HOKKAIDO UNIVERSITY

Title	The Polyethyleneglycol Dilemma: Advantage and Disadvantage of PEGylation of Liposomes for Systemic Genes and Nucleic Acids Delivery to Tumors
Author(s)	Hatakeyama, Hiroto; Akita, Hidetaka; Harashima, Hideyoshi
Citation	Biological & Pharmaceutical Bulletin, 36(6), 892-899 https://doi.org/10.1248/bpb.b13-00059
Issue Date	2013-06
Doc URL	https://hdl.handle.net/2115/53045
Type	journal article
File Information	36_b13-00059.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Current Topics

Polyethyleneglycol: A Classical but Innovative Material

The Polyethyleneglycol Dilemma: Advantage and Disadvantage of PEGylation of Liposomes for Systemic Genes and Nucleic Acids Delivery to Tumors

Hiroto Hatakeyama,* Hidetaka Akita, and Hideyoshi Harashima

Laboratory of Innovative Nanomedicine, Faculty of Pharmaceutical Sciences, Hokkaido University; Sapporo, Hokkaido 060–0812, Japan.

Received January 19, 2013

Gene and nucleic acid therapy is expected to play a major role in the next generation of agents for cancer treatment. We have recently developed a multifunctional envelope-type nano device (MEND) for use as a novel nonviral gene delivery system. The modification of polyethyleneglycol (PEG), *i.e.*, PEGylation, is a useful method for achieving a longer circulation time for the delivery of MEND to a tumor *via* the enhanced permeability and retention (EPR) effect. However, PEGylation strongly inhibits cellular uptake and endosomal escape, which results in significant loss of activity of the delivery system. For successful nucleic acid delivery for cancer treatment, the crucial problem associated with the use of PEG, *i.e.*, the "PEG dilemma" must be resolved. In this review, we describe the development and applications of MEND and discuss various strategies for overcoming the PEG dilemma based on the manipulation of both pharmacokinetics and intracellular trafficking of cellular uptake and endosomal release. To increase cellular uptake, target ligands including proteins, peptides, antibodies and aptamers that recognize molecules specifically expressed on tumors are first introduced. Second, cleavable PEG systems are described. The cleavage of PEG from carriers was achieved in response to the intracellular environment as well as the tumor microenvironment, which improvs cellular uptake and endosomal escape. Then, endosomal fusogenic peptides are discussed. Finally, pH-sensitive liposomes using pH-sensitive lipids are described.

Key words liposome; small interfering RNA; tumor; multifunctional envelope-type nano device; drug delivery system

1. INTRODUCTION

Liposomes are now well-recognized drug delivery vehicles that can be used in cancer therapy.[1] From the viewpoint of practical use, systemic administration is desirable because it can be performed easily. After intravenous administration, a liposome is adsorbed by biological components such as serum proteins (opsonins) in the systemic circulation. An opsonized liposome is recognized by the mononuclear phagocytic system (MPS) located in the liver and spleen, originally known as the reticuloendothelial system (RES), which functions as an important host defense mechanism.[2] In the early 1990s, it was reported that the modification of liposomes with polyethyleneglycol (PEG), *i.e.*, PEGylation, allowed them to circulate for remarkably long periods of time in the blood circulation after intravenous administration.[3–5] The PEG moiety forms an aqueous layer on the surface of liposomes and provides stabilization of the lipid bilayer and steric hinderance, resulting in the inhibition of protein adsorption and less recognition by macrophages. In general, the modification of neutral liposomes with approximately 5 mol% PEG-lipid in the total lipid content allows long-term circulation of liposomes in the blood after intravenous administration.[6,7] The term "stealth" was applied to these liposomes because of their ability to evade interception by the immune system in much the same way as the stealth bomber is able to evade detection by radar.[8]

Matsumura and Maeda found that high molecular-weight (≥40 kDa), long-circulating macromolecules as well as various long-circulating nanoparticulate pharmaceutical carriers are capable of spontaneous accumulations in various pathological sites, such as solid tumors and infracted areas, *via* the so-called enhanced permeability and retention (EPR) effect.[9,10] This effect is based on the fact that the pathological neovasculature of tumor tissue, unlike normal tissues, contains a discontinuous or absent basement membrane, making it "leaky."[11] This allows large macromolecules and even small particles to extravasate and accumulate in the interstitial tumor space. Such accumulation is also facilitated by the lack of a lymphatic system, which is responsible for the drainage of macromolecules from tissues. It is well known that long-circulating liposomes with an average diameter of 100–200 nm accumulate efficiently in tumor tissues *via* the EPR effect, which is known as "passive targeting."[12–14] Advances in long-circulating liposomes accumulating in tumors *via* the EPR effect allow liposomes to be used clinically in tumor chemotherapy. Doxil (Caelyx), PEGylated liposomal doxorubicin with 100 nm in diameter, accumulates in tumors at high levels and has fewer side effects compared with free doxorubicin, and is used in the treatment of acquired immunodeficiency syndrome (AIDS)-related Kaposi's sarcoma, ovarian cancer, and breast cancer.[15] Anticancer agent-incorporating liposomal products are also being studied for possible clinical trials.[16,17]

The authors declare no conflict of interest.

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

© 2013 The Pharmaceutical Society of Japan

2. THE APPLICATION OF PEGYLATED LIPOSOMES FOR DELIVERING NUCLEIC ACIDS TO CANCER: PEG DILEMMA

The use of genes and functional nucleic acids in medicine such as plasmid DNA (pDNA), oligodeoxynucleotide, small interfering RNA (siRNA) or anti-microRNA (miRNA) oligonucleotides represents a promising new approach for treating a variety of genetic and acquired diseases, including cancer.[18–20] For functional nucleic acids to be therapeutically efficacious, nano carriers must deliver their cargos to the appropriate intracellular compartment where functional nucleic acids act, *i.e.*, the nucleus or cytosol.[21] However, the physical properties of nucleic acids such as highly hydrophilic macromolecules with negative charges restrict their binding to the cell surface and passive diffusion across lipophilic cell membranes. This means that the most important and most difficult challenge in gene and nucleic acid therapy is the issue of delivery. Numerous nonviral delivery strategies have been developed to circumvent this problem, and some of them have been successfully used for the introduction of nucleic acids into cells both *in vitro* and *in vivo*. To condense negatively charged nucleic acids into delivery vehicles, most nanoparticles contain cations such as cationic polymers or lipids.[22,23] Cationic liposomes have been among the more efficient synthetic gene delivery system reagents *in vitro* since the late 1980s. The first successful *in vitro* transfection with a cationic lipid was described by Felgner *et al.* in 1987, when *N*-[1-(2,3-dioleyloxy)-propyl]-*N*,*N*,*N*-trimethylammonium chloride was synthesized to form a complex of cationic liposomes with pDNA, *i.e.*, a lipoplex.[24] Following the success of the lipid in gene transfection, numerous cationic lipids, such as 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), *N*,*N*-dimethyl-*N*-[2-(sperminecarboxamido)ethyl]-2,3-bis(dioleyloxy)-1-propaniminium pentahydrochloride, dioctadecylamidoglycylspermine, 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]cholesterol and diC14-amidine have been synthesized and used for transfection.[25–28]

As a novel liposomal delivery system for nucleic acids, we have developed a multifunctional envelope-type nano device (MEND).[29–31] The ideal MEND consists of nucleic acids in free form or as a condensed or complexed core with a polycation and a lipid envelope structure equipped with the various functional devices, such as PEG, specific target ligands, and cell-penetrating peptides to manipulate both *in vivo* pharmacokinetics and intracellular trafficking. For *in vivo* systemic administration, a lipid envelope of MEND is composed of DOTAP as a cationic lipid and dioleoylphosphatidylethanolamine (DOPE) and cholesterol as helper lipids. After entering the blood stream, the cationic charged surface of MEND immediately interacts with anionic serum proteins and cells in blood, resulting in accumulation in the liver and spleen. To prevent MEND from recognition by the RES, the cationic surface should be masked by greater amounts of PEG (10–15 mol% of total lipids) than neutral liposomes (*ca.* 5 mol% of total lipids), since cationic charged surfaces strongly interact with anionic plasma proteins.[32–35]

PEGylated MEND achieves long-term circulation in the blood and accumulates in tumors *via* the EPR effect as expected. However, the surface aqueous phase formed by the PEG moiety inhibits the interaction of MEND with the target cell surface. As a result, cellular uptake is nil or minimal. Furthermore, PEGylation improves the stability of the lipid envelope of MEND, which results in poor endosomal escape *via* membrane fusion and in degradation of cargos in lysosomes, the digestive compartments.[36,37] These serious issues in the use of PEG in gene and nucleic acid delivery to cancer are referred to as the "PEG dilemma."[32,33] Therefore, a successful gene and nucleic acid delivery for cancer treatment requires a rational strategy and the design of carrier systems to overcome the issues associated with the use of PEG.

3. STRATEGIES FOR OVERCOMING THE PEG DILEMMA

3.1. Enhancement of Cellular Uptake The cause of the PEG dilemma should be clarified o develop strategies to overcome it. The positive charge on the surface of the lipoplexes ensures their efficient binding to the cell surface. Anionic glycoproteins such as heparan sulfate proteoglycans have been proposed to play a role in the interactions of lipoplexes with the cell surface.[38,39] Therefore, one reason for issues associated with the use of PEG could be explained by steric hindrance conferred by PEG which inhibits the interactions between the surface positive charge of lipoplexes and anionic molecules on the cell surface and subsequent cellular uptake of lipoplexes. The first method for overcoming this problem is to display ligands for receptors on the surface of targeted cells on PEGylated carriers. This would be expected to improve the selectivity, binding and uptake of the carriers by targeted cells and is known as "active targeting." Target molecules are carefully selected based on their characteristics such as specificity, expression level on tumor cells, and ability to internalize ligand-modified PEGylated nanoparticles.[40] Proteins, vitamins, peptides, antibodies and antibody fragments, and nucleic acids (aptamers) are employed as target ligands.

Transferrin (Tf), an iron-binding glycoprotein, and antibodies have been widely utilized as target ligands for drug and nucleic acid delivery to target to Tf receptors that are overexpressed by tumor cells.[41–46] Folate receptors are also overexpressed in many types of tumor cell while their distribution in normal tissues is minimal,[47] and folate-modified liposomes enhanced the cellular uptake and antitumor efficacy of encapsulated drugs and nucleic acids.[48–55] A hyaluronan-modified PEGylated liposome was attempted to target a melanoma overexpressing CD44, which is a surface receptor and binds to hyaluronan.[56,57] Huang and coworkers demonstrated that a PEGylated liposome-polycation-DNA nanoparticle (LPD) modified with anisamide on top of PEG efficiently delivered siRNA to lung carcinomas with high expression levels of sigma receptors, which resulted in luciferase gene silencing in metastatic tumors compared with nontargeted PEGylated LPD.[58,59]

Antibodies have been widely investigated as ligands for the tumor targeting of liposomes due to their high specificity and affinity for target molecules.[60] Antibody-modified PEGylated liposomes, *i.e.*, immunoliposomes, are prepared by attaching the specific monoclonal immunoglobulin G (IgG) antibody, Fab′ fragment, and recombinant single-chain variable fragment on top of the PEG chain. A well-demonstrated target of immunoliposomes is human epidermal growth factor (EGF) receptor (EGF-R) type2 (HER2).[61–65] The EGF-R,

which is upregulated in tumors including epithelial tumors, glioblastoma, and hepatocellular carcinoma, is another widely investigated target of immunoliposomes.[66,67] Incorporation of EGF into PEGylated nanoparticles was also demonstrated for targeting to the EGF-R.[68–70] Other molecules overexpressed on tumors such as nucleosome, CD19, membrane type 1 matrix metalloproteinase (MT1-MMP), GAH, GD2, and heparin-binding EGF-like growth factor were targeted by immunoliposomes.[71–77] Aptamers are single-stranded DNA or RNA, can specifically bind molecules, and are beginning to be used as targeting ligands for drug delivery systems.[78–81]

Peptides containing specific motifs in sequence which recognize specific molecules have been identified using phage-displayed peptide libraries. Arginine-glycine-aspartic acid (RGD) is the most typical motif and has been used for the targeted delivery of drugs and genes because of its ability to recognize integrins that are expressed on both tumor cells and neovascular endothelial cells.[82] The RGD motif has more frequently been utilized for targeting tumor endothelial cells.[83–87] Another peptide ligand, the asparagine-glycine-arginine (NGR) motif peptide, is identified as a ligand for CD13 or aminopeptidase N overexpressed on tumor endothelial cells and tumor cells.[88] NGR motif peptides have been successfully used to deliver drugs as well as liposomes to tumor vasculature.[89–92] Oku and colleagues determined that the alanine-plorine-arginine-plorine-glycine (APRPG) motif peptide specifically binds to tumor angiogenic vessels and demonstrated the utility of APRPG-modified PEG liposomes.[93,94]

3.2. De-PEGylation After glycoproteins are recognized by lipoplexes, endocytosis or endocytosis-like pathways occur for their internalization. Because endosomes are fused with lysosomes, digestive compartments, lipoplexes, and their cargos should escape enzymatic degradation. Therefore, endosomal release of cargos such pDNA and siRNA into the cytoplasm is regarded as a crucial step in achieving successful transgene expression and gene silencing. Endosomal escape of lipoplexes is mediated by membrane fusion or disruption of endosomes.[95,96] PEGylation confers steric stability on complexes and inhibits fusion with the endosomal membrane. Therefore, valid strategies for removing the PEG moiety from complexes at the target site in response to the local environment are expected to improve cellular uptake and the subsequent endosomal escape of carriers. Intracellular environments with a low pH in endosomes/lysosomes are employed as triggers of PEG cleavage.[97–102] Szoka and colleagues constructed pH-sensitive PEG lipids containing an orthoester linkage, and a transfection experiment showed greater luciferase expression of pH-sensitive lipoplexes as compared with stable PEGylated ones.[97] In addition to the pH gradient, an intracellular reducing environment and enzymes are considered alternative stimulating triggers.[103–105] Most of the current cleavable PEG devices were designed to be cleaved in response to some feature of the intracellular microenvironment. Therefore, a cleavable PEG-lipid triggered in a tumor-specific manner would be beneficial for tumor gene delivery. To achieve a tumor-specific cleavable PEG system, the enzymes specifically expressed in a tumor are focused on, such as matrix metalloproteinase (MMP).[33,34,106,107] The MMP substrate peptide was inserted between PEG and DOPE as a linker, and the resulting conjugated PEG-peptide-DOPE ternary conjugate is referred to as PPD.[33,34] In comparison with noncleavable PEG-lipid, liposomal pDNA or siRNA formulation modified with PPD exhibited significantly increased gene expression or gene silencing in subcutaneously xenografted tumors after intravenous injection as a result of enhanced cellular uptake and endosomal escape.

3.3. Acceleration of Endosomal Escape *via* Membrane Fusion Alternative methods to increase the endosomal escape of PEGylated carriers are the acceleration of either membrane fusion or disruption of the endosomal membrane. The endosomal disruption mechanism has been extensively utilized to accelerate endosomal escape in gene delivery. The high transfection efficiency of poly(ethyleneimine) (PEI) can be attributed to its buffering effect or the "proton sponge effect" due to its secondary and tertiary amines.[108] Wagner and co-workers conjugated a pH-responsive endosomolytic peptide, melittin, with PEGylated PEI *via* an acid-labile dimethylmaleic anhydride (DMMAn) linker. The resulting conjugate showed an enhanced gene transfer and silencing effect. This can be attributed to the enhanced lytic activity at acidic pH, which triggered the destabilization of endosomal membranes.[109,110]

The pH-sensitive fusogenic membrane peptide GALA (WEAALAEALAEALAEHLAEALAEALEALAA) was developed based on the endosomal escape mechanism of the influenza virus, an envelope-type RNA virus.[111] A 30-amino acid GALA contains a glutamic acid-alanine-leucine-alanine sequence that is repeated four times. GALA undergoes structural change to the α-helix under acidic conditions like endosomes/lysosomes, which could induce membrane fusion. The introduction of GALA to PEGylated MEND enhanced the silencing activity of siRNA after *in vitro* and *in vivo* intratumoral injection.[112,113] However, when injected into the blood circulation, the GALA-modified PEGylated MEND was eliminated rapidly. We hypothesized that this was caused by the recognition of GALA by serum proteins because GALA was not masked completely. Therefore, we developed a new, shorter version of GALA (shGALA: WEAALAEALAEALAEHLAEALA). After systemic administration, PEGylated MEND modified with shGALA showed systemic stability and subsequent tumor accumulation *via* the EPR effect which were comparable to those of PEGylated MEND, with an enhanced gene-silencing effect in tumors.[35] We also reported that the acceleration of endosomal escape of PEGylated MEND by GALA could minimize unfavorable immunological stimulation. Nucleic acids such as pDNA and siRNA have the ability to stimulate the immune system through recognition by Toll-like receptors (TLRs)-3, -7/8, or -9 expressed in endosomes.[114] Due to trapping of PEGylated MEND in endosomes, the exposure time of encapsulated pDNA or siRNA to TLRs in endosomes is prolonged, which leads to excess stimulation of TLRs and enhanced expression of type I interferons (IFNs). GALA modification diminished the expression of type I IFNs.[35,115]

3.4. pH-Sensitive Cationic Lipids As mentioned above, a cationic characteristic of nucleic acid carriers requires considerable PEG modification to avoid interactions with the biological milieu. Another strategy for overcoming the PEG dilemma is offered by pH-sensitive cationic devices. pH-Sensitive liposomes have been reported from the 1980s. pH-Sensitive liposomes composed of phosphatidylethanolamine was protonated in endosomes/lysosomes, which stimulated the release of cargos into the cytoplasm.[116,117] Bailey and Cullis

reported the synthesis of an ionizable aminolipid 1,2-dioleoyl-3-dimethylammonium propane (DODAP), and utility of ionizable liposomes for nucleic acid delivery.[118,119] The head group of DODAP is a tertiary amine responsible for cationization at acidic pH. Since liposomes containing DODAP are rendered neutral at physiological pH, the net cationic lipid content of the liposome is reduced in the systemic circulation. This pH sensitivity allows liposomes to circulate stably in the blood with a little amount of PEG modification (*ca.* 5 mol%).

A series of ionizable aminolipids were synthesized and utilized for nucleic acid delivery to tumors as a formulation of stable nucleic acid-lipid particles or ionizable lipid nano particles.[120–122] Recently, we have designed a new pH-sensitive cationic lipid, YSK05 (1-methyl-4,4-biz[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yloxy]piperidine).[123] The apparent pK_a value of MEND containing YSK05 (YSK05-MEND) was around 6.5, indicating that YSK05-MEND was positively charged at the early endosome stage. Despite lower cellular uptake in YSK05-MEND, the *in vitro* silencing activity of YSK05-MEND was 100-fold higher than that of cationic MEND composed of DOTAP (DOTAP-MEND). This was attributed to the more efficient membrane fusion activity of YSK05-MEND compared with DOTAP-MEND. For tumor targeting *via* the EPR effect, the systemic stability of YSK05-MEND modified with PEG-lipid was evaluated. The result revealed a lower amount of PEG (*ca.* 5 mol%) was sufficient for YSK05-MEND to circulate in the blood, unlike cationic DOTAP-MEND.[124] This suggests that YSK05-MEND is a promising system for use in the *in vivo* delivery of nucleic acids to tumors. As another type of pH-sensitive lipid, we reported SS-cleavable proton-activated lipid-like material (ssPalm), which contains two tertiary amines that are positively charged at acidic pH and a disulfide bond that can be cleaved in a reducing environment.[125] MEND containing ssPalm was positively charged with an apparent pK_a value of 6.2. Furthermore, the envelope structure is designed to be degraded in response to the reductive environment in the cytosol. As a result, MEND exhibited higher transgene expression comparable to that developed with conventionally used cationic lipids (*i.e.*, DOTAP) with lower cellular uptake. Collectively, pH-sensitive liposomes represent an alternative strategy to achieve sufficient nucleic acid delivery to tumors *via* the EPR effect.

4. CONCLUSION

As described above, various strategies have been developed to resolve the crucial problem associated with the use of PEG in the delivery of nucleic acids to tumors, *i.e.*, the PEG dilemma, by the manipulation of both pharmacokinetics and intracellular trafficking. In the near future, we have no doubt that the principles established as described above will offer safe, stable, effective, tumor-specific nanomedicines for successful clinical applications of new types of cancer therapies.

Acknowledgments The studies reported here were supported in part by a Grant-in-Aid for Young Scientists (B) from the Japan Society for the Promotion of Science (JSPS); Core Research for Educational Science and Technology (CREST) from the Japan Science and Technology Agency (JST); a Grant-in-Aid for Scientific Research on Innovative Areas "Nanomedicine Molecular Science" (No. 2306) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan and Special Education and Research Expenses of MEXT of Japan; and a Grant for Industrial Technology Research (financial support to young researchers) from the New Energy and Industrial Technology Development Organization (NEDO).

REFERENCES

1) Torchilin VP. Recent advances with liposomes as pharmaceutical carriers. *Nat. Rev. Drug Discov.*, **4**, 145–160 (2005).
2) Scherphof GL, Dijkstra J, Spanjer HH, Derksen JTP, Roerdink FH. Uptake and intracellular processing of targeted and nontargeted liposomes by rat Kupffer cells *in vivo* and *in vitro*. *Ann. N. Y. Acad. Sci.*, **446** (1 *Macromolecule*), 368–384 (1985).
3) Klibanov AL, Maruyama K, Torchilin VP, Huang L. Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. *FEBS Lett.*, **268**, 235–237 (1990).
4) Blume G, Cevc G. Liposomes for the sustained drug release *in vivo*. *Biochim. Biophys. Acta*, **1029**, 91–97 (1990).
5) Allen TM, Hansen C, Martin F, Redemann C, Yau-Young A. Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives *in vivo*. *Biochim. Biophys. Acta*, **1066**, 29–36 (1991).
6) Papahadjopoulos D, Allen TM, Gabizon A, Mayhew E, Matthay K, Huang SK, Lee KD, Woodle MC, Lasic DD, Redemann C, Martin FJ. Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 11460–11464 (1991).
7) Uster PS, Allen TM, Daniel BE, Mendez CJ, Newman MS, Zhu GZ. Insertion of poly(ethylene glycol) derivatized phospholipid into pre-formed liposomes results in prolonged *in vivo* circulation time. *FEBS Lett.*, **386**, 243–246 (1996).
8) Čeh B, Winterhalter M, Frederik PM, Vallner JJ, Lasic DD. Stealth® liposomes: from theory to product. *Adv. Drug Deliv. Rev.*, **24**, 165–177 (1997).
9) Matsumura Y, Maeda H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent SMANCS. *Cancer Res.*, **46**, 6387–6392 (1986).
10) Maeda H, Sawa T, Konno T. Mechanism of tumor-targeted delivery of macromolecular drugs, including the EPR effect in solid tumor and clinical overview of the prototype polymeric drug SMANCS. *J. Control. Release*, **74**, 47–61 (2001).
11) McDonald DM, Choyke PL. Imaging of angiogenesis: from microscope to clinic. *Nat. Med.*, **9**, 713–725 (2003).
12) Yuan F, Leunig M, Huang SK, Berk DA, Papahadjopoulos D, Jain RK. Microvascular permeability and interstitial penetration of sterically stabilized (stealth) liposomes in a human tumor xenograft. *Cancer Res.*, **54**, 3352–3356 (1994).
13) Maruyama K, Ishida O, Takizawa T, Moribe K. Possibility of active targeting to tumor tissues with liposomes. *Adv. Drug Deliv. Rev.*, **40**, 89–102 (1999).
14) Ishida O, Maruyama K, Sasaki K, Iwatsuru M. Size-dependent extravasation and interstitial localization of polyethyleneglycol liposomes in solid tumor-bearing mice. *Int. J. Pharm.*, **190**, 49–56 (1999).
15) Davis ME, Chen ZG, Shin DM. Nanoparticle therapeutics: an emerging treatment modality for cancer. *Nat. Rev. Drug Discov.*, **7**, 771–782 (2008).
16) Immordino ML, Dosio F, Cattel L. Stealth liposomes: review of the basic science, rationale, and clinical applications, existing and potential. *Int. J. Nanomedicine*, **1**, 297–315 (2006).
17) Allen TM, Cullis PR. Liposomal drug delivery systems: from concept to clinical applications. *Adv. Drug Deliv. Rev.*, **65**, 36–48 (2013).

18) Elsabahy M, Nazarali A, Foldvari M. Non-viral nucleic acid delivery: key challenges and future directions. *Curr. Drug Deliv.*, **8**, 235–244 (2011).
19) Pai SI, Lin YY, Macaes B, Meneshian A, Hung CF, Wu TC. Prospects of RNA interference therapy for cancer. *Gene Ther.*, **13**, 464–477 (2006).
20) Pecot CV, Calin GA, Coleman RL, Lopez-Berestein G, Sood AK. RNA interference in the clinic: challenges and future directions. *Nat. Rev. Cancer*, **11**, 59–67 (2011).
21) Kamiya H, Akita H, Harashima H. Pharmacokinetic and pharmacodynamic considerations in gene therapy. *Drug Discov. Today*, **8**, 990–996 (2003).
22) Schaffert D, Wagner E. Gene therapy progress and prospects: synthetic polymer-based systems. *Gene Ther.*, **15**, 1131–1138 (2008).
23) Tseng YC, Mozumdar S, Huang L. Lipid-based systemic delivery of siRNA. *Adv. Drug Deliv. Rev.*, **61**, 721–731 (2009).
24) Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM, Danielsen M. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 7413–7417 (1987).
25) Leventis R, Silvius JR. Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles. *Biochim. Biophys. Acta*, **1023**, 124–132 (1990).
26) Behr JP, Demeneix B, Loeffler JP, Perez-Mutul J. Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 6982–6986 (1989).
27) Gao X, Huang L. A novel cationic liposome reagent for efficient transfection of mammalian cells. *Biochem. Biophys. Res. Commun.*, **179**, 280–285 (1991).
28) Ruysschaert JM, el Ouahabi A, Willeaume V, Huez G, Fuks R, Vandenbranden M, Di Stefano P. A novel cationic amphiphile for transfection of mammalian cells. *Biochem. Biophys. Res. Commun.*, **203**, 1622–1628 (1994).
29) Kogure K, Moriguchi R, Sasaki K, Ueno M, Futaki S, Harashima H. Development of a non-viral multifunctional envelope-type nano device by a novel lipid film hydration method. *J. Control. Release*, **98**, 317–323 (2004).
30) Kogure K, Akita H, Harashima H. Multifunctional envelope-type nano device for non-viral gene delivery: concept and application of programmed packaging. *J. Control. Release*, **122**, 246–251 (2007).
31) Nakamura T, Akita H, Yamada Y, Hatakeyama H, Harashima H. A multifunctional envelope-type nanodevice for use in nanomedicine: concept and applications. *Acc. Chem. Res.*, **45**, 1113–1121 (2012).
32) Hatakeyama H, Akita H, Harashima H. A multifunctional envelope type nano device (MEND) for gene delivery to tumours based on the EPR effect: a strategy for overcoming the PEG dilemma. *Adv. Drug Deliv. Rev.*, **63**, 152–160 (2011).
33) Hatakeyama H, Akita H, Kogure K, Oishi M, Nagasaki Y, Kihira Y, Ueno M, Kobayashi H, Kikuchi H, Harashima H. Development of a novel systemic gene delivery system for cancer therapy with a tumor-specific cleavable PEG-lipid. *Gene Ther.*, **14**, 68–77 (2007).
34) Hatakeyama H, Akita H, Ito E, Hayashi Y, Oishi M, Nagasaki Y, Danev R, Nagayama K, Kaji N, Kikuchi H, Baba Y, Harashima H. Systemic delivery of siRNA to tumors using a lipid nanoparticle containing a tumor-specific cleavable PEG-lipid. *Biomaterials*, **32**, 4306–4316 (2011).
35) Sakurai Y, Hatakeyama H, Sato Y, Akita H, Takayama K, Kobayashi S, Futaki S, Harashima H. Endosomal escape and the knockdown efficiency of liposomal-siRNA by the fusogenic peptide shGALA. *Biomaterials*, **32**, 5733–5742 (2011).
36) Mishra S, Webster P, Davis ME. PEGylation significantly affects cellular uptake and intracellular trafficking of non-viral gene delivery particles. *Eur. J. Cell Biol.*, **83**, 97–111 (2004).
37) Remaut K, Lucas B, Braeckmans K, Demeester J, De Smedt SC. PEGylation of liposomes favours the endosomal degradation of the delivered phosphodiester oligonucleotides. *J. Control. Release*, **117**, 256–266 (2007).
38) Mislick KA, Baldeschwieler JD. Evidence for the role of proteoglycans in cation-mediated gene transfer. *Proc. Natl. Acad. Sci. U.S.A.*, **93**, 12349–12354 (1996).
39) Mounkes LC, Zhong W, Cipres-Palacin G, Heath TD, Debs RJ. Proteoglycans mediate cationic liposome-DNA complex-based gene delivery *in vitro* and *in vivo*. *J. Biol. Chem.*, **273**, 26164–26170 (1998).
40) Allen TM. Ligand-targeted therapeutics in anticancer therapy. *Nat. Rev. Cancer*, **2**, 750–763 (2002).
41) Ishida O, Maruyama K, Tanahashi H, Iwatsuru M, Sasaki K, Eriguchi M, Yanagie H. Liposomes bearing polyethyleneglycol-coupled transferrin with intracellular targeting property to the solid tumors *in vivo*. *Pharm. Res.*, **18**, 1042–1048 (2001).
42) Suzuki R, Takizawa T, Kuwata Y, Mutoh M, Ishiguro N, Utoguchi N, Shinohara A, Eriguchi M, Yanagie H, Maruyama K. Effective anti-tumor activity of oxaliplatin encapsulated in transferrin-PEG-liposome. *Int. J. Pharm.*, **346**, 143–150 (2008).
43) Hatakeyama H, Akita H, Maruyama K, Suhara T, Harashima H. Factors governing the *in vivo* tissue uptake of transferrin-coupled polyethylene glycol liposomes *in vivo*. *Int. J. Pharm.*, **281**, 25–33 (2004).
44) Ogris M, Walker G, Blessing T, Kircheis R, Wolschek M, Wagner E. Tumor-targeted gene therapy: strategies for the preparation of ligand-polyethylene glycol-polyethylenimine/DNA complexes. *J. Control. Release*, **91**, 173–181 (2003).
45) Pirollo KF, Rait A, Zhou Q, Hwang SH, Dagata JA, Zon G, Hogrefe RI, Palchik G, Chang EH. Materializing the potential of small interfering RNA *via* a tumor-targeting nanodelivery system. *Cancer Res.*, **67**, 2938–2943 (2007).
46) Davis ME, Zuckerman JE, Choi CH, Seligson D, Tolcher A, Alabi CA, Yen Y, Heidel JD, Ribas A. Evidence of RNAi in humans from systemically administered siRNA *via* targeted nanoparticles. *Nature*, **464**, 1067–1070 (2010).
47) Ross JF, Chaudhuri PK, Ratnam M. Differential regulation of folate receptor isoforms in normal and malignant tissues *in vivo* and in established cell lines. Physiologic and clinical implications. *Cancer*, **73**, 2432–2443 (1994).
48) Gabizon A, Tzemach D, Gorin J, Mak L, Amitay Y, Shmeeda H, Zalipsky S. Improved therapeutic activity of folate-targeted liposomal doxorubicin in folate receptor-expressing tumor models. *Cancer Chemother. Pharmacol.*, **66**, 43–52 (2010).
49) Lee RJ, Low PS. Folate-mediated tumor cell targeting of liposome-entrapped doxorubicin *in vitro*. *Biochim. Biophys. Acta*, **1233**, 134–144 (1995).
50) Lu Y, Wu J, Wu J, Gonit M, Yang X, Lee A, Xiang G, Li H, Liu S, Marcucci G, Ratnam M, Lee RJ. Role of formulation composition in folate receptor-targeted liposomal doxorubicin delivery to acute myelogenous leukemia cells. *Mol. Pharm.*, **4**, 707–712 (2007).
51) Yamada A, Taniguchi Y, Kawano K, Honda T, Hattori Y, Maitani Y. Design of folate-linked liposomal doxorubicin to its antitumor effect in mice. *Clin. Cancer Res.*, **14**, 8161–8168 (2008).
52) Reddy JA, Abburi C, Hofland H, Howard SJ, Vlahov I, Wils P, Leamon CP. Folate-targeted, cationic liposome-mediated gene transfer into disseminated peritoneal tumors. *Gene Ther.*, **9**, 1542–1550 (2002).
53) Leamon CP, Cooper SR, Hardee GE. Folate-liposome-mediated antisense oligodeoxynucleotide targeting to cancer cells: evaluation *in vitro* and *in vivo*. *Bioconjug. Chem.*, **14**, 738–747 (2003).
54) Kim SH, Mok H, Jeong JH, Kim SW, Park TG. Comparative evaluation of target-specific GFP gene silencing efficiencies for antisense ODN, synthetic siRNA, and siRNA plasmid complexed with PEI-PEG-FOL conjugate. *Bioconjug. Chem.*, **17**, 241–244 (2006).
55) Guo W, Lee RJ. Efficient gene delivery *via* non-covalent complexes of folic acid and polyethylenimine. *J. Control. Release*, **77**, 131–138 (2001).

56) Eliaz RE, Nir S, Marty C, Szoka FC Jr. Determination and modeling of kinetics of cancer cell killing by doxorubicin and doxorubicin encapsulated in targeted liposomes. *Cancer Res.*, **64**, 711–718 (2004).
57) Peer D, Margalit R. Tumor-targeted hyaluronan nanoliposomes increase the antitumor activity of liposomal doxorubicin in syngeneic and human xenograft mouse tumor models. *Neoplasia*, **6**, 343–353 (2004).
58) Li SD, Huang L. Targeted delivery of antisense oligodeoxynucleotide and small interference RNA into lung cancer cells. *Mol. Pharm.*, **3**, 579–588 (2006).
59) Li SD, Chono S, Huang L. Efficient oncogene silencing and metastasis inhibition *via* systemic delivery of siRNA. *Mol. Ther.*, **16**, 942–946 (2008).
60) Manjappa AS, Chaudhari KR, Venkataraju MP, Dantuluri P, Nanda B, Sidda C, Sawant KK, Murthy RS. Antibody derivatization and conjugation strategies: application in preparation of stealth immunoliposome to target chemotherapeutics to tumor. *J. Control. Release*, **150**, 2–22 (2011).
61) Goren D, Horowitz AT, Zalipsky S, Woodle MC, Yarden Y, Gabizon A. Targeting of stealth liposomes to erbB-2 (Her/2) receptor: *in vitro* and *in vivo* studies. *Br. J. Cancer*, **74**, 1749–1756 (1996).
62) Shmeeda H, Tzemach D, Mak L, Gabizon A. Her2-targeted PEGylated liposomal doxorubicin: retention of target-specific binding and cytotoxicity after *in vivo* passage. *J. Control. Release*, **136**, 155–160 (2009).
63) Park JW, Hong K, Kirpotin DB, Colbern G, Shalaby R, Baselga J, Shao Y, Nielsen UB, Marks JD, Moore D, Papahadjopoulos D, Benz CC. Anti-HER2 immunoliposomes: enhanced efficacy attributable to targeted delivery. *Clin. Cancer Res.*, **8**, 1172–1181 (2002).
64) Kirpotin DB, Drummond DC, Shao Y, Shalaby MR, Hong K, Nielsen UB, Marks JD, Benz CC, Park JW. Antibody targeting of long-circulating lipidic nanoparticles does not increase tumor localization but does increase internalization in animal models. *Cancer Res.*, **66**, 6732–6740 (2006).
65) Smith B, Lyakhov I, Loomis K, Needle D, Baxa U, Yavlovich A, Capala J, Blumenthal R, Puri A. Hyperthermia-triggered intracellular delivery of anticancer agent to HER2(+) cells by HER2-specific affibody (ZHER2-GS-Cys)-conjugated thermosensitive liposomes (HER2(+) affisomes). *J. Control. Release*, **153**, 187–194 (2011).
66) Mamot C, Drummond DC, Noble CO, Kallab V, Guo Z, Hong K, Kirpotin DB, Park JW. Epidermal growth factor receptor-targeted immunoliposomes significantly enhance the efficacy of multiple anticancer drugs *in vivo*. *Cancer Res.*, **65**, 11631–11638 (2005).
67) Beuttler J, Rothdiener M, Müller D, Frejd FY, Kontermann RE. Targeting of epidermal growth factor receptor (EGFR)-expressing tumor cells with sterically stabilized affibody liposomes (SAL). *Bioconjug. Chem.*, **20**, 1201–1208 (2009).
68) Kullberg EB, Nestor M, Gedda L. Tumor-cell targeted epiderimal growth factor liposomes loaded with boronated acridine: uptake and processing. *Pharm. Res.*, **20**, 229–236 (2003).
69) Shir A, Ogris M, Wagner E, Levitzki A. EGF receptor-targeted synthetic double-stranded RNA eliminates glioblastoma, breast cancer, and adenocarcinoma tumors in mice. *PLoS Med.*, **3**, e6 (2006).
70) Schaffert D, Kiss M, Rödl W, Shir A, Levitzki A, Ogris M, Wagner E. Poly(I:C)-mediated tumor growth suppression in EGF-receptor overexpressing tumors using EGF-polyethylene glycol-linear polyethylenimine as carrier. *Pharm. Res.*, **28**, 731–741 (2011).
71) ElBayoumi TA, Torchilin VP. Tumor-targeted nanomedicines: enhanced antitumor efficacy *in vivo* of doxorubicin-loaded, long-circulating liposomes modified with cancer-specific monoclonal antibody. *Clin. Cancer Res.*, **15**, 1973–1980 (2009).
72) Ishida T, Kirchmeier MJ, Moase EH, Zalipsky S, Allen TM. Targeted delivery and triggered release of liposomal doxorubicin enhances cytotoxicity against human B lymphoma cells. *Biochim. Biophys. Acta*, **1515**, 144–158 (2001).
73) Hatakeyama H, Akita H, Ishida E, Hashimoto K, Kobayashi H, Aoki T, Yasuda J, Obata K, Kikuchi H, Ishida T, Kiwada H, Harashima H. Tumor targeting of doxorubicin by anti-MT1-MMP antibody-modified PEG liposomes. *Int. J. Pharm.*, **342**, 194–200 (2007).
74) Atobe K, Ishida T, Ishida E, Hashimoto K, Kobayashi H, Yasuda J, Aoki T, Obata K, Kikuchi H, Akita H, Asai T, Harashima H, Oku N, Kiwada H. *In vitro* efficacy of a sterically stabilized immunoliposomes targeted to membrane type 1 matrix metalloproteinase (MT1-MMP). *Biol. Pharm. Bull.*, **30**, 972–978 (2007).
75) Matsumura Y, Gotoh M, Muro K, Yamada Y, Shirao K, Shimada Y, Okuwa M, Matsumoto S, Miyata Y, Ohkura H, Chin K, Baba S, Yamao T, Kannami A, Takamatsu Y, Ito K, Takahashi K. Phase I and pharmacokinetic study of MCC-465, a doxorubicin (DXR) encapsulated in PEG immunoliposome, in patients with metastatic stomach cancer. *Ann. Oncol.*, **15**, 517–525 (2004).
76) Di Paolo D, Ambrogio C, Pastorino F, Brignole C, Martinengo C, Carosio R, Loi M, Pagnan G, Emionite L, Cilli M, Ribatti D, Allen TM, Chiarle R, Ponzoni M, Perri P. Selective therapeutic targeting of the anaplastic lymphoma kinase with liposomal siRNA induces apoptosis and inhibits angiogenesis in neuroblastoma. *Mol. Ther.*, **19**, 2201–2212 (2011).
77) Nishikawa K, Asai T, Shigematsu H, Shimizu K, Kato H, Asano Y, Takashima S, Mekada E, Oku N, Minamino T. Development of anti-HB-EGF immunoliposomes for the treatment of breast cancer. *J. Control. Release*, **160**, 274–280 (2012).
78) Kang H, O'Donoghue MB, Liu H, Tan W. A liposome-based nanostructure for aptamer directed delivery. *Chem. Commun.* (Camb.), **46**, 249–251 (2010).
79) Cao Z, Tong R, Mishra A, Xu W, Wong GC, Cheng J, Lu Y. Reversible cell-specific drug delivery with aptamer-functionalized liposomes. *Angew. Chem. Int. Ed. Engl.*, **48**, 6494–6498 (2009).
80) Dhar S, Gu FX, Langer R, Farokhzad OC, Lippard SJ. Targeted delivery of cisplatin to prostate cancer cells by aptamer functionalized Pt(IV) prodrug-PLGA-PEG nanoparticles. *Proc. Natl. Acad. Sci. U.S.A.*, **105**, 17356–17361 (2008).
81) Mann AP, Bhavane RC, Somasunderam A, Liz Montalvo-Ortiz B, Ghaghada KB, Volk D, Nieves-Alicea R, Suh KS, Ferrari M, Annapragada A, Gorenstein DG, Tanaka T. Thioaptamer conjugated liposomes for tumor vasculature targeting. *Oncotarget*, **2**, 298–304 (2011).
82) Arap W, Pasqualini R, Ruoslahti E. Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. *Science*, **279**, 377–380 (1998).
83) Schiffelers RM, Ansari A, Xu J, Zhou Q, Tang Q, Storm G, Molema G, Lu PY, Scaria PV, Woodle MC. Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle. *Nucleic Acids Res.*, **32**, e149 (2004).
84) Murphy EA, Majeti BK, Barnes LA, Makale M, Weis SM, Lutu-Fuga K, Wrasidlo W, Cheresh DA. Nanoparticle-mediated drug delivery to tumor vasculature suppresses metastasis. *Proc. Natl. Acad. Sci. U.S.A.*, **105**, 9343–9348 (2008).
85) Anand S, Majeti BK, Acevedo LM, Murphy EA, Mukthavaram R, Scheppke L, Huang M, Shields DJ, Lindquist JN, Lapinski PE, King PD, Weis SM, Cheresh DA. MicroRNA-132-mediated loss of p120RasGAP activates the endothelium to facilitate pathological angiogenesis. *Nat. Med.*, **16**, 909–914 (2010).
86) Kibria G, Hatakeyama H, Ohga N, Hida K, Harashima H. Dual-ligand modification of PEGylated liposomes shows better cell selectivity and efficient gene delivery. *J. Control. Release*, **153**, 141–148 (2011).
87) Yonenaga N, Kenjo E, Asai T, Tsuruta A, Shimizu K, Dewa T, Nango M, Oku N. RGD-based active targeting of novel polycation liposomes bearing siRNA for cancer treatment. *J. Control. Release*, **160**, 177–181 (2012).
88) Pasqualini R, Koivunen E, Kain R, Lahdenranta J, Sakamoto M,

Stryhn A, Ashmun RA, Shapiro LH, Arap W, Ruoslahti E. Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. *Cancer Res.*, **60**, 722–727 (2000).
89) Pastorino F, Brignole C, Di Paolo D, Nico B, Pezzolo A, Marimpietri D, Pagnan G, Piccardi F, Cilli M, Longhi R, Ribatti D, Corti A, Allen TM, Ponzoni M. Targeting liposomal chemotherapy *via* both tumor cell-specific and tumor vasculature-specific ligands potentiates therapeutic efficacy. *Cancer Res.*, **66**, 10073–10082 (2006).
90) Moffatt S, Wiehle S, Cristiano RJ. A multifunctional PEI-based cationic polyplex for enhanced systemic p53-mediated gene therapy. *Gene Ther.*, **13**, 1512–1523 (2006).
91) Takara K, Hatakeyama H, Ohga N, Hida K, Harashima H. Design of a dual-ligand system using a specific ligand and cell penetrating peptide, resulting in a synergistic effect on selectivity and cellular uptake. *Int. J. Pharm.*, **396**, 143–148 (2010).
92) Takara K, Hatakeyama H, Kibria G, Ohga N, Hida K, Harashima H. Size-controlled, dual-ligand modified liposomes that target the tumor vasculature show promise for use in drug-resistant cancer therapy. *J. Control. Release*, **162**, 225–232 (2012).
93) Asai T, Miyazawa S, Maeda N, Hatanaka K, Katanasaka Y, Shimizu K, Shuto S, Oku N. Antineovascular therapy with angiogenic vessel-targeted polyethyleneglycol-shielded liposomal DPP-CNDAC. *Cancer Sci.*, **99**, 1029–1033 (2008).
94) Katanasaka Y, Ida T, Asai T, Maeda N, Oku N. Effective delivery of an angiogenesis inhibitor by neovessel-targeted liposomes. *Int. J. Pharm.*, **360**, 219–224 (2008).
95) Xu Y, Szoka FC Jr. Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry*, **35**, 5616–5623 (1996).
96) Hafez IM, Maurer N, Cullis PR. On the mechanism whereby cationic lipids promote intracellular delivery of polynucleic acids. *Gene Ther.*, **8**, 1188–1196 (2001).
97) Li W, Huang Z, MacKay JA, Grube S, Szoka FC Jr. Low-pH-sensitive poly(ethylene glycol) (PEG)-stabilized plasmid nanolipoparticles: effects of PEG chain length, lipid composition and assembly conditions on gene delivery. *J. Gene Med.*, **7**, 67–79 (2005).
98) Walker GF, Fella C, Pelisek J, Fahrmeir J, Boeckle S, Ogris M, Wagner E. Toward synthetic viruses: endosomal pH-triggered deshielding of targeted polyplexes greatly enhances gene transfer *in vitro* and *in vivo*. *Mol. Ther.*, **11**, 418–425 (2005).
99) Oishi M, Nagatsugi F, Sasaki S, Nagasaki Y, Kataoka K. Smart polyion complex micelles for targeted intracellular delivery of PEGylated antisense oligonucleotides containing acid-labile linkages. *ChemBioChem*, **6**, 718–725 (2005).
100) Shin J, Shum P, Thompson DH. Acid-triggered release *via* dePEGylation of DOPE liposomes containing acid-labile vinyl ether PEG-lipids. *J. Control. Release*, **91**, 187–200 (2003).
101) Masson C, Garinot M, Mignet N, Wetzer B, Mailhe P, Scherman D, Bessodes M. pH-sensitive PEG lipids containing orthoester linkers: new potential tools for nonviral gene delivery. *J. Control. Release*, **99**, 423–434 (2004).
102) Murthy N, Campbell J, Fausto N, Hoffman AS, Stayton PS. Design and synthesis of pH-responsive polymeric carriers that target uptake and enhance the intracellular delivery of oligonucleotides. *J. Control. Release*, **89**, 365–374 (2003).
103) Zalipsky S, Qazen M, Walker JA 2nd, Mullah N, Quinn YP, Huang SK. New detachable poly(ethylene glycol) conjugates: cysteine-cleavable lipopolymers regenerating natural phospholipid, diacyl phosphatidylethanolamine. *Bioconjug. Chem.*, **10**, 703–707 (1999).
104) Ishida T, Kirchmeier MJ, Moase EH, Zalipsky S, Allen TM. Targeted delivery and triggered release of liposomal doxorubicin enhances cytotoxicity against human B lymphoma cells. *Biochim. Biophys. Acta*, **1515**, 144–158 (2001).
105) Zhang JX, Zalipsky S, Mullah N, Pechar M, Allen TM. Pharmaco attributes of dioleoylphosphatidylethanolamine/cholesterylhemisuccinate liposomes containing different types of cleavable lipopolymers. *Pharmacol. Res.*, **49**, 185–198 (2004).
106) Terada T, Iwai M, Kawakami S, Yamashita F, Hashida M. Novel PEG-matrix metalloproteinase-2 cleavable peptide-lipid containing galactosylated liposomes for hepatocellular carcinoma-selective targeting. *J. Control. Release*, **111**, 333–342 (2006).
107) Harris TJ, von Maltzahn G, Derfus AM, Ruoslahti E, Bhatia SN. Proteolytic actuation of nanoparticle self-assembly. *Angew. Chem. Int. Ed. Engl.*, **45**, 3161–3165 (2006).
108) Akinc A, Thomas M, Klibanov AM, Langer R. Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. *J. Gene Med.*, **7**, 657–663 (2005).
109) Boeckle S, Fahrmeir J, Roedl W, Ogris M, Wagner E. Melittin analogs with high lytic activity at endosomal pH enhance transfection with purified targeted PEI polyplexes. *J. Control. Release*, **112**, 240–248 (2006).
110) Meyer M, Philipp A, Oskuee R, Schmidt C, Wagner E. Breathing life into polycations: functionalization with pH-responsive endosomolytic peptides and polyethylene glycol enables siRNA delivery. *J. Am. Chem. Soc.*, **130**, 3272–3273 (2008).
111) Li W, Nicol F, Szoka FC Jr. GALA: a designed synthetic pH-responsive amphipathic peptide with applications in drug and gene delivery. *Adv. Drug Deliv. Rev.*, **56**, 967–985 (2004).
112) Sakurai Y, Hatakeyama H, Akita H, Oishi M, Nagasaki Y, Futaki S, Harashima H. Efficient short interference RNA delivery to tumor cells using a combination of octaarginine, GALA and tumor-specific, cleavable polyethylene glycol system. *Biol. Pharm. Bull.*, **32**, 928–932 (2009).
113) Hatakeyama H, Ito E, Akita H, Oishi M, Nagasaki Y, Futaki S, Harashima H. A pH-sensitive fusogenic peptide facilitates endosomal escape and greatly enhances the gene silencing of siRNA-containing nanoparticles *in vitro* and *in vivo*. *J. Control. Release*, **139**, 127–132 (2009).
114) Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat. Immunol.*, **11**, 373–384 (2010).
115) Hatakeyama H, Ito E, Yamamoto M, Akita H, Hayashi Y, Kajimoto K, Kaji N, Baba Y, Harashima H. A DNA microarray-based analysis of the host response to a nonviral gene carrier: a strategy for improving the immune response. *Mol. Ther.*, **19**, 1487–1498 (2011).
116) Connor J, Yatvin MB, Huang L. pH-sensitive liposomes: acid-induced liposome fusion. *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 1715–1718 (1984).
117) Straubinger RM, Düzgünes N, Papahadjopoulos D. pH-Sensitive liposomes mediate cytoplasmic delivery of encapsulated macromolecules. *FEBS Lett.*, **179**, 148–154 (1985).
118) Bailey AL, Cullis PR. Modulation of membrane fusion by asymmetric transbilayer distributions of amino lipids. *Biochemistry*, **33**, 12573–12580 (1994).
119) Semple SC, Klimuk SK, Harasym TO, Dos Santos N, Ansell SM, Wong KF, Maurer N, Stark H, Cullis PR, Hope MJ, Scherrer P. Efficient encapsulation of antisense oligonucleotides in lipid vesicles using ionizable aminolipids: formation of novel small multilamellar vesicle structures. *Biochim. Biophys. Acta*, **1510**, 152–166 (2001).
120) Heyes J, Palmer L, Chan K, Giesbrecht C, Jeffs L, MacLachlan I. Lipid encapsulation enables the effective systemic delivery of polyplex plasmid DNA. *Mol. Ther.*, **15**, 713–720 (2007).
121) Judge AD, Robbins M, Tavakoli I, Levi J, Hu L, Fronda A, Ambegia E, McClintock K, MacLachlan I. Confirming the RNAi-mediated mechanism of action of siRNA-based cancer therapeutics in mice. *J. Clin. Invest.*, **119**, 661–673 (2009).
122) Lee JB, Zhang K, Tam YY, Tam YK, Belliveau NM, Sung VY, Lin PJ, LeBlanc E, Ciufolini MA, Rennie PS, Cullis PR. Lipid nanoparticle siRNA systems for silencing the androgen receptor in human prostate cancer *in vivo*. *Int. J. Cancer*, **131**, E781–E790 (2012).
123) Sato Y, Hatakeyama H, Sakurai Y, Hyodo M, Akita H, Harashima H. A pH-sensitive cationic lipid facilitates the delivery of liposomal

siRNA and gene silencing activity *in vitro* and *in vivo*. *J. Control. Release*, **163**, 267–276 (2012).
124) Sakurai Y, Hatakeyama H, Sato Y, Hyodo M, Akita H, Harashima H. Gene silencing *via* RNAi and siRNA quantification in tumor tissue using MEND, a liposomal siRNA delivery system. *Mol. Ther.*, (2013), in press.
125) Akita H, Ishiba R, Hatakeyama H, Tanaka H, Sato Y, Tange K, Arai M, Kubo K, Harashima H. A neutral envelope-type nanoparticle containing pH-responsive and SS-cleavable lipid-like materials as a carrier for plasmid DNA. *Adv. Healthcare Mater.*, in press.