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RESEARCH PAPER

Lipid Encapsulation of Cationic Polymers in Hybrid Nanocarriers Reduces Their Non-Specific Toxicity to Breast Epithelial Cells

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ABSTRACT

Purpose Clinical application of cationic polymers for delivery of nucleic acids has been limited by their toxicity. The purpose of this study is to evaluate whether the polymer-in-lipid hybrid nanotechnology recently developed for controlled siRNA delivery can tackle this toxicity issue by reducing exposure of the cellular components to free cationic polymers.

Methods Lipid-polymer hybrid nanocarriers (LPNs) encapsulating complexes of hexadecylated polyethylenimine (H-PEI) and biologically inactive siRNA in lipids were prepared at different lipid-polymer ratios. Comparative toxicity of these LPNs and unencapsulated cationic materials on breast epithelial cell lines MDA-MB-231 and MCF-10a was evaluated.

Results Even at a low lipid-polymer ratio (3:1 w/w), encapsulation of H-PEI improved its LC_{50} values measured within hours by 3–5 fold, and caused less reduction in the colony-formation rates in 10–14 days. The observed reductions in the acute and delayed carrier toxicity were associated with significantly less membrane damages, improved mitochondrial functions, reduced reactive oxidative species production, and lower caspase-3 activity (all $p < 0.05$) without sacrificing the siRNA transfection efficiency.

Conclusions This study has validated the hybrid nanotechnology for controlled RNA delivery from a toxicological perspective. This is especially valuable if local or long-term RNA therapy is intended for which low carrier toxicity is essential.

KEY WORDS cationic polymer · hybrid nanocarrier · lipid · nonviral delivery · toxicity

ABBREVIATIONS

DSPE	1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine
DSPE-PEG	1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[(polyethylene glycol)-2000]
DSPE-PEG-folate	1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[folate(polyethylene glycol)-2000]
H-PEI	hexadecylated polyethylenimine
LPN	lipid-PEI hybrid nanocarrier
PEI	polyethylenimine
PLGA	poly(lactic- co -glycolic acid)
siRNA	small-interfering RNA

INTRODUCTION

DNA or RNA therapy often relies on the use of carriers. A broad range of carrier systems, typically of submicron size, have been developed to prevent the degradation, improve the biodistribution profiles, and enhance the transfection performance of therapeutic genes or oligonucleotides (please see reviews (1–4) and references therein). While the focus has often been on their delivery and transfection performance, their inherent toxicity should not be overlooked. Since the death of a patient in response to adenovirus gene carrier in 1999 (5), there has been increased awareness of the carrier toxicity. In addition to identifying safer viruses for gene delivery (6,7), researchers have begun to extensively study the use of nonviral nanocarriers (8,9). Although in principle, the nonviral systems are relatively safe alternatives, they still frequently demonstrate significant intrinsic toxicity (10–12). Hence,

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carrier toxicity is often considered a critical factor limiting the clinical application of nucleic acid therapy, and it is important to develop new, convenient and cost-effective strategies to overcome this limitation.

Many of these nonviral nanocarriers are made of biodegradable cationic materials, one most commonly used class being polyethylenimine (PEI) and its derivatives (13–15). The dense positive charges of these materials not only allow stable non-covalent complexation with the anionic nucleic acids and facilitate their condensation, they also promote interaction with the negatively charged cell membranes and improve transfection (13,14). However, the strong cell- cationic carrier interactions are also notorious for causing non-specific cell toxicity (11–15). As these toxic effects are usually concentration-dependent and time-dependent, we expect an even higher risk of cationic carrier toxicity if local nucleic acid therapy (*e.g.* loco-regional breast cancer treatment) is intended because of the high carrier concentration in a confined compartment, or if extended treatment is implicated for chronic disease conditions (16,17).

Several strategies have been previously studied to reduce the toxicity of these cationic materials, and to some extent, also enhance their transfection performance at the same time. These strategies include the use of degradable cross-linkages, synthesis of more linear polymers such as linear PEI, grafting polymers with hydrophobic moieties, and incorporating liposomal ingredients with cationic polymers to form polysomes (15,18–22). Recently, our group has integrated some of these strategies and developed "lipid-polymer hybrid nanocarriers" (LPNs) (23,24), in which a cationic polymer with hydrophobic groups, *e.g.* hexadecylated derivative of linear PEI, was complexed with small-interfering RNA (siRNA) and encapsulated into a matrix of mostly solid lipids. This "drug/polymer-in-lipid" hybrid design has been previously demonstrated by us and other groups to provide several benefits from the drug delivery and therapeutic perspectives, *e.g.* controlled release of diverse small molecule drugs (25–28). For LPNs, the hybrid design was shown to result in reduced extracellular release of siRNA, controlled, steady intracellular siRNA release kinetics and extension of the gene silencing effects, so the application of the short-lived siRNA therapy can be expanded for management of chronic, tough-to-treat diseases (23,24,29).

The present study focuses on investigating the toxicological implications of this hybrid approach. There was a recent report on successful use of lipid coating to improve the immunocompatibility of the encapsulated PLGA polymers by reducing their direct interaction with the host immune system (30). This lends additional credence to our hypothesis that this approach will also avoid sudden, full-scale exposure of the cellular components to free cationic polymer molecules, so their non-specific cell damaging effects can be ameliorated.

Studies have shown that the toxic effects of cationic gene materials can be acute and/or delayed (10,11). The acute carrier toxicity is frequently associated with cell membrane damages and can take place within hours soon after cell-carrier interaction, whereas the delayed effects are often related to the loss of mitochondrial functions and apoptosis which typically occur two days after transfection or later. The majority of studies of nucleic acid nanocarriers tend to focus on the acute toxicity. As LPNs were previously shown to gradually release siRNAs within the transfected cells for seven days or more (23,24), the role of delayed toxicity may become even more crucial. This will be delineated in this study by carefully distinguishing the delayed toxic effects from the acute component.

MATERIALS AND METHODS

Chemicals and Reagents

Non-fluorescent siRNA negative control (Non-targeting siControl #3) and FAM-labeled negative control #1 siRNA (a siRNA tagged with a derivative of FITC emitting more stable fluorescence) were purchased from Dharmacon (Chicago, IL) and Ambion (Austin, TX), respectively. Linear 2500 Da PEI and branched 25 kDa PEI were purchased from Polysciences (Warrington, PA). The linear PEI was dissolved in dichloromethane-methanol (4:1 v/v), the solution filtered and solvents removed in vacuum for hydrophobic modification. 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[folate (polyethylene glycol)-2000] (DSPE-PEG-folate), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE), 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (DSPE-PEG) and cholesterol were ordered from Avanti Polar Lipids (Alabaster, AL). Tripalmitin and triolein were purchased from TCI America Chemicals (Boston, MA) and purified by recrystalization in ethanol before use. RiboGreen RNA Quantitation Reagent and Lipofectamine-2000 were purchased from Invitrogen (Carlsbad, CA), Ac-DEVD-AMC from Enzo Life Sciences (Plymouth Meeting, PA), Advanced Protein Assay reagent from Cytoskeleton (Denver, CO) and other chemicals from Sigma-Aldrich (St. Louis, MO). Water used for all RNA works was RNAase-free.

Cell Cultures

The non-specific intrinsic toxicity of drug or gene delivery systems are commonly evaluated in cancer cell lines to take advantage their fast growth and responses (31–33). Meanwhile, we also desired to observe the toxicity effects of LPNs in non-cancerous breast cells. Hence, the carriers were tested on both non-cancerous human breast epithelial MCF-10a cell line and human breast cancer MDA-MB-231 (MDA231) cell line (American Type Culture Collection, Manassas, VA).

MCF-10a were maintained in MEBM medium supplemented with MEGM CC-3150 kit (Lonza/Clonetics Corp) and 100 ng/ml cholera toxin. MDA231 cells were cultivated in colorless RPMI 1640 medium supplemented with 10% fetal bovine serum, 50,000 units penicillin G and 50,000 μg streptomycin. Cells were incubated at standard cell culture conditons (37°C, humidified atmosphere of 5% CO_2 in air) and passaged every 5 to 7 days for maintenance. Cells used for experiments were 10th to 25th passages.

Hexadecylated PEI and Nanocarrier Preparation

Hexadecylated PEI (*i.e.* H-PEI) and LPNs were prepared as described before (24). In brief, H-PEI was prepared as follows. 0.945 g 1-bromohexadecane in dichloromethane was added to a solution of 1 g PEI and 1 ml triethylamine in 95:5 v/v dichloromethane/methanol. The resulting mixture was stirred for 48 h at room temperature, concentrated under vacuum, purified by dialysis against 50% ethanol (5 cycles × 1 L) followed by RNAase free water, adjusted to pH 4–5 with HCl/NaOH and lyophilized before use. The substituted H-PEI product is represented by the stoichiometric formula $(C_2H_4N)_{59}(C_{16}H_{33})_{6.9}$ as previously determined (24).

To prepare LPN, H-PEI was complexed with 15 nmol siRNA (FAM-siRNA or non-fluorescent siRNA negative control) in dichloromethane at an N/P ratio of 15:1. The siRNA/H-PEI complex was added to a lipid mixture containing tripalmitin: cholesterol: triolein: DSPE: DSPE-PEG: DSPE-PEG-folate in 1.5: 2.5: 2: 2.5: 1.3: 0.2 w/w ratio in 0.4 ml dichloromethane at different polymer to lipid ratios (1:15, 1:7 or 1:3 w/w ratio to prepare $LPN_{1:15}$, $LPN_{1:7}$, $LPN_{1:3}$). 0.05 mg of fluorescent, rhodamine-DPPE was also included for LPN quantification. LPNs were formed by solvent evaporation/ emulsification technique. The above mixture was dispersed in 3 ml of RNAase free water, following by 3 cycles × 1 min sonication (40 kHz, 120 V, Bransonic 3510, Danbury, CT) and stirring at 1200 rpm in vacuum overnight at room temperature. Free siRNA molecules were removed by columns containing Sephadex G-25.

Blank LPN (LPN_{blank}) was prepared without H-PEI. Unencapsulated siRNA/PEI or siRNA/H-PEI complexes were prepared by the direct complexation method (34). Thin films of PEI or H-PEI were sonicated for 3 min to form polymer aqueous suspension. Polyplexes were formed by incubating the dispersed polymer with siRNA at room temperature for 20 min at an N/P ratio of 15:1. Lipofectamine/siRNA complexes were prepared as per manufacturer's instruction.

Size Distribution and Zeta Potential Measurement

Particle size and zeta potential values of different carrier formulations were measured by photon correlation spectroscopy using Malvern Zetasizer NanoZS90 (Worcestershire, UK). For each measurement, nanocarriers were dispersed in 2 ml distilled water and fifteen successive cycles were run. Size distribution by intensity was used.

Release Kinetics of Polymer Molecules from LPN

Release kinetics of H-PEI from the LPN formulations were measured as described by Bertschinger *et al.* (35). 200 μl suspension containing 8 mg carrier was added to 4800 μl medium (enzyme-free buffer: 0.1 M PBS at pH 7.4; buffer with enzyme: 0.1 M citrate buffer at pH 4.8 containing 500 U/ml lysosomal acid lipase) in a dialysis bag (Spectrapor, MWCO: 25 kDa). The bag was immersed in 95 mL release medium (blank PBS or citrate buffer) at 37°C with magnetic stirring. At each predetermined time point, 800 μl of medium was sampled and incubated with 200 μl Advanced Protein Assay reagent for 30 min at room temperature. The resulting colored product was spectrophotometrically measured at 590 nm. Standard curve was prepared using free H-PEI solution. Experiments were repeated in triplicate.

Trypan Blue Exclusion Assay

Cells grown on 24-well plates in antibiotic-free medium were treated with different carrier formulations loaded with siRNA negative control for 5 h at standard culture conditions. Trypsinized cells were stained with 0.4% trypan blue in PBS and stained, non-viable cells were counted in a hemacytometer.

Clonogenic Assays

Cells grown in 10-cm culture dishes in antibiotic-free medium were treated with different formulations loaded with siRNA negative control for 5 h at standard culture conditions. Cells were washed with PBS, re-incubated in fresh medium for an additional 12 h, trypsinized and seeded into culture dishes at density of 100 or 1000 viable cells per dish (counted cells unstained by trypan blue). Cells were incubated at standard culture conditions in drug-free medium for 10–14 days until visible cell colonies were formed from proliferation of the seeded cells (>50 cells/ colony). The colonies were fixed and stained with 0.5% methanol solution of methylene blue and their numbers counted. The fraction of cells grown into viable colonies, *i.e.* plating efficiency (%), is calculated as: $\text{normalized plating efficiency} = (\text{number of viable colonies} \times 100\%)/(\text{number of cells seeded})$. Results are normalized against the untreated control and expressed as normalized plating efficiency.

Flow Cytometry

Transfection efficiencies of different formulations were measured by flow cytometry in combination with trypan blue quenching in accordance to the method previously described (24). Cells were transfected by incubating with different carriers loading 40 nM fluorescent FAM-labeled siRNA or non-fluorescent siRNA control for 5 h. They were then trypsinized, resuspended in PBS containing 0.5% FBS, and treated with 0.4% trypan blue for 30 min to quench the extracellular fluorescence. Flow cytometry was performed using FACSCalibur (BD Bioscience) with excitation wavelength at 488 nm, and the resulting FAM-siRNA fluorescence was detected using FL1 channel. 10,000 events were recorded for each measurement. Data were analyzed by FlowJo software (version 7.6.4, Tree Star, Ashland, OR).

To confirm the sustained siRNA release feature of LPNs in the cell lines we tested, the experiment was repeated 7 days after transfection to quantify the siRNA that remained in the cells. To further confirm the lack of significant autofluorescence from the LPNs' lipids/polymer, we have treated the cells with $LPN_{15:1}$ carrying non-fluorescent siRNA control. The histograms obtained were similar to the untreated control (data not shown for brevity). The fluorescence measured was therefore primarily originated from the transfected FAM-siRNA.

Lactate Dehydrogenase (LDH) Assay

The effects of nanocarriers on membrane integrity were evaluated using LDH assay. Cells grown on 24-well plates were treated with carriers loading siRNA negative control for 5 h at standard culture conditions. 100 μl supernatant from each well was then withdrawn, centrifuged and transferred to a 96-well plate, followed by addition of 100 μl LDH reaction mixture per well (LDH cytotoxicity detection kit, Roche, Indianapolis, IN). After 30 min incubation at 37°C in darkness, the absorbance at 500 nm was measured for quantification of the free LDH leaked from the damaged cells. Blank medium and 5 mg/ml PEI served as the negative (0% damage) and positive controls (100% damage), respectively.

MTT Assay

Cells were grown overnight in 96-well plates at 4000 cells/well in antibiotic-free medium. Cells were treated with carriers loaded with negative-siRNA for 5 h and then washed with PBS. Transfected cells were re-incubated in 100 μl fresh carrier-free medium at standard culture conditions for 48 h or 7 days. For the 7-day group, cells were trypsinized on day 5, 4000 viable cells reseeded per well and further grown for 2 more days. At the end of incubation, cells were incubated with 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 37 C for 2 h followed by 200 μl DMSO per well for 1 h. Concentrations of the solubilized metabolized dye formed in viable cells were measured by a SpectraMax microplate reader at 570 nm with a reference wavelength at 690 nm.

Detection of Reactive Oxidative Species (ROS)

Levels of intracellular ROS, primarily superoxides, were measured using dihydroethidium method as reported (36). Briefly, dihydroethidium is oxidized by intracellular superoxides to ethidium which emits fluorescence for quantification. Cells were treated with different carriers delivering non-fluorescent siRNA negative control for 5 h. After 48 h or 7 days, cells were treated with 5 μM dihydroethidium in colorless RPMI-1640 medium for 30 min, washed with PBS twice, and the ethidium fluorescence was measured using excitation/emission at 520 nm/610 nm emission. Cell number is used to normalize the results for comparison.

Caspase-3 Assay

The activity of caspase-3, the key effector protease that triggers apoptotic death (37) was measured using fluorescence method. Cells were treated with different formulations loaded with non-fluorescent siRNA control for 5 h at standard culture conditions. After 24 h, cells were trypsinized and resuspended in lyse buffer containing 10% sucrose, 0.1% CHAPS, 100 mM HEPES, pH 7.4, protease inhibitor cocktail (Sigma-Aldrich), and 10 mM dithiothreitol. The lysate containing 20 μg total protein was incubated with the caspase-3 fluorogenic substrate Ac-DEVD-AMC. The caspase activity was measured using excitation/emission at 350 nm/450 nm. The % changes in fluorescence comparing to the untreated control are presented.

Statistical Analysis

The significance of differences was assessed using one-way ANOVA with Tukey's post-hoc tests (for comparison of multiple groups) and Student's t-test (for comparison between two data points). $p<0.05$ was considered significant.

RESULTS

Nanocarrier Properties

Table 1 summarizes the particle size and zeta potential data of different LPN formulations. Overall, the LPNs were smaller than unencapsulated polyplexes and are all around 200 nm in diameter. This is consistent with previous studies of gene delivery systems involving lipids (38), in which the presence of lipid/phospholipids could help pack the

Table I Size and Zeta Potential Measurements of the Carriers

Carrier	Particle diameter (nm).		Zeta potential (mV),
	z-average value	PDI	
siRNA/H-PEI polyplexes	302.6 ± 51.7	0.45 ± 0.18	+31.7 ± 9.3
Blank LPN (no H-PEI)	161.5 ± 20.5	0.22 ± 0.02	−29.8 ± 6.3
$LPN_{15:1}$	215.5 ± 26.9	0.24 ± 0.01	−20.1 ± 5.2
$LPN_{7:1}$	253.6 ± 32.5	0.29 ± 0.07	−13.9 ± 9.3
$LPN_{3:1}$	226.3 ± 22.2	0.26 ± 0.04	+16.8 ± 5.0

Particle diameter based on distribution by intensity. Mean ± S.D. presented (N ≥ 3). PDI = polydispersity index. All formulations carry non-fluorescent siRNA negative control

polyplexes more tightly and prevent their aggregation. And all three LPNs with different lipid-to-polymer ratios (15:1, 7:1, 3:1 w/w) had similar particle size values ($p > 0.05$ comparing the three formulations). The carrier size therefore should not be a deciding factor of their toxicity.

Encapsulation of the siRNA/H-PEI with increasing amount of lipids turned the zeta potential value from highly positive (+31.7 ± 9.3 mV, in the absence of lipids), moderately positive ($LPN_{3:1}$: +16.8 ± 5.0 mV) to fairly negative ($LPN_{15:1}$: −20.1 ± 5.2 mV), indicating that the polymers' cationic charges were increasingly screened out by the electrostatically negative lipids. We have also used Ribogreen dye binding method (24) to determine the siRNA encapsulation efficiencies of the three LPNs and H-PEI. The measured values were in the range of 80–88%. As siRNA is not the focus of this study and such small differences should not affect toxicity, details are not shown.

Controlled Release of Cationic Polymer Molecules from LPN

Figure 1 presents the profiles of H-PEI from LPNs in regular PBS buffer and acidic citrate buffer containing enzyme lysosomal acid lipase. The dotted lines indicate the diffusion of free H-PEI solution which served as the positive control. All LPNs released the H-PEI molecules more slowly than the unencapsulated H-PEI/siRNA polyplex in the enzyme-free PBS. Their release rates were higher in the enzyme containing buffer, but remained considerably lower than the unencapsulated polyplex. In both release medium, LPN encapsulated with more lipids (*e.g.* $LPN_{15:1}$) released the polymer more slowly than the less encapsulated LPN (*e.g.* $LPN_{3:1}$).

Hybrid Approach Substantially Reduced Acute Cell Toxicity

Figure 2 presents the dose effect of different formulations on cell viability as measured with trypan blue exclusion assay (left panel: MDA231, right panel: MCF-10a). Results have been normalized against the untreated control. Because our primary goal is to evaluate the effect of lipid encapsulation on the polymer toxicity, the various formulations were compared on the basis of the quantity of PEI/siRNA instead of the full carrier (*e.g.* because the same quantity of $LPN_{15:1}$ contained only ≈ one/fifth of PEI/siRNA in $LPN_{3:1}$, the relative concentration of $LPN_{15:1}$ to $LPN_{3:1}$ used was ≈ 5:1 for fair comparison; same in other assays).

After normalization of the polymer quantity, it was shown that the acute toxicities of the encapsulated formulations were several fold lower than the unencapsulated polyplexes ($p < 0.05$). The LC_{50} values of unencapsulated PEI, unencapsulated H-PEI, $LPN_{15:1}$, $LPN_{7:1}$, and $LPN_{3:1}$ were estimated as 17, 22, 92, 94, 72 μg/ml PEI for MDA231 cells, and 10, 19, 173, 132, 95 μg/ml PEI for MCF-10a cells, respectively. Two key trends were observed. First, comparing the three LPNs to the two unencapsulated polyplexes, the LPN formulations were significantly less toxic ($p > 0.05$) to both cell types. Second, comparing the responses of the non-cancerous MCF-10a cells and cancerous MDA231 cells, the MCF-10a cells appeared to tolerate the LPNs particularly well. The LD_{50} values of LPNs increased by 32% to 88% in MCF-10a, while the LD50 of PEI and H-PEI remained low.

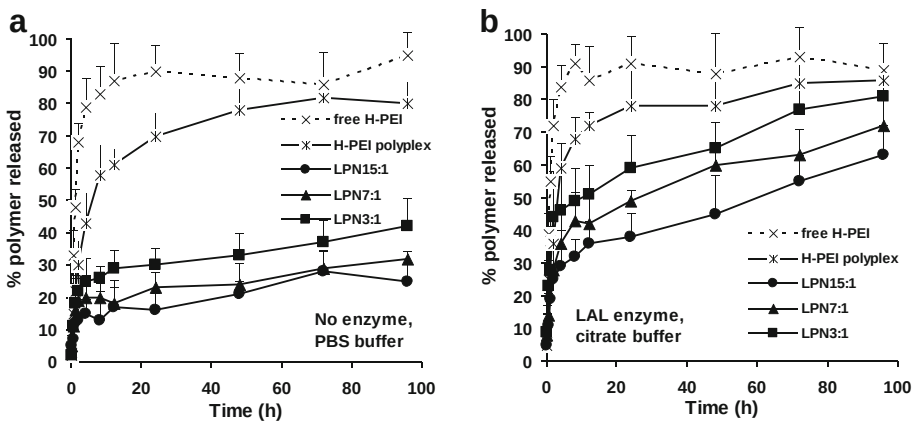

Fig. 1 Time profiles of polymer release from LPN (**a**) in PBS at pH 7.4 and (**b**) in citrate buffer at pH 4.8 containing lysosomal acid lipase. Free H-PEI: H-PEI solution containing no siRNA. Mean values + S.D. ($N=3$) presented.

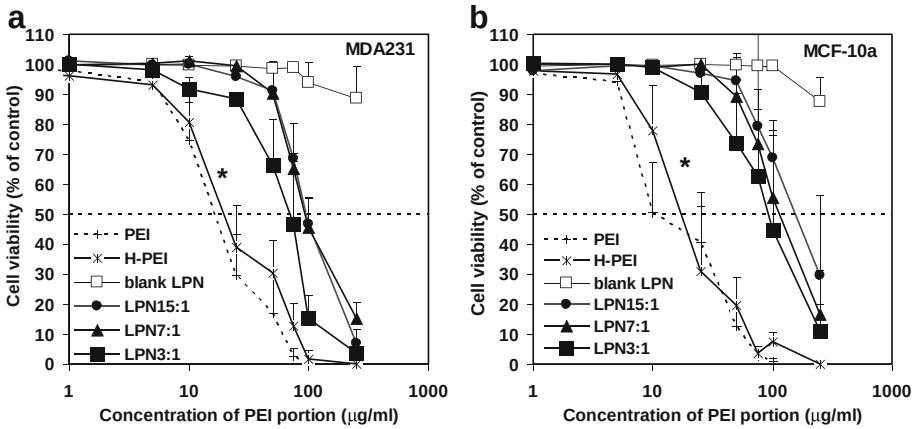

Fig. 2 Trypan blue exclusion assay measuring the acute carrier toxicity to (**a**) MDA231 cells and (**b**) MCF-10a cells. Different treatments are compared based on the quantity of hexadecylated PEI (H-PEI) included. Results are normalized against the untreated control data and expressed as % cell viability (means + S.D., $N=3$, triplicate per experiment). * indicates significant difference ($p<0.05$) comparing H-PEI and PEI polyplexes to all three LPN groups. The dashed line in each graph highlights the 50% cell viability to indicate the LC_{50} values at the intersections.

Lipid Encapsulation of Polymer Significantly Reduced Delayed Carrier Toxicity

Figure 3 presents the clonogenic assay data. The plating efficiency values presented here have been normalized against the untreated control (so 100% plating efficiency means there would be no long-term toxic effects on cell proliferation). In general, encapsulation of H-PEI to form hybrid nanocarriers moderately but significantly reduced the toxicity. LPNs caused significantly less inhibitive effects on proliferation (*i.e.* higher plating efficiency, $p<0.05$) of both MDA231 (left panel) and MCF-10a cells (right panel) than unencapsulated polyplexes and lipofectamine (except $LPN_{3:1}$, which was only moderately less toxic than lipofectamine in MDA231). Blank LPN did not inhibit cell proliferation (plating efficiency around 100%).

LPNs Efficiently Taken up by Breast Epithelial Cells

Figure 4 presents the flow cytometry measurements of the siRNA transfection efficiency (left panels: representative histograms; right panels: summary of transfection efficiency data). Figure 4a shows the data obtained immediately after transfection. LPNs were taken up by both cell types at comparable or better efficiency (68–83%) when compared to the unencapsulated H-PEI and Lipofectamine (42–55%). In Fig. 4b, we compared selected groups on day 7 after transfection and showed that substantially more LPN-treated cells retained

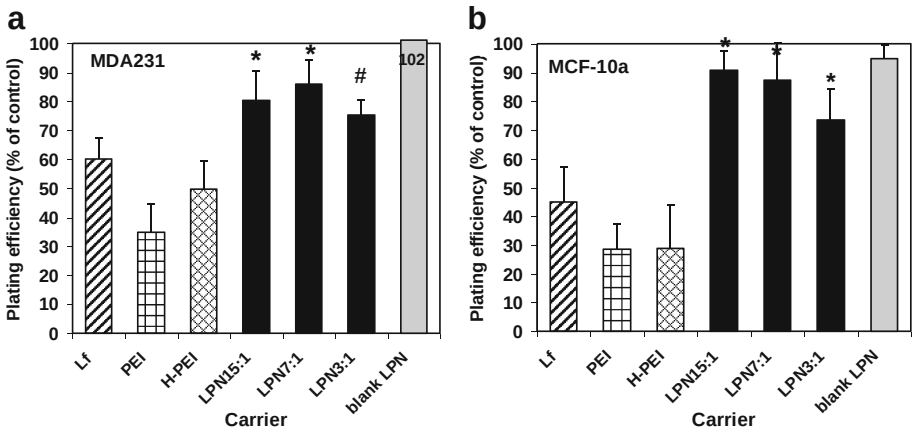

Fig. 3 Clonogenic assay measuring the effects of delayed carrier toxicity on the proliferation of (**a**) MDA231 cells and (**b**) MCF-10a cells. Different treatments containing 5 μg/ml H-PEI are compared. Results are normalized against the untreated control data and expressed as plating efficiency (as means + S.D., $N=3$, duplicate per experiment), so 100% means the lack of any effects on long-term colony formation. The plating efficiency of blank LPN treated MDA231 cells is 102 ± 6. * indicates significant difference ($p<0.05$) comparing to both PEI, H-PEI and Lf (lipofectamine-2000) groups, # indicates significant difference ($p<0.05$) only to PEI and H-PEI but not Lf.

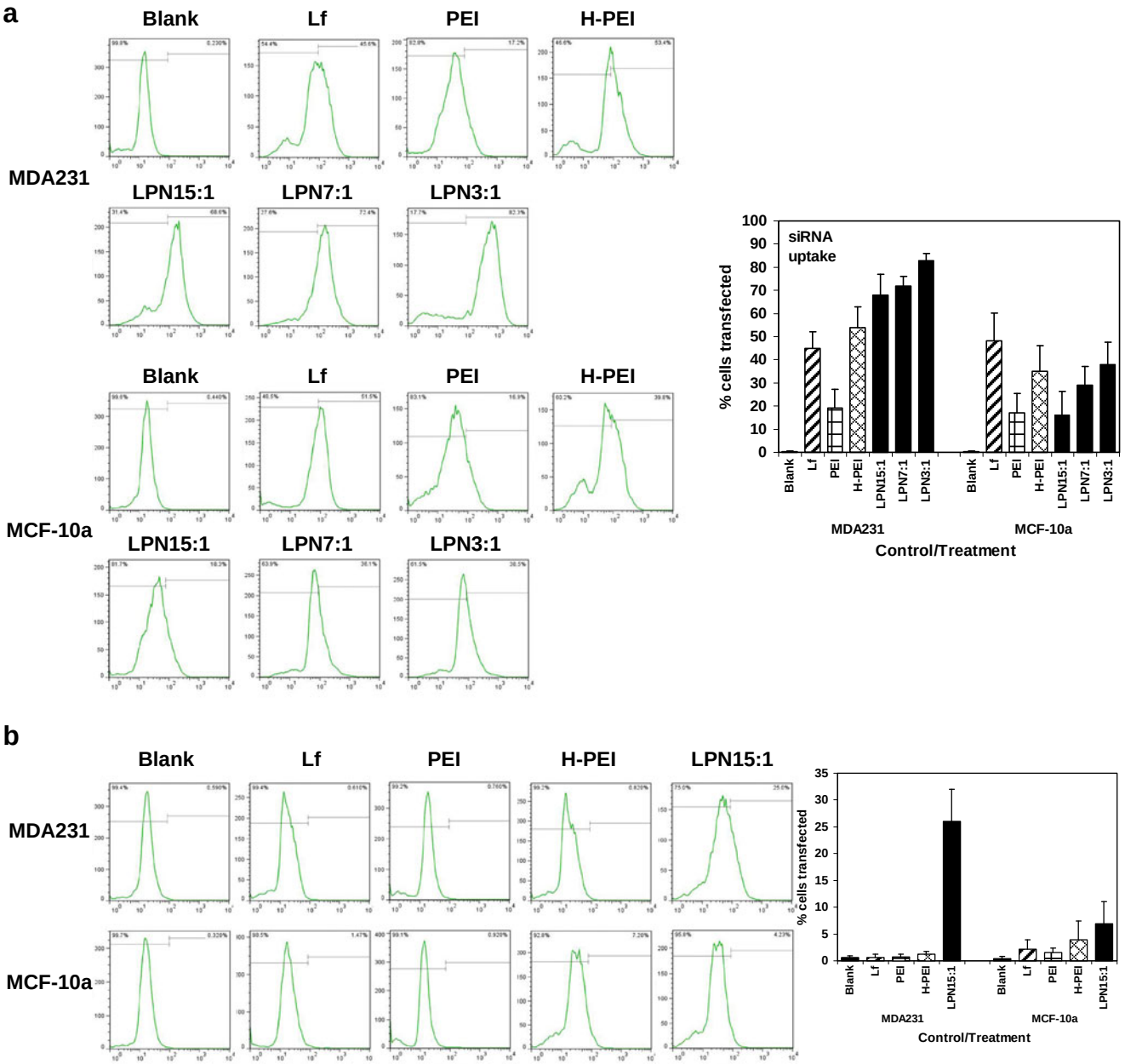

Fig. 4 Flow cytometry data showing the siRNA transfection efficiency (**a**) right after treatment and (**b**) 7 days after treatment. The left panels present representative histograms whereas the right panel the summary of data (means + S.D. *N*=3). MDA231 cells and MCF-10a cells were treated with 40 nM fluorescent 3-FAM-siRNA carried by different formulations including Lf (lipofectamine-2000), PEI, H-PEI (hexadecylated PEI) and the three LPN systems. 10,000 events per measurement were recorded.

detectable cellular levels of siRNA when compared to the H-PEI and Lipofectamine groups.

Reduced Membrane Damage Effects

Figure 5 shows increasing levels of LDH leaked from the cells treated with different carrier formulations. The relative toxicity in terms of LDH leakage was in the order of PEI and H-PEI >> $LPN_{3:1}$ > $LPN_{7:1}$ and $LPN_{15:1}$ in both cell types (left panel: MDA231, right panel; MCF-10a), and no significant toxicity was observed with blank LPN. The general trend is therefore identical to that demonstrated in the trypan blue exclusion assay data (Fig. 2).

Reduced Mitochondrial Toxicity and ROS Generation in 48 h

Figure 6 presents the MTT assay results obtained 48 h (Figs. 6a, b) and 7 days (Fig. 6c, d) after transfection (left panels: MDA231, right panels; MCF-10a). A higher MTT

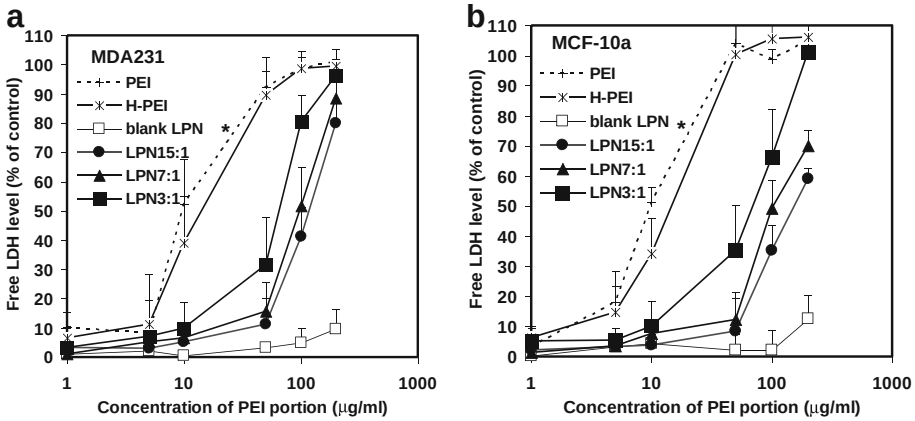

Fig. 5 Lactase dehydrogenase (LDH) assay measuring the effects of different carriers on the cell membrane integrity of (**a**) MDA231 cells and (**b**) MCF-10a cells. Different treatments are compared based on the quantity of hexadecylated PEI included. Levels of LDH released are normalized against the untreated control data and expressed as means + S.D., $N=3$ (duplicate per experiment). * indicates significant difference ($p<0.05$) comparing PEI and H-PEI to all three LPN groups.

absorbance indicates higher mitochondrial activity to metabolize the MTT dye. At 48 h, the relative toxicity of different formulations was similar to the clonogenic assay data. LPNs resulted in significantly higher MTT absorbance than the two encapsulated formulations, indicating that the encapsulation of polymers has reduced their mitochondrial toxicity. On day 7, the toxicity has subsided and the differences between different groups became insignificant.

Figure 7 presents the ROS assay data. At 48 h, significant elevations in cellular ROS levels from baseline were detected in both types of cells after treatment with the unencapsulated formulations (*i.e.* lipofectamine, PEI and H-PEI in Fig. 7a and b). No such elevation was observed in the LPN formulations except $LPN_{3:1}$ in MDA231. Similar to the MTT assay data, the ROS stimulation effects became insignificant on Day 7 (Fig. 7c and d).

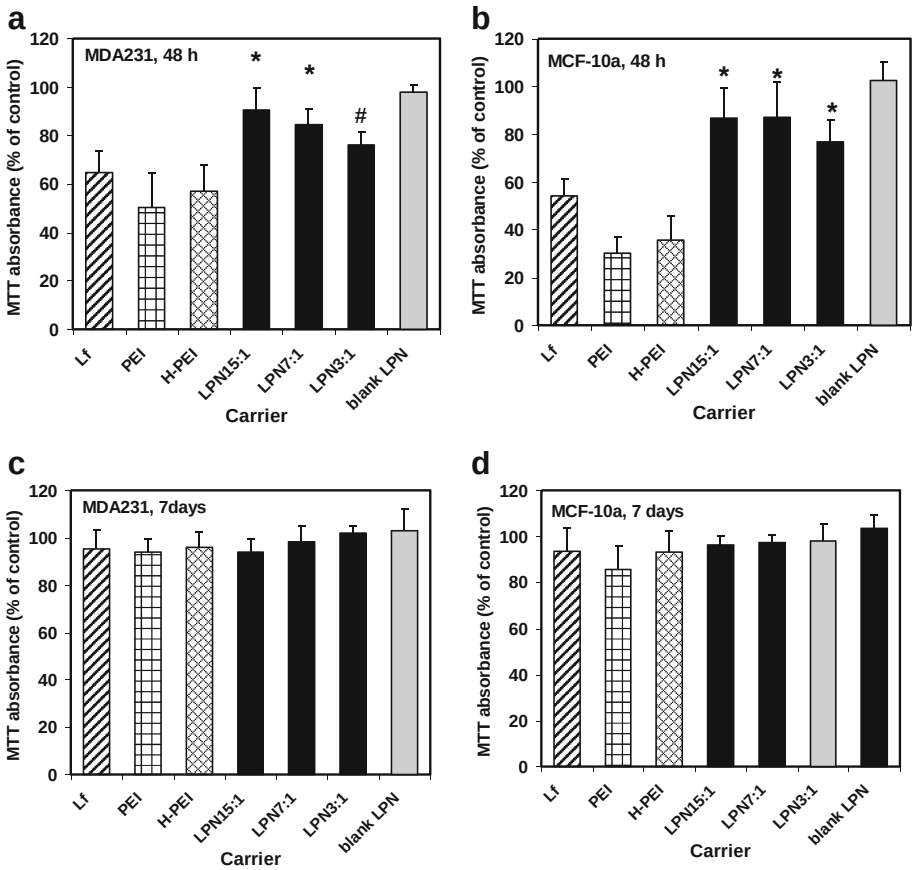

Fig. 6 MTT assay measuring the toxic effects of different carriers on the metabolic functions of mitochondria in MDA231 cells and MCF-10a cells at 48 h (**a, b**) or 7 days (**c, d**) after treatment. MTT absorbance values are normalized against the untreated control data and expressed as means + S.D., $N=3$ (triplicate per experiment). * indicates significant difference ($p<0.05$) comparing to PEI, H-PEI and Lf (Lipofectamine-2000), # indicates significant difference ($p<0.05$) only to PEI and H-PEI but not Lf.

Lipid Encapsulation Suppressed Caspase-3 Activity

Figure 8 presents the measurements of cellular caspase-3 activity as indicated by the level of fluorescent AMC formed from caspase-3 mediated cleavage of Ac-DEVD-AMC. Percent changes in the fluorescence intensity as compared to the untreated group are shown. The three LPN formulations all resulted in lower caspase-3 activity ($p<0.05$) in both cell lines when compared to the unencapsulated formulations. We also took measurements on day 7 after transfection but no significant changes in caspase-3 level were noticed (data not shown).

DISCUSSION

With only a few exceptions (39), the majority of the current nonviral platforms designed for nucleic acid delivery are made of cationic polymers or lipids. Although these cationic materials are reasonably tolerated by diverse cell types, they still exhibit moderate but clearly noticeable dose-related toxicity even in well-controlled *in vitro* settings, not to mention *in vivo* or clinical conditions in which the local carrier concentration can be increased due to various reasons (*e.g.* concentrated in reticuloendothelial organs (40,41) or loco-regional cancer treatment (42)). An effective and convenient strategy to lower their general toxicity remains an unmet need. Physiological or neutral lipids, especially at solid state, have been shown to be less toxic biomaterials for carrier formulation even compared to the gold standard PLGA (43), and lipid-coated PLGA hybrid nanoparticles were recently demonstrated to be more immunocompatible than the conventional PLGA nanosystems (30). Along this concept, here we further demonstrated that by encapsulating a cationic polymer in mostly solid lipids to control its release rates, its non-specific cell toxicity can be significantly ameliorated.

Prior to the toxicity studies, we evaluated the release kinetics of free H-PEI polymer so the toxicological data could be better interpreted. The HPEI release kinetics were measured in enzyme-free PBS at pH 7.4 (Fig. 1a) and lysosomal acid lipase solution (Fig. 1b) to simulate the extracellular and intracellular environments that a RNA carrier typically encounters. The low release rates of all three LPNs in Fig. 1a (20–30% release after 100 h) confirmed the integrity of solid lipids as a barrier material to prevent premature discharge of the cationic polymer before transfection. Lysosomal lipase accelerated the polymer release from LPNs likely by promoting the degradation of this release rate limiting barrier. This was supported by the lack of response of lipid-free polyplexes to the enzyme. The release rates of LPNs remained lower than the polyplexes especially at high lipid-

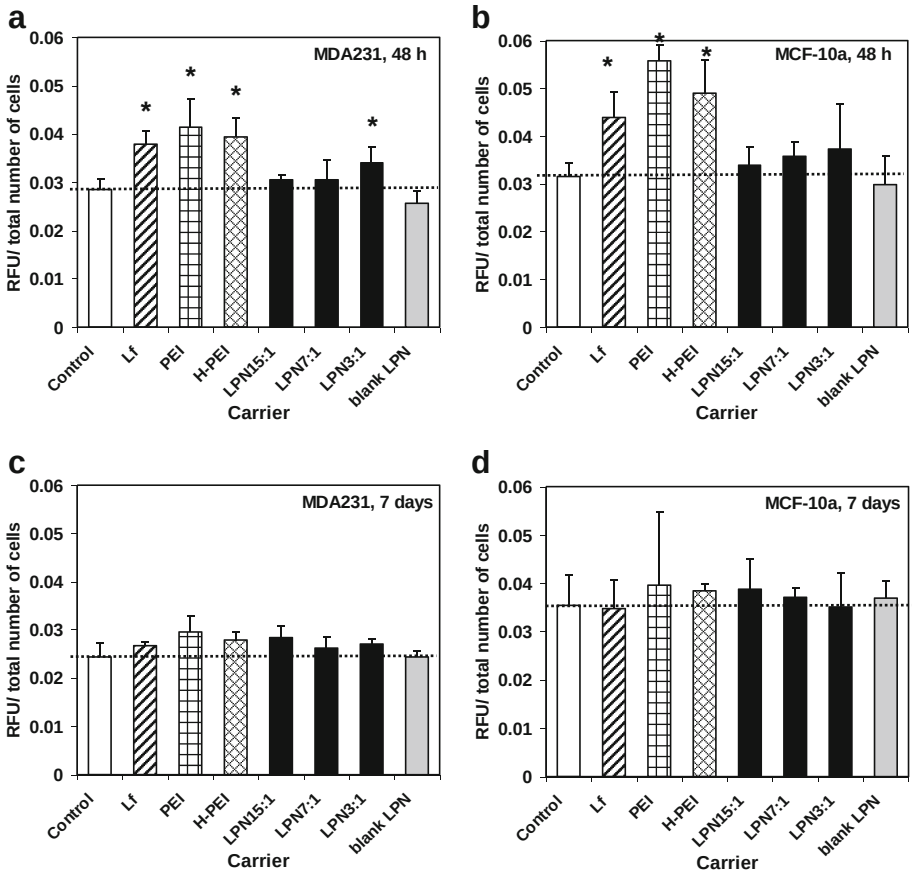

Fig. 7 ROS assay measuring the effects of different carriers on the rates of superoxide formation in MDA231 cells and MCF-10a cells at 48 h (**a, b**) or 7 days (**c, d**) after treatment. The fluorescence intensities of ethidium formed by oxidation of dihydroethidium are normalized for the cell number, and expressed as means + S.D., $N=3$ (duplicate per experiment). The dashed line in each graph highlights the baseline level of the untreated control and * indicates significant difference ($p<0.05$) comparing to this baseline value.

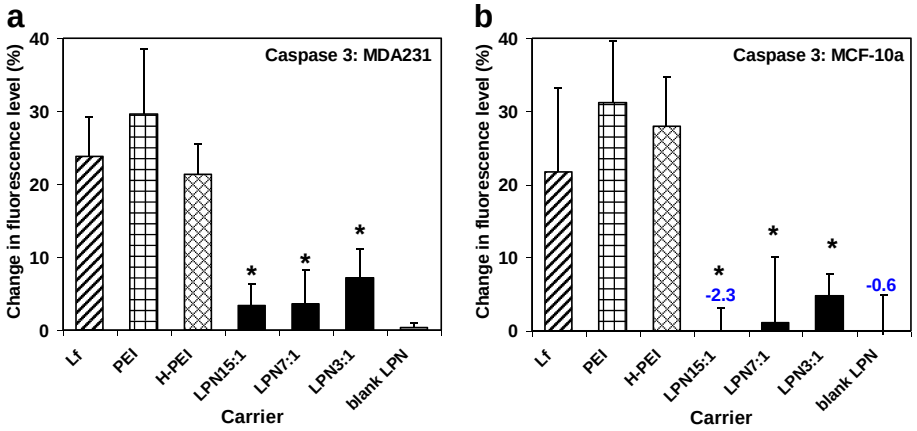

Fig. 8 Caspase-3 assay measuring the effects of various carriers on the cellular level of apoptotic effector caspase-3 in (**a**) MDA231 cells and (**b**) MCF-10a cells. Changes in the fluorescence intensity comparing to the untreated control are presented, and expressed as means + S.D., *N*=3 (duplicate per experiment). * indicates significant difference ($p < 0.05$) comparing to complexes of PEI, H-PEI and Lf (lipofectamine-2000) with negative siRNA control.

to-polymer ratio (*e.g.* $LPN_{15:1}$). The data thus also support the role of lipids in LPNs to prevent quick burst release of free cationic polymer molecules inside a cell, and hint at the feasibility of tailoring the lipid content in a nucleic acid carrier for optimizing the exposure of cells to the toxic cationic polymers.

As previously mentioned, nucleic acid carrier toxicity can be acute or delayed. We first performed the trypan blue exclusion assay to evaluate the acute toxicity that takes effect within hours (Fig. 2). The three encapsulated nanocarrier formulations (the three LPNs) were significantly less toxic to both cell types (MDA231 and MCF-10a) than the unencapsulated formulations (polyplexes of siRNA with PEI or H-PEI) as indicated by their multi-fold higher LC_{50} values. This demonstrates the general toxicity reduction effect of lipid encapsulation. In addition, $LPN_{3:1}$ was more toxic than the two relatively more encapsulated LPNs (*i.e.* $LPN_{7:1}$ and $LPN_{15:1}$) which released at lower rates (Fig. 1). Hence, the data clearly show that the polymer-in-lipid hybrid approach is able to substantially lower the acute cellular toxicity of the cationic polymer component. The fact that $LPN_{3:1}$ remained several fold less toxic than the unencapsulated H-PEI even when both formulations had positive zeta potential values (Table 1) suggests that the carrier surface charge was not the most crucial factor causing the observed toxicity; instead it was the availability of unencapsulated polymer molecules that could freely interact with the cell membrane which decided the acute cell death rate.

The clonogenic assay (Fig. 3) measured the delayed carrier toxicity component as it took 10–14 days for the cells to multiply into macroscopic colonies (>50 cells), and we have screened out the cells that became non-viable within 12 h after treatment. The acute effect was further eliminated by the choice of 5 μg/ml PEI as at this dosing level, <10% acute cell death was observed (Fig. 2) so we could focus on the delayed component. In both cell types, lipid encapsulation of polymer significantly reduced ($LPN_{15:1}$ and $LPN_{7:1}$) or at least resulted in comparable ($LPN_{3:1}$) long-term toxicity. As blank LPN did not inhibit cell proliferation, the observed delayed toxic effects were likely derived from the cationic polymer component and these effects were dampened by lipid encapsulation. The finding in this experiment is important as we were concerned that slowing down polymer release would merely delay the toxicity and even inflict chronic damages to the cells. Our data suggest that this should not be a concern. The cells tested appeared to tolerate the low level of steadily released cationic polymer.

Both cancer and a non-cancerous cell lines were tested. It should be pointed out that a gene or drug carrier is generally designed to be intrinsically non-toxic regardless of the cell types and rely mainly on the therapeutic molecules it carries to provide the desired treatment. However, data show that the three LPNs were noticeably better tolerated by the non-cancerous MCF-10a cells *versus* the cancerous MDA231 cells (Figs. 2 and 3; similar data using another cancer cell line MCF-7, see Supplementary Material). This is likely because LPNs were surface-tagged with folate moieties, so they were expected to be taken up more efficiently by the folate-receptor overexpressing breast cancer cells and less so by the non-cancerous cells (44). While this could be considered a beneficial feature of LPNs for cancer treatment, it brought up another concern: is the observed toxicity reduction effect of the hybrid approach a mere consequence of inefficient cellular uptakes of LPNs?

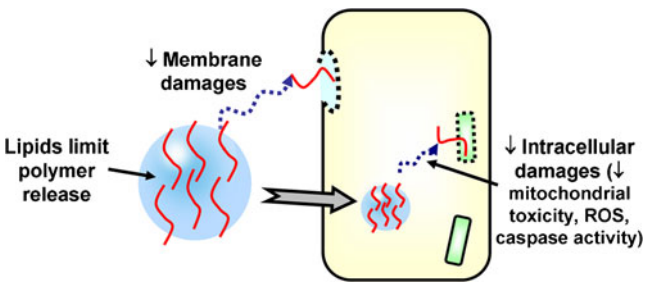

Fig. 9 Scheme summarizing the two major effects of hybrid nanotechnology for alleviating the cell toxicity of cationic polymers. LPN prevents acute damages of the cell membrane caused by sudden, full-scale exposure to free polymer molecules, and keeps the intracellular level of the free polymer molecules reasonably low to avoid harmful events (e.g. cell organelle damages) that may trigger delayed cell toxicity.

Flow cytometry was performed to address this concern. The results in Fig. 4 indicate that while the MCF-10a cells were less efficiently transfected by the more encapsulated LPNs ($LPN_{15:1}$ and $LPN_{7:1}$) as predicted, the uptake efficiencies of LPN3:1 and H-PEI polyplex were actually comparable. LPNs were in fact taken up more efficiently than the unencapsulated polyplexes by the MDA231 cells, likely an outcome of folate-receptor mediated uptake. In addition, as indicated by the higher fluorescence levels in LPN groups on day 7 comparing to the H-PEI and Lipofectamine groups (Fig. 4b), we can conclude that LPNs also resided longer in the cells due to their slow release properties. Overall, the low toxicity of LPNs in these cases suggests that the diminished LPN uptake or rapid decline in their intracellular level can be ruled out as the primary cause of the observed toxicity reduction. By controlling and evening out the release of free polymer molecules, the lipid encapsulation feature of LPNs provides a means to reduce the polymer's general toxicity.

A series of assays were conducted to further understand the possible causes underlying the observed toxicity reduction effects. Acute toxicity is frequently caused by direct cell membrane damages. This was confirmed by the close similarity between the results of the LDH leakage assay (Fig. 5) and the trypan blue exclusion assay. Lipid encapsulation of cationic polymers has reduced their membrane damaging effects by several fold. Considering the high uptake of LPNs to MDA231, this was made possible likely because the polymer molecules were released gradually only after the carriers were internalized and the lipids degraded by endosomal/ lysosomal enzymes as shown in Fig. 1 and as previously reported (23). This low level of free polymer was not sufficient to damage the cell membranes to cause noticeable acute toxicity.

Cationic polymers are known to cause damages to the cellular organelles, especially mitochondria. These damages were assessed using MTT assay (Fig. 6), which relies on the mitochondrial metabolic activity to reduce the MTT dye, and ROS assay (Fig. 7), which measures the ROS generated often associated with organelle damages. The close resemblance between the trends shown in MTT and ROS assays at 48 h and clonogenic assay obtained 10–14 days after treatment, together with the lack of signs of toxicity on Day 7, suggest that the majority of damages were already done in the first few days. These led to increased caspase activities (Fig. 8) that are essential for apoptotic cell death. Lipid encapsulation could prevent the polymers from releasing too quickly to overwhelm the cells, so the above events that are often associated with delayed cell toxicity were significantly suppressed.

All in all, our findings support the use of a controlled release approach mediated by hybrid nanocarriers for nucleic acid delivery from a toxicological perspective. As we have proposed the use of siRNA-nanomedicine for loco-regional treatment of breast cancer and probably prostate cancer (23,24,45), the findings here indicate that the carrier toxicity should not be a limiting factor of the proposed treatments even though high local nanocarrier concentrations are implicated. This also bears significance if siRNA therapy is intended for long-term use for other chronic diseases, as repeated exposure of the healthy tissues to siRNA carriers is expected.

CONCLUSION

Figure 9 presents the general scheme summarizing the benefits of lipid encapsulation of cationic polymers by LPNs, which allow control over the release kinetics of and damages inflicted by the polymer components to cell membrane and intracellular organelles. Overall, substantial reduction in polymer-induced acute toxicity and significant decrease in delayed toxicity can be achieved especially in non-cancerous cells. This study has therefore validated the polymer-in-lipid hybrid nanotechnology for sustained, controlled RNA delivery from a toxicological perspective. This will be clinically valuable if the RNA therapy is intended for management of tough-to-treat disease conditions (*e.g.* breast cancer) using high dose levels or repeated dosing for which low carrier toxicity is essential. Our future works will further investigate this issue in *in vivo* models.

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