Published in final edited form as:

Int J Pharm. 2015 November 10; 495(1): 186–193. doi:10.1016/j.ijpharm.2015.08.079.

Improved pharmacokinetics and enhanced tumor growth inhibition using a nanostructured lipid carrier loaded with doxorubicin and modified with a layer-by-layer polyelectrolyte coating

Samuel V. Mussi¹, Gaurav Parekh², Pravin Pattekari², Tatyana Levchenko¹, Yuri Lvov², Lucas A.M. Ferreira³, and Vladimir P. Torchilin^{1,*}

¹Center for Pharmaceutical Biotechnology and Nanomedicine, 360 Huntington Avenue, 140 The Fenway, Northeastern University, Boston, MA 02115, USA

²Institute for Micromanufacturing, Louisiana Tech University, Ruston, LA, USA

³Department of Pharmaceutics, Faculty of Pharmacy, Federal University of Minas Gerais (UFMG), Av Antônio Carlos, 6627, Campus Pampulha, Belo Horizonte, Minas Gerais, 31270-901, Brazil

Abstract

A nanostructured lipid carrier (NLC) loaded with doxorubicin (DOX) has been shown to be cytotoxic against the human cancer cell lines A549 and MCF-7/Adr. In attempts to improve formulation characteristics, enhance pharmacokinetics and antitumor effects, we modified the surface of these NLC with an alternating layer-by-layer (LbL) assembly of polycation and polyanion polyelectrolytes and an additional coating with PEG using a simple method of core shell attachment. The formulation had a narrow size distribution, longer residence in the blood, lower accumulation in the liver, higher accumulation in tumors and a significant tumor growth inhibition effect. Thus, NLC-DOX nanopreparations complexes modified by LbL coating have the potential to enhance the anticancer effects of DOX against tumors.

Keywords

LbL; Doxorubicin; nanostructured lipid carrier; drug delivery; in vivo

1. Introduction

The advantages of lipid nanoparticles including solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) have been widely described earlier as promising drug delivery systems for the treatment of cancer. We have previously proposed the co-

*Corresponding author. Tel.: +1 617 373 3206; Fax: +1 617 373 7509. v.torchilin@neu.edu (V. P. Torchilin).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

encapsulation of doxorubicin (DOX) and docosahexaenoic acid (DHA) in SLN and NLC for the enhancement of drug antitumor effects (Mussi et al., 2014; Mussi et al., 2013). The SLN co-loaded with DOX and DHA were more cytotoxic than free drugs against the human lung A549 cancer cell line in monolayer cultures (Mussi et al., 2013). The developed NLC co-loaded with DOX and DHA were more cytotoxic against the sensitive MCF-7 breast cancer cell line and more potent against the drug resistant MCF-7/Adr cell line than the free drugs. The higher cytotoxicity against the MCF-7/Adr was also verified in a cultured spheroid model. It was suggested that the NLC overcame drug resistance due to a bypass of the efflux effect of the P-glycoprotein pump (Mussi et al., 2014; Zhang et al., 2008).

To further improve the anticancer effect of the NLC co-loaded with DOX and DHA and to improve the colloidal stability by narrowing the particle size distribution, we proposed the surface modification of the nanoparticles with a layer-by-layer (LbL) technique involving an alternate adsorption of polycation and polyanion on a charged substrate (Shutava et al., 2012). This layer-by-layering is a simple method for encapsulation of micro- and nano-structures (Shutava et al., 2013). The LbL film acts to promote a controlled release of drug by controlling the diffusion of drug from the solid core to the external medium. NLC loaded with DOX and DHA have a sufficient surface negative charge to support the LbL architecture. (Mussi et al., 2014). An another approach was made in which a complex of DOX and dextran sulphate (DS) were loaded in SLN. They were further LbL coated with chitosan as the positive polyelectrolyte (PE) and hyaluronic acid as negative PE (Ramasamy et al., 2014). LbL polymeric films are typically uniform and sufficiently adherent to charged surfaces (Parekh et al., 2014; Shutava et al., 2012).

Recently, a technique without a washing step for polyelectrolyte adsorption has been developed based on the addition of each shell layer in a precise amount, determined by titration, that leads to almost complete charge reversals ($\pm 15\text{mV}$). This avoids intermediate washing after each layering and significantly reduces the process time. Additionally, the reduction of total polyelectrolytes used lowers the cost of the whole process (Shutava et al., 2012). Its recent application in encapsulation of drugs with low solubility such as paclitaxel (Shutava et al., 2012) and camptothecin (Parekh et al., 2014; Shutava et al., 2012) improved their colloidal stability and cytotoxicity.

For this study, we modified the surface of NLC co-loaded with DOX and DHA by the LbL technique with polymers containing PEG and evaluated the drug release, characteristics of the nanoparticles, cytotoxicity against human and murine cancer cell lines, and the *in vivo* biodistribution and tumor growth inhibition.

2. Materials and Methods

2.1. Materials

Doxorubicin hydrochloride was purchased from LC Laboratories (Woburn, MA). Triethanolamine (TEA), oleic acid (OA), polyvinyl pyrrolidone, 10 kDa (PVP), the negative polyelectrolyte (PE) and heparin sulfate (HEP) were purchased from Sigma-Aldrich (St. Louis, MO). Positive PE, block-copolymers of poly-L-lysine with polyethylene glycol (PLB) of different molecular weights (PLL[16 kDa]-b-PEG[5 kDa] (PLB16-5) and PLL [16

kDa]-b-PEG[20 kDa] (PLB16-20)) were purchased from Alamanda Polymers Inc. (Huntsville, AL). Extra PEG coating was done with methoxy poly ethylene glycol 5kDa, Succinimidyl valeric acid (mPEG5kDa-SVA) from Laysan Bio Inc. (Arab, AL). Glyceryl behenate (Compritol 888 ATO®) was kindly provided by Gattefossé (Rhône-Alpes, France). Monooleate of sorbitan ethoxylated (Super refined™ polysorbate 80; Tween™ 80) and docosahexaenoic acid (DHA) as triglyceride (Incromega™ DHA 500TG) were kindly provided by Croda Inc. (Edison, NJ). Dulbecco's modified eagle medium (DMEM), antibiotic stock solution (10,000 I.U. of penicillin + 10,000 µg/mL of streptomycin) and 0.25% Trypsin-EDTA were purchased from Corning/Mediatech, (Manassas, VA). Heat inactivated fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Atlanta, GA). Centrifugation devices used were Amicon® Ultra-4 100 kDa; Millipore; (Billerica MA). The Cell Titer Blue assay kit and CytoTox 96® Non-Radioactive Cytotoxicity Assay were purchased from Promega (Madison, WI). All other chemicals were of analytical grade.

2.2. Preparation of formulations

The preparation and the composition of the formulations have been previously described (Mussi et al., 2014). Briefly, for a batch of 5 ml of formulation, the oily phase (OP) was prepared with 100 mg of Tween 80, 10 mg of oleic acid, 5 mg of doxorubicin, 6 mg of triethanolamine and 150 mg of matrix lipid composed of 110 mg of Compritol and 40 mg of DHA (0.4% w/v). This formulation is referred to as "NLC-DOX". The aqueous phase (AP) was composed of purified water. After preparation, the pH was adjusted to 7.0-7.5 and a final volume of 5mL. First, the OP and the AP were heated separately to 80°C. After OP melting, the AP was added drop-wise into the OP and homogenized using a glass rod for 1 min. This emulsion was immediately subjected to intense probe sonication (8 watts) for 5 minutes, using a high intensity ultrasonic processor (Fisher F60 Sonic Dismembrator Fisher F-6; Fisher Scientific, Pittsburgh, PA). The pH was adjusted to 7.0 with 0.1M HCl and 0.1 M NaOH. The formulation was stored at 4°C, protected from light in a nitrogen atmosphere.

2.3. Layer by layer assembly

NLC-DOX (3 mL, with approx. 1 mg/mL of DOX encapsulated) was added to a small glass bottle. To form NLC-DOX-PVP (NDP), a minimum of 4 mg of PVP was added with simultaneous sonication, for a final concentration of 1.31 mg/mL. The sonication was continued for a further 10 min to stabilize these NLC cores. Zeta potential measurements confirmed the negative charge (−15 to −20 mV), necessary for a proper LbL poly electrolyte (PE) assembly. The first layer assembled, was with a positive PE (~4.8 mg of PLB16-5) added to the NDP mixture under continuous sonication for 2-3 min at 40 kHz. Similarly, a total of 2.5 bi-layers of alternating HEP/ PLB16-5 were assembled by adding lower amounts of each PE, ~1.9 mg, with the final outermost layer of HEP. Sonication for 2 to 3 min was used for each layer assembled. The complete process was conducted in a deionized (DI) water medium, maintained at pH 7.4. This formulation was termed "NLC-DOX-LbL". For an extra-PEGylated sample, termed "NLC-DOX-LbL-PEG", a dry powder of mPEG5kDa-SVA was added directly to the above LbL formulation (NLC-DOX-LbL) with PLB16-5 as the outermost LbL layer. Sonication was done for 2-3 min at 40 kHz in cold water at 10°C to ensure a temperature lower than the melting point of the OP, i.e. 80°C (Mussi et al., 2014). The succinimidyl valerate (SVA) group in mPEG5kDa-SVA was an N-hydroxyl

succinimidyl ester group (-NHS ester) conjugated to methoxy PEG. It readily conjugates mPEG groups with excess -NH₂ groups on a surface side by releasing free NHS into the medium (Hermanson, 2008). The final concentration of mPEG5kDa-SVA added to the dispersion was 20 mg/mL. The mixture was sonicated for 5 min, at 40 kHz and then kept overnight for ageing (Hermanson, 2008; Shutava et al., 2012). The mixture was spun at 20,800× g (14,000 RPM) for 10 min, and the supernatant was collected; the pellet was discarded. The NDP LbL formulation has a lipid-based core. Thus, it suspended in the supernatant. This supernatant, NDP(PLB16-5/HEP)2.5/mPEG was finally termed “NLC-DOX-LbL-PEG”. For the LbL sample with no extra PEGylation, 3 bilayers of (PLB16-5/HEP) were assembled, i.e. HEP as the outermost layer was similarly washed. The supernatant obtained, NDP(PLB16-5/HEP)3.0 was termed “NLC-DOX-LbL”. Zeta potential was measured after each additional layer deposition to confirm complete charge reversal between -15 and +15 mV.

All the formulations, including NLC-DOX, were further purified prior the *in vitro* and *in vivo* studies to remove the unloaded DOX and the unattached polymers by ultrafiltration using Amicon® Ultra-4 with a cutoff of 100 kDa (Millipore). About 2 ml of formulations were added to the devices and centrifugation performed for 15 min at 2,655×g (5,000 RPM) and 4°C. The retentate was resuspended in a 5% glucose solution.

2.4. Characterization of formulations

The mean particle diameter and zeta potential were measured by dynamic light scattering (DLS) and electrophoretic mobility, respectively, using the Zeta PLUS particle size analyzer (Brookhaven Instruments; Holtsville, NY). All measurements were performed in triplicate.

The encapsulation efficiency (EE) and drug loading (DL) of doxorubicin in NLC were determined by an ultrafiltration method using Amicon® Ultra-0.5 mL, 100 kDa centrifugal devices (Millipore) with the membrane passivated with a 5% aqueous solution of TweenTM 20 to eliminate doxorubicin binding as previously described (Mussi et al., 2013). The EE and DL were calculated using the following equations:

$$EE(\%) = (C_T - C_{Ap}) / C_T \times 100$$

$$DL_{(mg/g)} = W_{DL} / W_{NP}$$

Where, C_T = total doxorubicin concentration in NLC, C_{AP} = doxorubicin concentration in the aqueous phase (non-encapsulated), W_{DL} = mg of drug loaded in nanoparticles and W_{NP} = g of nanoparticles (lipids).

Briefly, the C_T, C_{AP} and W_{DL} were evaluated as follows. The C_T was determined by dissolving a 200 µL aliquot of NLC dispersion in 5 mL of a mixture of tetrahydrofuran (THF)/methanol (MeOH) 4:6 v/v, centrifugation for 10 min at 2,400×g and analysis of the supernatant by spectrophotometry at 480 nm (UV-mini 1240; Shimadzu, Japan). The C_{AP} was evaluated from an aliquot of the aqueous phase separated from the NLC dispersion by

ultrafiltration (10 min at 2,400 g), dilution with THF/MeOH and analysis by UV-visible. The W_{DL} was derived using the calculated $EE \times \text{total mg of doxorubicin added}$.

2.5. Colloidal stability study

The colloidal stability was checked by diluting the dispersion 100 times in PBS, pH 7.4. The hydrodynamic size was measured by particle size analysis on the 1st, 7th and 14th storage day for both LbL and PEGylated samples. The average values were from 20 readings taken on each sample to confirm the particle size distribution (PSD).

2.6. SEM sample preparation

The dispersion was diluted 10 times in distilled water, pH 7. A drop of each sample was allowed to dry on a clean silicon wafer at room temperature. Before performing SEM imaging, a 12 nm thickness of gold was sputtered at 40 mA and run for 30 sec. The FE-SEM was observed at 1.5 kV to prevent burning of polymer coated samples.

2.7. Transmission electron microscopy

Nanoparticles were negatively stained with 1.5% uranyl acetate, and images were taken using a JEOL TEM-1010 transmission electron microscope (JEOL USA, Inc., Peabody, MA) operating at an acceleration voltage of 80 kV. The images were captured at a 20,000 $\times$ magnification.

2.8. Release of doxorubicin from NLC

The release study was performed by analysis of dialysate in PBS, pH 7.4 (Mussi et al., 2014). Cellulose ester membrane dialysis tubing with a cutoff size of 100 KDa (Spectrum Laboratories; Rancho Dominguez, CA) were filled with 3 mL of formulations loaded with doxorubicin or doxorubicin aqueous solution (~ 0.5 mg/mL) diluted in the buffer 1:2, sealed and incubated with 50 ml of the media for up to 24 h at 37°C, with continuous shaking at 250 RPM. At various time points, 1 mL of solution was withdrawn, replaced with an equal volume of the media. The doxorubicin concentrations were measured spectrofluorimetrically with a Synergy HT Multi-Mode Microplate Reader (Biotek; Winnooski, VT) at 485/590nm ex/em wavelengths. The values were plotted as percentage of cumulative drug release.

2.9. In vivo studies

2.9.1. Cell culture and tumor growth—The cell line 4T1 (murine mammary carcinoma) was purchased from The American Type Culture Collection (ATCC; Manassas, VA). The cells were grown and maintained in RPMI, pH 7.4, supplemented with 10% (v/v) FBS and 1% of penicillin/streptomycin stock solution in a humidified, 5% (v/v) CO₂ atmosphere at 37°C.

For subcutaneous injection over the left flank region, a 100 μ L suspension of 2×10^6 4T1 cells in RPMI serum free were injected in 6-8 week old BALB/c mice for biodistribution and tumor growth inhibition studies. The time for the initial detection of a tumor was about 7 days when tumors were about 100-150 mm³. Mice were regularly monitored and allowed free access to food and water following an animal care protocol (13-1242 R) approved by

the Northeastern University Institutional Animal Care and Use Committee in accordance with the “Principles of laboratory animal care”, NIH publication no. 85-23, revised in 1985). Tumor volumes were calculated using the formula $V = 0.5 (\text{length} \times \text{width}^2)$ by caliper measurements of tumors at regular time intervals.

2.9.2. Biodistribution—Groups of 4-5 animals bearing 4T1 tumors were injected on day +7 with a single dose equivalent of 5 mg/kg of DOX. The treatments were as follows: (1) Free DOX; (2) NLC-DOX; (3) NLC-DOX-LbL and (4) NLC-DOX-LbL-PEG via the lateral tail vein. At 0.5, 1, 2 and 4 h post-injection, blood was collected from anesthetized mice by cardiac puncture into a heparinized (1 U) tube. The blood was centrifuged at 1000 g for 10 min at 4°C followed by collection of the upper plasma layer and storage at –80°C until use. Liver and tumors were harvested for DOX analysis. The amount of the DOX was quantified as previously described by Wang, et al. (Wang et al., 2014). Briefly, weighed tissue homogenates of 10% (w/v) or 25% plasma (200 µL) were added to a mixture of 100 µL of 10% (v/v) Triton X-100, 200 µL of water, and 1.5 mL of acidified isopropanol (0.75 N). Following extraction of DOX overnight at –20°C, samples were warmed to room temperature, vortexed for 5 min, centrifuged at 15,000 g for 20 min and stored at –80°C until analysis. DOX was quantified fluorometrically (ex/em 470/590 nm). Standard curves were prepared using tissues or plasma of mice not treated with DOX.

2.9.3. Tumor growth inhibition—The treatments of tumor-bearing mice started 7 days after 4T1 cell inoculation. The first two doses were of 2.5 mg DOX/kg every 2 days and then 3 doses of 1.25 mg DOX/kg every 2 days administered via tail vein. Each treatment group consisted of 5-6 mice. Tumor volume was estimated as $V = 0.5 (\text{length} \times \text{width}^2)$ every 2 days from the beginning of treatments. The Relative Tumor Volume (RTV) was calculated by using the equation $RTV = V_{\text{day}12}/V_{\text{day}0}$, where $V_{\text{day}12}$ = Volume at the last day of the treatment and $V_{\text{day}0}$ = Volume at Day 0 of the treatment. At +12 days after the first injection, mice were sacrificed, and tumors were excised and weighed. The control group was treated with PBS, pH 7.4.

3. Results

3.1. NLC characterization

Figure 1 and Table 1 show the hydrodynamic sizes and zeta potential of nanoparticles with each layer's addition. At the first polymer addition, the average size increased from 89 nm to 127 nm and then remained unchanged. The final average size for NLC-DOX-LbL was about 138 nm and for NLC-DOX-LbL-PEG it was 128 nm. These samples showed a particle size distribution with a single sharp peak and a very low polydispersity. Polyelectrolytes in the LbL assembly have a combined electrolytic repulsion property, which improves the colloidal stability of the nanostructures. It thus significantly decreased the polydispersity index (PDI), with high monophasic distribution.

Figure 2 shows the SEM and TEM images. Both NLC-DOX-LbL and NLC-DOX-LbL-PEG samples are irregularly shaped with an average diameter of 150 nm and 100 nm, respectively. The extra-PEGylated NLC were more uniformly spherical, smaller in size and had less standard deviation. The larger, more irregular particles represent agglomerates

formed by polyelectrolytes unbound to the nanoparticles floating freely in the dispersion. The oppositely charged polyelectrolytes themselves agglomerate together. The bright white halos seen in the SEM images around most structures represent an edge effect caused by generation of secondary electrons from the curved circumferences of spherical structures (Reimer, 1998). There is an apparent difference in sizes observed between Figures 1 and 2. The measurements shown in Figure 1 were taken immediately after the preparation, while the SEM images reported in Figure 2 were taken 7 days after sample preparation.

The zeta potential of the NLC-DOX lipid core was highly negative, (~ -40 mV, Table 1). For this reason, the initial amount of PE (PLB16-5) required to reverse surface charge (4.8 mg) was high. For subsequent layers, the amount of HEP or PLB16-5 needed was lower, (2.4 mg) for complete charge reversal. After washing of each LbL sample, there were slight changes in surface charge due to separation of loosely bound electrolytes. We verified the formation of some precipitates, which may have been due to interaction of free oppositely charged polymers in the solution. They were removed with the pellet during the centrifugation steps. The conjugation of mPEG-SVA to amine groups of the NDP (PLB/HEP)2.5 was confirmed by a reduction of the zeta potential from high to low positive values, i.e. +5 to +2 mV (See Figure 1).

The colloidal stability of the extra PEGylated sample was better compared to the LbL coated NDP cores (Figure 3). There was a gradual increase in the particle size distribution (PSD) values between the 1st and 14th day of the study for both LbL-coated and extra PEGylated NDP cores. After 14 days, LbL samples showed a bimodal PSD with a polydispersity of 0.158 and maximum peaks at 229 nm (100) and 91 nm (38). In extra PEGylated samples, the PEG tails outside the LbL assembly improved the colloidal stability due to its steric hindrance property (Hermanson, 2008) as indicated by its narrow unimodal PSD of 178 nm with polydispersity of 0.108 after 14 days. This indicated a decrease in polydispersity, i.e. 0.291 of NLC-DOX to 0.108 of NLC-DOX-LBL-PEG after 14 days.

3.2. Drug release

The results of the drug release assay performed by dialysis with PBS, pH 7.4 are included in Figure 4. The released doxorubicin from NLC-DOX was $22 \pm 1\%$ in the first 30 min and increased to $35 \pm 2\%$, $49 \pm 2\%$, and $57 \pm 4\%$ at 1 h, 2 h and 4 h, respectively, following which there was no significant release for up to 24 h. In contrast, for NLC-DOX-LbL and NLC-DOX-LbL-PEG, the release at 30 min was $10 \pm 1\%$ and $9 \pm 1\%$, respectively and increased to $21 \pm 1\%$ in 1 h. The release profiles of NLC-DOX-LbL and NLC-DOX-LbL-PEG were very similar and showed improved capacity to retain the drug compared to NLC-DOX ($35 \pm 2\%$ and $37 \pm 0\%$ at 2 h, $41 \pm 3\%$ and $47 \pm 1\%$ at 4 h, respectively). Release of free DOX was about 80% in 2 hours and in 24 h 90% of Free DOX had been released.

3.3. Pharmacokinetic studies

An evaluation of the drug concentration in plasma, liver and tumor for mice injected i.v. with Free DOX, NLC-DOX, NLC-DOX-LbL and NLC-DOX-LbL-PEG is shown in Figure 5. The plasma concentrations of Free DOX and NLC-DOX were 6.5 ± 0.9 $\mu\text{g/mL}$ and 8.8 ± 1.1 $\mu\text{g/mL}$ at 30 min; at 2 h, they were 3.8 ± 0.8 $\mu\text{g/mL}$ and 5.3 ± 0.7 $\mu\text{g/mL}$, and at 4 h, 2.2

$\pm 0.5 \mu\text{g/mL}$ and $4.4 \pm 0.5 \mu\text{g/mL}$ respectively. NLC-DOX-LbL and NLC-DOX-LbL-PEG retained a higher plasma DOX concentration over the 4 h test period of $10.4 \pm 0.6 \mu\text{g/mL}$ and $12.5 \pm 0.5 \mu\text{g/mL}$ at 30 min, of $8.6 \pm 0.6 \mu\text{g/mL}$ and $11.5 \pm 1\%$ at 2 h, and $5.6 \pm 0.1 \mu\text{g/mL}$ and $8.7 \pm 0.7 \mu\text{g/mL}$ by 4 h, respectively (Figure 5A). The percent of injected dose in plasma over this time period is consistent with the relatively greater retention of DOX for the NLC-DOX-LbL and NLC-DOX-LbL-PEG over Free DOX and NLC-DOX (Figure 5B).

Figure 5C shows the level of accumulation of DOX in the liver and its decline over a 4 h period. It remained highest for the NLC-DOX treatment. NLC-DOX-LbL and NLC-DOX-LbL-PEG treatments resulted in a lower accumulation than NLC-DOX and Free DOX. At 4 h, the levels were $0.13 \pm 0.01 \mu\text{g/g}$, $0.51 \pm 0.02 \mu\text{g/g}$, $0.18 \pm 0.03 \mu\text{g/g}$ and $0.23 \pm 0.06 \mu\text{g/g}$ for Free DOX, NLC-DOX, NLC-DOX-LbL and NLC-DOX-LbL-PEG, respectively.

Figure 5D represents the accumulation of DOX formulations in mouse tumors. At 30 min after administration, there was no difference of drug concentration in tumors among the groups. However, by 2 h, NLC-DOX-LbL-PEG was higher in concentration compared to Free DOX. At 4 h, the concentrations in tumors for all NLC-DOX treatments was clearly greater than for Free DOX. Additionally, LbL-containing formulations were approximately double the NLC-DOX levels ($p < 0.05$). No difference was observed between NLC-DOX-LbL and NLC-DOX-LbL-PEG.

3.4. Tumor Growth Inhibition

The evaluation of the effect of treatment on tumor volumes in mice is outlined in Figure 6A. At the end of the treatment (day 12), the relative tumor volumes (RTV) were 10.7 ± 3.4 , 8.3 ± 2 , 6.5 ± 1.0 , 3.6 ± 0.3 and 2.9 ± 0.1 for vehicle controls, Free DOX, NLC-DOX, NLC-DOX-LbL and NLC-DOX-LbL-PEG, respectively. The more efficient treatments for inhibition of tumor growth contained NLC-DOX, which was most apparent by day 12. Plain NLC-DOX was somewhat more effective than Free DOX. The NLC-DOXs formulated with LbLs clearly ($p < 0.05$) inhibited tumor growth.

Figure 6B shows the tumor weights after excision at day +12. The tumors of mice treated with NLC-DOX-LbL and NLC-DOX-LbL-PEG had a consistently lower weight. There was no tumor weight difference among Vehicle Controls, Free DOX and NLC-DOX and little effect of DOX. These results were consistent with the tumor volume data.

3.5. Toxicity

Mice were monitored for signs of toxicity using observations of behavior and body weight changes. Figure 7 describes the variation of mouse weight from a day 0 baseline over the 12 day treatment period. The Control group maintained a consistent increase in body weight of about a gram. With the exception of the NLC-DOX-LbL-PEG, which had an initial weight loss followed by a late recovery, DOX-treated groups had only small weight fluctuations.

4. Discussion

NLC have been described previously as a promising drug delivery system for enhanced cancer therapy (Joshi and Muller, 2009; Yang et al., 2013; Zhang et al., 2008). Among the

advantages of the NLC is a retention of the drug in the lipid core that promotes controlled drug release (Muller et al., 2002; Radtke and Müller, 2001). Previous data indicated that NLC-DOX enhanced cytotoxicity in human cancer lines, such as MCF-7/Adr and A549 (Mussi et al., 2014; Mussi et al., 2013). Thus, we proposed that a NLC co-loaded with doxorubicin and DHA, modified with LbL would have improved the pharmacokinetics and inhibit tumor growth.

We verified a narrow size distribution for the NLC modified with LbL. The best results were seen with the extra-PEGylated formulation, NLC-DOX-LbL-PEG. The attachment of PEG polymers may have promoted their organization as a one-sized population. Ramasamy et al. (Ramasamy et al., 2014) also verified a more narrow size distribution after LbL modification of SLN in somewhat larger nanoparticles. They also reported a slower DOX release with the modified SLN. This was attributed to the attachment of polymers to the surface of nanoparticles with the generation of a porous barrier for the release of the DOX. This may likewise explain the results obtained for our drug release assay, where the NLC-DOX-LbL and NLC-DOX-LbL-PEG showed lower DOX release than the plain formulation, NLC-DOX.

We proposed that the modification of nanoparticles with LbL would alter the pharmacokinetics, prolong the circulation time and promote tumor targeting by an EPR effect. This effect was seen with the biodistribution studies. The formulations with the LbL polymers attached (NLC-DOX-LbL and NLC-DOX-LbL-PEG) showed longer residence in the blood for DOX compared to free drug and the plain NLC-DOX formulation. Similar results were observed for solid lipid nanoparticles loaded only with DOX and modified with layer-by-layering, but using different polymers (Ramasamy et al., 2014).

The lower accumulation in the liver of LbL formulations may have been associated with a generally low level of toxicity, while the associated higher concentration of DOX in tumors enhanced the antitumor effects. In addition to the higher retention of the drug by these formulations, another factor to consider is related to the fact that the attached polymers are very hydrophilic, and this may have protected these nanoparticles from the mononuclear phagocytic system (Torchilin, 2006).

The potential antitumor effect of these formulations was evaluated by their effect on tumor growth. The formulations NLC-DOX-LbL and NLC-DOX-LbL-PEG were more effective in tumor growth inhibition as expected. Their greater longevity in the blood circulation and higher accumulation of their DOX in tumors are consistent with an enhanced antitumor effect. However, the extra-PEGylated NLC-DOX-LbL-PEG did not improve the anti-tumor effect compared as NLC-DOX-LBL. In this case, PEGylation did not increase the blood residence time.

Mice treated with Free DOX, NLC-DOX, NLC-DOX-LbL exhibited little change in body weight during treatments. Animals receiving NLC-DOX-LbL-PEG did lose some weight initially but fully recovered. This pattern may have been associated with the group's highest initial plasma level followed by a dose reduction to once per day.

The NLC-DOX-LbL formulations show promise in terms of the formulation characteristics and possible therapeutic antitumor activity compared to the Free DOX and plain NLC-DOX.

5. Conclusion

Our proposed core shell nanostructured lipid carrier (NLC) loaded with doxorubicin and DHA and modified with LbL show the size, zeta potential and stability characteristics needed for a controlled release after parenteral administration. The NLC-DOX-LbL and NLC-DOX-LbL-PEG had a more narrow better size distribution, a more sustained DOX release, improved pharmacokinetics, a higher plasma concentration, lower accumulation in the liver with a higher accumulation in tumors, and an enhanced effect on tumor growth inhibition. Thus, the attachment of LbL to the surface of NLC loaded with DOX represents a potential improvement in anticancer therapy. The steps in the process of LbL coating can be reduced by optimizing conditions with the exact amount of polyelectrolyte needed per layer so that multiple layers can be assembled with fewer steps more rapidly and efficiently.

Acknowledgements

This study was supported by the NIH grant 5R01CA134951 to Vladimir P. Torchilin. The authors gratefully acknowledge Dr. Alfred Gunasekaran for SEM imaging and Ms. Debbie Woods, Mr. Davis Beilly for other assistances at Institute for Micromanufacturing, Louisiana Tech University, Dr. Can Sarisozen, Dr. Gemma Navarro and Dr. Aditi Jhaveri are thanked for their assistance with the *in vivo* studies, and Dr. Rajiv Kumar for TEM images.

References

- Hermanson, G. Bioconjugate Techniques. 2nd ed.. Academic Press; San Diego, CA: 2008.
- Joshi MD, Muller RH. Lipid nanoparticles for parenteral delivery of actives. Eur. J. Pharm. Biopharm. 2009; 71:161–172. [PubMed: 18824097]
- Muller RH, Radtke M, Wissing SA. Nanostructured lipid matrices for improved microencapsulation of drugs. Int. J. Pharm. 2002; 242:121–128. [PubMed: 12176234]
- Mussi SV, Sawant R, Perche F, Oliveira MC, Azevedo RB, Ferreira LA, Torchilin VP. Novel nanostructured lipid carrier co-loaded with doxorubicin and docosahexaenoic acid demonstrates enhanced in vitro activity and overcomes drug resistance in MCF-7/Adr cells. Pharm. Res. 2014; 31:1882–1892. [PubMed: 24522814]
- Mussi SV, Silva RC, Oliveira MC, Lucci CM, Azevedo RB, Ferreira LA. New approach to improve encapsulation and antitumor activity of doxorubicin loaded in solid lipid nanoparticles. Eur. J. Pharm. Sci. 2013; 48:282–290. [PubMed: 23178339]
- Parekh G, Pattekari P, Joshi C, Shutava T, DeCoster M, Levchenko T, Torchilin V, Lvov Y. Layer-by-layer nanoencapsulation of camptothecin with improved activity. Int. J. Pharm. 2014; 465:218–227. [PubMed: 24508806]
- Radtke M, Müller RH. NLC-nanostructured lipid carriers: the new generation of lipid drug carriers. New Drugs. 2001; 2:48–52.
- Ramasamy T, Tran TH, Choi JY, Cho HJ, Kim JH, Yong CS, Choi HG, Kim JO. Layer-by-layer coated lipid-polymer hybrid nanoparticles designed for use in anticancer drug delivery. Carbohydr. Polym. 2014; 102:653–661. [PubMed: 24507332]
- Reimer, L. Scanning electron microscopy, Physics of image formation and microanalysis. Springer Verlag; New York, N.Y.: 1998.
- Shutava, T.; Pattekari, P.; Parekh, G.; Lvov, Y. Improving efficiency of Layer-by-Layer coating on nanosized particles with non-washing assembly technique, Physics, Chemistry and Applications of Nanostructures; The International Conference Nanomeeting World Scientific; Minsk, Belarus. 2013; p. 267-270.

- Shutava TG, Pattekari PP, Arapov KA, Torchilin VP, Lvov YM. Architectural layer-by-layer assembly of drug nanocapsules with PEGylated polyelectrolytes. *Soft Matter*. 2012; 8:9418–9427. [PubMed: 23144650]
- Torchilin VP. Multifunctional nanocarriers. *Adv Drug Deliv Rev*. 2006; 58:1532–1555. [PubMed: 17092599]
- Wang T, Hartner WC, Gillespie JW, Praveen KP, Yang S, Mei LA, Petrenko VA, Torchilin VP. Enhanced tumor delivery and antitumor activity in vivo of liposomal doxorubicin modified with MCF-7-specific phage fusion protein. *Nanomedicine*. 2014; 10:421–430. [PubMed: 24028893]
- Yang XY, Li YX, Li M, Zhang L, Feng LX, Zhang N. Hyaluronic acid-coated nanostructured lipid carriers for targeting paclitaxel to cancer. *Cancer Lett*. 2013; 334:338–345. [PubMed: 22776563]
- Zhang XG, Miao J, Dai YQ, Du YZ, Yuan H, Hu FQ. Reversal activity of nanostructured lipid carriers loading cytotoxic drug in multi-drug resistant cancer cells. *Int. J. Pharm*. 2008; 361:239–244. [PubMed: 18586075]

Figure 1.

Hydrodynamic size and zeta potential along the LbL assembly process and extra PEGylation of NLC-DOX-DHA.

Figure 2.
SEM and TEM images: (A) SEM of NLC-DOX-LbL (outermost layer being HEP); (B) SEM of NLC-DOX-LbL-PEG (with extra PEGylation), (C) TEM of NLC-DOX-LbL and (D) NLC-DOX-LbL-PEG.

Figure 3. Colloidal stability of NLC-DOX, NLC-DOX-LbL and extra NLC-DOX-LbL-PEG. The maximum peak values of the Particle size Distribution (PSD) are indicated as in Multimodality Size Distribution (MSD) summary with a maximum intensity - weighted size obtained from a Non-negatively constrained Least Squares (NNLS) algorithm.

Figure 4. Doxorubicin release in complete PBS, pH 7.4 by dialysis using a cellulose ester membrane (100 KDa MWCO). The released doxorubicin measured in the media was analyzed by fluorescence. N = 3; Mean $\pm$ SD; *P < 0.05 for the comparison of NLC-DOX-LbL/ NLC-DOX-LbL-PEG against Free DOX.

Figure 5.

Evaluation of DOX concentration in plasma (**A, B**), liver (**C**) and tumor (**D**) after administration of Free DOX, NLC-DOX, NLC-DOX-LbL and NLC-DOX-LbL-PEG. The time points for collection of samples were 30 min, 2 h and 4 h. N = 4-5; Mean $\pm$ SD; *P < 0.05.

Figure 6. Evaluation of the relative tumor volume (RTV) (A) and tumor weight excised at day 12 (B). The mice were inoculated with 4T1 cells and treated with PBS, Free DOX, NLC-DOX, NLC-DOX-LbL or NLC-DOX-LbL-PEG. N= 5-6. Average $\pm$ SD. One way ANOVA was used considered day by day. The post test was Newman-Keuls.

Figure 7.
Variations of the mouse body weights by treatment. N = 3 to 6. Mean $\pm$ SD. One way ANOVA with Newman-Keuls post test.

Table 1

Zeta potential and hydrodynamic size of NLC-DOX, NLC-DOX-LbL and NLC-DOX-LbL-PEG.

Sample	Zeta potential, mV	Average hydrodynamic diameter, nm	Polydispersity index	Max peak/s, nm (differential intensity)
NLC-DOX	-40 ± 2	89 ± 3	0.291	63 (100), 149 (80)
NLC-DOX-LbL	-14 ± 2	138 ± 2	0.092	115 (100)
NLC-DOX-LbL-mPEG	5 ± 3	128 ± 1	0.098	129 (100)