon Huh-7 Luc-Neo cells with Lipofectamine, (galactosylated or non-galactosylated) lipoplexes, or no transfection agents (None). Transfected cells were washed with the PBS at 4 h and 24 h post-transfection. Luciferase activity in the cell lysates was measured by the dual-luciferase assay reporter system at 24 h after transfection. Control denotes HCV genome expression without PNA transfection, to which all other values were normalized. The data indicated are the average of 2 independent experiments with standard deviation.

cules into the cytoplasm, where the antagomir PNA is likely to sequester miRNA-122 and suppress IRES-translation of the HCV genome.

Discharge of PNA from the PNA-DNA Hybrid in the Endosomal Environment. Next we wanted to investigate whether the antagomir PNA was displaced from the PNA-DNA hybrids at low pH, which mimics the endosomal environment. Since galactose receptor-mediated endocytosis is a probable mechanism for introduction of galactosylated gene carrier into cells,[36] the endosome/lysosome is a place the PNA-DNA hybrid is likely to encounter in the cytoplasm. Therefore, PNA dissociated from the PNA-DNA hybrid at low pH should be effective as an antagomir agent in the cytoplasm. We examined the discharge of PNA from the PNA-DNA complex at various pH conditions by native PAGE (Fig. 6(a)). To inhibit reannealing of the displaced ^{32}P-labeled DNA to PNA, an excess of trap DNA with same sequence as the annealing DNA without the ^{32}P-labeling was included in the reaction mixture after the pH change. As the pH decreased, the amount of PNA dissociated from the PNA-DNA hybrid increased. At pH 4, most of the PNA (about 80%) was discharged from the PNA-DNA hybrid (Fig. 6(b)). In contrast, incubation of the PNA-DNA hybrid complex at neutral pH (pH 7.4) did not dissociate the PNA from the complex, and less than half of the PNAs remained as a hybrid. These results suggest that the PNA hybridized to DNA was readily dissociated from the PNA-DNA hybrid at

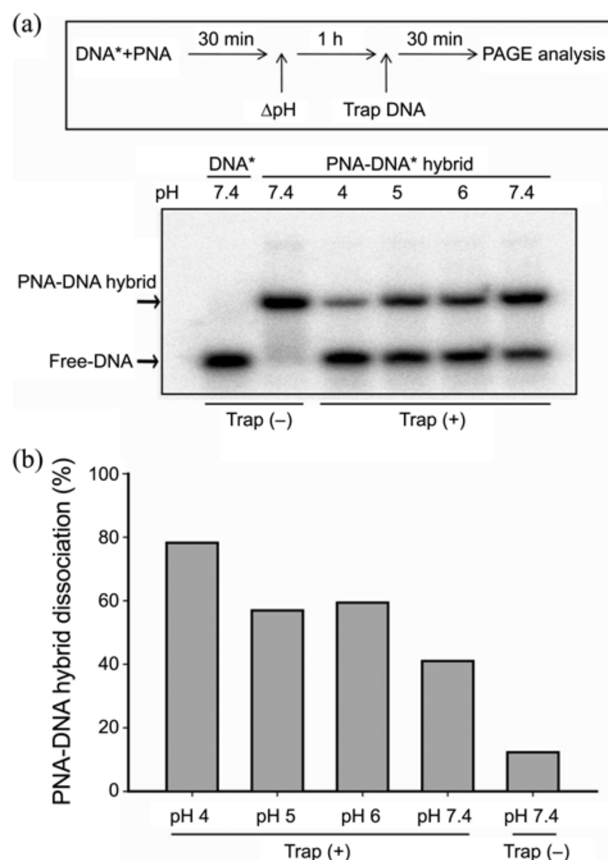

Figure 6. Dissociation of PNA from the PNA-DNA hybrid at low pH. PNA was mixed with ^{32}P-labeled DNA and incubated in HEPES buffer (pH 7.4) for 30 min. Citrate buffer (pH 2.0) was added to the mixture to adjust the pH and further incubated. After incubation for 1 h, trap DNA was added to the mixture. (a) The reaction scheme is shown in the upper panel. Radioactive bands corresponding to the PNA-DNA hybrid and the free ssDNA were resolved by 10% non-denaturing PAGE. (b) The radioactivity in the bands was quantified, and the percentage of free DNA dissociated from the PNA-DNA hybrid molecules are shown in the graph.

endosomal pH and it exists as a single strand. Because oligopeptides are known to be capable of escaping through the endosomal membrane,[36] the released PNA can be transported from the endosome into the cytoplasm. Therefore, the antagomir PNA released into the cytoplasm presumably binds to the target microRNA (miR-122), acting as an effective antiviral suppressor of HCV genome translation in hepatocytes.

In this study, an antagomir PNA targeting miR-122, which is required for translation of the HCV genome in hepatocytes, was hybridized to a DNA oligonucleotide and complexed with cationic liposome for cellular delivery of PNA. The galactosylated ceramide-containing cationic liposome was capable of hepatocyte-specific delivery of PNA-DNA hybrid molecules. The PNA-DNA hybrid was readily dissociated in the low pH endosomal environment. The antagomir PNA delivered to hepatocytes harboring the HCV replicon efficiently suppressed HCV expression genome. In particular, galactosylation of liposome significantly enhanced

the cell binding of the lipoplexes and PNA delivery to hepatic cells. According to the data presented in this study, PNA can be readily delivered to hepatocytes using complementary DNA oligonucleotide, cationic liposome-containing galactosylated ceramide, and a novel cationic lipid, DMKE. Transfection of PNA-DNA hybrid molecules with galactosylated cationic liposome into hepatocytes suggests that galactosylated cationic liposome can be utilized as an efficient carrier of antagomir PNA oligonucleotides as antiviral agents *in vivo*.

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