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A Lipo-PEG-PEI complex for encapsulating curcumin that enhances its antitumor effects on curcumin-sensitive and curcumin-resistance cells

Yu-Ling Lin, MS[a,1], Yen-Ku Liu, PhD[a,1], Nu-Man Tsai, PhD[b,c,2], Jui-Hung Hsieh, MS[d], Chia-Hung Chen, MS[a], Ching-Min Lin, MS[a], Kuang-Wen Liao, PhD[a,e,*,2]

[a]*Institute of Molecular Medicine and Bioengineering, National Chiao Tung University, Hsinchu, Taiwan*
[b]*School of Medical Laboratory and Biotechnology, Chung Shan Medical University, Taichung, Taiwan*
[c]*Department of Pathology and Laboratory, Chung Shan Medical University Hospital, Taichung, Taiwan*
[d]*Institute of Electronics, National Chiao-Tung University, Hsinchu, Taiwan*
[e]*Department of Biological Science and Technology, National Chiao Tung University, Hsinchu, Taiwan*

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Abstract

A cationic liposome-PEG-PEI complex (LPPC) was used as a carrier for the encapsulation of hydrophobic curcumin to give curcumin/LPPC. Curcumin/LPPC had an average size less than 270 nm and a zeta potential of approximately 40 mV. The LPPC encapsulation efficiency for curcumin was about 45%. The authors found it surprising that the cytotoxic activity of the curcumin/LPPC was fivefold higher than curcumin when tested on curcumin-sensitive cells and 20-fold more active against curcumin-resistant cells. Curcumin/LPPC treatment caused a cell cycle arrest at G2/M phase, which rapidly resulted in apoptosis. The increased cytotoxic activity of curcumin/LPPC is likely attributable to its rapid accumulation in the cell. In vivo, administration of curcumin/LPPC inhibited about 60 – 90% of tumor growth in mice bearing CT-26 or B16F10 cells. These results demonstrate LPPC encapsulation technology is able to enhance the effects of antitumor drugs. Use of this technology may provide a new tool for cancer therapy, especially for drug-resistant cancer.

From the Clinical Editor: This team of investigators used a cationic liposome-PEG-PEI complex (LPPC) to encapsulate curcumin. The different delivery method resulted in the five-fold increase of cytotoxic activity against curcumin-sensitive cells and twenty-fold against curcumin-resistant cells.

Key words: Lipo-PEG-PEI complex; Curcumin; Cytotoxicity; Apoptosis; Antitumor

Due to the low bioavailability of chemotherapeutic agents at the site of lesion particularly so in lesser accessible sites of the body (for example, joints, brain, posterior segment of the eye etc.) In addition, traditional antitumor drugs lack selectivity when distinguishing between malignant and normal cells. Due to these circumstances, side effects of chemotherapeutic agents are common, including renal tubular damage, peripheral neuropathy and hematological disease.[1-3] To increase both the activity and the selectivity of such drugs, various nanocarriers, such as liposomes, polymer nanoparticles (NPs), lipid-based NPs, iron oxide NPs and biodegradable microspheres, have been developed as pharmaceutical carrier systems to improve the specific delivery of drugs to tumors.[4-9]

Certain biodegradable polymers can be used to modify the surface of liposomes. These modified liposomes are able to enhance the delivery of drugs, increase drug uptake by target cells and reduce drug toxicity with respect to nontarget organs.[10] Polyethylene glycol (PEG), a low-toxicity polymer, can be used to modify the surface of liposomes. These modifications serve to prolong the circulation of the liposomes by inhibiting phagocytosis by mononuclear phagocytes[10] as well as by reducing the uptake of the liposomes by the reticuloendothelial system.[11] Another polymer, polyethylenimine (PEI), has also been applied in biomedical research for the delivery of DNA into mammalian cells.[12] Cationic PEI-conjugated liposomes have been reported to effectively deliver DNA into the cytosol through the endosomal

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*Corresponding author: Department of Biological Science and Technology, National Chiao Tung University. Hsinchu 30068, Taiwan.
E-mail address: liaonms@pchome.com.tw (K.-W. Liao).

[1] Yu-Ling Lin and Yen-Ku Liu contributed equally to this work.
[2] Dr. Nu-Man Tsai and Dr. Kuang-Wen Liao contributed equally to this work.

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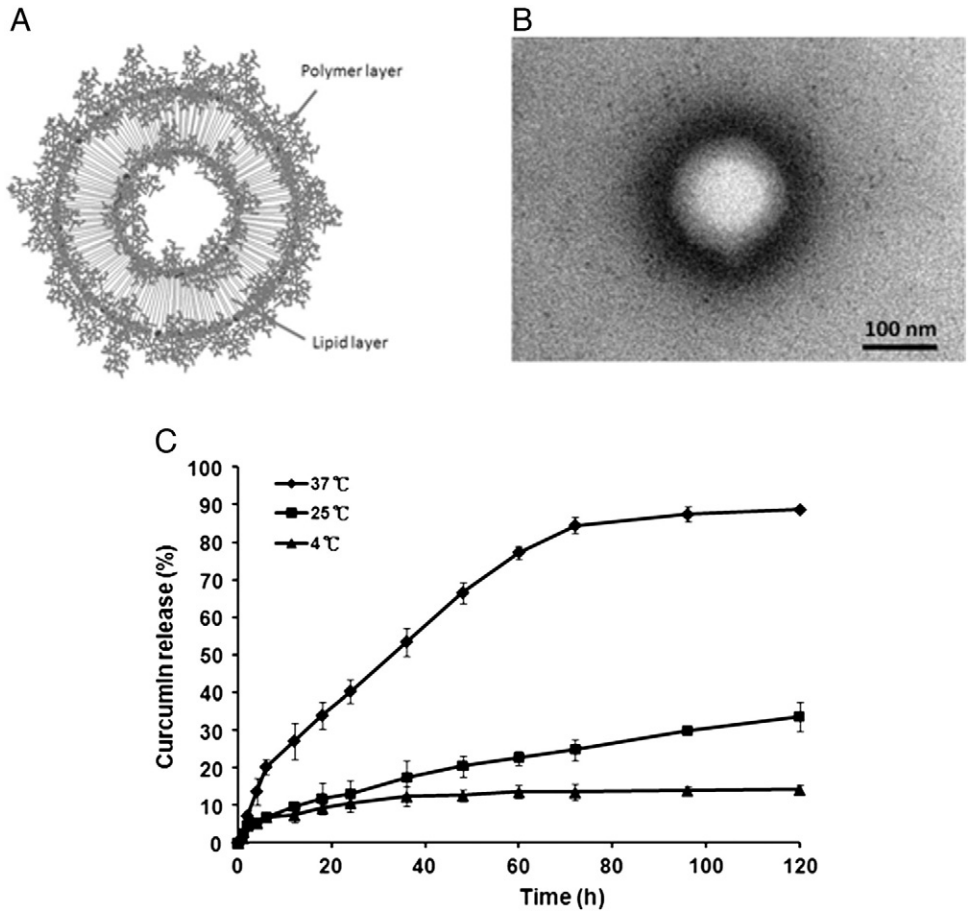

Figure 1. Microstructure of LPPC and drug release from LPPC. **(A)** Schematic illustration of the microstructure of LPPC. **(B)** The TEM images showed the microstructure of LPPC, which consisted of dense circular regions and darkish hair-like projections on the surface. **(C)** Drug release rates for curcumin/LPPC. Curcumin/LPPC complexes were incubated at 4, 25 or 37°C for 120 h. As the incubation time increased, the percentage of curcumin in each supernatant was measured and compared with the total amount of curcumin (n = 6).

pathway.[13] Furthermore, Dr. Fewell et al synthesized a lipopolymer, termed PEG-PEI-CHOL (PPC), which consists of a low molecular weight branched PEI covalently conjugated with methoxyPEG and cholesterol. This polymer was used to encapsulate murine IL-12 transgenes, and when combined with chemotherapeutic agents, the treatment resulted in a significant inhibition of tumor growth.[14] Therefore, surface modification with the biodegradable polymers PEG and PEI appears to enhance the delivery of liposomes along with the target drug they contain.

One clinical trial has shown curcumin (diferuloylmethane) to be a natural phytochemical that is nontoxic for humans even at doses as high as 8 g per day for 3 months.[15] Curcumin has potent antiproliferative and anti-inflammatory activities against a variety of tumors both in vitro and in vivo via diverse signaling pathways that affect tumor growth, including nuclear factor-κB (NF-κB), PI3K/Akt, activator protein-1 (AP-1) and cyclooxygenase-2 (COX-2) signaling.[16-19] However, curcumin is currently limited in its clinical utility because of its poor solubility.[20] Many nanocarriers, including lipid-based NPs, polymer NPs and biodegradable microspheres, have been developed to increase the solubility and bioavailability of curcumin.[4-8]

We recently reported the development of a polycationic liposome complex containing PEI and PEG (LPPC) that can capture the targeting proteins via a noncovalent linkage without defluxion.[21] This technology may have many potential uses, such as drug targeting in combination with functional immunoglobulin. In this study, we evaluated the potential of LPPC to act as a drug carrier by encapsulating curcumin (curcumin/LPPC). We also demonstrated that the cytotoxic activity of curcumin/LPPC was higher in a variety of cancer cell lines, including curcumin-sensitive and -resistant cells, in comparison with nonencapsulated curcumin. We found that curcumin/LPPC treatment was able to arrest the cell cycle at the G2/M phase and induce apoptosis at a low dose by facilitating a rapid delivery of the drug into the cells. In vivo, curcumin/LPPC treatment also resulted in a significant inhibition of tumor growth, which may be due to the higher accumulation of curcumin/LPPC than nonencapsulated curcumin in the tumor area. Thus, these results suggest that LPPC may serve as an effective drug carrier and as a useful anticancer tool.

Methods

Materials

For this research, 1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine (DOPC) and 1,2-Dilauroyl-*sn*-Glycero-3- Phosphocholine

Table 1
Effects of curcuminal/LPPC on proliferation in different cell lines*

Cell lines	Curcumin (μM)	Curcumin liposome (μM)	Curcumin/LPPC (μM)
Mouse			
B16/F10	8.2 ± 1.0†	7.8 ± 1.3	1.1 ± 0.1
LL-2	10.8 ± 2.3	9.9 ± 0.1	1.4 ± 0.2
CT-26	7.9 ± 0.8	6.8 ± 0.8	1.2 ± 0.1
JC	11.0 ± 1.5	9.3 ± 0.1	1.3 ± 0.1
Human			
HepG2	12.2 ± 1.1	10.0 ± 0.4	1.7 ± 0.2
HT-29	12.9 ± 1.2	10.9 ± 1.0	1.5 ± 0.1
HeLa	17.7 ± 7.0	10.0 ± 0.2	1.2 ± 0.2
Curcumin-resistant cells			
A549	30.0 ± 9.5	12.5 ± 0.4	1.4 ± 0.1
CT26/cur-r	27.3 ± 4.6	ND	1.3 ± 0.1
B16F10/cur-r	24.0 ± 8.5	ND	1.3 ± 0.2
Normal cells			
PBMC	15.2 ± 4.1	ND	9.9 ± 1.1
MS1	21.1 ± 6.4	ND	11.7 ± 1.5
SVEC4-10	15.7 ± 3.7	ND	9.0 ± 0.5

ND: non-detection.
* Inhibition of cells exposed to IC_{50} levels of curcumin. IC_{50}: concentration at which cell growth was inhibited by 50%.
† All values are mean ± SD of 3 independent experiments (n = 6).

(DLPC) were purchased from Avanti Polar Lipids (Alabaster, Alabama). curcumin (≧ 94% purity), PEG (MW 8000) and polyethyleneimine (branched PEI, MW 25,000) were purchased from Sigma chemicals (Sigma-Aldrich, St. Louis, Missouri).

Preparation of the Lipo-PEI-PEG complex (LPPC), curcumin/LPPC and curcumin liposome

Equal weights of 25 mg DOPC and DLPC in 1 ml of chloroform were mixed with or without 2.65 mg curcumin and coated onto a round-bottom flask by a rotary evaporator (EYELA, N-1000S, Tokyo, Japan) at 37°C to yield a thin lipid film. The lipid films were then hydrated by steam for 2 hours, and then 5 ml of PEG-PEI solution (675 mg PEI and 220 mg PEG-8000 in deionized water) was added into the container. The lipid films were vigorously resuspended by vortexing for 10 minutes and the suspension was extruded 9 times through a LiposoFast extruder (Avestin Inc., Ottawa, Canada) with a 200 nm mesh. The extrusive suspensions were then diluted 50-fold in deionized water and centrifuged at 5900*g* for 5 minutes to remove any unincorporated substances. Finally, the pellets were resuspended with deionized water, and both types of particles — curcumin/LPPC and empty LPPC — were stored at 4°C until needed. Before use, both types of lipoplexes were warmed to room temperature (25–27°C). For the preparation of curcumin liposomes, curcumin liposomes devoid of PEI and PEG were prepared as described above.

Microstructure of curcumin/LPPC

Ten microliters of curcumin/LPPC (200 μg/ml) was dropped and dried onto a formvar/carbon membrane without staining, and morphologic features of curcumin/LPPC particles were observed by transmission electron microscopy (TEM, JEM 1230; JEOL, Tokyo, Japan) at 100 kV.

Encapsulation efficiency of curcumin in LPPC

After the preparation of curcumin/LPPC, the curcumin/LPPCs were centrifuged and harvested. The amount of curcumin remaining in the supernatant of the solution was then measured by a spectrophotometer (Amersham Biosciences, Uppsala, Sweden) at 432 nm. The encapsulation efficiency was calculated using the following equation:

$$\text{Encapsulating efficiency (\%)} = [(\text{Total amount of curcumin} - \text{Nonencapsulated curcumin})/\text{Total amount of curcumin}] \times 100\%$$

In vitro curcumin release from LPPC

The in vitro release of curcumin from LPPC was evaluated using a dialysis bag diffusion technique.[22] Curcumin/LPPCs (18.5 mg of curcumin/LPPC contained 1 mg total lipid) were suspended in 1 ml of phosphate-buffered saline (PBS, pH 7.4). The curcumin/LPPC solution was then placed into a dialysis bag (Spectra/Por, Spectrum Laboratories Inc., Rancho Dominguez, California) with a 6 – 8 kDa molecular weight cutoff and was immersed into 20 ml of PBS at 4 or 37°C with continuous stirring. In addition, 1 ml of the sample was collected from the incubation medium and measured for curcumin concentration as described above. The release rate was calculated as follows:

$$\text{Release rate (\%)} = (\text{Released curcumin}/\text{Total curcumin in LPPC}) \times 100\%$$

Cells and culture conditions

Four different murine cancer cell lines were used: B16F10 melanoma cells, LL2 lung carcinoma cells, CT26 colorectal adenocarcinoma cells and JC breast adenocarcinoma cells. In addition, 4 different human cancer cell lines were also used: HepG2 hepatocellular carcinoma cells, A549 lung carcinoma cells, HT-29 colorectal adenocarcinoma cells and HeLa cervical cancer cells. All cell lines were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Curcumin-resistant B16F10 (B16F10/Cur-r) and curcumin-resistant CT26 (CT26/Cur-r) cells were generated by continuously culturing the parental tumor cells in growth media containing 5 μM curcumin. All cells were grown in a humidified atmosphere with 5% CO_2 at 37°C, subcultured with a 0.1% trypsin, 2 mM EDTA solution, and maintained in either Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, California) or RPMI 1640 (Invitrogen) supplemented with 10% heat inactivated fetal bovine serum (Gibco BRL, Gaithersburg, Maryland) and 1% penicillin/streptomycin/amphotericin (PSA, Biological Industries, Beithaemek, Israel).

Cytotoxicity of curcumin/LPPC

Cells were seeded into 96-well tissue culture plates at a concentration of 2×10^4 cells/100 μl/well. Subsequently, the cells were treated with serial dilutions of either nonencapsulated curcumin, empty LPPC, liposome curcumin or curcumin/LPPC. After 48 hours of incubation, the cell viability of each line was

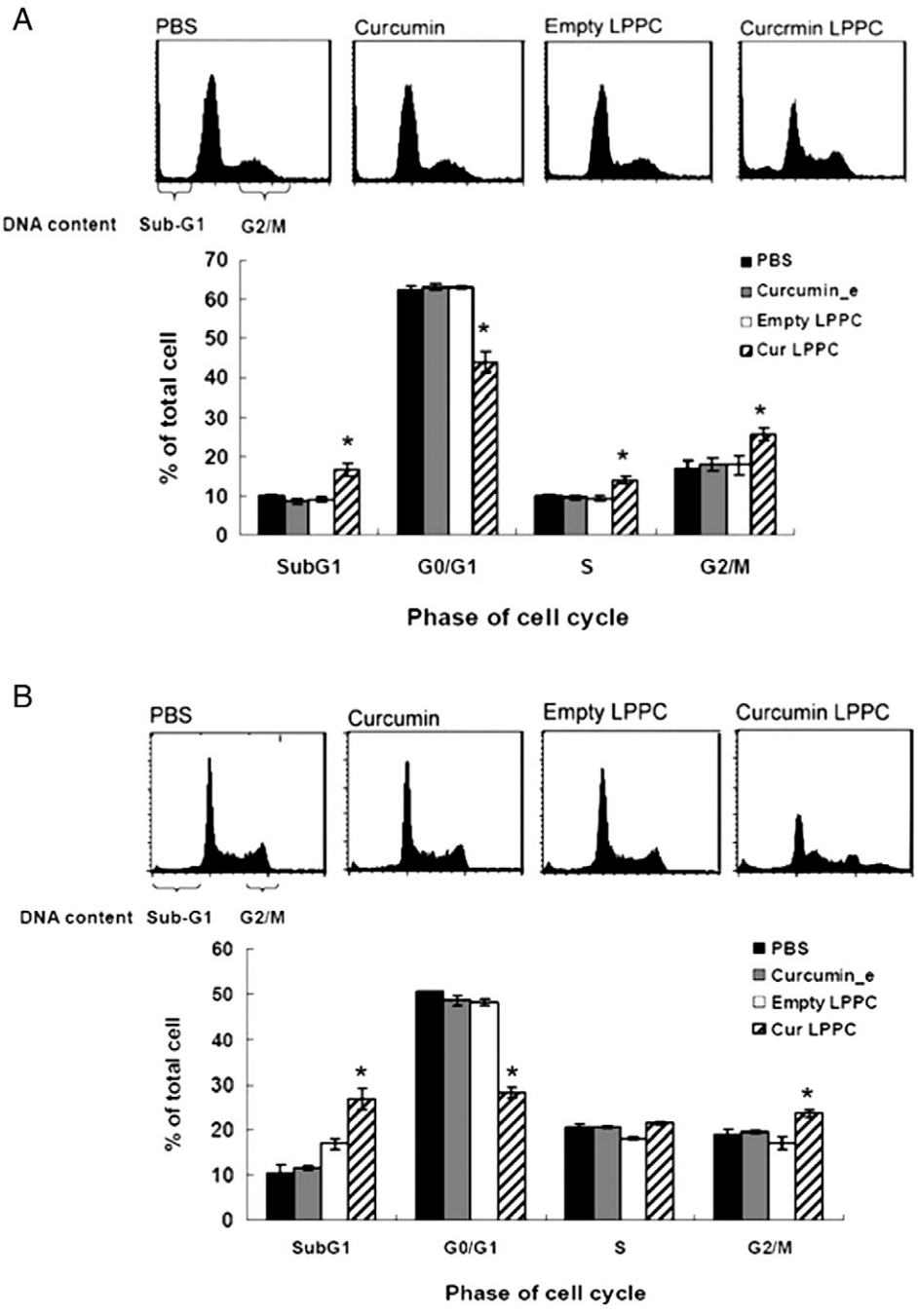

Figure 2. The effect of curcumin/LPPC on the cell cycle and apoptosis. **(A)** CT26 cells and **(B)** B16F10 cells were treated with curcumin, LPPC or curcumin/LPPC. Only curcumin/LPPC was able to increase the proportion of cells in G2/M and sub-G1 phase as compared to PBS-treated cells. The bar graphs show the cell cycle distribution and the percentage of cells in each phase; these were obtained by calculation using the Modfit software. A significant difference compared to the PBS group is indicated by * ($P < 0.05$).

determined by the MTT colorimetric assay (Sigma-Aldrich). Cell viabilities were plotted as a percentage of the untreated control, and the inhibitory concentration at 50% cell survival (IC_{50}) of each reagent was determined from the dose effect curve.

Cell cycle analysis

After either a 16- or 12-hour incubation with PBS, curcumin, LPPC or curcumin/LPPC, approximately 1×10^6 CT26 or B16F10 cells were harvested from monolayer cultures by low-speed centrifugation. The cell pellets were washed with PBS and fixed in 3 ml of 70% ethanol at 4°C for 30 minutes. After centrifugation at 400*g*, the fixed cells were then resuspended with 1 ml of DNA staining buffer (containing 5% Triton-X 100, 0.1 mg/ml RNase A and 4 μg/ml propidium iodide) and incubated for 30 minutes at room temperature. Using a Becton-Dickinson FACScan (Becton Dickinson, Mountain View, California, 10,000 cells were analyzed for DNA content, and

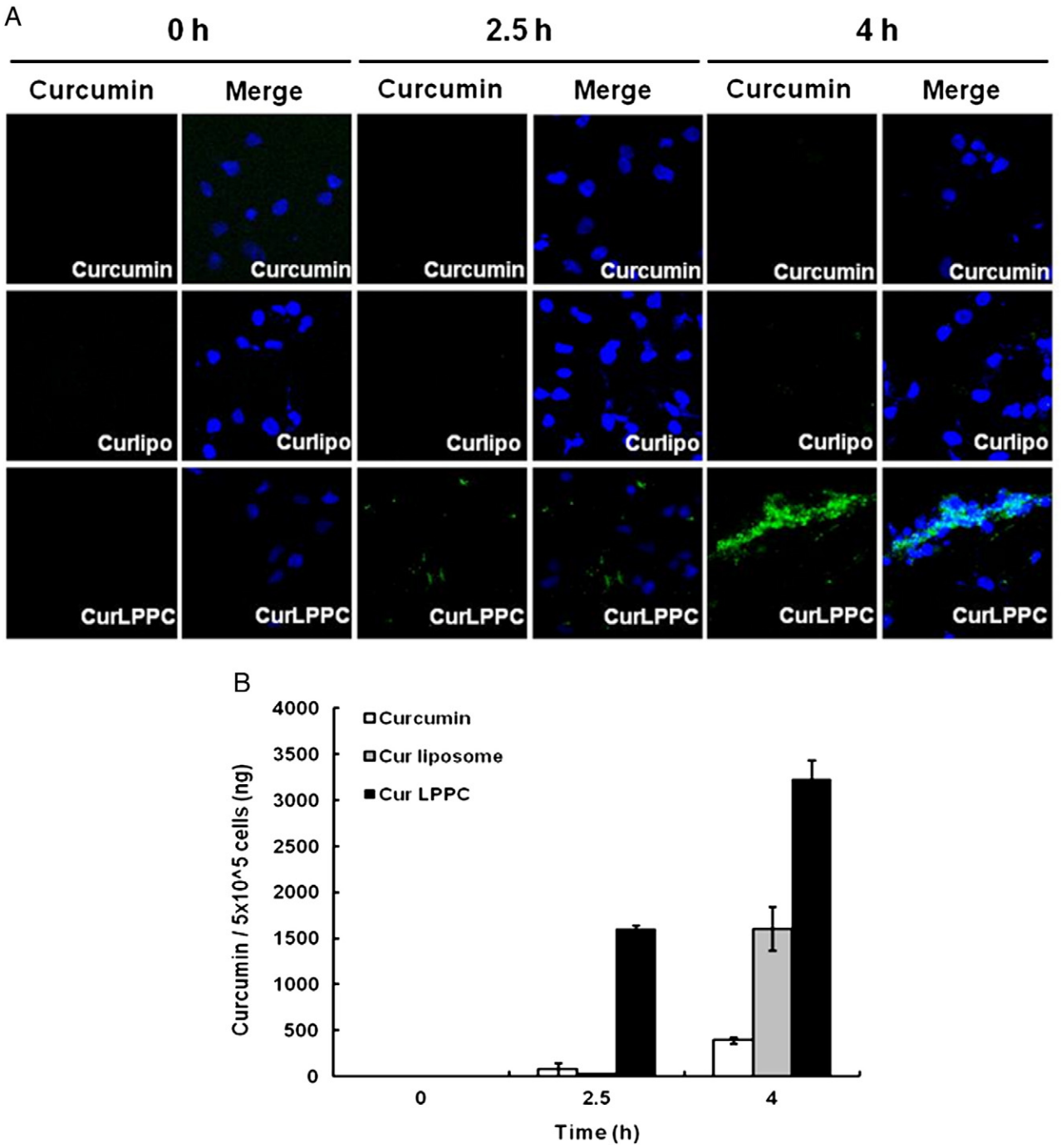

Figure 3. The delivery effect of curcumin/LPPC. **(A)** Uptake of curcumin/LPPC in cells. After treatment with curcumin, curcumin liposome or curcumin/LPPC, the treated A549 cells were photographed under a confocal microscope at the indicated time intervals. Nuclei were stained blue with DAPI, and cellular distribution of curcumin is shown as green fluorescence in the cytosol. **(B)** Curcumin accumulation in cells. After treatment with curcumin, curcumin liposomes or curcumin/LPPC, the treated A549 cells were lysed and cellular curcumin was extracted by chloroform and measured by a spectrophotometer at 420 nm.

the cell cycle distribution was determined using Modfit software (Becton Dickinson).

Measuring curcumin/LPPC delivery in vitro

Because curcumin can serve as a fluorophore (Ex = 480 nm –530 nm), the uptake of curcumin into cells was measured by confocal microscope or spectrophotometry. To measure curcumin uptake by confocal microscope, A549 cells were seeded onto glass coverslips (Nalge NUNC International, Rochester, New York) at a density of 2×10^5 cells per well and cultured in 2 ml of growth medium overnight. The next day, the growth media was removed, and cells were treated with 2 ml of serum-free medium containing curcumin, curcumin/liposome or

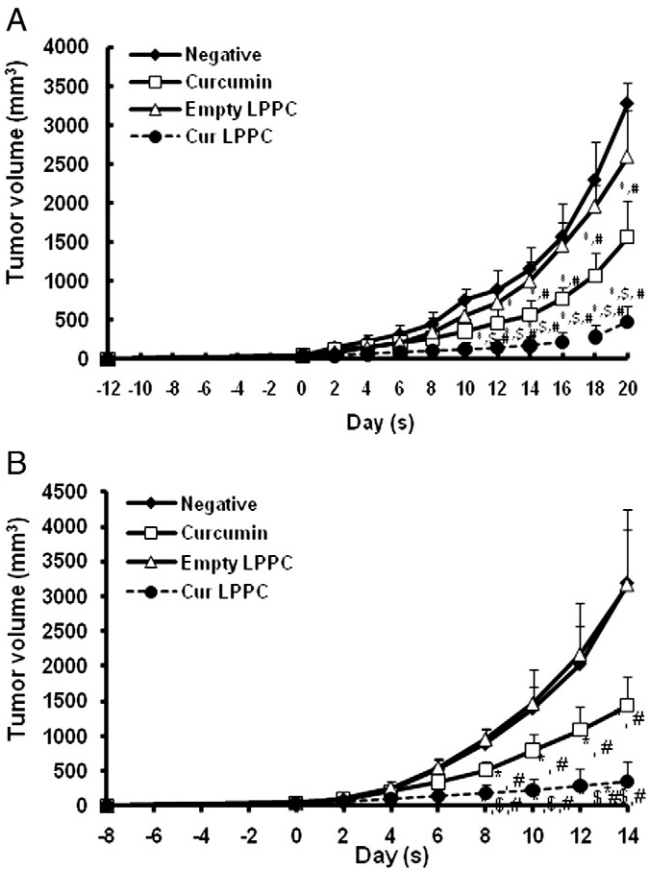

Figure 4. The effects of curcumin/LPPC on tumor growth in vivo. **(A)** Balb/c mice bearing CT26 cells (n = 6/group) were treated with a low dose of curcumin, LPPC or curcumin/LPPC (2.1 mg/kg curcumin) by IV injection once every 3 days. **(B)** C57BL/6J mice bearing B16F10 tumors (n = 8/group) were treated with a high dose of curcumin or curcumin/LPPC (40 mg/kg curcumin) by IV injection once every 3 days. Tumor volume was measured every 2 days after treatment. Significant differences after treatment were found for the curcumin/LPPC-related group compared with the negative control group (∗: $P < 0.001$), the curcumin-treated group ($: $P < 0.01$) and the LPPC-treated group (#: $P < 0.01$), in terms of tumor size. **(C)** Active caspase-3 and curcumin accumulation were observed within the tumor area.

curcumin/LPPC at a final curcumin concentration of 2 μM. After incubation at 37°C for 0, 2.5 and 4 hours, the media were removed from the cells, washed with PBS, fixed with 4 w/w% paraformaldehyde in PBS and imaged at 400× magnification using a Zeiss LSM 510 META confocal microscope (Carl Zeiss, Thornwood, New York). Each condition was performed in triplicate.

To measure the accumulation of curcumin by spectrophotometry, 5×10^5 cells/well were treated with free curcumin, curcumin liposome or curcumin/LPPC at the IC_{50} concentration. After incubation at 37°C for 0, 2.5 and 4 hours, the cells were lysed, and curcumin was extracted by chloroform and measured by a spectrophotometer (Amersham Biosciences) at 420 nm.

Antitumor activity of curcumin/LPPC

Female C57BL/6J mice (4 – 6 weeks of age) were inoculated subcutaneously with 1×10^6 B16F10 cells in 100 μL PBS. Once a tumor mass of 35 mm^3 was established, the animals were treated with curcumin/LPPC (2.1 mg/kg curcumin, IV; this dosage was calculated as twofold IC_{50} for encapsulated curcumin/LPPC in vitro divided by 70% body weight), curcumin (2.1 mg/kg curcumin, i.v.) and LPPC once every 3 days. Subsequently, the tumor volumes were measured using a caliper every 2 days, and the volume was calculated using the formula: volume (mm^3) = length × width × high.

Statistical analysis

All results were analyzed using the SAS statistical software package (SAS Institute Inc., Cary, North Carolina) and expressed as mean ± SD. The t-test was used when comparing 2 independent samples, and the ANOVA test was used when comparing multiple samples. Differences that had a $P < 0.05$ were considered to be statistically significant.

Results

The characteristics of curcumin/LPPC

The morphology and microstructure of the LPPC and curcumin/LPPC were examined by TEM. According to the TEM images, an analysis of the structure of LPCC revealed a structure that is similar to the scheme in Figure 1, *A*. LPPC had a roughly spherical shape with hair-like projections on the surface (Figure 1, *B*). The sizes and zeta potential values of the LPPC and curcumin/LPPC were also measured (Table S1). The diameters of the LPPC and curcumin/LPPC ranged from 258 to 269 nm, and the zeta potential values for both types of particles were approximately 40 mV. The encapsulation efficiency of curcumin in LPPC was determined to be 45 ± 0.2%.

The in vitro drug release of curcumin from the curcumin/LPPC was measured at varying temperatures. These results demonstrated that after 120 hours of incubation time, only 10 – 15% of the curcumin was released from curcumin/LPPC at 4°C, and 30% of the curcumin was released from curcumin/LPPC at 25°C. However, nearly 90% of the curcumin from curcumin/LPPC had been released into the medium after 120 hours at 37°C (Figure 1, *C*).

The cytotoxic activity of curcumin/LPPC against tumor cells

To further determine whether the encapsulation of curcumin into curcumin/LPPC could enhance its cytotoxicity, 10 different cancer cell lines were analyzed for cell viability after curcumin or curcumin/LPPC treatment. The results indicated that the IC_{50} levels for nonencapsulated curcumin ranged from 7.9 μM (CT26 cells) to 30 μM (A549 cells); however, treating the cells with curcumin/LPPC resulted in a lower IC_{50} for all 10 cancer cell lines tested. We found it interesting that the IC_{50} levels for curcumin/LPPC in all cancer cell lines were quite similar, ranging from 1.0 μM to 1.7 μM (Table 1), in comparison with the much wider range observed for the nonencapsulated curcumin. In the case of curcumin-resistant cells, the IC_{50} of nonencapsulated curcumin for the 2 parental cell lines (CT26 and B16F10) rose from 8 μM to more than 20 μM after drug

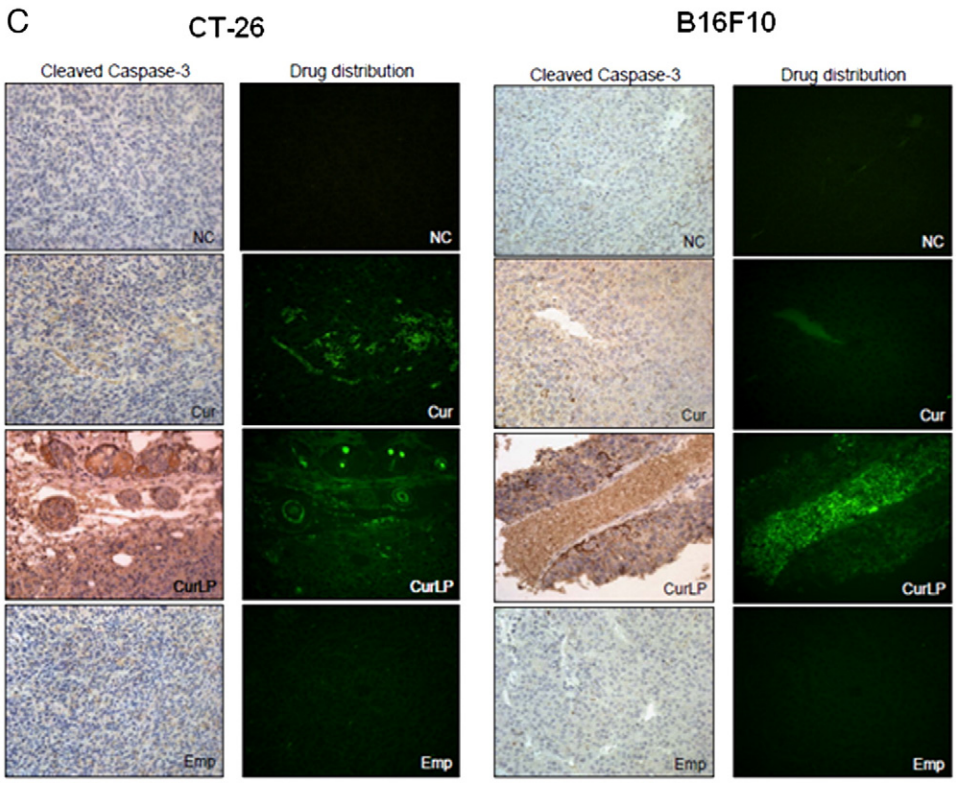

Figure 4. (*continued*).

selection, but the cytotoxic activity of curcumin/LPPC in the 2 curcumin-resistant cell lines was the same as in their parental counterparts. Thus, curcumin/LPPC was able to enhance the cytotoxic activity of curcumin between 3.9- and 20-fold for both curcumin-sensitive and curcumin-resistant cells. These results were dramatic when compared with other delivery methods, such as ordinary liposomes (Table 1) or other nanocarriers. In addition, the IC_{50} levels of curcumin/LPPC ranged from 9 μM to 11.7 μM for normal cells, such as PBMC, MS-1 and SVEC4-10 cells (Table 1). In this study, empty LPPC did not have cytotoxic effects on all cancer cell lines at the dosage equivalent to the IC_{50} of curcumin/LPPC.

The effects of curcumin/LPPC on the cell cycle and cell death

To measure the effects of curcumin/LPPC on the cell cycle, CT26 and B16F10 cells were treated with curcumin/LPPC at their IC_{50} concentrations and analyzed by flow cytometry. Treatment with curcumin/LPPC increased the proportion of sub-G1 and G2/M phase cells (Figure 2, *A* and *B*). In contrast, treatment with the nonencapsulated curcumin or empty LPPC did not cause any significant changes in the cell cycle distribution under similar conditions. To further investigate the resulting effect of this cell cycle arrest, a TUNEL assay was used to confirm that curcumin/LPPC induced tumor cell death by apoptosis (Figure S1A and B). Treatment with curcumin/LPPC, but not nonencapsulated curcumin, caused both the CT26 and B16F10 cells to become apoptotic. Additionally curcumin/LPPC, but not nonencapsulated curcumin, significantly increased the caspase-3 activity in CT26 and B16F10 cells (Figure S1C).

The delivery efficiency of curcumin/LPPC

To observe changes in delivery efficiency, the cellular accumulation of nonencapsulated curcumin, curcumin liposome or curcumin/LPPC was determined by confocal microscopy. A549 cells, which are highly resistant to curcumin treatment (IC_{50} = 30 μM), were treated with the same concentration of either nonencapsulated curcumin, liposome-encapsulated curcumin or curcumin/LPPC, and the accumulation of curcumin in the cytosol was measured over time. The results showed that curcumin in the cells incubated with nonencapsulated curcumin for 4 hours was undetectable (Figure 3, *A*). For the liposome-encapsulated curcumin, liposomes could accumulate in the cytosol after a 4-hour incubation. However, the curcumin/LPPC could rapidly accumulate in the cytosol over a 2.5-hour incubation. Furthermore, after determining the amount of curcumin in the cells, curcumin/LPPC treatment showed 1.5 μg more curcumin accumulated in cells within the 2.5 hours than cells treated with nonencapsulated curcumin alone, and curcumin/LPPC treatment continued to gradually increase cellular curcumin levels over longer times(Figure 3, *B*). From these results, one can infer that the polymers in LPPC not only help lipids to encapsulate curcumin but that they also enhance the delivery of curcumin into cells to increase the cytotoxic efficacy of the drug.

The in vivo suppression of tumor growth by curcumin/LPPC

Mice bearing CT26 tumors were treated with curcumin (2.1 mg/kg), empty LPPC or curcumin/LPPC (containing 2.1 mg/kg curcumin) once every 3 days by IV injection. After 10 days, the animals treated with curcumin/LPPC showed a significant suppression of CT26 tumor growth compared with the untreated controls, nonencapsulated curcumin or empty LPPC (Figure 4, *A*). This result was retested using another malignant tumor cell line, B16F10. After 8 days of treatment, significant suppression of tumor cell growth by curcumin/LPPC was also observed (Figure S2). In this experiment, nonencapsulated curcumin displayed no inhibitory effect on B16F10 tumor growth with an effect similar to that found in the untreated control group. In addition, high doses of curcumin (40 mg/kg) could inhibit tumor growth as previously reported.[8] At this high dosage, curcumin/LPPC (containing 40 mg/kg curcumin) inhibited about 90% B16F10 tumor growth (Figure 4, *B*). These results show that curcumin/LPPC was more effective than nonencapsulated curcumin on inhibiting malignant tumor growth.

Pathological sections of the treated CT26 and B16F10 tumors were further examined for correlation between curcumin accumulation and the induction of caspase-dependent apoptosis (Figure 4, *C*). When compared with nonencapsulated curcumin treatment, curcumin/LPPC treatment increased caspase-3 activity and the accumulation of curcumin in the tumor area, corresponding to an induction of apoptosis in the tumor. These results confirm that LPPC was effective at delivering the curcumin to the cells and, thus, induced a higher therapeutic efficacy of curcumin in CT-26 and B16F10 cells.

Discussion

In this study, LPPC was found to provide an advanced encapsulation that was able to efficiently deliver curcumin into tumor cells. When compared with nonencapsulated curcumin, curcumin/LPPC not only dramatically increased the cytotoxic activity of curcumin in 10 different cancer cells in vitro (from 3.9- to 20-fold, Table 1), but was also able to inhibit 60% to 90% of subcutaneous tumor growth in vivo (Figure 4). We found it interesting that curcumin/LPPC could also suppress the growth of curcumin-resistant cells (Table 1). Various encapsulating methods have been shown to modestly elevate the antiproliferative effect of curcumin from 0.1- to 1.7-fold.[5,6,8,23-26] Additionally, other encapsulating methods, such as albumin-bound curcumin NPs, curcumin-rubusoside complexes and magnetic NPs, demonstrated only cytotoxic activities similar to nonencapsulated curcumin.[27-29] However, in this study, curcumin/LPPC showed a significant improvement in the cytotoxic efficacy of curcumin in comparison with previous approaches. The increased antiproliferative effect of curcumin/LPPC may result from the ability of curcumin/LPPC to more efficiently arrest cells at G2/M phase (Figure 2) and, thus, increase the induction of apoptosis (Figure S1). Furthermore, the enhanced cytotoxicity of curcumin/LPPC may be due to its increased ability to rapidly penetrate and accumulate in cells (Figure 3). This high level of efficient transport into cells is supported by the fact that LPPC is a good shuttle carrier for drugs across the cell membrane. LPPC has been shown to be an excellent drug transporter, capable of delivering large quantities of encapsulated molecules across the cell membrane quickly, which would likely explain the benefits observed by curcumin/LPPC treatment both in curcumin-sensitive and curcumin-resistant tumor cells.

Curcumin is a hydrophobic molecule like many other antitumor molecules, such as taxol and doxorubicin. A number of completed and ongoing clinical trials of curcumin, including studies in colon cancer (phase I/II), pancreatic cancer (phase II/III), cervical cancer (phase II/III), oral cancer (phase II/III), rectal cancer (phase II) and multiple myeloma,[30] have revealed that curcumin is safe for patients and has activity against variant cancers. However, curcumin is limited in its clinical utility because of its poor solubility.[20] The technique of encapsulating curcumin into different NPs has been used to try to overcome this problem.[27-29] Mourtas et al conjugated curcumin with PEG/DSPC/DPPC to efficiently form nanoliposomes, which indicated the structures of curcumin conjugation to form the curcumin-decorated liposomes are stable in media in the presence of plasma proteins.[31] Unlike other protocols for drug encapsulation, LPPC efficiently encapsulates curcumin without conjugation. Therefore, the encapsulation of curcumin by LPPC allows curcumin to form an aqueous suspension due to the hydrophilic polymers on the LPPC surface. Thus, LPPC-encapsulated curcumin has the added advantage of enhanced solubility and can be used directly without any organic solvent, allowing it to be given by IV administration. Many nanocarriers, including liposomes, polymer NPs, lipid-based NPs and biodegradable microspheres, have been developed to increase the solubility and bioavailability of curcumin.[4-8] In this context, NP encapsulation, such as that used with LPPC in this study, not only provides enhanced drug activity but also offers increased hydrophobic drug solubility.

In this study, LPPC-encapsulated curcumin clearly and significantly increased the inhibition of subcutaneous tumor growth; however, the mechanisms behind this inhibition may be many. One possible explanation of the curcumin/LPPC growth-inhibition may involve tumor vasculature. It has been proposed that the leakiness of tumor blood vessels may increase the access of therapeutic vehicles to the tumor mass. Usually, tumor blood vessels are structurally abnormal and have endothelial gaps that range in size from 200 nm to 2 μm.[32] In addition, cationic liposomes, which are promising carriers for tumorigenic targeting, have been found to selectively target the angiogenic endothelial cells in tumors.[33] Thus, curcumin/LPPC, a cationic polymer-liposome of about 270 nm, should be able to accumulate near tumor cells due to the leakiness of tumor vasculature and selectively deliver curcumin to the tumor area. The efficient delivery ought to result in highly selective cytotoxicity against the tumor via the angiogenic endothelial cells. In addition, drug-loaded LPPC should be able to release the drug in the tumor area in a stable and controlled manner, similar to the results observed with curcumin/LPPC release in vitro (Figure 1*C*). This controlled

release varies from other encapsulation approaches that tightly encapsulate curcumin and prevent easy drug release from the micelles.[5,25] Finally, the nature of LPPC, which allows efficient transmembrane transportation (Figure 3), should give rise to the highly efficient penetration of the drug into tumor cells.

Curcumin/LPPC was shown to increase cell cycle arrest at the G2/M phase (Figure 2), resulting in rapid apoptosis after treatment (Figure S1). We find it interesting that curcumin/LPPC treatment was found to cause polyploidization at G2/M phase of B16F10 cells (Figure 2, *B*), which could be due to a disruption of the mitotic spindle structure,[34-37] an inhibition of microtubule assembly, a reduction of GTPase activity and/or an induction of tubulin aggregation.[38] Curcumin has also been reported to affect the expression of genes involved in the G2/M arrest of the cell cycle, including the downregulation of BUB1B, MAD2L1 and HDAC1.[37] Thus, curcumin may serve as a good cancer therapeutic; however, it is currently not a strong chemotherapeutic drug due to its low cytotoxicity in cancer cells. This study demonstrated that the encapsulation of curcumin by LPPC enhances the cytotoxicity of curcumin by up to 20-fold. This improvement in efficacy may help the drug be accepted as having clinical utility.

Curcumin with the reverse activity can also abolish the multidrug resistance (MDR) of cancer cells by downregulating the intracellular levels of three major ATP-binding cassette super families of transporters, such as P-glycoprotein (P-gp), breast cancer resistance protein (ABCG2) and multidrug resistance associated protein (MRP-1).[39,40] For example, curcumin has been shown to increase the sensitivity of drug-resistant cancer cells to vincristine,[41] paclitaxel[42] and doxorubicin.[43] which promote apoptosis. Thus, curcumin may be used as a chemosensitizer to make MDR cells more sensitive to antitumor drugs. However, cancer cells may also develop a curcumin-resistant phenotype after long-term treatment with the drug. We treated CT26 and B16F10 cells with 5 μM of curcumin for 2 weeks, resulting in the formation of curcumin-resistant cells (CT26/cur-r and B16F10/cur-r cells) and altering the curcumin IC_{50} values from 8 μM to 25 μM. Moreover, certain tumor cells naturally have a curcumin-resistant property, such as A549 cells (IC_{50} = 30 μM). Therefore, curcumin's reverse activity for sensitizing drug-resistant cells may be canceled during chemotherapeutic treatment for curcumin-resistant cells. In this study, LPPC encapsulation improved curcumin cytotoxicity approximately 17- to 20-fold for curcumin-resistant cells, in comparison with only a 1.4-fold increase in cytotoxicity for curcumin liposomes. Therefore, curcumin/LPPC treatment may provide a more efficient reverse activity for chemotherapy in curcumin-resistant cells.

In brief, this study showed that LPPC encapsulation improves the cytotoxicity of curcumin against cancer cells through rapid internalization. Our findings show that LPPC is a good vector for the encapsulation of hydrophobic antitumor drugs, such as curcumin, and it appears to increase the cytotoxicity of curcumin in vivo. Future studies are underway to determine the potential of LPPC encapsulation as a means to enhance the in vivo selective delivery of curcumin using antitumor antibodies on the LPPC surface.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.nano.2011.06.011.

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