



Unmodified drug used as a material to construct nanoparticles: delivery of cisplatin for enhanced anti-cancer therapy

Shutao Guo, Lei Miao, Yuhua Wang, 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, USA

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ABSTRACT

The poor solubility of cisplatin (CDDP) often presents a major obstacle in the formulation of CDDP in nanoparticles (NPs) by traditional methods. We have developed a novel method for synthesizing CDDP NPs taking advantage of its poor solubility. By mixing two reverse microemulsions containing KCl and a highly soluble precursor of CDDP, cis-diaminedihydroplatinum (II), we have successfully formulated CDDP NPs with a controllable size (in the range of 12–75 nm) and high drug loading capacity (approximately 80 wt.%). The formulation was done in two steps. The pure CDDP NPs were first stabilized for dispersion in an organic solvent by coating with 1, 2-dioleoyl-sn-glycero-3-phosphate (DOPA). Both x-ray photoelectron spectroscopy and ¹H NMR data confirmed that the major ingredient of the DOPA-coated NPs was CDDP. After purification, additional lipids were added to stabilize the NPs for dispersion in an aqueous solution. The final NPs contain a lipid bilayer coating and are named Lipid-Pt-Cl (LPC) NPs, which showed significant antitumor activity both *in vitro* and *in vivo*. Thus, CDDP precipitate serves as the major material for assembling the novel NPs. This unique method of nanoparticle synthesis may be applicable in formulating other insoluble drugs.

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1. Introduction

Cisplatin (CDDP) is a chemotherapy drug commonly used in the clinical setting. In clinics, maximum tolerated dose (MTD) of CDDP is significantly limited by nephrotoxicity [1,2]. To improve patient care, carboplatin and oxaliplatin are administered, while altering the chloride leaving groups of CDDP with 1,2-diaminocyclohexane or an oxalate ligand compromises outcome [1].

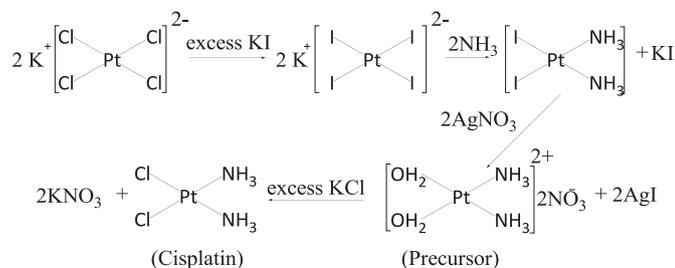
In order to maintain the efficacy and reduce the nephrotoxicity of cisplatin, nanoparticulate CDDP formulations are very promising. Nanoparticulate CDDP formulations have been achieved through chelating CDDP with polymers and NPs [3–5], loading of a pro-drug in the poly(lactide-co-glycolide) (PLGA) nanoparticles (NPs) or encapsulating CDDP into liposomes [3,6–13]. For example, CDDP was loaded into PLGA NPs by exploiting double emulsion technique, while the encapsulation and loading efficacy were low and burst release was often observed [14]. Dhar et al. utilized a prodrug strategy, i.e., modified the hydrophobicity of CDDP, and therefore improved the encapsulation of CDDP into PLGA NPs [13,15,16]. Kataoka et al. alternatively chelated CDDP positively charged platinum species to carboxylate-rich copolymers with a drug loading of 30 wt.% and showed a strong relationship between the therapeutic efficacy and the size of carrier [3,4]. Lipoplatin, a

liposomal formulation, employed electrostatic interaction to load positively charged platinum into negatively charged 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol sodium salt (DPPG)-lipid micelles [17,18]. For Lipoplatin, reverse micelles were mixed with premade liposomes and homogenized by extrusion. Drug loading of Lipoplatin was reported to be 8.9 wt.%. However, these formulations were for either prodrug or charged platinum, but not for native CDDP.

While the synthesis of CDDP (Scheme 1) is a well-documented reaction in the field of inorganic chemistry [19], the poor solubility of CDDP in both water and organic solvents significantly hinders the development of nanoparticulate formulations in a manner similar to the formulation of nanoparticles with hydrophobic drugs [20,21]. In our lab, we have recently developed a Lipid coated Calcium Phosphate (LCP) platform to deliver diverse bioactive molecules, such as DNA, silencing RNA and gemcitabine triphosphate [22–24]. An outer layer of a cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP) and high density of poly(ethylene glycol) (PEG) was coated on the calcium phosphate cores. The cationic lipid DOTAP allows the nanoparticles to be internalized by tumor cells more efficiently and to subsequently escape from the lysosomes. Additionally, a high density of PEGylation will help the nanoparticles avoid reticuloendothelial system (RES) system, improving drug pharmacokinetics and drug bioavailability. We found both components are critical for the successful delivery of drugs into tumors.

Herein, we wish to replace calcium phosphate by CDDP as the core in order to make a CDDP nanoparticulate formulation. Our new formulation would be favorable due to its high drug loading capacity. Another aspect is that, our platform might be applicable to the manufacture of many other CDDP analog nanoparticulate formulations. Our platform is

* Corresponding author. Tel.: +1 919 843 0736; fax: +1 919 966 0197.
E-mail address: leafh@unc.edu (L. Huang).



Scheme 1. Classic synthesis route of CDDP.

expected to improve the solubility of platinum based drug candidates with poor solubility, such as cis-diamminedibromoplatinum(II) and cis-diamminediodoplatinum(II). Therefore, we hypothesize that: (1) CDDP can be encapsulated as a nanoprecipitate in a microemulsion and stabilized in an organic solvent with 1, 2-dioleoyl-sn-glycero-3-phosphate (DOPA); (2) DOPA-coated CDDP NPs can be further dispersed into aqueous solution by adding lipids to form the outer leaflet of the coating bilayer; (3) the lipid bilayer-coated CDDP NPs will show anti-cancer activity *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials

Lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Dulbecco's Modified Eagle Medium (DMEM), L-glutamine, penicillin G sodium, streptomycin and fetal calf serum were purchased from Gibco®. 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[anisamide (polyethylene glycol)-2000] (DSPE-PEG-AA) was synthesized in our laboratory as previously reported [25]. 1-Hexanol was purchased from Alfa Aesar. Igepal® CO-520, triton™ X-100, cyclohexane, cisplatin and silver nitrate were obtained from Sigma-Aldrich (St. Louis, MO) without further purification.

2.2. Cell lines

1205Lu cells were cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 20 mM of L-glutamine, 100 U/mL of penicillin G sodium, and 100 mg/mL of streptomycin at 37 °C in an atmosphere of 5% CO₂ and 95% air.

2.3. Synthesis of cis-[Pt(NH₃)₂(H₂O)₂](NO₃)₂ precursor

To a suspension of CDDP (60 mg, 0.20 mmol) in 1.0 mL water, AgNO₃ (66.2 mg, 0.39 mmol) was added. The mixture was heated at 60 °C for 3 h and then stirred overnight in a flask protected from light with aluminum foil. Afterwards, the mixture was centrifuged at 16,000 rpm for 15 min to remove the AgCl precipitate. The solution

was then filtered using a 0.2 μm syringe filter. The concentration of cis-[Pt(NH₃)₂(H₂O)₂](NO₃)₂ was measured using inductively coupled plasma mass spectrometry (ICP-MS) and adjusted to 200 mM.

2.4. Preparation of LPC NPs

The synthesis route of LPC NPs is described in Scheme 2. First, 100 μL of 200 mM cis-[Pt(NH₃)₂(H₂O)₂](NO₃)₂ was dispersed in a solution composed of mixture of cyclohexane/Igepal CO-520 (71:29, V:V) and cyclohexane/triton-X100/hexanol (75:15:10, V:V:V) to form a well-dispersed, water-in-oil reverse micro-emulsion. Another emulsion containing KCl was prepared by adding 100 μL of 800 mM KCl in water into a separate 8.0 mL oil phase. One hundred microliters of DOPA (20 mM) was added to the CDDP precursor phase and the mixture was stirred. Twenty minutes later, the two emulsions were mixed and the reaction proceeded for another 30 min. After that, 16.0 mL of ethanol was added to the micro-emulsion and the mixture was centrifuged at 12,000g for at least 15 min to remove the cyclohexane and surfactants. After being extensively washed with ethanol 2–3 times, the pellets were re-dispersed in 3.0 mL of chloroform and stored in a glass vial for further modification.

To prepare the final NPs, 1.0 mL of LPC NPs core, 100 μL of 20 mM DOTAP/Cholesterol (molar ratio 1:1) and 50 μL of 10 mM DSPE-PEG-2000 or DSPE-PEG-AA were combined. After evaporating the chloroform, the residual lipids were dispersed in 1.0 mL of d-H₂O.

2.5. Characterization of NPs

The zeta potential and particle size of LPC NPs were determined using a Malvern ZetaSizer Nano series (Westborough, MA). Transmission electron microscopy (TEM) images were acquired using a JEOL 100CX II TEM (JEOL, Japan). The LPC NPs were negatively stained with 2% uranyl acetate.

The drug-loading capacity and platinum content were measured using Varian 820-MS (Palo Alto, CA). Samples were digested in 70% HNO₃ and diluted in water to a final acid content of 2%. Platinum concentration was determined using the ¹⁹⁵Pt isotope.

The composition of DOPA-coated CDDP NPs was studied using X-ray photoelectron spectroscopy (XPS) (Kratos Axis Ultra DLD X-ray Photoelectron Spectrometer) and NMR (Varian Inova 400 NMR Spectrometer).

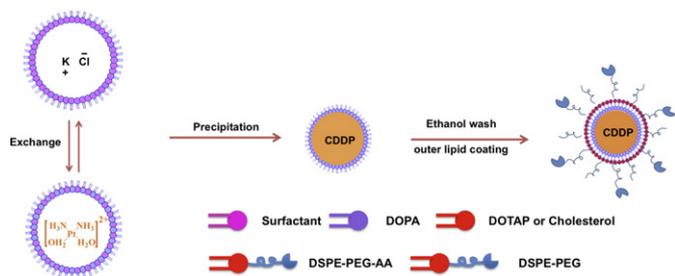
2.6. Cell toxicity assay

1205Lu cells were seeded in 96-well plates at a density of 2000 cells/well and incubated in 10% FBS of DMEM containing 100 U/mL penicillin, and 100 mg/mL streptomycin for 20 h. Then, the medium was removed and replaced by Opti-MEM containing CDDP or LPC NPs. Forty-eight hours later, a CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) kit containing the tetrazolium compound MTS was used to assay cell viability according to the manufacturer's protocols.

2.7. Cellular uptake

1205Lu cells (2 × 10⁵) were seeded in 35 mm MatTek glass bottom dishes (MatTek Corporation, MA) 20 h before experiments. The cells were treated with NBD-PE labeled LPC NPs at a concentration of 100 μM platinum (Pt) at 37 °C for 4 h. The cells were subsequently washed twice with PBS. The nucleus was stained with Hoechst 33342 (Sigma, St Louis, MO), and lysosomes were stained by lysotracker red (Invitrogen, Carlsbad, CA). Then, the sample was observed by Olympus FV 1000-MPE microscope (Olympus, Japan).

To measure the amount of Pt in cells, cells were washed and lysed in order to determine their uptake of NPs. Samples were digested in 70% HNO₃ and diluted in water to a final acid content of 2%.



Scheme 2. Synthesis of lipid bilayer-coated CDDP NPs using a reverse microemulsion method.

2.8. In vivo anticancer efficacy evaluation

Animals were maintained in the Center for Experimental Animals (an AAALAC accredited experimental animal facility) at the University of North Carolina. All procedures involving experimental animals were performed in accordance with the protocols approved by the University of North Carolina Institutional Animal Care and Use committee and conformed to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23, revised 1985). Female athymic nude mice, 5–6 weeks old and weighing 18–22 g, were supplied by the University of North Carolina animal facility. 1205Lu xenograft tumors were developed through subcutaneous injection of approximately 5 million 1205Lu cells in female nude mice. 2.0 mg/kg of Pt was administered weekly by IV injection for CDDP and LPC NPs groups. Tumor growth and body weight were monitored. Tumor volume was calculated using the following formula: $TV = (L \times W^2)/2$, with W being smaller than L . Finally, mice were sacrificed by CO₂ inhalation. Tumors were collected after treatment and were formalin fixed and processed for TUNEL assay.

2.9. TUNEL assay

The tumors were fixed in 4.0% paraformaldehyde (PFA) and subsequently paraffin-embedded, to be sectioned at the UNC Lineberger Comprehensive Cancer Center Animal Histopathology Facility. To detect apoptotic cells in tumor tissues, a terminal deoxynucleotidyl

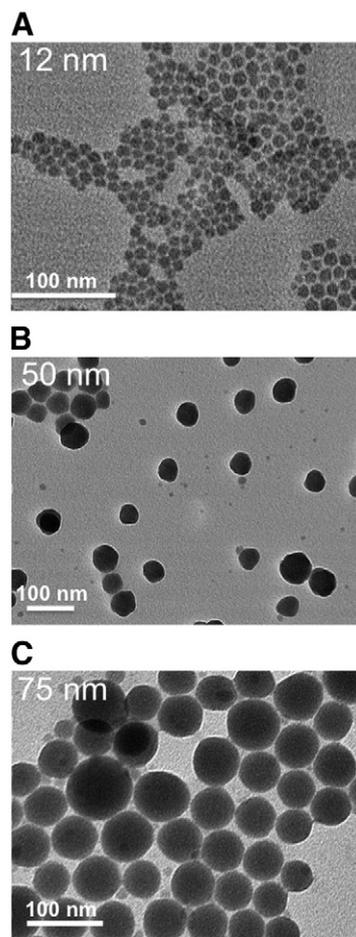


Fig. 1. TEM images of DOPA-coated, CDDP NPs prepared using different surfactant systems. The surfactants used to create the microemulsion were a mixture of Igepal-520 system (Igepal-520: cyclohexane = 30:70 (v/v)) and Triton X-100 system (Triton X-100: Hexanol: cyclohexane = 15:10:75 (v/v/v)); the volume ratio of Igepal-520 system to Triton X-100 system was 6:2 (A), 2:6 (B) and 0:8 (C) respectively.

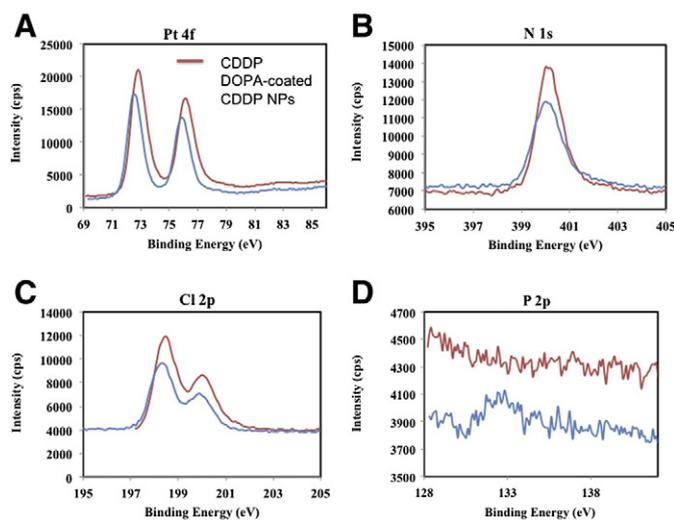


Fig. 2. XPS study of Pt 4f (A), N 1s (B), Cl 2p (C) and P 2p (D) on CDDP and DOPA-coated CDDP NPs.

transferase dUTP nick end labeling (TUNEL) assay, using a DeadEnd™ Fluorometric TUNEL System (Promega, Madison, WI), was performed, following the manufacturer's protocol. Cell nuclei, which were stained with green fluorescence, were defined as TUNEL-positive nuclei. TUNEL-positive nuclei were monitored using a fluorescence microscope (Nikon, Tokyo, Japan). The cell nuclei were stained with 4, 6-diaminidino-2-phenyl-indole (DAPI) (Vectashield, Vector Laboratories, Inc., Burlingame, CA). To quantify TUNEL-positive cells, green-fluorescence-positive cells were counted in three images taken at 40× magnification.

2.10. Statistical analysis

Quantitative data were expressed as mean ± SEM. The analysis of variance is completed using a one-way ANOVA. $P < 0.05$ was considered statistically significant.

3. Results and discussions

3.1. Synthesis and characterization of DOPA-coated CDDP Cores

CDDP NPs were synthesized in microemulsion during the reaction between KCl and its highly soluble $\text{cis-}[\text{Pt}(\text{NH}_3)_2(\text{H}_2\text{O})_2](\text{NO}_3)_2$ precursor. To synthesize stable CDDP precipitates, DOPA, which is known to strongly interact with the platinum cation at the interface [26–28], was used. To maximize the yield of CDDP NPs, an excess of KCl was used to inhibit hydrolysis equilibrium. After CDDP was precipitated, CDDP cores were then coated with a hydrophobic layer of DOPA (Scheme 2). DOPA-coated CDDP NPs were purified in a manner similar to that of silica NPs, which are also synthesized in microemulsion. Ethanol was added to destroy the emulsion and precipitate CDDP NPs. DOPA-coated CDDP NPs precipitate was then collected by centrifugation and readily dispersed in chloroform, toluene or hexane. By adjusting the composition of the surfactant system, the size of the NPs can be altered between 12 and 75 nm in diameter (Fig. 1).

X-ray photoelectron spectroscopy (XPS) was used to confirm the composition of DOPA-coated CDDP NPs (Fig. 2). The ratio of N:Pt:Cl was 2:1:1.8, and a small amount of phosphorous element was also present, as expected from the presence of DOPA (Fig. 2D). In addition, no potassium element was found. We also confirmed the composition of DOPA-coated CDDP NPs using ¹H NMR spectra in DMF-d₇ (shown in Fig. 3). The data illustrated that the major peaks of DOPA-coated NPs were consistent with those of CDDP; and therefore, we can conclude that NPs are core-shell structures with CDDP as the core and DOPA as

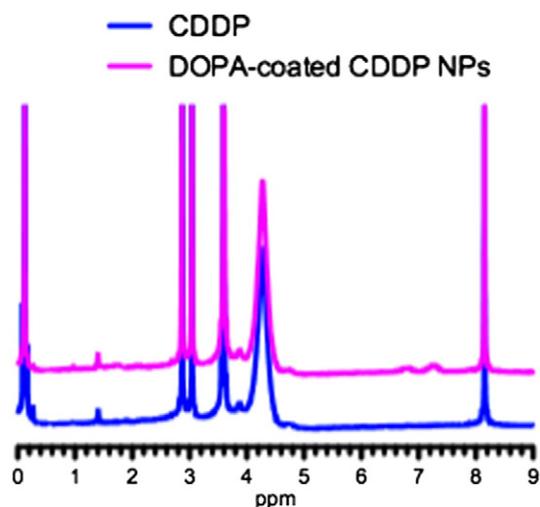


Fig. 3. ^1H NMR spectra of CDDP and DOPA-coated CDDP NPs in DMF- d_7 .

a coating layer. The DOPA-coated CDDP NPs were expected to have high drug loading capacity. We used ICP-MS to measure the content of Pt and used NBD-labeled DOPA to measure the amount of DOPA on the surface of CDDP NPs. The results indicated that the yield of DOPA-coated CDDP NPs was approximately 45 ± 3 wt.%. The NPs were also characterized with drug loading capacity as high as 93 ± 2 wt.%.

3.2. Facile surface engineering of hydrophobic DOPA-coated CDDP NPs with outer leaflet lipids

To further disperse the hydrophobic DOPA-coated CDDP NPs into aqueous solution, additional lipids composed of DOTAP, cholesterol, DSPE-mPEG and DSPE-PEG-AA (molar ratio 4:4:1:1) were used. These lipids self-assembled in water into the outer leaflet of the bilayer through a hydrophobic interaction with DOPA-coated CDDP NPs as a template [29,30]. The DOPA layer served as inner leaflet of the asymmetrical bilayer coating CDDP core. Composition of outer leaflet lipids was carefully chosen to contain steric lipid (DSPE-mPEG) for prolonged circulation of NPs in the blood stream [31], DSPE-PEG-anisamide for vivid uptake of NPs by the tumor cells and DOTAP for rupturing endosomes [32,33].

The final NPs are named Lipid-Pt-Cl (LPC) NPs. LPC NPs were then purified *via* centrifugation to remove free liposomes formed as the result of excess outer leaflet lipids. LPC NPs were negatively stained with uranyl acetate and examined using TEM (Fig. 4A). The clear core/membrane structure revealed the bilayer coating the surface of LPC NPs. As shown in Fig. 4A, size of the particles determined using TEM was approximately 15 nm, which was smaller than the results obtained from dynamic light scattering (DLS) (Fig. 4B). Zeta potential of the NPs was $+15 \pm 0.3$ mV and DLS results showed that distribution of

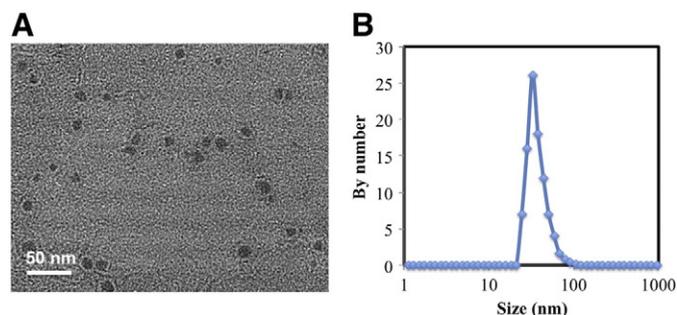


Fig. 4. TEM image of CDDP NPs negatively stained using uranyl acetate (A) and size distribution by DLS (B) of LPC NPs.

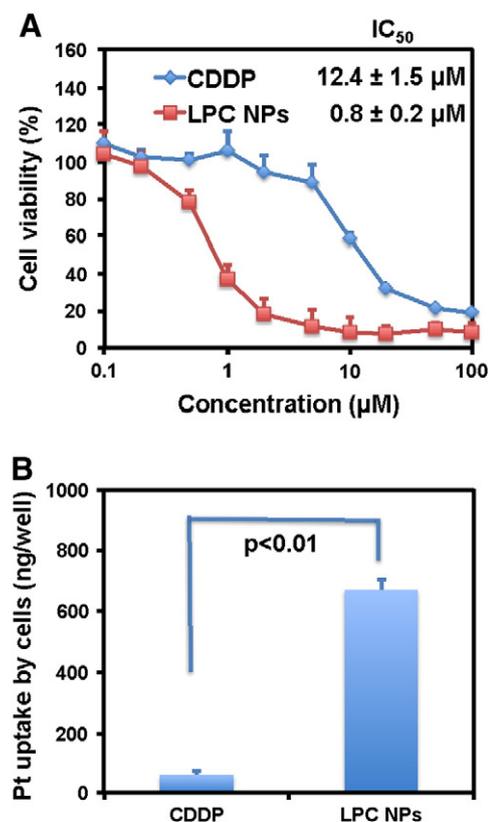


Fig. 5. Cytotoxicity of free CDDP and LPC NPs in 1205Lu cells (A) and the amount of cell uptake of CDDP and LPC NPs in 1205Lu tumor cells quantified using ICP-MS (B). Data are expressed as the amount of the Pt drug associated with cells incubated with $100 \mu\text{M}$ CDDP or LPC NPs in 24 well plates. Each bar represents the mean \pm SEM of three independent experiments. The analysis of variance was completed using a one-way ANOVA.

LPC NPs was narrow, with a PDI of 0.15 ± 0.02 . The drug loading of LPC NPs was approximately 82 ± 5 wt.%. Overall, these results indicate LPC NPs are well dispersed in aqueous solution and characterized by a high drug loading.

Previously, a similar formulation, Lipoplatin, employs electrostatic interaction to load positively charged platinum into negatively charged DPPG-lipid micelles. Reverse micelles were mixed with premade liposomes and homogenized by extrusion [18]. Drug loading was reported to be inefficient, yielding a loading of 8.9 wt.% cisplatin [17]. In contrast, LPC NPs employed a microemulsion system to synthesize a hydrophobic, pure cisplatin-based nanoparticle with tunable size for customized delivery, which further surface-functionalized with amphiphilic lipids to ensure successful evasion of reticuloendothelial system and efficient tumor targeting. LPC NPs achieved high loading capacity due to the solid, cisplatin-based core, producing nanoparticles containing up to 80 wt.% drug. In addition, DOPA-coated CDDP NPs were prepared with the hydrophobic surface in organic solvent, allowing versatile coating and surface modification for a variety of purposes, in a manner similar to quantum dots and iron nanoparticles. We believe our platform may be of significant value to the research community as an alternative approach to develop nanoparticles made of the drug itself rather than loaded with drug as cargos.

3.3. Cell toxicity and cellular uptake of LPC NPs in 1205Lu cells

To test the anticancer efficacy of LPC NPs, we evaluated the performance of LPC NPs in 1205Lu melanoma cancer cells. DOPA-coated CDDP NPs with size of 12 nm were used to prepare LPC NPs for the evaluation of anti-cancer effect. As shown in Fig. 5A, the IC_{50} of CDDP and LPC NPs in 1205Lu cells was $12.4 \pm 1.5 \mu\text{M}$ and $0.80 \pm 0.2 \mu\text{M}$ respectively. Additionally, the empty liposome vesicles having a

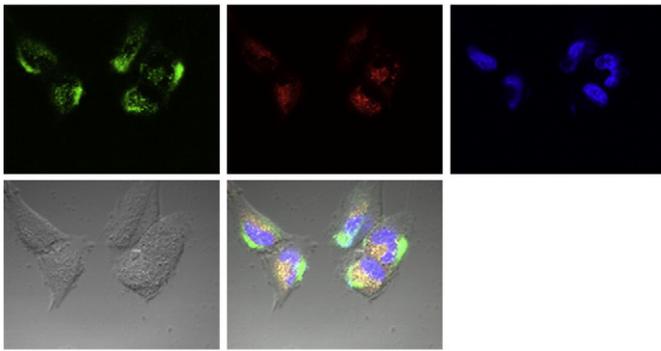


Fig. 6. Cell uptake of LPC NPs in 1205Lu cell line imaged by confocal microscopy. The LPC NPs were labeled with NBD-PE lipid (green). Lysosome (red) and nucleus (blue) were stained by LysoTracker-Red and Hoechst 33342, separately.

composition similar to that of the coating bilayer of LPC did not show any cytotoxicity (data not shown). The cellular uptake of LPC NPs was studied using confocal microscopy and ICP-MS. We labeled LPC NPs using fluorescent 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt) (NBD-PE) lipid and incubated them with cells. Some of the LPC NPs were co-localized with lysosomes as shown by the yellow areas (Fig. 6). However, while a large number of NPs were endocytosed, they showed little accumulation in the lysosomes; a phenomenon that is facilitated by the capability of DOTAP to enhance endosome escape. Quantitative data indicated that cellular uptake of LPC NPs was more efficient than free CDDP (Fig. 5B). LPC NPs delivering CDDP showed 11-fold increase in drug internalization over free CDDP, which explained the low IC_{50} of LPC NPs.

3.4. *In vivo* anti-cancer efficacy of LPC NPs on 1205Lu xenograft tumor and TUNEL assay

Efficacy of LPC NPs in a xenograft tumor model was further evaluated. Fig. 7A illustrates that free CDDP only had a partial effect on tumor growth inhibition at the dose and dose schedule adopted in

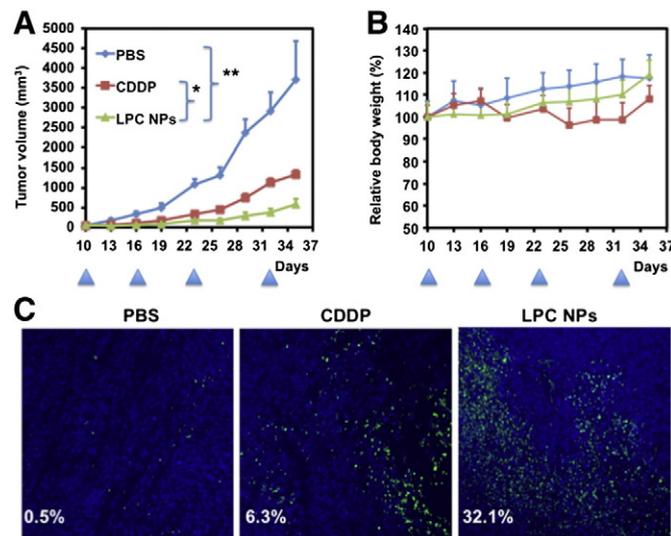


Fig. 7. Effects of free CDDP and LPC NPs on growth of 1205Lu tumors (A) and relative body weight (B). Free CDDP and LPC NPs were administered intravenously at a dose of 2.0 mg/kg Pt. After mice were sacrificed, tumor tissue was sectioned for TUNEL assay (C). The arrowheads indicate the time of injection. Tumor volume (TV) was calculated using the following formula: $TV = (L \times W^2)/2$, with $W < L$. The results are shown as means \pm SEM (error bars) of 5 animals per group and are representative of two independent experiments. The analysis of variance is completed using a one-way ANOVA. * $P < 0.05$; ** $P < 0.01$.

the experiment. In contrast, tumor growth was significantly suppressed when LPC NPs were administered intravenously. In addition, mice in the treatment groups did not exhibit significant weight loss (Fig. 7B). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay is a method for detecting DNA fragmentation occurring in apoptosis by labeling the terminal end of nucleic acids. TUNEL images (Fig. 7C) indicate that LPC NPs induced noticeably more apoptosis ($32.1 \pm 3.6\%$) than free CDDP ($6.3 \pm 0.8\%$), which is consistent with the observed efficacy of tumor inhibition. All in all, our data suggest that LPC NPs are both effective and safe in treating 1205Lu melanoma tumors. Biodistribution and lack of nephro- and hepatotoxicities of LPC NPs have been studied and published elsewhere [34].

4. Conclusions

Successful fabricated CDDP NPs are characterized with high drug loading capacity and optimal aqueous dispersibility. Engineered via the microemulsion method and coated with DOPA and additional outer leaflet layers, LPC NPs exhibited significant antitumor effects both *in vitro* and *in vivo*. By adjusting the fabrication parameters, size of CDDP NPs can also be altered between 12 and 75 nm for optimal tumor accumulation. Note that CDDP is the major ingredient of LPC. As far as we know, this is a good example of using an unmodified drug as a material for the assembly of NPs. Synthesis of LPC NPs can be applied to formulate other insoluble drugs.

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