Published in final edited form as:

Mol Pharm. 2013 November 4; 10(11): 4391–4395. doi:10.1021/mp400028m.

How Does the Cell Overcome LCP Nanoparticle-Induced Calcium Toxicity?

Yu-Cheng Tseng, **Angela Yang**, and **Leaf Huang***

Division of Molecular Pharmaceutics and Center for Nanotechnology in Drug Delivery, Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7571, USA

Abstract

To address the question of how cells respond to the possible Ca^{2+} toxicity caused by the release of Ca^{2+} into the cytoplasm by LCP nanoparticles, a series of *in vitro* and *in vivo* studies using Ca^{2+} pump inhibitors were conducted. The results indicated that two major Ca^{2+} pumps on the plasma membrane and the mitochondrial membrane, respectively, were able to rapidly respond to the elevated cytosolic Ca^{2+} concentration and prevent Ca^{2+}-induced apoptosis or necrosis. However, exposure to specific inhibitors of calcium pumps would cause LCP-treated H460 cells to undergo necrosis both *in vitro* and *in vivo*. These results demonstrated that the Ca^{2+} delivered by LCP was not toxic to cells when the cells contain functional Ca^{2+} pumps.

Keywords

LCP; nanoparticle toxicity; intracellular calcium; calcium indicator

INTRODUCTION

The LCP (Lipid/Calcium/Phosphate) nanoparticles (NPs) are a versatile formulation for *in vivo* siRNA, chemical drug (gemcitabine phosphates, manuscripts in submission), and cDNA plasmid delivery. There were two generations of LCP NPs.[1–4] The first generation (LCP-I) was a proof-of-concept formulation showing the feasibility of utilizing microemulsion technology to create calcium-phosphate (CaP) nanoprecipitate cores that entrap siRNA in a reverse microemulsion system. After collecting the CaP-siRNA cores, sodium citrate was used to stabilize them and to provide a negative surface charge. Cationic liposomes were then added, forming a lipid bilayer coating around the cores, to which DSPE-PEG_{2000} (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-poly(ethylene glycol)2000) could be post-inserted for protection of the surface. However, the preparation of the LCP-I was limited by the inclusion of an un-scalable column chromatography step for purification. The formulation was additionally limited by the fact that only cationic lipids could be incorporated into the lipid coating.

*Corresponding Author: Prof. Leaf Huang, Division of Molecular Pharmaceutics, Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, 1315 Kerr Hall CB# 7571, Chapel Hill, NC 27599-7571, leafh@unc.edu.

Author Contributions

The manuscript was written through contributions of both authors. Both authors have given approval to the final version of the manuscript.

Supporting Information Available

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The LCP-II formulation was an improvement to the preparation of LCP-I through the introduction of dioleoylphosphatydic acid (DOPA) as the inner leaflet lipid. Utilizing the microemulsion technology in a similar manner as in LCP-I preparation, the CaP core of the LCP-II was coated with a single DOPA layer. The phosphate head group of DOPA provides a strong binding interaction with the CaP core and prevents the core from aggregation. The acyl chain of DOPA also provided a hydrophobic surface that enabled an extensive wash with ethanol and storage in $CHCl_3$. Additionally, the core could then be coated with the lipids of choice as an outer leaflet to form an asymmetric lipid bilayer. For example, the cationic 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) lipid was often used as an outer leaflet due to its ability to destabilize the endosomes. Neutral dioleoylphosphatidylcholine (DOPC) with 20% DSPE-PEG on the other hand, provides prolonged circulation time, and was used for tumor and lymph node imaging after intravenous injection (manuscript in submission).

The well-established method of calcium phosphate transfection for plasmid DNA delivery[5] is similar to the process through which the CaP cores in LCP NPs precipitate with siRNA (or DNA). Furthermore, the CaP-siRNA or CaP-DNA co-precipitates are acid sensitive. After cellular internalization, the co-precipitates will dissolve in the acidic endosomal environment to induce an osmotic lysis and release the trapped siRNA (or DNA). This mechanism was designed to improve the release of siRNA into the cytoplasm.[1] This CaP dissolution and endosome escape was demonstrated further using a calcium sensing dye, Fura-2, to visualize the elevated cytoplasmic calcium concentrations.[4]

Compared to the LPD (Lipid/Protamine/DNA) formulation that was previously developed in our lab, the gene silencing activities of LCP-I and LCP-II were significantly improved. Luciferase silencing experiments *in vitro* showed that the IC_{50} was 5 nM for LCP-II, 50 nM for LCP-I, and 200 nM for LPD. The gene silencing effect *in vivo* demonstrated in an NCI-H460 xenograft model expressing luciferase showed that when a dose of 1.2 mg/kg siRNA is delivered in LCP-II, LCP-I, or LPD formulations, the knockdown of luciferase *in vivo* was ~65, ~50, and ~25% efficient, respectively.[1–4]

Under physiological conditions, the extracellular Ca^{2+} concentration is ~1.2 mM and the cytosolic concentration is ~0.1 μM. The cytosolic Ca^{2+} concentration is associated with almost all cellular signaling and metabolic events, including cell growth and proliferation, cellular motility, contractility, and neuronal transmission.[6,7] Ca^{2+} also plays important roles in regulating several enzyme activities and ion channels.[8,9] Low to moderate elevation of the Ca^{2+} concentration (0.2~0.4 μM) triggers apoptosis and higher concentrations of Ca^{2+} (>1 μM) are associated with necrosis.[10–14] For these reasons, it is necessary to address if the Ca^{2+} delivered by LCP is toxic to cells.

A low cytosolic concentration of Ca^{2+} is carefully maintained by several Ca^{2+} pumps on plasma membrane, mitochondria, and endoplasmic reticulum (ER). Plasma membrane Ca^{2+} ATPase (PMCA) consumes ATP to extrude cellular Ca^{2+} with a high affinity, therefore playing a major role in Ca^{2+} homeostasis and signaling. Inside of the cell, Ca^{2+} is stored within the mitochondria and ER. When there is an inward flux of Ca^{2+}, the mitochondrial calcium uniporter (MCU) should be the one to initiate the lowering of cytoplasmic Ca^{2+} with a fast speed. PMCA, although with a slower speed, is the one eventually responsible for lowering the cytoplasmic Ca^{2+} to resting homeostasis. The SERCA (sarco/endoplasmic reticulum Ca^{2+}-ATPase) on the ER, however, is not likely to take up Ca^{2+} due to the fact that ER is already replete with Ca^{2+}.[15] To evaluate the role of SERCA when dosing the cells with LCP, we have tested thapsigargin (a SERCA inhibitor) in cells treated with LCP. No obvious LCP toxicity enhancement was observed when H460 cells were treated with LCP and thapsigargin, indicating SERCA was not significantly involved in the homeostasis in

this case (supplement figure 1). Based on the known calcium homeostasis mechanism and our results with thaspsigargin, we hypothesized that when cells take up LCP and release Ca^{2+} into the cytoplasm, Ca^{2+} pumps on the plasma membrane and the mitochondria, but not the one on ER, will rapidly respond to the elevated cytosolic Ca^{2+} concentration and prevent the cells from Ca^{2+} induced apoptosis or necrosis.

LCP-II with DOTAP/Cholesterol as an outer leaflet coating was prepared for the *in vitro* study using the protocol (manuscript in submission) slightly modified from the original one.[1] Briefly, two reverse water-in-oil emulsions containing $CaCl_2$ and Na_2HPO_4 were mixed to form CaP nanoprecipitate which was stabilized by DOPA. After washing by centrifugation, additional lipids containing DOTAP/Cholesterol were added to complete the assembly of NPs. The prepared nanoparticles were ~25 nm in diameter and their zeta-potentials were ~50 mV.

Fura-2, a ratiometric fluorescent dye that is sensitive to Ca^{2+}, was used to study our hypothesis.[4] Fura-2 was the first widely-used calcium indicator, and remains very popular especially in the neuroscience field. Free Fura-2 that is not bound to Ca^{2+} has a peak in the excitation wavelength at 380 nm. Upon binding with Ca^{2+}, there is a blue-shift in the excitation peak, to 340 nm. The emission wavelength remains unchanged at 510 nm.

To examine our hypothesis we chose two specific Ca^{2+} pump inhibitors for the two major Ca^{2+} pumps, respectively. The plasma membrane Ca^{2+} ATPase (PMCA) inhibitor, Caloxin 2A1, is a peptide (Val-Ser-Asn-Ser-Asn-Trp-Pro-Ser-Phe-Pro-Ser-Ser-Gly-Gly-Gly-NH_2, purchased from American Peptide Company) developed by using the phage display technology.[16–18] The specific inhibitor of the mitochondrial calcium uniporter, Ru360 (EMD Millipore), is a cell-permeable oxygen-bridged dinuclear ruthenium amine complex that binds to mitochondria with a high affinity (K_d = 340 pM) and blocks Ca^{2+} uptake into mitochondria at IC_{50} = 184 pM *in vitro*.[19,20]

H460 human lung cancer cells were pre-loaded with Fura-2 AM (Molecular Probes) using the manufacturer's protocol. After Fura-2 loading, H460 cells were treated with Ru360 at ~500 pM starting 30 min before the addition of LCP (2.5 mM as of calcium) and was present throughout the whole ratio-imaging experiment. Divalent cation-free PBS was used as the culture medium to avoid the interference of Ca^{2+} from the regular RPMI-1640 medium. Caloxin 2A1 at 1 mM was added 10 min before the addition of LCP and was presented throughout the ratio imaging experiment. The live ratio images of the cells were taken using an inverted Nikon ECLIPSE TE2000 microscope. This microscope was designed with a rapid-switch excitation shutter, multifunction, time-lapse capabilities and dual cameras that are ideal for Fura-2 experiment. A cell-culture chamber with temperature (37 °C) and humidity (saturation) controls, as well as a 5% CO_2 supply, was used to maintain cell viability. We recorded ratio imaging for 10 sec, paused for the addition of LCP, and then resumed recording for an additional 8 min.

During the ratio imaging, red pseudo-color was applied to the fluorescent signal when an excitation of 380 nm was used. Green pseudo-color was applied when using 340 nm as the excitation. Red and green images were then superimposed and adjusted to be in red color before the addition of LCP (Figure 1). In the group without inhibitors some cells turned green following the addition of LCP, indicating an elevated Ca^{2+} concentration in the cytosol. Note that the cells were incubated with LCP throughout the imaging experiment. The processes through which the cells took up LCP and pumped Ca^{2+} were both continuous. As a result, some cells actually switched color several times during the observation period.

As we hypothesized, when the Ca^{2+} pumps were inhibited, the cells lost their ability to cope with the elevated cytosolic Ca^{2+} concentration. The cells turned green immediately and rarely turned back to red. Furthermore, most cells started to lose their fluorescence, indicating a loss of the Fura-2 dye. After imaging, we observed the morphology of the cells using the phase contrast mode of the microscope. Most cells were swollen, which is a typical sign of cell necrosis. Loss of cell membrane integrity is also a typical sign of necrosis. As indicated in Figure 1, cells treated with only one inhibitor showed partial effects. PMCA was more important in managing the Ca^{2+} toxicity caused by LCP; Caloxin 2A1 alone seemed to have more effect than Ru360 alone (Figure 1). Empty DOTAP liposomes at the same DOTAP concentration as was in LCP did not cause any color change (data not shown), indicating that the observed change in Ca^{2+} concentration was not due to the cationic lipid.

Assuming X gram of tissue *in vivo* has a volume similar to that of the X mL of PBS used in the *in vitro* experiment, i.e. the density of the tissue is approximately 1 g/mL, the concentration of LCP used in this *in vitro* ratio-imaging experiment was calculated as ~50% injected dose per gram tissue (ID/g) in the tumor after *in vivo* intravenous dosing. A comparable accumulation level is rarely achievable *in vivo*. The H460 cells were exposed to LCP as a single layer cell culture, which is also a condition not achievable in the tumor. Thus, we conclude that the Ca^{2+} delivered by LCP is unlikely to cause apoptosis or necrosis *in vivo*. Several *in vivo* studies delivering siRNA with the LCP formulation also reported minimal toxicities in the animal models.[2,3] Nevertheless, we have proceeded to test the hypothesis *in vivo*.

Next, we tested the Ca^{2+} induced toxicity *in vivo*. Nude mice bearing H460 xenografts on the right, hind leg were used. The two Ca^{2+} pump inhibitors together were administered through intratumoral (IT) injection. The LCP NPs used in the *in vivo* experiment were PEGylated. With DOTAP/Cholesterol as an outer leaflet, 15% DSPE-PEG_{2000} and 5% DSPE-PEG_{2000}-Anisamide (a targeting ligand for the sigma receptor)[1] were added for protection of the surface and enhancement of the cellular uptake by H460 tumor cells. The PEGylated LCP NPs were ~25 nm in diameter and their zeta-potential was ~15 mV. The mice were given LCP through either intravenous (IV) or IT injection. At 2 h post injection, the mice were sacrificed and the tumors were fixed with formalin and sectioned for H&E staining.

As Figure 2 indicates, there was no significant sign of necrosis in tumors that were not treated, treated only with inhibitors or treated only with LCP delivered through IV injection. Obvious cell necrosis was observed in tumors receiving an IT injection of inhibitors and either an IV or IT injection of LCP. An additional mouse received an IT injection of both inhibitors and LCP that was not PEGylated; this mouse exhibited the most severe necrosis in the tumor (Figure 2F). PEGylation of LCP was necessary for IV injection; un-PEGylated LCP did not accumulate in the tumor (data not shown). When administered through IT injection, un-PEGylated LCP should interact more strongly with the tumor cells than the PEGylated LCP. Thus, the result of the *in vivo* experiment confirmed our hypothesis that no significant tumor cell necrosis can be induced by LCP unless the Ca^{2+} pumps of the tumor cells are inhibited.

We conclude that in the normal condition, cells were able to manage the elevated cytosolic Ca^{2+} delivered by LCP by removing the Ca^{2+} with two major Ca^{2+} pumps on the plasma membrane and mitochondria, respectively (Figure 3). The elevated Ca^{2+} concentration was only a transient event and was not toxic to the cells. Previous projects completed in this lab regarding the delivery of siRNA to tumors and of genes to liver hepatocytes also indicated that minimal toxicity existed *in vivo*.[2,3] The current study has provided mechanistic insight to these previous observations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Sources

Work supported by NIH grants CA129835, CA149363, and CA151652

We thank Dr. Neal Kramarcy at Michael Hooker Microscopy Facility, University of North Carolina at Chapel Hill for assisting the *in vitro* Fura-2 experiment.

ABBREVIATIONS

LCP	Lipid/Calcium/Phosphate
NP	nanoparticle
CaP	calcium phosphate
DOPC	dioleoylphosphatidylcholine
DOPA	dioleoylphosphatydic acid
DSPE-PEG$_{2000}$	(1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-poly(ethylene glycol)2000)
LPD	Lipid/Polycation/DNA
PMCA	Plasma membrane Ca^{2+} ATPase
MCU	mitochondrial calcium uniporter
ER	endoplasmic reticulum
MICU1	mitochondrial calcium-uptake 1

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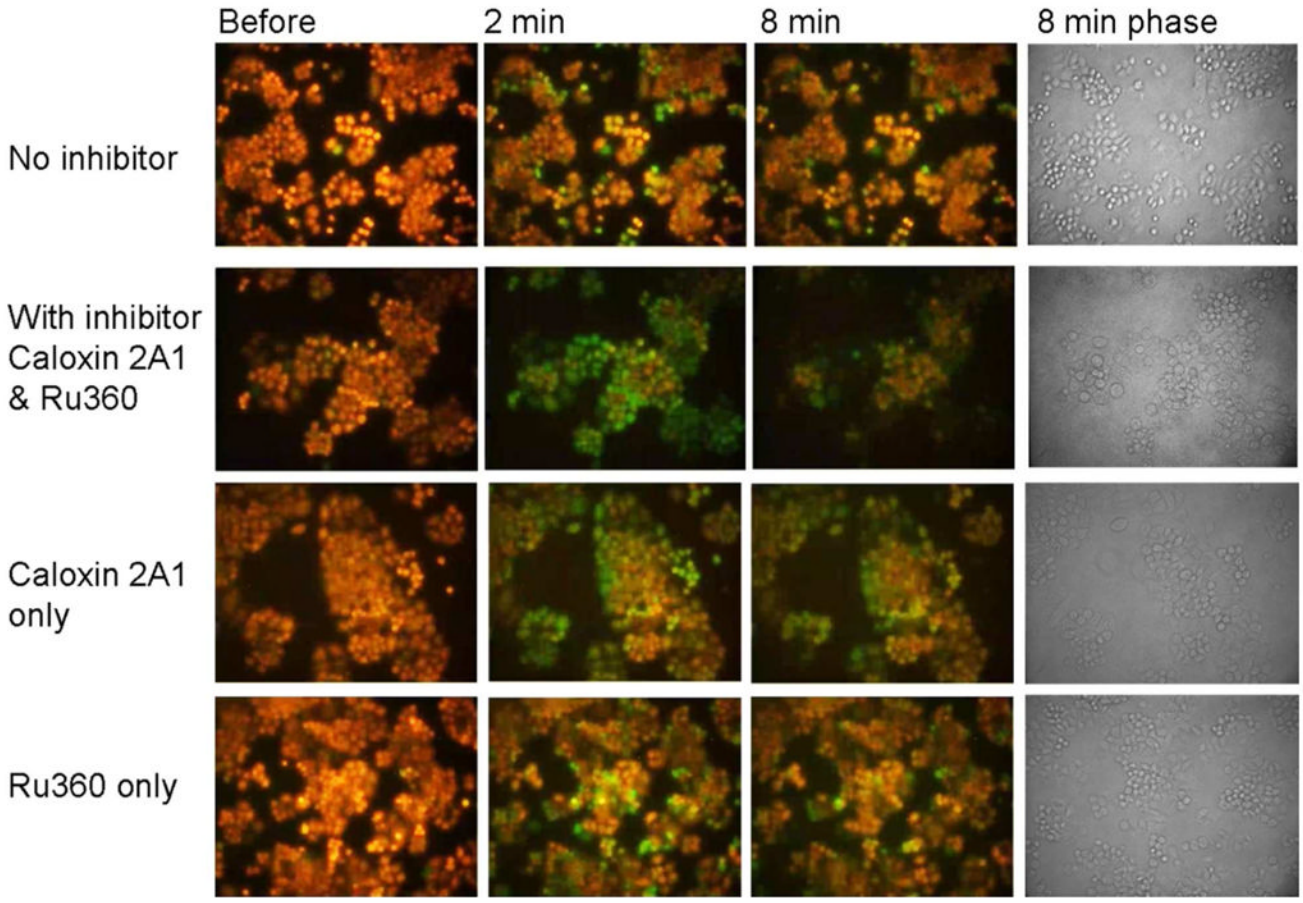

Figure 1. Calcium pumps are important for Ca^{2+} toxicity management

Shown here are stills taken from the recorded video of H460 cells at the indicated time points for four different treatment groups. Red color indicates low intracellular Ca^{2+} concentration under physiological conditions. Green color indicates elevated intracellular Ca^{2+} concentration. Phase contrast images taken at 8 min are included to show round, swollen cells.

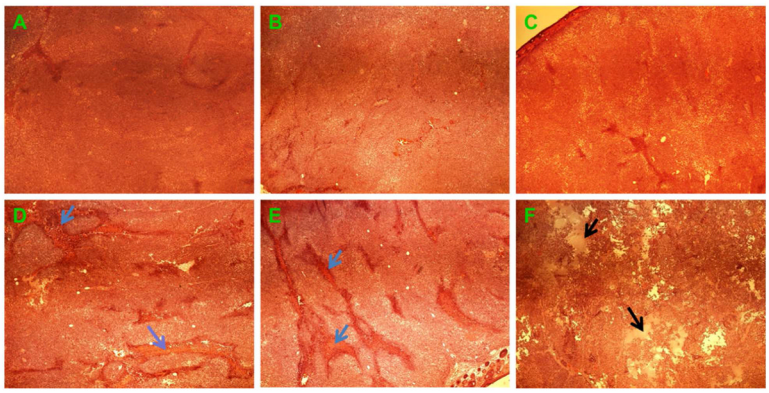

Figure 2. Tumor necrosis *in vivo* which has been induced by LCP with Ca^{2+} pump inhibitors

H460 subcutaneous tumor sections with H&E staining showing that LCP toxicity was only observed in the presence of Ca^{2+} pump inhibitors. (A) Control H460 tumor without any treatment. (B) Tumor that has received an IT injection of two Ca^{2+} pump inhibitors. (C) Tumor that has received an IV injection of LCP. (D) Tumor that has received an IT injection of two Ca^{2+} pump inhibitors and an IV injection of LCP. (E) Tumor that has received an IT injection of two Ca^{2+} pump inhibitors and an IT injection of LCP. (F) Tumor that has received an IT injection of two Ca^{2+} pump inhibitors and an IT injection of LCP without PEGylation. Necrotic lesions were recognized as the “ghost” cells morphology on the H&E-stained sections. Blue and black arrows indicate necrotic and severely necrotic regions, respectively.

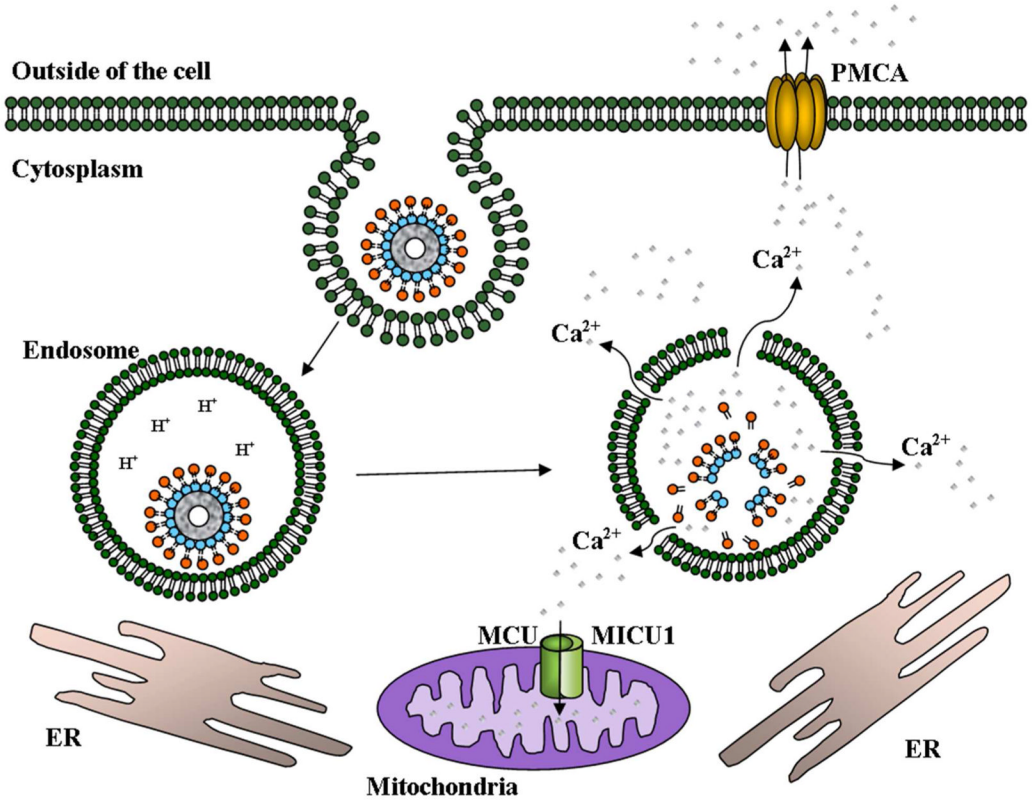

Figure 3. Schematic illustration of the Ca^{2+} removing mechanism after LCP dosing

The elevated cytosolic Ca^{2+} delivered by LCP was rapidly removed by the PMCA on the plasma membrane and the MCU on the mitochondria. Mitochondrial calcium-uptake 1 (MICU1) is a calcium sensor that regulates the Ca^{2+}-influx capacity of MCU.