Published in final edited form as:

Eur J Pharm Biopharm. 2016 November ; 108: 68–75. doi:10.1016/j.ejpb.2016.08.014.

A biodistribution study of solid lipid-polyethyleneimine hybrid nanocarrier for cancer RNAi therapy

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Abstract

Solid lipid-polymer hybrid nanocarrier (LPN) was previously reported to achieve high siRNA transfection efficiency and induce sustained RNAi-based chemosensitizing effect at cellular level. In this study, our objectives are to evaluate the *in vivo* biodistribution of LPNs in a prostate cancer model and determine the factors that potentially affect tumor penetration by LPNs. The LPN formulation with the highest transfection efficiency (64%) and stability was selected for the study. Mice bearing tumors of PC-3M cells were treated with LPNs labeled with IR780 or AF647-siRNA. Near infrared imaging showed that LPNs achieved favorable *in vivo* biodistribution with high tumor/low organ ratios. LPN accumulation was also observed in liver metastatic tissue. Result of extravasation study confirmed that encapsulated siRNA molecules were able to escape into the tumor tissue at the extravascular area. When LPN levels in large (volume > 750 mm³) and small (<500 mm³) tumors were compared, no significant difference was observed. However, both docetaxel pretreatment (72 h before LPN) and concurrent docetaxel treatment significantly enhanced the tumor LPN levels by 3.9- and 3.1-fold, respectively (both $p < 0.01$). In conclusion, LPN is a promising carrier system to deliver RNAi therapy to solid malignancies that also receive chemotherapy.

Graphical abstract

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Keywords

Biodistribution; Solid tumor; Lipid nanoparticles; Hybrid nanocarrier; siRNA; Drug delivery; Cancer therapy; Tumor priming; Chemotherapy

1. Introduction

RNA interference (RNAi) has been widely studied for therapeutic purpose since its discovery [1]. The use of RNAi triggers such as small-interfering RNA (siRNA) for treatment of malignancies is particularly appealing considering the possibility to silence the expression of essentially any oncogenic or drug resistance gene product in a potent, reversible and customizable manner [2,3]. However, the development of RNAi therapeutics has encountered various issues during the bench-to-bedside translation process [4]. Only until recent years encouraging clinical progress with a few RNAi products were reported, and this has led to renewed interest in this class of therapeutics [4–6]. Reviews on the tortuous path of the development of RNAi therapeutics suggested that suboptimal RNA delivery remains to be one of the rate-limiting factors [6,7]. Hence, for development of any RNAi-based therapeutics, it is of foremost importance to adequately evaluate and optimize their delivery and biodistribution

The delivery issue is especially critical in developing systemic RNAi treatment of solid tumors. For efficient tumor delivery, the RNAi therapeutics need to stay in circulation for sufficient time, achieve high vascular escape at the tumor site, penetrate and accumulate well in the tumor tissues, and transfect the cancer cells and escape into the cytoplasm efficiently [8,9]. Meanwhile, the accumulation of RNAi agents in the non-target organs should be kept as low as possible to minimize potential toxicity [10]. By delivering RNAi therapeutics in a well-designed nanocarrier, the passive (through enhanced permeation and retention effect) and active targeting properties (by surface decoration with cancer targeting moieties) of the carrier may help achieve significant tumor RNA drug levels in a relatively specific manner [7,11]. These advantages have been reported in the successful clinical development of stable nucleic acid lipid particles (known as SNALPs or sometimes LNPs). Significant accumulation of SNALPs in solid tumors was reported in several studies [12–14].

The success of SNALPs highlights the advantages of using lipids and phospholipids as the main ingredients to carry RNAi therapeutics. Nanosystems that include solid lipids, *e.g.* solid lipid nanoparticles and nanostructured lipid carriers, are particularly appealing for drug delivery. In addition to the high biocompatibility, high biodegradability, low immunogenicity and good interaction with cell membranes that are the hallmarks of lipid-based devices, the solid lipid components may also provide sustained, controlled drug release [11,15]. To expand on the application of the conventional solid lipid nanoparticles, our group has previously developed a new hybrid nanocarrier known as solid lipid–polymer hybrid nanocarriers (LPNs) for siRNA therapy of cancer [16,17]. Hybrid nanocarriers are highly versatile because of the possibility to combine the various advantages conferred by different class of materials [18,19]. In LPNs, the polymeric component, polyethylenimine (PEI), formed complex with siRNA and is incorporated in solid lipid components after hydrophobic modification [18]. PEI has been a frequently studied class of polycationic polymers for gene transfection because of their ability to protect nucleic acids, good interaction with cell surfaces and excellent endosomal escape effect [20]. In our previous studies, we have demonstrated that in LPNs, the lipid components not only reduced the toxicity of PEI by more than half, they also prevented premature release of siRNA for days, reduced siRNA degradation by up to 21%, significantly enhanced the transfection efficiency of PEI, and helped to achieve sustained intracellular siRNA release [16,17,21]. The target gene product survivin was consequently knocked down by LPNs to less than 20% of the baseline level for at least a week. In short, the LPNs have demonstrated strong potential for sustained RNAi nanotherapy.

With the physicochemical properties and performance at cell level already demonstrated, our key objectives in the present study are to evaluate the LPN biodistribution in an animal tumor model and investigate the effects of tumor size and chemotherapy co-treatment, two factors that may likely affect the LPN accumulation in the solid tumor target. The tumor model used in this study was initiated with a highly metastatic human prostate cell line PC-3M, which is known for expression of epidermal growth factor (EGF) receptor [22,23]. Prior to the biodistribution study the EGF level on LPN was therefore first optimized to ensure that good cancer targeting and transfection performance were preserved at cell level, so we could focus on the *in vivo* delivery studies. The findings in this study not only are valuable for the clinical development of LPNs, they may also provide the researchers with good information when designing other solid lipid-based or hybrid nanosystems for nucleic acid delivery to solid malignancies.

2. Materials and Methods

2.1. Chemicals and reagents

Tripalmitin and triolein were purchased from TCI America Chemicals (Boston, MA). 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG) were ordered from Avanti Polar Lipids (Alabaster, AL). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[(polyethylene glycol)-3400] maleimide (DSPE-PEG-maleimide) was purchased from Nanocs (New York, NY, USA). Negative-siRNA (Non-targeting

siControl #3, 5'-AUGUAUUGGCCUGUAUUAG-3') was purchased from Dharmacon (Chicago, IL), and Rhodamine-siRNA (5'-UUCUCCGAACGUGUCACGUdTdT-3', rhodamine conjugated) and AF647-siRNA (5'-UUCUCCGAACGUGUCACGUdTdT-3', Alexa Fluor 647 conjugated) were purchased from Qiagen (Valencia, CA). Linear PEI (2500 Da) was purchased from Polysciences (Warrington, PA), dissolved in dichloromethane-methanol (4:1 v/v), filtered and lyophilized before use. PEI was modified by grafting hexadecyl groups to form hexadecylated PEI and purified using the method previously reported [16]. Other chemicals including human EGF and IR780 dye were bought from Sigma-Aldrich (St. Louis, MO). Water used for all RNA works was RNAase-free.

2.2. Cell Cultures

PC-3M, the metastatic sub-line of human prostate epithelial PC3 cells, was a gift donated from Dr. Raymond Bergen at Northwestern University. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 50,000 units penicillin G and 50,000 µg streptomycin at standard cell culture conditions (37 °C, humidified atmosphere of 5% CO₂ in air). Cells were passaged every 5 to 7 days and used for experiments from 10th to 25th passages.

2.3. EGF conjugation and nanocarrier preparation

DSPE-PEG-EGF was prepared with EGF and DSPE-PEG₃₄₀₀-maleimide using standard thiolation protocol [24]. EGF in TEA buffer (50 mM triethanolamine, 150 mM NaCl, 1mM EDTA, pH 8.0) was incubated with 2-iminothiolane at 5:1 molar ratio at room temperature for 1 h, dialyzed (MWCO: 4 kDa, tube-O-dialyzer, G-Biosciences, St. Louis, MO) with excess MES buffer (50 mM MES, 2 mM EDTA, pH 6.0) to desalt, and then added to DSPE-PEG-maleimide in HEPES buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) and incubated at room temperature for 2 h. Free EGF was removed by dialyzing (MWCO: 8 kDa) the product in double distilled water three times. DSPE-PEG-EGF was lyophilized for LPN preparation.

Non-targeting LPNs were prepared using emulsification-solvent evaporation technique as previously reported [16,17]. 15 nmole siRNA was precomplexed with 0.7 mg hexadecylated PEI in a dichloromethane solution containing 2 mg triolein. The mixture was added mixture of lipids including cetyl palmitate, tripalmitin, triolein, DSPC and DSPE-PEG2000 at molar ratio of 40:15:18:15:12 in dichloromethane. For LPN with EGF decoration, same molar amount of DSPE-PEG2000 was substituted with DSPE-PEG-EGF. Nanocarriers were formed by sonicating the mixture in 3 ml of 5% dextrose solution (3 cycles x 1 min, 40kHz, 120V, Branson 3510, Danbury, CT) followed by magnetic stirring at 1200 rpm in vacuum at room temperature.

For *in vivo* biodistribution study, LPNs (loading siControl as a dummy RNA) were prepared with inclusion of a lipophilic near-infrared dye IR780 (at ratio of 4.5 nmole dye per nmole siRNA). To rule out premature release of IR780 from the nanoparticles, 10 mg LPN containing IR780 was dialyzed in 50 mL of cell culture medium at 37 °C for 48 h. Spectrophotometric analysis of the medium showed less than 4% dye was released within this period, indicating that the dye was well retained in LPNs, and was useful for imaging and monitoring of the nanocarrier distribution.

2.3. Size, zeta potentials and serum stability of LPNs

Photon correlation spectroscopy was used to evaluate nanoparticle size and zeta potential (Malvern Zetasizer NanoZS90, Worcestershire, UK) of LPNs with different EGF densities. For each measurement, 50 µg (for size) or 100 µg (for zeta potential) sample of nanocarriers was dispersed in 2 ml distilled water and fifteen successive cycles were run at 25 °C. A minimum of three samples were measured. Data based on the distribution by intensity were reported. To evaluate if samples of LPN could remain well dispersed in the experimental conditions, suspensions of nanocarriers were incubated in phosphate buffered saline (pH7) supplemented with 10% FBS at 37 °C and stirred at 100 rpm for up to 72 h. Their size and zeta potential values were similarly measured.

2.4. Transfection efficiency of LPNs

PC-3M cells at 70–80% confluence cultured in antibiotic-free medium were treated with different nanoformulations of LPN dual-labeled with 1% FITC-DSPE and 50 nM AF647-siRNA for 5 h. Commercial transfecting agent Transfectamine-2000 was included as a positive control. Cells were rinsed twice with PBS, trypsinized, fixed with 10% buffered formalin, and subjected to flow cytometry using FACSCalibur (BD Bioscience, USA) with laser excitation wavelength at 488 nm and fluorescence detection at FL1- and FL4 channels. 10,000 events were recorded for each measurement. Data were analyzed with FlowJo software (version 7.6.4, Tree Star, Inc., Ashland, OR). Transfection efficiency was calculated as (number of cells with fluorescence level past the cutoff x 100%)/(total number of cells analyzed).

2.5. Animal tumor model

Male athymic mice (5–6 weeks old) purchased were housed and acclimatized for 7 days. Animals were cared for in accordance with institutional guidelines (ACUP 303–A4) as well as The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans <http://www.wma.net/e/policy/b3.html>. Animals had access to food and water *ad libitum*. Exponentially growing PC-3M cells were mixed in a 1:1 ratio with Matrigel (Becton Dickinson, Bedford, MA) and a 0.1 mL suspension containing 2×10^6 cells were injected subcutaneously on the back flank area of each mouse to initiate tumor growth.

2.6. LPN biodistribution study

14 days after tumor initiation, animals were randomized into groups of 3–4 animals and given treatment (LPN containing 1 nmole of siControl labeled with 4.5 nmole IR780) or control (normal saline for injection) by tail vein injection. 24 h or 48 h after treatment, animals were sacrificed, tumor samples and vital organs such as liver, lung, kidney, heart and spleen will be excised and near infrared fluorescence from LPN captured using Odyssey Infrared Imaging System (Li-cor Biosciences, Lincoln, NE). The fluorescence was analyzed by ImageJ software (US National Institutes of Health, Bethesda, MD, as public-domain freeware). The fluorescence intensity in an organ was calculated as the total number of pixels whose intensity exceeded the baseline threshold (typically 400), multiplied by the mean fluorescence intensity value of these pixels. The data were normalized by weight of

the organ or tissue, and expressed as % of total dose. To estimate the fluorescence of total dose of LPNs (containing 4.5 nmole IR780), the intensity of 1% of LPN dose (containing 0.045 nmole IR780) was measured and the value multiplied by 100.

2.7. LPN accumulation in metastatic site

Animals were treated with LPN 14 days after tumor initiation and then sacrificed. As liver is one of the commonest sites of prostate cancer metastasis, the livers were examined for any visible metastases. The positive ones were imaged for detection of LPNs in the metastatic tissue.

2.8. Extravasation and tumor penetration

siRNA extravasation was studied as previously described [25]. On day 14, a tumor-bearing mouse was injected with an IV dose of LPNs with rhodamine-siRNA. 24 h after LPN treatment, it was followed by tail vein injection of green perfusion marker 3,3'-diheptyloxacarbocyanine iodide (1 mg/kg in 75:25 dimethyl sulfoxide:phosphate-buffered saline) to outline the blood vessels. Animals were euthanized 2 min after the marker injection, and tumor samples were excised, cryo-sectioned and mounted on cover slides and viewed with an Axiostar Plus epifluorescence microscope (Carl Zeiss, Oberkochen, Germany) using Insight camera model 8.0 for image capture. The perfusion marker and siRNA were imaged at green channel (484 nm/504 nm) and red channel $\lambda_{\text{ex}}/\lambda_{\text{em}}=535$ nm/610 nm), respectively. Images were analyzed using Spots Advanced imaging software (v. 4.6, Diagnostic Instrument, Sterling Heights, MI).

2.9. Effects of tumor size and docetaxel treatment on LPN accumulation in tumors

Large tumors may be more difficult for nanoparticles to access. In this study, the effect of tumor size on tumor LPN accumulation was evaluated by comparing the tumor levels of LPNs in the large tumors (tumor volume > 750 mm³) *versus* small tumors (tumor volume <500 mm³).

As patients with metastatic cancer almost always receive chemotherapy, we also evaluated the effect of chemotherapy treatment on the tumor level of LPN. Animals were treated with 5 mg/kg docetaxel by intraperitoneal injection, and LPN distribution in the tumor was evaluated. Three groups were compared: (1) docetaxel-pretreated group, in which docetaxel was administered 72 h prior to LPN treatment; (2) docetaxel concurrent group, in which the animals receive the docetaxel and LPN treatments at the same time; (3) LPN only group, in which no docetaxel was administered.

2.10. Statistical analysis

The results were expressed as mean $\pm$ SD. Mann-Whitney U-test was performed for statistical analysis due to the small sample size. Unless otherwise specified, differences were considered statistically significant at $p<0.05$.

3. Results

3.1 Screening for optimal LPN for biodistribution studies

Non-targeted LPNs and EGF decorated LPNs containing different EGF levels were evaluated for their average diameter, size polydispersity (PDI), and zeta potential by photon correlation spectroscopy measurements. Table 1 summarizes the results. Low to intermediate level EGF conjugation (EGF-to-nanoparticle ratios from 0 to 0.4 %w/w) did not significantly affect the nanoparticle size. They were within the range of 108–125 nm in diameter and were mono-dispersed (PDI all below 0.2). LPNs with EGF content beyond 0.4 %w/w showed signs of particle aggregation probably due to diminishing of the overall surface charge by EGF decoration.

Figure 1A and 1B presents the effects of serum-supplemented medium to the size and PDI values of these nanoformulations up to 72 h. Except the one with the highest EGF decoration (1.5 %w/w) which showed significant difference of the average diameter after incubation, all LPN formulations remained well dispersed with no sign of significant size growth or aggregation.

These nanoformulations were then screened for their transfection efficiency using flow cytometry (Fig. 2). PC-3M cells were treated with nanocarriers dual-labeled with fluorescent AF647-siRNA and FITC-DSPE phospholipid. Dual labeling ensured that both the lipid component and siRNA component were taken up by the cancer cells to count as a positive event. As shown in Fig. 2D, the degree of EGF decoration clearly affected the siRNA transfection. We obtained $\approx 60\%$ (up to 64%) uptake efficiency at 0.4% EGF level by weight, *versus* 18–29% efficiency using non-targeted LPN. For comparison, the transfection efficiency was about 30% using the commercial transfection agent Lipofectamine-2000. Further increase in EGF level resulted in substantial decline of siRNA transfection.

Overall, the screening process showed that LPNs with 0.4% EGF demonstrated a combination of reasonable size, good stability and high transfection efficiency. This formulation was therefore selected for subsequent animal studies.

3.2. Distribution of LPN in tumor-bearing animals

Fig. 3 presents the imaging result of mice bearing PC-3M prostate cancers. Fig. 3A shows high accumulation of LPNs in the tumor area (indicated by the red box) 24 h after LPN treatment. To more accurately evaluate the relative levels of LPNs in the tumor and various organs, they were excised and imaged *ex vivo* (Fig. 3B). After normalizing the fluorescence by the weight, LPNs were shown to accumulate well in the tumor at levels significantly higher ($p < 0.05$) than the organs such as liver, spleen and lung. There was a modest decrease in the tumor LPN level 48 h post-treatment but it was statistically not significant ($p > 0.05$).

Fig. 4 shows that LPNs were able to distribute and accumulate into the metastatic site in liver (as indicated by the blue circle). The LPN level in the metastase was noticeably higher than the surrounding normal liver tissue. However, in this study out of the five animals studied, only one could successfully develop metastatic tissue large enough for imaging purpose, so we did not have enough subjects to perform further statistical analysis.

3.3. Extravasation of siRNA

Fig. 5 shows the distribution of fluorescent-siRNA (green) in tumor tissue. The red fluorescence outlines the endothelial cells of the blood capillaries. The image indicates that the siRNA molecules carried by LPNs distributed well outside the blood capillaries into the extravascular tumor tissue.

3.4. Effects of tumor size and chemotherapy on tumor accumulation of LPN

Figs. 6A and 6B compare the LPN levels in smaller and larger tumors. In this study, the average size of the smaller tumors was $382 \pm 62 \text{ mm}^3$ and that of the larger tumors was $810 \pm 97 \text{ mm}^3$ (mean $\pm$ SD, $n=3$ per group). Although there was a modest difference between the fluorescence levels after normalization by the tumor weight, no significant difference between the two groups was observed ($p>0.05$). The data indicated that LPNs distributed similarly in small and large tumors.

The effect of chemotherapy treatment on the tumor levels of LPNs was studied. Fig. 7 shows that the LPN levels in both docetaxel groups (docetaxel pretreated group and docetaxel concurrent group) were visibly higher than the no docetaxel group. Quantitative analysis indicates that both docetaxel pretreatment (72 h before LPN) and concurrent docetaxel treatment significantly enhanced the tumor LPN levels by 3.9- and 3.1-fold, respectively (both $p<0.01$). However, although the docetaxel pretreated group was apparently more effective, there is no significant difference between the two docetaxel groups, suggesting that the timing of docetaxel treatment can have some impact on tumor penetration but the impact is likely moderate.

4. Discussion

Effective systemic RNAi nanotherapy of cancer requires strong performance of the nanocarrier at the body level (good distribution of the RNA/nanocarrier from the administration site to the tumor site), tumor level (adequate extravascular escape of the nanocarrier and good penetration within the tumor tissue) and cell level (efficient transfection including uptake by the cancer cells and endosomal escape) [4,5,7]. Our group has previously reported strong performance of the hybrid nanosystem LPN at the cancer cell level, with high transfection efficiency and sustained knockdown effect on survivin being demonstrated [16,17]. The findings in this study further show that a properly screened LPN system also performs satisfactorily at the other two levels.

Before proceeding to the *in vivo* components, the first stage of this study was to screen for the desirable level of targeting moieties on LPN to ensure optimal size, stability and siRNA transfection at cell level. From Table 1 and Figs. 1 and 2, it is evident that decoration with intermediate level of the targeting moieties (*i.e.* 0.4% EGF) could confer the nanosystem with favorable size range and stability in the presence of serum, and achieve the highest siRNA transfection efficiency (~64%, approximately doubled the performance of Lipofectamine, 30%). This LPN formulation was therefore considered suitable so we could focus on the biodistribution studies.

Fig. 3 shows high tumor level of LPNs. The decrease in their level from 24 h to 48 h was moderate, indicating that the LPNs were well retained there. As one of the major concerns of any cancer drug therapy is whether the drug can access the metastatic site as well as the primary tumor, we also explored this area. Unfortunately, the metastatic rate of our tumor model was substantially lower than expected even though PC-3M is already a highly metastatic cell line [26]. Only one animal demonstrated a well formed metastase, and systemic study was therefore unavailable. Nonetheless, given the limited sample, LPNs were shown well-accumulated in the liver metastase and their local concentration was noticeably higher than in the surrounding normal liver tissue (Fig. 4). Hence, our finding, at a minimum, suggests feasibility of using LPNs for RNAi therapy of metastatic cancer diseases.

It may be questioned whether the observed siRNA/LPN level accumulated in tumor is sufficient for RNAi therapy, and too high in organs such as the lungs and livers that may lead to toxic effects. Considering that 0.0057% dose/mg is equivalent to 5.7% dose per gram by tumor weight or approximately 5% dose per ml by tumor volume, and for a typical *in vivo* study the siRNA dose is 1 to 3 nmole per mouse, this will translate into 50–150 nM in terms of siRNA tumor concentration, which has exceeded the level generally required for *in vitro* RNAi activity of LPN as previously reported [17]. The two organs with the second and third highest LPN levels were the lung (~0.0022% dose/mg) and liver (~0.0012% dose/mg), which approximately translated into one-third and one-fifth of the LPN concentration in the tumors. As our study of LPN on non-cancer cells has previously demonstrated that LPN led to minimal toxicities even up to 0.5 mg/mL (containing over 5 nmole siRNA) [21], we expect at the observed levels the toxicities will be modest at most.

There remained two questions given the observed favorable biodistribution pattern of LPNs. First of all, while knowing that LPNs can distribute to and accumulate in the tumor, can they successfully carry the siRNA into the extravascular region where the tumor tissue is located? The second question is how do we further improve the tumor accumulation of LPNs. Tumor is well known to be a heterogeneous structure and nanocarriers may not have access to all parts of a tumor at equal efficiency. As shown in Fig. 3, some areas in the tumors were relatively dim, indicating that there were some spots in the tumors much harder to be accessed by LPNs, even with active targeting strategy implemented.

To address the first question, an extravasation study was performed. As shown in Fig. 5, the fluorescent siRNA were found well-distributed in the tumor tissue outside the blood capillary. It should be emphasized that for any RNAi therapeutics to be useful for cancer treatment, they need to access and physically interact with the cancer cells. Our finding has ruled out the possibility that the LPN/siRNA were just trapped in the tumor vasculature.

For the second question, two factors that may potentially affect the tumor LPN levels were studied. The first is the tumor size. When a solid tumor is large, the high interstitial pressure may retard the penetration by therapeutic agents including nanomedicine [27]. However, our finding (Fig. 6) shows that there is no clear correlation between the tumor size and LPN accumulation in the tumor. It is likely that the smaller tumors were less vascularized, and the

two effects (higher interstitial pressure versus lower vascularization) may have balanced out in this study.

Most patients with advanced, metastatic cancers receive chemotherapy. Hence, it is foreseeable that if RNAi therapeutics are to be clinically used in cancer patients, they will very likely to be used in combination with chemotherapeutic drugs. We previously showed that RNAi therapeutics are effective in serving as a “helper” of conventional chemotherapy, for instance, as a chemosensitizer to restore the drug sensitivity of the chemo-resistant cancer cell sub-populations [16,28]. In this study that focuses on LPN distribution, we evaluated the effect of chemotherapy on LPN tumor accumulation. Docetaxel was chosen because it is the first line chemotherapeutic drug for metastatic prostate cancer. Fig. 7 shows that docetaxel treatment significantly enhanced the average tumor LPN levels, and the “dim spots” in the tumors without docetaxel were mostly eliminated. This is consistent with the previous findings, which reported enhanced penetration of large molecules and liposomes into solid tumors that were pretreated with chemotherapy agents such as doxorubicin and paclitaxel [29,30]. It was suggested that this strategy, termed as “tumor-priming” by these researchers, is caused by expanded interstitial space subsequent to apoptosis-induced cancer cell shrinkage [29]. As it takes time (24–72 h) for cell apoptosis to occur, it was indicated that chemotherapy pretreatment may be superior to concurrent use at least *in vitro*. In this study, the finding not only demonstrated that this strategy can also be implemented with docetaxel and LPNs, we also showed that while pretreatment may have an edge over concurrent treatment, this is likely moderate at least in the case of LPNs, which are stable enough to last till the initiation of apoptosis. The implication is that given the right nanoformulations, the tumor-priming strategy can be implemented with more flexibility in terms of the timing of dosing.

Finally, it should be noted that this is an imaging-based study in which near-infrared fluorescence imaging was extensively used. Like any other imaging analyses there are inherent limitations, most notably non-linear correlation between the fluorescence intensity and drug/dye level in high concentration range. Although we have performed preliminary experiments to minimize this issue, some saturation spots still occurred (*e.g.* in Fig. 6) because the localized LPN concentrations were too high. In future studies, we will consider performing radiolabel quantification to further validate the findings in this study. However, the findings here still provide us with a good picture about the general trend of the siRNA/LPN *in vivo* biodistribution and the factors that may have significant impact on it, which is already valuable for future development of RNAi nanodelivery technology.

5. Conclusion

Hybrid nanosystems have high versatility and are known to deliver a wide range of anticancer drugs with diverse physicochemical properties. Few studies, however, focused on their *in vivo* biodistribution aspect especially regarding their tumor penetration properties. In this study, we not only demonstrated that a properly screened LPN system can efficiently deliver siRNA to tumor sites including the metastatic ones and carry the siRNA into extravascular tissue, and accumulate significantly less in the healthy organs, we also showed that combination of LPNs with chemotherapy administered a few days before or at the same

time can substantially enhance both the average level and uniformity of their distribution in the tumor tissue. LPNs are therefore a promising carrier system for RNAi therapy of solid malignancies, and LPN based RNAi therapy may work better in the context of combination therapy with conventional chemo-treatment.

Acknowledgments

This research was supported by a National Institute of Health/National Cancer Institute R01 grant (grant no. R01CA168917). We also thank Dr. Raymond Bergen at Northwestern University for donating us PC-3M cell line.

ABBREVIATIONS

DSPC	1,2- distearoyl-sn-glycero-3-phosphocholine
DSPE-PEG	2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]
EGF	epidermal growth factor
LNP	lipid nanoparticle
LPN	lipid-polymer hybrid nanocarrier
PEI	polyethyleneimine
RNAi	RNA interference
siRNA	small-interfering RNA
SNALP	stable nucleic acid lipid particle

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Figure 1. Effects of EGF decoration on LPN on (A) serum stability in terms of particle diameter; (B) serum stability in terms of polydispersity index (PDI). * $p < 0.05$ versus 0 h.

Figure 2. Screening for LPNs with high siRNA transfection efficiency in PC-3M cells. Representative flow cytometry charts including (A) LPN without EGF decoration; (B) LPN with inadequate EGF (0.1%); (C) LPN with optimal EGF (0.4%). Results are summarized in (D). Mean $\pm$ S.D (n=3)

Figure 3. Biodistribution of IR780 labeled LPNs in murine tumor model. (A) Representative image of the distribution of LPNs in whole animal (red dashed box indicates tumor area); (B) Representative fluorescence image of LPN in various organs and tumor (from left to right: liver, kidney, spleen, lung, heart, tumor). (C) Quantification of LPN levels after normalization by the tissue weight. * $p < 0.05$ versus all other groups.

Figure 4.
Image of LPN accumulation (in green) in cancer metastase in liver (red background). The blue circle indicates the location of the metastase.

Figure 5.
Image of fluorescent siRNA (in green) in a section of tumor with blood capillary stained (in red) indicating extravasation of the siRNA carried by LPNs.

Figure 6. Comparison of LPN distribution in large (upper panel) and small (lower panel) tumors. (A) presents the images of tumors and (B) compares the fluorescence intensity. Mean $\pm$ S.D (n=3)

Figure 7.

Effect of docetaxel co-treatment on LPN accumulation in tumors. No docetaxel (upper), pretreated with docetaxel 72 h prior to LPN administration (middle) and docetaxel concurrently administered with LPN (lower) were compared. (A) presents the images of tumors and (B) compares the fluorescence intensity. Mean $\pm$ S.D (n=3) * $p < 0.01$ versus no docetaxel

Table 1

Screening for LPN formulation with favorable physicochemical properties (size, polydispersity and zeta potential).

%w/w of EGF in nanoparticles	No EGF (<i>i.e.</i> non- targeted)	0.05%	0.1%	0.2%	0.4%	0.8%	1.5%
Average diameter (nm)	121 ± 13	125 ± 19	110 ± 9	108 ± 16	119 ± 19	120 ± 22	122 ± 27
Polydispersity index, PDI	0.179 ± 0.016	0.152 ± 0.023	0.177 ± 0.013	0.189 ± 0.022	0.198 ± 0.025	0.212 ± 0.037	0.254 ± 0.051
Average zeta potential (mV)	+26 ± 5	+20 ± 5	+20 ± 3	+14 ± 5	+13 ± 4	+12 ± 9	+9 ± 9