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Self-Assembled Lipid Nanomedicines for siRNA Tumor Targeting

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Abstract

Lipid-based nanoparticle technology has developed from chemical drug carrier into an efficient multifunctional siRNA tumor targeting delivery system. In this review, we start with an overview of the lipid-based nanomedicine history and the two classes of lipidic vectors for DNA or siRNA delivery. Then we discuss the features of lipid-based nanomedicine that lead to effective tumor targeting and the principles behind. We also discuss nanoparticle surface modification, classes of tumor targeting ligands, and other state-of-the-art strategies for enhancing endosome release primarily focused on lipid-based systems. At the end, we show that multifunctional self-assembled lipid-based nanoparticles could also be versatile delivery vehicles for cancer molecular imaging probes.

Keywords

Self-Assembly; Lipid; Liposome; Nanoparticles; Nanomedicine; siRNA; Tumor Targeting; Endosome Escape

1. INTRODUCTION

Drugs formulated in liposomes are considered the very first class of nanomedicine used in clinics. Liposomes are artificial cell-like vesicles that have an aqueous compartment inside the surrounding one or multiple lipid bilayers. The bilayer usually consists of a lipid component (usually a cationic lipid and/or a fusogenic lipid) and cholesterol. Some may further contain a polyethylene glycol-lipid conjugate for surface protection.[1] The aqueous compartment and the lipid bilayer have both been used to carry drugs. By formulating doxorubicin into liposomal dosage form, it can increase the tumor uptake and reduce the cardio-toxicity.[2,3] For the drugs with very poor solubility such as paclitaxel, loading the drug into the bilayer compartment can increase the delivering dose.[4,5] For some drugs, such as camptothecins that are not stable under physiological pH, formulation into liposomes can also protect them from degradation.[6,7] Furthermore, it can improve the pharmacokinetic profile primarily by increasing the circulation time of the drug.[8]

Lipid-based systems have also been developed for poly- or oligo-nucleotide delivery for decades. In 1987, Felgner et al.[9] showed that lipofection, i.e., cationic lipid mediated transfection, is more efficient for delivering DNA into cells than calcium phosphate[10,11] or

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DEAE-dextran.[12] Later, various cationic lipid formulations such as the popular Lipofectamine[13,14] or cardiolipin analogs[15,16] have been developed and used extensively for *in vitro* DNA or siRNA delivery.

An important milestone for lipid-based nanomedicines is the clinical trial for liposome-mediated gene therapy conducted in 1992. This clinical trial used a liposome formulation consisting of a cationic derivative of cholesterol, 3-β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl] cholesterol and dioleoylphosphatidylethanolamine (DC-Chol/DOPE) to transfer a xenogenic MHC class I antigen gene to the cutaneous melanoma lesions.[17] Although the transfection efficiency, the duration of expression, and the overall therapeutic effect was not as promising as anticipated, no adverse clinical effects were observed.[18] There were other clinical trials for cystic fibrosis using other cationic lipids.[19,20]

The discovery of RNA interference has brought a new category of therapeutics that can be used for genetic diseases,[21–25] viral infections,[26–31] or cancers by inhibiting various pathways. Compared to delivering plasmid DNA for expressing short-hairpin RNA (shRNA) in cells, delivering synthetic siRNA can silence protein expression and is more favorable in terms of drug delivery. The great advantage of siRNA therapy is that the site of action is the cytoplasm, not the nucleus. Lipid-based systems and other non-viral vectors are excellent vehicles for siRNA delivery.

2. CLASSES OF LIPIDIC VECTORS

DNA or siRNA delivery systems can be divided into viral and non-viral vector systems. Based on the type of the target disease, local or systemic administration is a factor of consideration. Viral vectors have been used for both systemic and local administrations. The strategy is, by genetic engineering, replacing pathogenic viral genes with desired genes or shRNA expression cassette. One great advantage of using viral vectors is that some viral vectors such as lentiviral and retroviral vectors can achieve stable long term expression due to their host genome-insertion nature.[32] In addition, viral vectors are generally more efficient in terms of expression level. However, immunogenicity and other safety issues are always the major concerns of using viral based systems, especially in humans. For the field of tumor targeting siRNA therapy, since stable long term expression is not needed and the site of action for siRNA is only the cytoplasm, non-viral vectors are more favorable.

There are different types of non-viral vectors for siRNA or DNA delivery such as polymers, block co-polymers, proteins, or peptides.[33–36] Various designs have been established based on either chemical conjugation or self-assembly processes. Self-assembled nanomedicines are more desirable due to their easy preparation and potential for scale-up manufacturing. Desimone et al.[37–40] established an imprint lithographic technique called PRINT™ (Particle Replication In Non-wetting Templates) for nanoparticle production. A variety of materials including synthetic polymers, hydrogels, active pharmaceutical ingredients, and proteins[41] could be made into shape-specific, monodisperse, and surface modifiable nanoparticles.[37] Bioactive agents including proteins, DNA, and small-molecule therapeutics[42] have also been encapsulated into PRINT™ nanoparticles. There are also other non-viral physical methods such as hydrodynamic injection,[43–45] electroporation,[46–48] and particle bombardment[49] that

could be used for local DNA or siRNA delivery. In this review, we will primarily focus on lipid-based self-assembled nanoparticles with tumor as the target disease.

2.1. Lipoplex

There are two main types of self-assembled lipid nanomedicines, one is the traditional type that formed simply by mixing positively charged liposome with negatively charged DNA or siRNA to make a complex. These types of reagents have been extensively used for *in vitro* gene transfection or silencing. The other type is a more sophisticated lipopolyplex nanoparticle such as the LPD (liposome-polycation-DNA) nanoparticles designed in our lab in the mid 90s.[50]

Verma et al. reported the first lipoplex mediated *in vivo* tumor siRNA delivery via intraperitoneal (i.p.) injection to a HCT116 colon cancer xenograft model with commercially available Oligofectamine (Invitrogen, Carlsbad, CA). They showed successful β-catenin expression reduction and HCT116 tumor growth inhibition.[51] Sorensen et al. used DOTAP (N-[1-(2,3-dioleoyloxy)]-N-N-N trimethyl ammonium propane) liposomes to make lipoplex for systemic siRNA delivery.[52] They showed inhibited exogenous green fluorescent protein expression in liver and spleen via systemic intravenous (i.v.) injection and endogenous tumor necrosis factor expression in macrophages via i.p. injection.

The problem for lipoplex is that the complex is not very stable. Especially when diluted in the blood circulation after injection. The lipoplex is usually made fresh immediately before use. Also, the works mentioned above did not really target siRNA to solid tumors via i.v. injection. To achieve siRNA solid tumor targeted delivery via systemic i.v. injection, a more sophisticated system that can produce a nanoparticle stable long enough before reaching the solid tumor is required.

2.2. LPD

Unlike a liposome that has an aqueous phase inside the particle, LPD nanoparticles consist of a solid core inside of the lipid bilayer.[53] The core formation is a self-assembly process driven by charge–charge interaction. In the LPD formulation designed in our lab, we use FDA approved protamine with the help of a high molecular weight calf thymus DNA to condense DNA or siRNA into a solid core.[54,55] Protamine is a highly positive charged arginine-rich nuclear protein from salmon sperm. Its natural function is to replace histones in the haploid phase of spermatogenesis and stabilize the DNA. With slightly excess amounts of negatively charged DNA or siRNA to positively charged protamine, the solid core remains negatively charged and thus allows further coating with positively charged DOTAP/cholesterol cationic liposomes. The self-assembled LPD nanoparticles were further modified by post-inserting either DSPE-PEG for surface protection or DSPE-PEG-anisamide for targeting to the sigma receptor (Fig. 1).[54–56]

With a similar approach, Harashima group used poly-L-lysine to condense shRNA encoding plasmid DNA or siRNA into their octaarginine modified Multifunctional Envelope type Nano Device (MEND) (Fig. 2). The octaarginine function for cell penetration will be discussed later. In this work, over 80% of luciferase gene expression silencing in HeLa cells was reported.[57,58]

Besides lipoplex and LPD formulation, Zimmermann et al. used a stable nucleic acid lipid particles (SNALP) formulation consisting of 3-*N*-[(ω-methoxypoly(ethyleneglycol)$_{2000}$) carbamoyl]-1, 2-dimyristoyloxy-propylamine (PEG-C-DMA), 1,2-dilinoleyloxy-*N*,*N*-dimethyl-3-aminopropane, (DLin DMA), 1,2-distearoyl-sn-glycerol-3-phosphocholine (DSPC) and cholesterol, in a 2:40:10:48 molar ratio to deliver siRNA against apolipoprotein B (*apoB*) in the liver. In this formulation, the siRNA was encapsulated within the liposomes. More than 80% silencing of *apoB* mRNA and apoB-100 protein could be achieved with a single 1 mg/kg dose in non-human primate.[59]

3. FEATURES THAT LEAD TO EFFECTIVE TUMOR TARGETING

For targeting nanoparticles to solid tumors, there are several important barriers that have to be overcome. The pharmacokinetic and pharmacodynamic profiles of nanoparticles are completely different from conventional small chemical drugs or some protein drugs that are usually eliminated or metabolized by the kidneys, liver, or lungs.[60] Nanoparticles are cleared from the blood circulation primarily by the reticuloendothelial system (RES), especially the Kupffer cell in the liver [61] and the macrophages in the spleen. After injecting the nanoparticles into the blood circulation, opsonins such as IgM, IgG, fibronectins, or complement C3 will absorb to their surface. Phagocytic cells will recognize the opsonins and will rapidly and effectively take up the opsonized nanoparticles. The uptake of the nanoparticles by the tumor is a slower and less efficient process. Thus, the RES uptake represents a major “kinetic barrier” for drug delivery to the tumor by nanoparticles. Once the nanoparticles arrive at the tumor, there are other “physical barriers” preventing the cargo drugs from entering the cytoplasm. With appropriate design, self-assembled lipid nanomedicines have been successfully used for siRNA tumor targeting delivery. Li et al.[55,62] showed that by taking advantage of the enhanced permeability and retention (EPR) effect of the tumor (see below), PEGylated LPD could accumulate up to 60–80% injected dose per gram of tissue in the H460 lung cancer xenograft model (Fig. 3). With the help of a targeting ligand–anisamide, significant siRNA uptake and almost complete oncogene silencing and significant tumor growth inhibition *in vivo* were observed. By delivering MDM2, *c*-myc, and VEGF siRNA combination, significant pulmonary metastasis inhibition in a B16F10 murine melanoma model was also observed (Fig. 4). In the following sections, we will discuss in detail how self-assembled nanoparticles overcome these barriers.

3.1. EPR Effect, PEGylation, Optimal Size

Tumor cells are rapidly differentiating and growing cells. They require a large amount of nutrient supply. Angiogenesis as induced by growth factors, e.g., VEGF, is important for tumor growth.[63] Neovessels in the tumor are usually leaky and not well organized. However, the degree of leakiness is highly tumor dependent and could vary significantly. Matsumura and Maeda[64] discovered that due to the leakiness of the vasculature in the solid tumor, macromolecules and colloidal nanoparticles that are too big to penetrate normal blood vessels could penetrate these leaky vasculature and accumulate at the tumor site. This is so called Enhanced Permeability and Retention (EPR) effect. Lacking lymphatic drainage might also contribute to the enhanced retention effect.[64–66] To take advantage of the EPR

effect, nanoparticles must be within an optimal size range. The optimum diameter should be around 100 nm.[67] However, it is dependent on the leakiness of the tumor vasculature.

Nanoparticles need to stay in the blood circulation long enough to overcome the kinetic barrier for extravasating the leaky tumor vasculature. As previously mentioned, the primary elimination mechanism for nanoparticles is the uptake by phagocytic cells after opsonization of the nanoparticles. Modifying the nanoparticle surface with carbohydrate or polyethylene glycol (PEG) is a common strategy for protecting and shielding the surface charge.[68] Studies have shown that PEGylated colloids[69,70] and stealth liposomes[55] could stay in the blood circulation up to 6–10 h in mice and 40 h in humans.[71]

3.2. Targeting Ligands

EPR effect is important in guiding the nanoparticles to tumor tissue, but EPR effect is not enough for delivering siRNA into the cancer cells. There still remains two physical barriers, cell membrane and endosome membrane, that prohibit siRNA from entering the cytoplasm. Drugs or siRNA that stay outside of the cancer cells are not bio-available and will not show therapeutic effect. Nanoparticles with a structure too stable may stay in the tumor extracellular matrix without releasing payload drugs. For example, a stealth liposomal-cisplatin formulation (SPI-077) accumulated efficiently at the tumor site, but it showed minimum therapeutic effect compared to free cisplatin.[72] In order to prompt cancer cells to take up nanoparticles, targeting ligands are needed for triggering receptor mediated endocytosis. There are various types of targeting ligands being used for tumor targeting, including peptides, proteins, antibodies (including Fab, scFv, etc.), aptamers, and small molecular weight ligands, etc.

3.2.1. Peptide Ligands—Binding motifs between ligands and receptors usually involves only several amino acids. Based on this concept, investigators have establish phage display libraries to select special amino acid sequences that show strong binding affinities to tissues, cells, or organs of interests.[73] This method has been established and improved for decades. Increasing numbers of peptide ligands have been identified with high affinities against neo-vasculature, various kinds of cancer cells, proteins, receptors, organs and even lymphatic vessels.[74] For example, the RGD (Arg-Gly-Asp) motif that shows great binding around 1 nM has been used for targeting various drugs or nanoparticles to either tumor neo-vasculature or cancer cells that express integrin $\alpha_v\beta_3$ cell surface receptor.[75–77] NGR (Asn-Gly-Arg) peptide targeting aminopeptidase *N* (APN, CD13) is another example for peptide ligand.[78] There are also other non-specific cell penetrating peptides for drug delivery systems. They include the famous HIV-1 Tat, *Drosophila* Antennapedia transcription factor, herpes simplex virus type-1 VP22 transcription factor, or even simple oligoarginine (R8, R9) peptides.[79,80]

3.2.2. Antibodies (Including Fab, scFv, etc.)—Antibodies (mostly IgG) have been extensively used in biological laboratories due to their high binding affinity to specific epitopes. Humanized antibodies can be used solely or combined with other chemotherapy agents for cancer therapy. Two great examples are the FDA approved anti-Her2/neu monoclonal antibody Herceptin® for breast cancer and the anti-VEGF monoclonal antibody

Avastin® for metastatic colorectal *cancer*. Taking advantage of their binding activity to cancer cell membrane proteins, Herceptin has also been used in targeting liposomes to breast cancer xenografts.[81–85]

IgG antibodies usually have a molecular weight around 150 kDa. In order to make them smaller to either increase the biological production efficiency or reduce the chances to generate immune response, several smaller versions of antibodies have been adapted.[86] For example, removal of the Fc region of the IgG to become the Fab fragment, or combination of the variable regions of both light and heavy chains into a single chain peptide antibody (scFv) is commonly used. Fab and scFv can further be engineered into dimer, trimer, or tetramer forms to provide stronger multivalent binding.

3.2.3. Transferrin—Transferrin is an iron transporting protein that can specifically react with its receptor (Tf receptor) that is expressed in various tissues. Due to the rapid growth of cancer cells, Tf receptors are over-expressed on various kinds of cancers. Anti-Tf receptor antibody[87,88] and transferrin have both been used as ligands for targeting liposomes to tumors[89,90] or even brain cells.[91]

3.2.4. Small Molecule Ligands (Folic Acid, Anisamide)—Small molecule ligands that have good binding affinity and specificity are also suitable for tumor targeting, although they are relatively rare. The advantages of using small molecule ligands compared to small peptides, proteins, or antibodies may include: easy synthesis, more tolerant to chemical modification/conjugation, low immunogenicity, and stable for long-term storage.

The vitamin, folic acid, is the high affinity natural ligand for the folate receptor which is over-expressed in a wide range of human cancers, including ovary, lung, breast, endometrium, kidney, and brain cancers. Drugs including protein toxins, chemotherapeutic agents, oligonucleotides, radioimaging/therapeutic agents, MRI contrast agents, liposomes,[92] etc. have been modified and targeted with folic acid to various tumors.[93–95] Anisamide[96] and haloperidol[97,98] are good small molecule ligands for cancer cells over-expressing the sigma receptor. They include melanoma, non-small cell lung carcinoma, breast tumors of neural origin, and prostate cancers.[97,99–101] The LPD tumor targeting work done in our lab uses mostly anisamide as the targeting ligand.[54–56,62]

3.2.5. Aptamers—Aptamers are nucleic acid-based ligands ranging in size from 20 to 80 bases (6 to 26 kDa). They were mostly identified through a procedure called “systemic evolution of ligands by exponential enrichment” (SELEX). Due to their unique nucleotide sequences, aptamers fold into unique 3D structures and are able to recognize, with high affinity, various molecules including proteins, sugars, phospholipids, or even small chemicals. One aptamer recognizing VEGF (Macugen®) has been approved by FDA as a therapeutic drug for the treatment of age-related macular degeneration (AMD).[102] The aptamer that recognizes the prostate-specific membrane antigen is so far the most successful tumor targeting aptamer. With this aptamer, poly(*d*,l-lactide-co-glycolide)-block-poly(ethylene glycol) (PLGA-b-PEG) nanoparticles,[103] aptamer-siRNA chimera,[104] and quantum dots[105] have been delivered to prostate cancer xenografts.

There are additional ligands under development such as protein scaffolds (e.g., affibody and monobody, which are protein domain-based frameworks).[106] The options for tumor targeting ligands will keep growing. Since some targeting ligands may have their biological functions after binding to the receptors, choosing them carefully is important. For example, if the ligand serves as an agonist, it may promote cancer cell growth. It might not be a good ligand for tumor targeting.

3.3. Endosome Escape, Proton Sponge Effect, H_{II} Phase, Ion-Pairs

Getting the nanoparticles endocytosed is not a major issue. The challenge that remains for the siRNA delivery field is how to bring the siRNA out of the endosome. For lipid-based systems, the mechanism through which cationic lipoplex can trigger endosome release has been proposed by Xu and Szoka.[107] They proposed that in the endosome, the cationic lipid of the lipoplex can form ion-pairs with the anionic endosomal membrane. By excluding the interfacial water molecules, the ion-pairs destabilize the endosomal membrane. Furthermore, binding of cationic lipid with anionic lipids can form the inverted hexagonal H_{II} phase, proposed by Cullis et al.,[108] and leads to membrane fusion and release of cargo. Generally speaking, cationic lipids with smaller and less charged head groups and more bulky acyl/alkyl chains favor the H_{II} phase formation.[109] This is probably the reason why DOTAP (containing two C18:1 acyl chains) is used quite often In liposome transfection formulation as a cationic lipid but DSTAP (1,2-distearyl-3-trimethylammonium propane, a close analog of DOTAP but with two C18:0 chains) is not.

Cationic lipid is not the only category of cationic molecules that can form ion-pairs with the endosomal membrane. Protein transduction domains such as HIV Tat, *Drosophila* Antennapedia transcription factor, herpes simplex virus type-1 VP22 transcription factor, or oligo-arginines (R8 or R9) also show similar activity. It is interesting to know that these peptides all have multiple arginines but not lysines in their sequences. Sakai and Matile[110] showed that this is because the charged groups of both the cationic guanidinium group of the poly-arginine and the anionic phosphate group of the endosomal membrane phospholipids contain delocalized electrons. They form stronger charge-charge interaction and hydrogen bonding than the interaction between phospholipid and lysine which does not contain delocalized electrons. Also, protamine used in the LPD formulation mentioned earlier also contains many arginines but not lysine.

Unlike cationic lipids that possess an intrinsic fusogenic property, polyplex formed by polymeric cationic carriers such as polyethyleneimine (PEI)[111–114] shows a “proton sponge effect” for endosome destabilization.[115–118] The polyplex has many crowded 1°-, 2°- and 3°-amines. Due to the crowding effect, these amines show different pKa within the endosomal pH range and serve as a buffering “proton sponge.” After endocytosis, the pH inside the endosome should drop from physiological pH 7.4 to around pH 5 during the endosome-lysosome maturation process. Due to the presence of the “proton sponge,” the pH would not drop as expected. The ATP-dependent proton-pump on the endosomal membrane would transport extra protons and chloride ions (counter ions), resulting in an increase in the osmotic pressure. Eventually, the endosome would swell and burst due to the large amount of water influx and the polyplex could be released. Polymers containing crowded histidines

(imidazoles) or morpholinos also show a similar buffering effect.[119] The buffering effect may also play a role in protecting siRNA from degradation during the early endosome to late endosome transport process.

Verkman et al.[118] did an elegant piece of work visually showing the accumulation of chloride ions in the endosomes and the release of chloride ions after endosomes burst. However, they did not show exactly that the cargo was efficiently released. The endosome burst and release of chloride ions does not necessary accompany the release of the cargo of the polyplex.

4. ENDOSOME ESCAPE (A PROGRESSING TECHNOLOGY)

Although some self-assembled lipid siRNA tumor targeting nanoparticles already show therapeutic effects in some xenograft mouse models, the endosome escape is still inefficient. Most of the siRNA delivered to tumor cells are still trapped inside the endosome compartment (Li et al. unpublished observation). There might be several reasons for the problem: PEG dilemma, lack of ion-pair formation, not small enough particle size, and insufficient de-assembly of the particles.

4.1. PEG Dilemma

As previous mentioned, PEGylation is the most commonly used method to protect the bare surface of nanoparticles. However, as PEG chains prevent the attachment of opsonins, they also impede the contact between nanoparticles and the target cells. Inside the endosome, PEG may also prohibit the interaction between the cationic lipids of the lipoplex and the anionic endosomal membrane. This is so called “PEG dilemma.”[120] Sophisticated designs such as tunable stealth liposomes,[121] cleavable PEG-lipid linker,[120] or acid labile PEG molecule might help dealing with this dilemma.

4.2. Enhance Endosome Escape

Boeckle et al.[122] have done an interesting study on the effect of free PEI in transfection. They compared the gene transfer efficiency between purified PEI-DNA polyplex and un-purified PEI-DNA polyplex mixture (containing unbound PEI molecules). The result shows that without the presence of unbound free PEI, the gene transfer efficiency decreased dramatically. By applying free PEI 4 h after purified PEI-DNA polyplex transfection, they could rescue the gene transfer efficiency, probably by helping the previously transfected purified PEI-DNA polyplex escape from the endosome. This shows that free unbound cationic polymers such as PEI or poly-arginine may play a critical role in disrupting endosomes by forming ion-pairs with the anionic endosomal membrane. Poly-arginine may have stronger activity than PEI due to their ion-pair formation activity described by Sakai and Matile.[110] If a nanoparticle formulation could sufficiently release free cationic polymers inside the endosome, there would be a great chance that the siRNA delivery be significantly improved.

4.3. Particle de-Assembly and Smaller Particles

Besides endosome escape, de-assembly of nanoparticles is also essential for sufficient siRNA release. If the structure of the nanoparticles is so stable that they will not release the siRNA inside, the siRNA will not be bio-available. De-assembly may take place either in the endosome or in the cytoplasm after the endosome escapes. Ideally, it should take place in the endosome with the release of endosome disrupting cationic materials, because, even though the endosome is disrupted or burst, the “hole” opened on the endosomal membrane may not be large enough to allow intact nanoparticles to pass through. The LPD nanoparticles established by our lab have a particle size around 120 ~ 150 nm. If the particle size could be smaller (perhaps under 100 nm), not only could they escape from the endosome more efficiently, the required siRNA dose for tumor killing may also decrease. Furthermore, they may reach those tumors with less leaky vasculatures.

5. THERANOSTIC NANOMEDICINES

Tumor imaging is a very powerful clinical technique for tumor detection and therapeutic effect monitoring. With the development of multifunctional nanoparticles that can carry both drugs and imaging agents in the same formulation,[123] monitoring cancer therapeutic effects while delivering the therapeutic agents at the same time has become possible. Self-assembled lipids-based nanoparticles could be one of these multifunctional delivery systems for both therapeutic siRNA and a diagnostic agent. This is the so called theranostic smart nanomedicines. Since tumor cells will receive therapeutic siRNA and the diagnostic agent at the same time, cell-specific real-time monitoring of the therapeutic event can be achieved. By monitoring whether the tumor is undergoing apoptosis in the early phase of a given treatment, physicians could decide to either continue the treatment or changing the treatment strategy.

Apoptosis is a complex mechanism that involves various pro-apoptotic and anti-apoptotic molecules inside the cell. There are several methods for detecting apoptosis *in vitro* now, such as staining for the appearance of phosphatidylserine (PS) using annexin V or detecting the activation of caspase-3 which is an early apoptosis event. Annexin V is a human placental anticoagulant protein with four repeats each containing a putative Ca^{2+} dependent binding site for PS. PS was originally distributed in the inner plasma membrane. During apoptosis, the asymmetry of the cell membrane is disrupted, which results in the flip-out of PS and can be stained with annexin V as a marker of apoptosis. Several reports have demonstrated that annexin V labeled with indium-111, technetium-99 m, iodine-123, iodine-124 or fluoride-18 can be used for *in vivo* study.[124–128]

Monitoring caspase-3 activity is another widely applied *in vitro* apoptosis monitoring method. Several reports and commercially available kits have been designed based on the peptide sequence DEVD (asp-glu-val-asp) found in poly-ADP-ribose-polymerase (*PARP*), a natural substrate of caspase-3. Linking the DEVD sequence with two fluorescent proteins for fluorescence resonance energy transfer (FRET) (Fig. 5),[129] a fluorophore with a quencher,[130] or two subunits of luciferase[131] as probes for apoptosis have been demonstrated *in vitro* or *in vivo*.

Many recent reports demonstrate that liposomes can be loaded with gadolinium (Gd)[132–134] or Maghemite (γ-Fe_2O_3) nanocrystals[135] for magnetic resonance imaging, quantum dots[136] for optical imaging, or^{64} Cu for positron emission tomography (PET) imaging.[137] Among all the imaging techniques, MRI can provide good resolution and PET is very sensitive with moderate resolution. Both of them have almost no tissue depth limitation, but, so far, they can only show the size and location of the tumor. Monitoring therapeutic efficacy based on the change in tumor size may not be early enough. On the other hand, taking advantage of the FRET with only fluorescence dyes or involving quantum dots as a donor,[138] fluorescence imaging is capable of generating a signal that would change its profile. Thus, it has the potential for apoptosis monitoring.

Fluorescence with the wave length within visible range has been routinely used in fluorescence microscopy or intravital microscopy. But when it comes to *in vivo* imaging of the whole animal without any invasive procedure, high absorbance or scattering of the visible light by the tissues becomes a major issue. Inside the tissue, hemoglobin is the primary absorber for visible lights; water and lipids are the major absorbers of the infrared light. However, the absorbance coefficients of hemoglobin, water, and lipids are small within the near infrared (NIR) range (around 600 ~ 900 nm). Besides, imaging in the NIR range can also reduce the auto-fluorescence background from tissue and thus provide improved signal to noise ratio. Using advanced imaging methods such as fluorescence molecular tomography (FMT) with near infrared fluorophores can provide 7 to 14 cm penetration in tissue,[139] which could be useful in clinical practice.

6. CONCLUDING REMARKS

Lipid-based nanomedicines have been known for their high biological compatibilities. Their pharmacokinetics and pharmacodynamics profiles are also well studied. This is a solid foundation for further development of advanced self-assembled lipid nanomedicines. The work done by our lab and other groups has shown that self-assembled lipid nanomedicines can specifically deliver siRNA to tumors in several xenograft and syngeneic models. Although the endosome escape of siRNA cargo still has room for improvement, the existing results are already promising. Finally, theranostic nanomedicine will be a new generation drug with high demand. Self-assembly nanoparticles are capable of carrying various cargos as long as these cargos meet the pre-requirement of the self-assembly process. Another advantage is that the manufacturing process of self-assembled lipid nanomedicines could be easily scaled-up. This also makes self-assembled lipid nanomedicines a versatile multifunctional delivery system for theranostic nanomedicine design.

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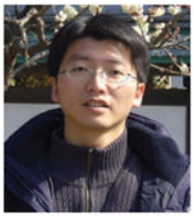
Biographies



Yu-Cheng Tseng received his bachelor's degree in Pharmacy and master's degree in Biochemistry and Molecular Biology from National Taiwan University. After finishing his military obligation, he worked as a research assistant for fifteen months at Institute of Biomedical Sciences, Academia Sinica in Taiwan before joining the Molecular Pharmaceutics Graduate Program in the School of Pharmacy, University of North Carolina at Chapel Hill. He is interested in molecular imaging and cancer targeted therapy with siRNA and peptide. Yu-Cheng Tseng is currently a Ph.D. graduate student in Dr. Leaf Huang's lab.

Leaf Huang Ph.D. is the Fred N. Eshelman Distinguished Professor and Chair, Division of Molecular Pharmaceutics in the School of Pharmacy, University of North Carolina at Chapel Hill. Dr. Huang's research has been in the area of gene therapy and targeted drug delivery. He has pioneered the liposome non-viral vector and has produced the vector for the first clinical trial in 1992. His current work centers on further improvement of liposome vectors for gene transfer in tumor, liver and lung. He also continues research in establishing a ligand targeted delivery system for *siRNA* and *peptides* for tumor growth inhibition and for peptide *vaccines* in treating cervical cancer. He has authored or co-authored more than 300 peer-reviewed papers and more than 120 reviews and book chapters. He is also the inventor or co-inventor of 16 US and foreign patents. In 2004, he received the Alec D. Bangham MD FRS Achievement Award, which is highest honor in the field of liposome research. Dr. Huang has also co-founded 4 biotech start-ups in the past.

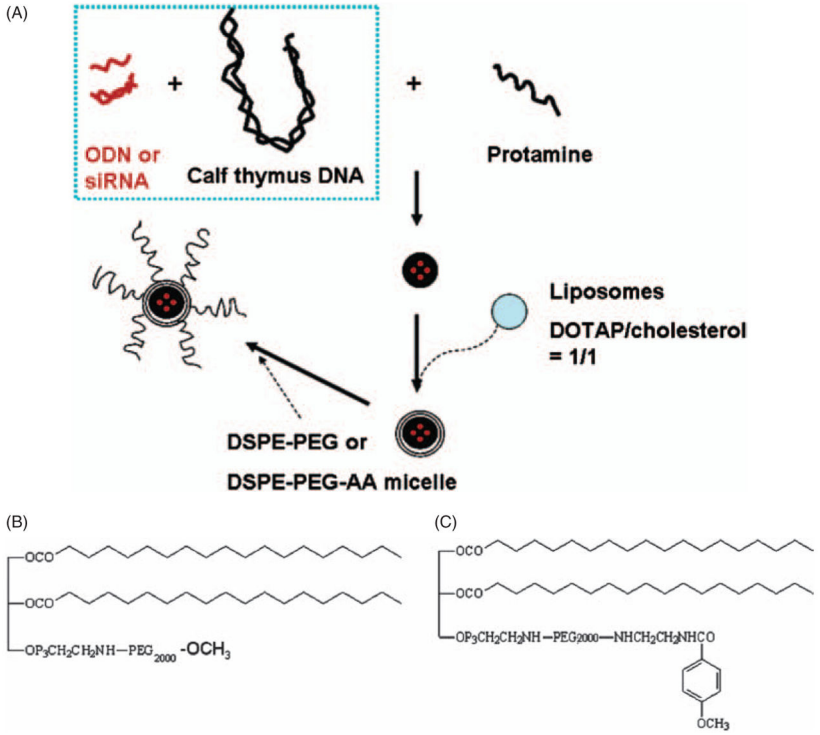


Fig. 1.

(A) Preparation of the PEG and PEG-anisamide (PEG-AA) modified LPD. (B) Chemical structures of DSPE-PEG_{2000} and (C) DSPE-PEG_{2000}-anisamide. Reproduced with permission from [54], S. D. Li and L. Huang, Targeted delivery of antisense oligodeoxynucleotide and small interference RNA into lung cancer cells. *Mol. Pharm.* 3, 579 **(2006)**. © 2006.

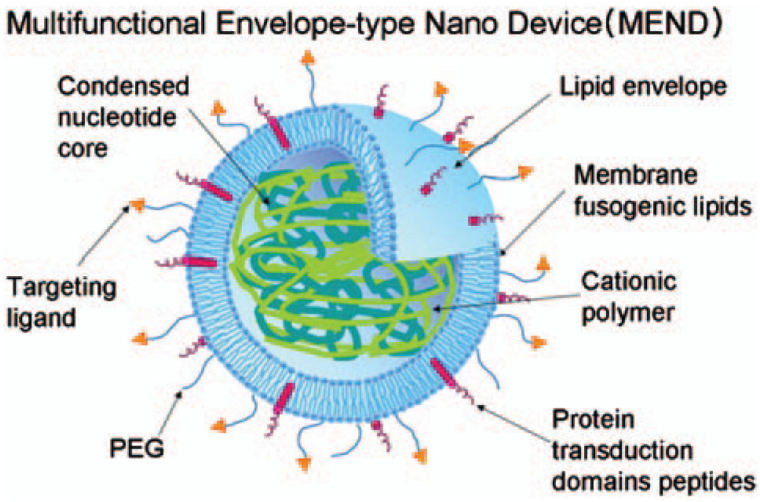


Fig. 2.

The multifunctional envelope-type nano device (MEND) has a condensed nucleotide core coated with lipid envelope. The lipid envelope contains membrane fusogenic lipids and is further modified with PEG, targeting ligand and protein transduction domain peptides. Reproduced width permission from [58], K. Kogure et al., Multifunctional envelope-type nano device (MEND) as a non-viral gene delivery system. *Adv. Drug Deliv. Rev.* 60, 559 **(2008)**. © 2006.

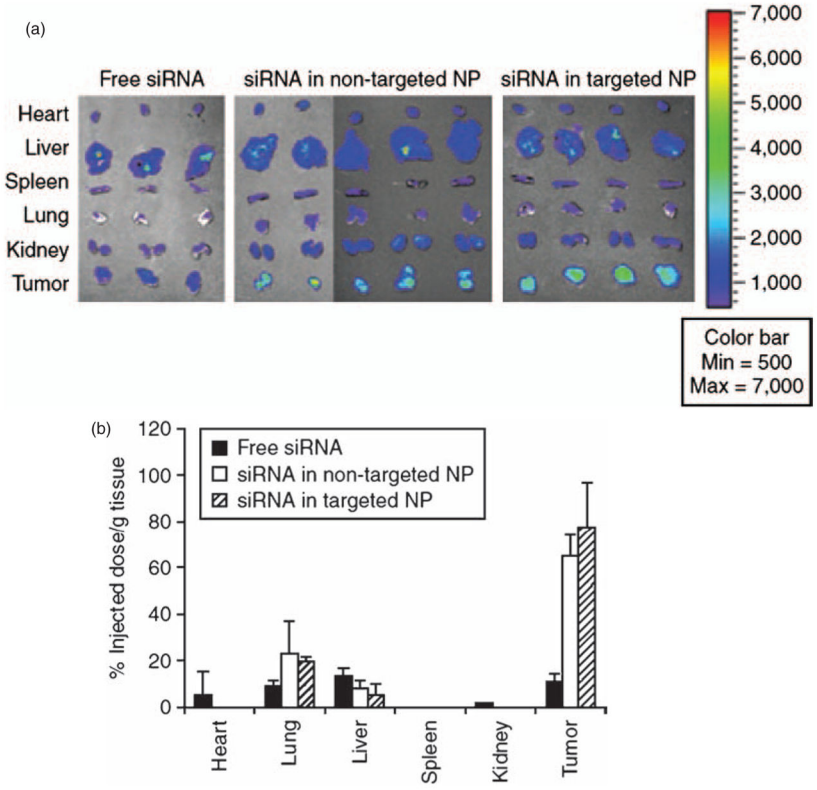


Fig. 3.

Tissue distribution study of siRNA formulated in different LPD formulations. (A) FAM-labeled siRNA was formulated into LPD formulations and i.v. injected into nude mice through tail vein. After 4 hours, tumor and major organs were collected. FAM fluorescence signals were detected by Xenogen IVIS-100 imaging system. (B) Quantitative results of FAM-siRNA tissue distribution. Data =mean ±SD, n = 3–4. NP, nanoparticles. Reproduced with permission from, [55], S. D. Li et al., Tumor-targeted delivery of siRNA by self-assembled nanoparticles. *Mol. Ther.* 16, 163 **(2008)**. © 2008.

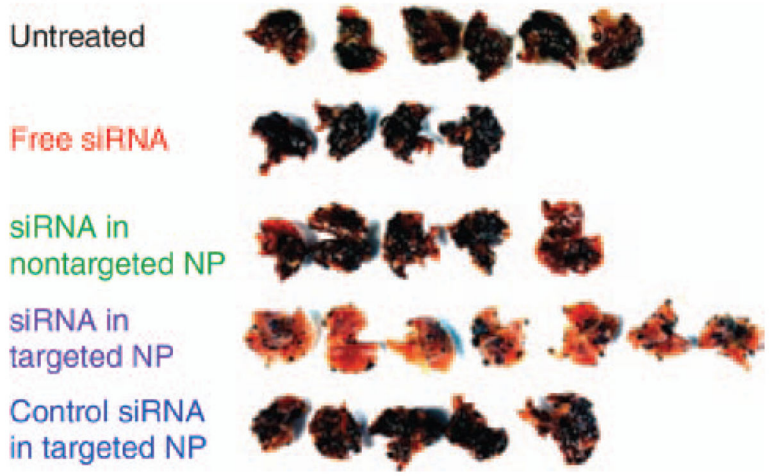


Fig. 4.

LPD nanoparticles (NP) mediated siRNA delivery for metastatic tumor growth inhibition. Lung–homing B16F10 melanoma cells were i.v. injected into mice. 10 days later, mice were i.v. injected with siRNA twice (0.45 mg/kg, MDM2/c-myc/VEGF = 1:1:1, weight ratio). After six days, the mice were sacrificed and observed for melanoma growth in the lung. Only the mice received siRNA in targeted NP showed significant tumor growth reduction. Reproduced with permission from [62], S. D. Li et al., Efficient oncogene silencing and metastasis inhibition via systemic delivery of siRNA. *Mol. Ther.* 16, 942 **(2008)**. © 2008.

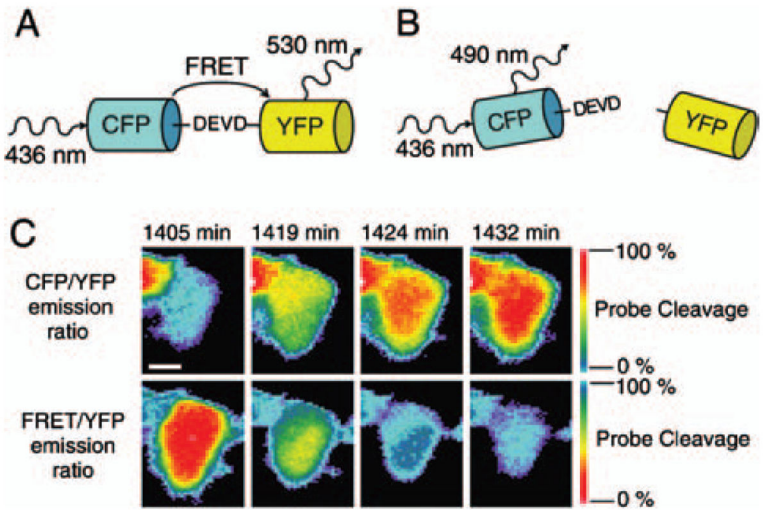


Fig. 5.

DEVD FRET probe containing DEVD as the specific cleavage site for caspase-3, Cyan Fluorescent Protein (CFP) as the FRET donor, and Yellow Fluorescent Protein (YFP) as the FRET acceptor. (A) Without the presence of caspase-3, CFP and YFP are linked by DEVD peptide. Upon CFP excitation, the energy was transferred to YFP by FRET. (B) With the presence of caspase-3, the DEVD linker was cleaved and the FRET between CFP and YFP was disappeared. (C) DLD-1 cell expressing DEVD FRET probe was treated with 1 μM staurosporine for inducing apoptosis. The changes of CFP/YFP and FRET/YFP emission ratio indicate the cleavage of the DEVD FRET probe. Reproduced with permission from [129], C. L. O'Connor et al., Intracellular signaling dynamics during apoptosis execution in the presence or absence of X-linked-inhibitor-of-apoptosis-protein. *Biochim. Biophys. Acta.* 1783, 1903 **(2008)**. © 2008.