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Comparative cellular pharmacokinetics and pharmacodynamics of siRNA delivery by SPANosomes and by cationic liposomes

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Abstract

Mechanistic understanding of intracellular trafficking is important for the development of small interfering RNA (siRNA) delivery vehicles. Here, we describe a novel methodology to quantitatively analyze nanocarrier-mediated disposition of siRNA. Cellular uptake and cytoplasmic release of siRNA over time were quantified by measuring the fluorescence intensities of fluorescently-labeled siRNAs and molecular beacons using flow cytometry. This method was used to investigate the cellular pharmacokinetics (PK) of siRNA delivery by SPANosomes (SP) and by cationic liposomes (CL). The results showed that the superior pharmacodynamic (PD) response of SP was because it enhanced transport of siRNA into the cytoplasm compared to the CL. The divergent cellular pharmacokinetic profiles of the two formulations were associated with different cellular entry pathways. These findings can facilitate the rational design of more efficient siRNA delivery vehicles in the future.

Keywords

RNA interference; siRNA; Nanoparticle; Cellular pharmacokinetics; Intracellular trafficking

Background

RNA interference (RNAi) is a potent and highly specific strategy for inhibiting gene expression¹ and an emerging therapeutic modality.² Nonviral nanocarriers (NCs) for siRNA delivery have the advantages of low immunogenicity and oncogenicity.^{3–5} Because siRNA molecules are designed to interact with the target mRNAs, they must enter the cytoplasm and integrate into the RNA-Induced Silencing Complex (RISC) in order to exert their biological effect.^{6,7} The intracellular trafficking of siRNA and the siRNA concentration at the site of action (cytoplasm) are determinants for its pharmacological (gene silencing) activity.^{6,7} Consequently, there is a pressing need to elucidate the relationship between the cellular pharmacokinetics and the biological activity of NC- delivered siRNA.^{8,9} Moreover,

the cellular pharmacokinetic studies can aid in identification of cellular barriers and the rate-limiting steps for siRNA delivery, thus guiding design of nonviral vectors in the future.

Previously, fluorescently-labeled DNA/siRNA have been used to investigate the intracellular trafficking of plasmid DNA (pDNA) and siRNA.^{8,10–12} For example, co-localization of fluorescently-labeled DNA with other markers for endocytic pathways was used to study subcellular distribution of the pDNA and its carriers.^{8,12,13} However, little quantitative information on the kinetics of intracellular trafficking of siRNA is currently available. Furthermore, fluorescently-labeled siRNA can provide only limited kinetic information regarding siRNA distribution in subcellular compartment. As a result, a quantitative methodology that offers both spatial and temporal resolution of siRNA inside the cells is needed to better understand the intracellular trafficking of NC/siRNA complexes.

Recently, a novel type of NCs called SPANosomes (SPs) was developed and evaluated by our laboratory.⁵ We demonstrated that the siRNA delivery efficiency of SPs was 6-fold higher than typical cationic liposomes (CLs).²² However, the cellular pharmacokinetics and pharmacodynamics of SPs and CLs were not studied in that report. In the present study, we proposed a novel strategy to easily and reliably quantify the amount of siRNA in the cytoplasm using molecular beacon (MB) as a mimic of siRNA. This quantification method was applied to analyze the cellular pharmacokinetics of siRNA for the SP formulation and of a typical CL formulation. We then correlated the pharmacokinetic parameters with the pharmacodynamic endpoint. Herein we demonstrated the importance of helper components of the formulation on the cellular pharmacokinetics and the biological activity of NCs.⁵

Methods

Material

1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) chloride and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were purchased from Genzyme Pharmaceuticals (Cambridge, MA, USA). Span 80[®], TPGS, and 10× PBS Solution (1.37 M NaCl, 0.027 M KCl, and 0.119 M Phosphate; pH 7.3 to 7.5 at 25 °C) were obtained from Fisher Scientific Inc. (Pittsburgh, PA, USA). The 10× PBS Solution was diluted with RNase free water 10 and 100 fold to obtain 1× and 0.1× PBS. The silencer[®] renilla luciferase siRNA (siLuc), control #1 siRNA (siNC), silencer[®] FAM[™]-labeled negative control #1 siRNA (FAM-siRNA), silencer[®] Cy3 labeled negative control #1 siRNA (Cy3-siRNA), quant-iT[™] riboGreen[®] RNA reagent, and QuantiT[™] OliGreen[®] ssDNA reagent were supplied by Life Technologies Corporation (Carlsbad, CA, USA). CellTiter 96[®] AQueous one solution cell proliferation (MTS) assay was purchased from Promega Corporation (Madison, WI, USA). The sequences and targets of the MBs were shown in Table 1.^{14,15} The MBs were custom-synthesized by Eurofins MWG Operon (Huntsville, AL, USA). All other reagents were of analytical grade.

Preparation of the SP and CL

SP and CL were prepared by the ethanol injection method reported previously by us.⁵ Briefly, stock solutions of DOTAP, DOPE, TPGS and Span 80 were prepared by dissolving individual lipid or surfactant in ethanol at 20 to 50 mg/ml. Stock solutions were then combined to achieve the desired lipid or surfactant molar ratio. The compositions (molar ratio) used for the SP and CL were DOTAP/Span 80/TPGS at 50:49:1 and DOTAP/DOPE/TPGS at 50:49:1, respectively. The ethanol solution was then rapidly injected to RNase free water to achieve a lipid/surfactant concentration of 2 mg/ml.

Size, zeta potential and siRNA incorporation efficiency

The particle sizes of the SP and CL were measured by dynamic light-scattering in a NICOMP Submicron Particle Sizer Model 370 (NICOMP, Santa Barbara, CA). The particle size was determined in the volume-weighted mode. The zeta (ζ) potentials of SP and CL were determined in $0.1\times$ PBS solution on a ZetaPALS instrument (Brookhaven Instruments Corp., Worcestershire, NY). The siRNA and MB incorporation efficiencies of the formulations were measured using the Quant-iTTM Ribo-Green[®] RNA Kit and Quant-iTTM OliGreen[®] ssDNA Kit, respectively, following the manufacturer's instructions.⁵

Cell culture

SK Hep-1 cells with stable luciferase expression (SK Hep-1 Luc) were grown in MEM culture medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μ g/mL streptomycin, at 37 °C in a humidified atmosphere containing 5% CO₂.

In vitro transfection and gene silencing activities of the SP and CL

SK Hep-1 Luc cells were grown until 80% confluency was reached. One day before treatment, the cells were seeded in a 96-well plate (2×10^4 cells/well) with 150 μ L per well of growth medium and incubated at 37 °C in a 5% CO₂ environment. In all transfection protocols, siRNA was combined with various amounts of SP or CL in serum free medium and incubated for about 15 min at room temperature prior to addition to the cells. Appropriate amounts of the SP or the CL were used to generate a vector/nucleic acid (VE/NA, w/w) ratio of 15.^{5,16} Cells were transfected with various concentrations of siLuc or siNC in 100 μ L serum free MEM growth medium for 4 h at 37 °C. After transfection, cells were washed twice and further incubated in complete culture medium for 48 h before analysis of the luciferase expression.

Prior to luciferase activity evaluation, 20 μ L/well MTS solution was added and the cells were incubated for 1 h at 37 °C. Absorbance at 490 nm was read using an automatic plate reader. The luciferase expression level was then measured by Promega Dual-GloTM Luciferase Assay System following the manufacturer's instructions. Briefly, cells were washed and lysed. The luciferase activity was measured on a luminometer. The relative luciferase expression was obtained by first normalizing the luciferase activity of each well by the cell number obtained from the MTS assay and then dividing by the average of normalized luciferase activities of the triplicate by that of the untreated control.

Quantifying the sub-cellular distribution of siRNA

Cells were transfected with SP/Cy3-siRNA or SP/Cy3-MB complexes in the presence of endosome marker, Alexa Fluor488-transferrin (A488-Tf, 0.1 mg/mL), for 1 h. After transfection, cells were washed 3 times with PBS and fixed in 4% formalin in PBS. For nuclear staining, cells were incubated with 5 mg/mL of Hoechst33342 for 10 min before washing with PBS. Co-localization of Cy3-siRNA or Cy3-MB with the A488-Tf was analyzed on a Flowview 1000 Laser Scanning Confocal Microscope (Olympus).

FAM-siRNA or Cy3-MB was diluted with unlabeled siNC or non-fluorescent control MB at various dilutions and complexed with SP to transfect SK Hep-1 Luc cells for 4 h. The cells were then washed three times with PBS and fixed in 4% formalin for flow cytometry analysis.

Cellular pharmacokinetics of the SP and the CL

FAM-siRNA and Cy3-MB were complexed with the SP or the CL to represent SP/siRNA or CL/siRNA complexes. For the studies, 6×10^4 SK Hep-1 Luc cells were seeded in a 24-well

plate one day before treatment. FAM-siRNA and Cy3-MB (GAPDH MB or Negative Control MB) were complexed with the SP or the CL as described above. Cells were treated with the complexes containing FAM-siRNA and Cy3-MB for 4 h at 37 °C, and then were washed three times with PBS. The transfection medium was then replaced with fresh culture medium. At various time points the cells were collected, washed, and fixed in 4% formalin. For dose-dependence study, cells were transfected with different concentrations of SP or CL complexes for 4 h, and then were washed and fixed. The mean fluorescence intensities (MFI) of FAM-siRNA and Cy3-MB were measured by flow cytometry on a Becton Dickinson FACSCalibur cytometer. The MFI of Cy3-MB was baseline corrected by subtracting the MFI of Negative Control MB treated cells collected at the same time point.

Cellular entry pathways of siRNA

The SP or CL was complexed with Cy3-siRNA as described above. SK Hep-1 Luc cells were seeded on round cover slips one day before transfection. SK Hep-1 Luc cells were transfected with SP/Cy3-siRNA or CL/Cy3-siRNA complexes for 1 h in the presence of pathway marker of clathrin-mediated endocytosis (A488-Tf, 0.1 mg/mL, Invitrogen), macropinocytosis (70 kDa FITC-dextran, 5 mg/mL, Sigma), or caveolae-mediated endocytosis (Alexa Fluor488-cholera toxin subunit B, A488-CT-B, 5 µg/mL, Invitrogen).^{17,18} After treatment, cells were washed 3 times with PBS and fixed in 4% formalin. Co-localization of Cy3-siRNA with the pathway markers was analyzed by using the Flowview 1000 Laser Scanning Confocal microscope (Olympus).

Pharmacokinetic and pharmacodynamic data analysis and statistics

MFI of FAM-siRNA and Cy3-MB at different time points were measured in transfection experiments. Pharmacokinetic parameters were obtained via non-compartmental analysis (NCA) by WinNonlin software (Pharsight, Sunnyvale, CA) using the average of three MFI measurements at the same time point. FAM-siRNA and Cy3-MB vs. time profiles were used to calculate pharmacokinetic parameters for cellular exposure and cytoplasm exposure, respectively. Major assumptions for pharmacokinetic parameter calculation are (1) the change of MFI represents the change of siRNA amounts inside the cell; (2) elimination rate of siRNA and MB are the same; (3) MBs bind to the target mRNAs immediately after being released into the cytoplasm.

Relative luciferase expression values (triplicates) were fitted to the inhibitory effect model ($I = I_{\max} - (I_{\max} - I_0) * [C_{\text{siRNA}} / (C_{\text{siRNA}} + IC_{50})]$) in WinNonlin, in order to obtain the pharmacodynamic parameters. Statistically significant differences between two groups were detected using Student's t-test in Microsoft Excel 2003 software (Microsoft, Redmond, WA). Results were considered significant at $p < 0.05$.

Results

Physical characteristics of the SP and CL formulations

The complexes of siRNA or MB with two NC formulations were assessed for particle size, zeta potential and incorporation efficiency (Table 2). Both formulations showed particle size 100 nm, zeta potential $> +20$ mV, and very high siRNA incorporation efficiency. The SP complexes had a smaller particle size, slightly higher zeta potential and siRNA incorporation efficiency compared to the CL, although the differences were minimal. Complexes containing siRNA or MB are similar in physical characteristics.

SP- and CL-mediated gene silencing

Figure 1, A and Table 3 illustrated the gene silencing activity of SP and CL. Cells treated with SP/siLuc and CL/siLuc decreased luciferase gene expression to 8.7% and 35.9% of

untreated cells, respectively (Figure 1, *A*). The SP had a higher silencing activity than the CL at the tested concentration range (5 to 100 nM). Both IC_{50} and I_{max} of the SP ($IC_{50} = 5.5$ nM, $I_{max} = 5.0\%$) were lower than those of the CL ($IC_{50} = 20.1$ nM, $I_{max} = 29.4\%$) (Table 3), indicating that the SP/siLuc complexes were more potent and efficacious in knocking down the luciferase gene than the CL/siLuc complexes. In addition, transfection of SP/siNC and CL/siNC did not cause luciferase down/upregulation (data not shown), suggesting that inhibition of the luciferase expression by the two formulations was selective.

The CL/siLuc complexes caused significant cytotoxicity (about 50% decrease in cell viability) at 100 nM, while the SP/siLuc complexes induced cytotoxicity that was not statistically significant at 100 nM (Figure 1, *B*). The data revealed that the helper component in the formulation contributed to the cytotoxicity of the NCs in a significant manner. Furthermore, the cytotoxicity appeared to affect the gene silencing effect of the CL, as the luciferase knockdown decreased to 47.3% at 100 nM compared to 64.1% at 80 nM, at which no significant cytotoxicity was observed. The maximum gene silencing effect (91.3% knockdown) for SP was also achieved at 80 nM, which was approximately the same as the 89.3% knockdown at 100 nM, suggesting the cytotoxicity effect did not significantly affect gene silencing activity of the SP. Interestingly, both formulations showed a protective effect on cell viability compared to the untreated control at low concentration. This is probably due to an artifact of the MTS assay, where low concentrations of NC/siRNA complexes can stimulate cell metabolic activity and increase the apparent cell viability beyond 100% compared to the untreated group.¹⁹

Quantification of the sub-cellular distribution of siRNA

Because siRNAs enter the RNAi pathway via forming the RISC in the cytoplasm,^{20,21} only the portion of siRNAs that is released into the cytoplasm from the NCs is “bioavailable” for the RNAi pathway. However, fluorescently labeled siRNA per se cannot accurately distinguish the sub-cellular location of the siRNA, when the total cellular fluorescence is measured by flow cytometry (Figure 2, *A*).⁵ MBs, which become fluorescent only upon entry into the cytoplasm and binding to mRNAs (Figure 2, *B*),^{5,15,22} have physiochemical properties, such as molecular structure, molecular weight, and charge, that are very similar to those of siRNA molecules. By assuming complete and immediate binding of MBs to their target mRNA, one can adopt the MB as a surrogate of siRNA to measure the amount of siRNA delivered into the cytoplasm, thereby delineating the cellular uptake and the cytoplasmic release processes of siRNA.²³ Figure 2 showed confocal microscopy images and schematic diagrams illustrating the sub-cellular distribution of the fluorescently labeled siRNA (Figure 2, *A*) and the MBs (Figure 2, *B*). The SP were complexed with Cy3-siRNA or Cy3-MB (red), and the endosomes and nuclei were stained with A488-Tf (green) and Hoechst33342 (blue), respectively. The yellow clusters, as indicated by white arrows, represent the co-localization of the Cy3-siRNA or Cy3-MB (red) and endosomes (green).

We observed that the Cy3-siRNA co-localized extensively with the endosomes (white arrows) and also was dispersed in the cytoplasm (purple arrows) (Figure 2, *A*). On the other hand, the Cy3-MB was primarily distributed in the cytoplasm and had minimal co-localization with the endosome marker (Figure 2, *B*). The images confirmed that the Cy3-siRNA was fluorescent when encapsulated in the NCs (purple arrows) as well as upon release into the cytoplasm (white arrows) (Figure 2, *A*). In contrast, the Cy3-MB was fluorescent only upon release into the cytoplasm.

To determine the amount of siRNA in the cell, we prepared SP complexes with a mixture of the FAM-siRNA and unlabeled siNC,¹² and measured the MFI of FAM-siRNA by flow cytometry after transfection. FAM-siRNA was used because it has a different fluorescent emission spectrum from that of Cy3, thus FAM-siRNA and Cy3-MB could be co-

encapsulated and detected simultaneously. The relative MFI decreased linearly ($R^2 = 0.985$) with decreased percentage of FAM-siRNA (Figure 3, *A*). The relative MFI of Cy3-MB also showed a linear relationship ($R^2 = 0.999$) with percentage of Cy3-MB in the mixture of Cy3-MB and non-fluorescent control MB (Figure 3, *B*). The data demonstrated that the MFI could be used to determine the amount of the siRNA or MB in the cells or the cytoplasm.¹²

Cellular pharmacokinetics of the SP/siRNA and CL/siRNA complexes

FAM-siRNA and Cy3-MB were mixed at a 1:1 molar ratio and complexed with SP or CL to transfect SK Hep-1 Luc cells. Figure 4 showed the $MFI_{FAM-siRNA}$ and MFI_{Cy3-MB} versus time profiles. Cellular pharmacokinetic profiles of $MFI_{FAM-siRNA}$ and MFI_{Cy3-MB} indicated the cellular exposure and cytoplasm exposure of siRNA, respectively. The derived pharmacokinetic and pharmacodynamic parameters are listed in Table 3. The shapes of the cellular pharmacokinetic profiles of the two formulations are quite different. For overall cellular exposure, the CL gave rise to a substantially higher $MFI_{FAM-siRNA}$ at the end of the 4 h transfection without the plateau period observed for the SP (Figure 4, *A*). The AUC_{0-24} for cellular exposures of the two formulations were more or less equivalent (Table 3). For the cytoplasm exposure, the peak MFI_{Cy3-MB} values of the two formulations were also comparable, while the AUC_{0-24} of the SP was 2.7-fold higher relative to the AUC_{0-24} of the CL for the cytoplasm exposure (Figure 4, *B* and Table 3).

To study the dose proportionality of the NCs, SK Hep-1 Luc cells were transfected with different concentrations of SP or CL complexes. The relative $MFI_{FAM-siRNA}$ and relative MFI_{Cy3-MB} after 4 h transfection were plotted against the siRNA concentration (Figure 5).²⁴ Figure 5, *A* and *C* demonstrated that the $MFI_{FAM-siRNA}$ for both formulations was proportional to siRNA concentration, indicating that both formulations exhibited linear pharmacokinetics for cellular exposure. However, the MFI_{Cy3-MB} plateaued for both NCs with increasing siRNA concentrations. Saturation of the MFI_{Cy3-MB} of the SP and the CL began at 85 nM and 15 nM, respectively, which suggested that the SP released the internalized siRNA more efficiently into the cytoplasm than the CL.

Cellular entry pathways of the SP- and CL-mediated siRNA delivery

To further elucidate the endocytic routes of the NC-mediated siRNA delivery, SK Hep-1 Luc cells were transfected with the SP/Cy3-siRNA or CL/Cy3-siRNA complexes (red) and were co-labeled the endocytic pathways with markers (green): clathrin-mediated endocytosis (A488-Tf), macropinocytosis (FITC-dextran), or caveolae-mediated endocytosis (A488-CT-B).^{17,25} The yellow clusters in Figure 6A1-A3 showed siRNA co-localized strongly with A488-Tf, FITC-dextran, and A488-CT-B, suggesting that SP/siRNA complexes entered the SK Hep-1 cell primarily through a combination of clathrin-mediated endocytosis, macropinocytosis, and caveolae-mediated endocytosis.^{23,25} This observation was consistent with our previous finding that multiple endocytic pathways were responsible for uptake of SP/siRNA complexes in MDA-MB-231 cells.^{5,25} In contrast, siRNA delivered with the CL only co-localized significantly with A488-Tf (Figure 6A1-A3), a common pathway marker for classical clathrin-mediated endocytosis, and showed no co-localization with either FITC-dextran or A488-CT-B.¹⁷ These results revealed that selection of the helper components, such as Span 80 and DOPE, is a critical factor in determining the cellular entry process of NCs.

Discussion

The siRNA NCs need to overcome a number of cellular barriers to successfully deliver siRNA to the site of action.²⁶⁻²⁸ Efficient delivery of siRNA requires functional synergy between cellular uptake, vesicular trafficking, vesicular escape, unpacking of NCs, and

cytoplasmic release of siRNA.²⁹ The cellular entry pathway and cellular pharmacokinetics determine the magnitude and duration of the siRNA exposure for the intracellular target, and ultimately govern the RNAi response.^{29,30} Depending on the relative efficiency and capacity, cellular uptake, intracellular transport, and cytoplasmic release, could each become a rate-limiting step and deterministic factor of the gene silencing activity.²⁹ Consequently, a global understanding of the pharmacokinetic/pharmacodynamic of siRNA NCs is of critical importance.⁹

Overall cellular exposure of siRNA has been widely used as a metric for target exposure of siRNA assuming that the amount of siRNA taken up by the cell was directly related to the amount of siRNA delivered into the site of action (cytoplasm). However, the siRNA disposition process behind this assumption could be invalid, because the overall cellular uptake of siRNA could be quite different from the amount of siRNA that escaped from the endocytic vesicles and entered into the cytoplasm.^{10,31} The disconnection between the overall cellular exposure and the exposure in the cytoplasm could explain why the overall cellular uptake did not predict the gene silencing activity of siRNA *in vitro*.^{10,31} It could also be a reason why plasma pharmacokinetics and target tissue accumulation of siRNA failed to predict gene silencing activity *in vivo*.³²

Previously, confocal microscopy images have been used to quantify the intracellular distribution of pDNA delivered by liposomes, polypeptide vectors¹² and polyplexes.⁸ However, due to the small number of cells that can be imaged and analyzed using image-based methods, the variability of the results was quite high.¹² Other method to quantitatively measure the spontaneous uptake of siRNA by mammalian cells involved using radio-labeled probes and complex procedures.³³ In the present study, a simple methodology was used to determine sub-cellular kinetics of NC-mediated disposition of siRNA using the MB as a mimic of siRNA. Because of the comparable physiochemical properties of siRNA and MB molecules, the physical characterization of the MB complexes were very similar to the siRNA complexes (Table 2). The MFI of fluorescently-labeled siRNA and MB was used as a measure of the total amount of siRNA in the cell and the amount of siRNA in the cytoplasm, respectively. A linear relationship between the amount of siRNA or MB and the corresponding MFI was found (Figure 3).

We then applied this quantitative assay to investigate the cellular pharmacokinetics of SP and CL based NCs, assuming that the elimination rate of MB occurs at the same rate as siRNA in the cytoplasm. This assumption was supported by the fact that the terminal half-lives of cellular exposure and cytoplasm exposure were similar for both formulations (Table 3). Although the terminal half-lives were estimated from the last 2 or 3 concentration-time points, which may not be very accurate, terminal half-lives of the CL and SP were quite different. The terminal half-life of the CL complexes was around 0.8 h, which was consistent with the published data for the half-life of naked siRNA.³⁴ Therefore, this half-life could reflect the kinetics of clearing of unprotected siRNA from the cell. In contrast, the terminal half-life of the SP complexes was around 2 h, which could result from the kinetics of unpacking and release of the siRNA inside the cell.³⁵ The slower elimination of the SP in the cytoplasm was responsible for its higher AUC₀₋₂₄ of SP compared with CL, since the maximum MFI_{Cy3-MB}'s of the two formulations were almost equal. Moreover, the equivalent AUC₀₋₂₄'s for cellular exposure of the two formulations did not correspond to the change in the pharmacodynamic parameters. Meanwhile, increase of AUC₀₋₂₄ for cytoplasm exposure correlated well to the increase of gene silencing activity (Table 3). This correlation between pharmacokinetic and pharmacodynamic parameters indicated that the classical exposure-response relationship held true for siRNA, and that previously reported poor pharmacokinetic/pharmacodynamic correlation^{10,11,32} was due to limitation of the analytical method used to measure the amount of siRNA at the target site. Our data also

suggested that the MFI data measured at a single time point should be interpreted cautiously,^{10,11} since a single MFI may or may not correlate with the actual exposure. The MFI-time profile, a better metric of exposure, should be captured whenever possible.

Further knowledge about the mechanistic differences of the cellular pharmacokinetics between NCs was gained by investigating the cellular entry pathways for the NCs. The confocal microscopy images showed that the CL-mediated siRNA delivery relied primarily on clathrin-mediated endocytosis as its major route of cellular entrance.^{36,37} The clathrin-coated vesicles have been reported to merge with late endosomes and lysosomes, where their contents were subject to exposure to acidic pH and enzymatic degradation.^{17,36}

Therefore, to achieve efficient siRNA delivery through this pathway, endosomal escape must occur before siRNA is transported to the enzyme-rich, low-pH environment.^{5,17,38} On the other hand, SP-mediated siRNA delivery into SK Hep-1 cells depended on a combination of multiple pathways, including clathrin-mediated endocytosis, macropinocytosis and caveolae-mediated endocytosis.⁵ Recently, macropinocytosis and caveolae-mediated endocytosis have been demonstrated as favorable pathways for siRNA delivery.^{38,39} The contents in the macro-pinosomes and caveosomes may not merge with late endosomes or lysosomes, thus not subject to enzymatic degradation.^{29,38,40} This provides a longer time window for release of the siRNA into the cytoplasm.^{5,17,38,39} As a result, the SP/siRNA complexes could reside in the vesicles for a prolonged period of time and showed slower elimination rate compared to CL/siRNA complexes. Moreover, multiple cellular entry pathways were less saturable, which explains why cytoplasm delivery of SP/siRNA complexes was saturated in a much higher concentration than CL/siRNA complexes.

Multiple methods have been used to target the favorable endocytic pathways for enhanced gene delivery. For example, avoidance of lysosomes could be achieved by controlling the particle size of polystyrene nanoparticles.^{41,42} Perfluorocarbon nanoemulsions, with markedly enhanced siRNA transfection efficacy over conventional transfection agents, were shown to enter the cell via a lipid-raft-mediated process.¹⁷ NC formulations with novel cationic lipid-like materials, which demonstrated remarkable siRNA delivery efficiency in vitro and in vivo, were also proven to alter the mechanism of delivery.^{18,39,43} We have recently reported that using non-ionic surfactant as a helper component can dramatically augment transfection efficiency of siRNA through multiple cellular uptake mechanisms.⁵ These data suggested the NCs and cellular membrane interactions play a deterministic role in cellular entry of NCs. In the present study, the SP and CL complexes had almost the same size and zeta potential, so their distinctive cellular trafficking mechanisms were unlikely due to their physiochemical differences. Instead, the helper component, Span 80 and DOPE could be responsible for differences in the cellular entry pathways and kinetics for the two NCs. Consequently, unlike traditional NC formulation strategy, which focused on promoting endosomal escape,³¹ incorporating components to target preferable internalization and trafficking machineries could be a better strategy for rational design of NC formulations. Serum proteins were known to bind to NCs and drastically affect the NCs-cell interactions.⁴⁴ The present transfection studies were carried out in serum free medium in vitro. Thus, the siRNA delivery mechanisms of the NCs in the serum in vitro and in vivo warrant further investigation. Moreover, since PEG component can alter serum protein and cell surface interaction with NCs, the stealthiness and toxicity of the current formulations could be improved via adjusting the density and stability of PEG on the surface of NCs.⁴⁴ Nevertheless our data revealed that introducing different helper components in the NCs could substantially influence the cellular entry pathways of NCs, which significantly altered the cellular pharmacokinetics of the NCs, ultimately leading to distinct biological activity of siRNA.

In conclusion, we have developed a quantitative and simple methodology to study cellular pharmacokinetics of siRNA by co-encapsulating the fluorescently-labeled siRNA and MB in NC and measuring the fluorescent signal by flow cytometry. This method formed the basis for mechanistic understanding of the uptake, unpacking and release kinetics for the formulations. The finding established a clear pharmacokinetic/pharmacodynamic correlation for NC/siRNA complexes, which was controversial in previous reports.^{10,11,32}

Moreover, our results suggested that NC formulations should contain components that target the preferable cellular entry pathways, in order to avoid the degradative fate and to enhance cytoplasm delivery of siRNA. Additional work is necessary to build a detailed pharmacokinetic/pharmacodynamic model that describes the kinetic pathways of NC-mediated disposition of siRNA, in order to effectively predict the exposure of siRNA to its target and enhance pharmacological effects.^{7,45}

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Figure 1.

Gene silencing activity and cytotoxicity of SP and CL. The SK Hep-1 Luc cells were transfected with various NC complexes for 4 h and incubated a further 44 h before measuring the luciferase activity. To calculate the relative luciferase expression, the luciferase activity for each well was first normalized by the cell number reading from the MTS assay and then divided by the average of normalized luciferase activities of the triplicate untreated control. **(A)** Dose-dependent luciferase gene silencing activity of SP/siLuc and CL/siLuc complexes. *Significant statistical differences ($p < 0.05$) of SP treated and CL treated group at the same siRNA concentration. **(B)** Dose-dependent cytotoxicity of SP/siLuc and CL/siLuc complexes. *Significant statistical differences ($p < 0.05$) of the treated group and the untreated group. Data are represented the mean $\pm$ S.D. ($n=3$).

Figure 2.

Schematic diagrams and confocal microscopic images showing subcellular distribution of fluorescently labeled siRNA or MB. The SP were complexed with Cy3-siRNA or Cy3-MB (red), and endosomes and nuclei were stained with Alexa Fluor488-transferrin (green) and Hoechst33342 (blue), respectively. The yellow clusters (white arrows), as indicated by white arrows, represented the co-localization of the Cy3-siRNA or Cy3-MB (red) and endosomes (green), while the diffused red fluorescence represented the Cy3-siRNA or Cy3-MB in the cytoplasm (purple arrows). **(A)** Cy3-siRNA produced fluorescent signal both inside the endosomes and in the cytoplasm. **(B)** Cy3-MB was quenched when they were encapsulated in the NCs and only became fluorescent after hybridization to target mRNA in the cytoplasm.

Figure 3. Linear relationship between the respective MFI and the amount of FAM-siRNA (**A**) or Cy3-MB (**B**). FAM-siRNA or Cy3-MB was diluted with unlabeled siNC or non-fluorescent control MB at various dilutions and complexed with SP to transfect SK Hep-1 Luc cells. The MFI was measured after transfection, normalized to the MFI of 100% FAM-siRNA or Cy3-MB, and plotted against the percentage of FAM-siRNA or Cy3-MB. Data are represented as mean $\pm$ S.D. (n=3).

Figure 4.

Cellular pharmacokinetic profiles of the SP/siRNA and CL/siRNA complexes. FAM-siRNA and Cy3-MB were complexed with SP or CL to transfect SK Hep-1 Luc cells. The cells were collected at different time points, washed and fixed by 4% formalin. The MFI of the cells was measured by flow cytometry, corrected for the baseline MFI, and plotted against time. **(A)** The cellular pharmacokinetic profile of $MFI_{FAM-siRNA}$, which represented the overall cellular exposure of siRNA. **(B)** The cellular pharmacokinetic profile of MFI_{Cy3-MB} , which corresponded to the cytoplasm exposure of siRNA. Data are represented as mean $\pm$ S.D. (n=3).

Figure 5.

Dose linearity of MFI after 4 h transfection versus siRNA concentration of the SP and CL formulations. FAM-siRNA and Cy3-MB were complexed with SP (A & B) or CL (C & D) to transfect SK Hep-1 Luc cells at different siRNA concentrations. After transfection, the cells were washed and fixed by 4% formalin. The MFI of the cells was measured by flow cytometry, normalized to the MFI of 100 nM siRNA, and plotted against siRNA concentrations. Data are represented as mean±S.D. (n=3).

Figure 6.

Cellular entry pathways for SP/siRNA and CL/siRNA complexes. The co-localization of SP/Cy3-siRNA or CL/Cy3-siRNA complexes with endocytic markers, A488-Tf (endosomes), FITC dextran (macropinosomes), and A488-CT-B (caveosomes), were investigated by confocal microscopy after 1 h incubation of the complexes (100 nM) and each marker with SK Hep-1 Luc cells. The Cy3-siRNA was shown in red (red arrows), the endocytic markers were shown in green, and co-localization of Cy3-siRNA and markers was shown in yellow (yellow arrows).

Table 1

Sequences and target mRNAs of MB used in this study

Name	Target mRNA	Sequence [*]	Modification
GAPDH MB	GAPDH	5'-CGACGGAGTCCTTCCACGATACCACGTCG-3'	5'-Cy3 and 3'-BHQ2
Negative Control MB	N/A	5'-GCGAGGGTAGATGCAGCCTTGTCTATACCCTCGC-3'	5'-Cy3 and 3'-BHQ2
Non-fluorescent Control MB	N/A	5'-GCGAGGGTAGATGCAGCCTTGTCTATACCCTCGC-3'	N/A

* All MBs were DNA-based oligonucleotides.

Table 2

Characterization of the SP and CL formulations

	SP		CL	
	SP/siRNA	SP/MB	CL/siRNA	CL/MB
Particle size (nm)	77.7±33.9	65.2±33.8	109.6±36.6	96.7±39.3
Zeta potential (mV)	28.5±1.30	32.4±1.83	24.7±2.20	23.0±2.30
Incorporation efficiency	90.8%	88.3%	86.9%	84.9%

Table 3

Cellular pharmacokinetic and pharmacodynamic parameters of the SP and CL.

Pharmacokinetic parameters ¹				Pharmacodynamic parameters ²			
Cellular exposure ³		Cytoplasm exposure ⁴		Gene silencing activity			
Relative AUC ₀₋₂₄	t _{1/2} ⁵ (h)	MFI _{max}	Relative AUC ₀₋₂₄	t _{1/2} ⁵ (h)	MFI _{max}	IC ₅₀ (nM)	I _{max} (%)
CL 100%	0.8	101.1	100%	0.8	21.4	20.1	29.6
SP 97%	2.0	49.5	268%	2.6	24.4	5.5	5.2

¹The cellular pharmacokinetic parameters were obtained by NCA using the pharmacokinetic data shown in Figure 4.

²The cellular pharmacodynamic parameters were obtained by fitting the gene silencing activity (Figure 1) to an I_{max} model.

³Cellular exposure was calculated from cellular pharmacokinetic data of FAM-siRNA.

⁴Cytoplasm exposure was calculated from cellular pharmacokinetic data of Cy3-MB.

⁵Terminal half-life was calculated by the NCA from the last 2 or 3 concentration-time points.