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RESEARCH ARTICLE

Production of limit size nanoliposomal systems with potential utility as ultra-small drug delivery agents

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Abstract

Previous studies from this group have shown that limit size lipid-based systems – defined as the smallest achievable aggregates compatible with the packing properties of their molecular constituents – can be efficiently produced using rapid microfluidic mixing technique. In this work, it is shown that similar procedures can be employed for the production of homogeneously sized unilamellar vesicular systems of 30–40 nm size range. These vesicles can be remotely loaded with the protonable drug doxorubicin and exhibit adequate drug retention properties *in vitro* and *in vivo*. In particular, it is demonstrated that whereas sub-40 nm lipid nanoparticle (LNP) systems consisting entirely of long-chain saturated phosphatidylcholines cannot be produced, the presence of such lipids may have a beneficial effect on the retention properties of limit size systems consisting of mixed lipid components. Specifically, a 33-nm diameter doxorubicin-loaded LNP system composed of 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC), 1,2-dipalmitoyl phosphatidylcholine (DPPC), cholesterol, and PEGylated lipid (DSPE-PEG2000) demonstrated adequate, stable drug retention in the circulation, with a half-life for drug release of ~12 h. These results indicate that microfluidic mixing is the technique of choice for the production of bilayer LNP systems with sizes less than 50 nm that could lead to development of a novel class of ultra-small drug delivery vehicles.

Keywords

Doxorubicin, herringbone micromixer, limit size nanoparticles, liposome, microfluidic mixing

History

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Introduction

Important features of lipid nanoparticle (LNP) drug carrier systems include ease of preparation, reproducibility, and efficient encapsulation and retention of the biologically active agent. However the LNP size is a critically important parameter. The ability to generate small (<50 nm) LNP can be highly desirable to optimize the biodistribution of the LNP carrier following intravenous (i.v.) injection. In this regard, the vast majority of LNP systems being used as drug delivery agents have primarily utilized particles of 80–100 nm diameter, largely because of the availability of formulation methods that produce LNP in that size range. To our knowledge, all clinically approved (to date) nanomedicines represent nanoparticulate carriers larger than 80 nm, examples include Doxil® (80-100 nm) (Gabizon, 2002), Marqibo® (100 nm) (Silverman & Deitcher, 2013), and Abraxane® (130 nm) (Green et al., 2006). It is well established that the enhanced permeation and retention (EPR) effect contribute to the passive tumor-targeting of nanoparticles with the size of 80–100 nm (Maeda et al., 2000). However, on one hand, numerous works have reported that whereas such ''large'' systems can often accumulate in the adjacent blood vessels and in the peripheral regions of solid tumors, there is limited penetration into tumor tissue itself, thus limiting the potency of the anticancer agent (Dreher et al., 2006; Jain et al., 2010; Kano et al., 2007; Perrault et al., 2009; Unezaki et al., 1996; Uster et al., 1998). On the other hand, it is widely recognized that smaller (≤50 nm) delivery agents may substantially improve penetration and retention within the tumor tissue (Cabral et al., 2011; Chauhan & Jain, 2013; Chauhan et al., 2011; Huo et al., 2013), provided that they are larger than 10 nm to avoid renal clearance. As particles in the size range of 10–50 nm can be expected to be the most promising carrier system in accessing extravascular target tissues, the synthesis of such systems is of intense interest for biomedical applications. A number of techniques aimed at production of smaller size vesicular LNP are available for decades, most of them can be described as ''top down'' approaches based on downsizing of previously formed larger structures (De Kruijff et al., 1975; Hope et al., 1986; Huang, 1969). Those methods (exemplified primarily by sonication) have many limitations, including, most importantly, lack of scalability. Other techniques to produce nanovesicular systems include ''bottom up'' approaches whereby LNP are formed by condensation of lipid from solution rather than by disrupting larger particles

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(Batzri et al., 1973; Jahn et al., 2007). This work will focus on a similar approach we have developed to achieve smaller LNP systems.

In an earlier report (Zhigaltsev et al., 2012), we demonstrated the use of microfluidic mixing employing a staggered herringbone micromixer (SHM) to produce LNP systems having either polar or non-polar cores (i.e., nanoemulsions and nanovesicles). In comparison to previous techniques to produce sub-50 nm vesicular LNPs, the SHM-aided micromixing process is considerably less harsh than sonication and is more robust than the ethanol dilution method (Batzri et al., 1973), as mixing can be performed in a precisely defined and controlled manner. The use of a SHM mixer provides an exponential increase in surface area between the two fluids with distance traveled, resulting in much faster diffusional mixing than the other micromixing approaches such as hydrodynamic flow focusing. Those findings allowed us to assume that at sufficiently fast mixing rates LNPs, formed by precipitation in response to the rapidly rising polarity of the medium, will adopt the smallest possible or ''limit size'' dimensions compatible with the physical properties of lipid constituents.

Within the limit size concept, combinations of different lipids result in LNPs with sizes specific to the particular lipid composition employed. For example, we found that pure POPC and POPC/cholesterol mixtures in the absence of PEGylated lipids give rise to limit size vesicles of approximately 20 and 45 nm diameter, respectively. In this work, we investigated other formulations (varying cholesterol and PEG-lipid content) in an attempt to identify a composition that would provide adequately stable drug retention in the circulation while not exceeding a 40 nm size limit. An optimal composition was identified that falls into the 30–40 nm size range and includes partially unsaturated phosphatidylcholine (POPC), saturated phosphatidylcholine (DPPC), cholesterol, and PEGylated component (DSPE-PEG2000).

Materials and methods

Materials

Lipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC), hydrogenated soy L-α-phosphatidylcholine (HSPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol)-2000] (DSPE-PEG2000) were obtained from Avanti Polar Lipids (Alabaster, AL). Cholesterol (Chol), sodium chloride, ammonium sulfate, and doxorubicin hydrochloride were from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). Tritiated [^{3}H]cholesteryl hexadecyl ether ([^{3}H]CHE) and [14-^{14}C]doxorubicin hydrochloride were purchased from Perkin-Elmer (Boston, MA).

Preparation of limit size vesicular LNPs

Limit-size vesicular LNPs were prepared by mixing appropriate volumes of lipid stock solutions in ethanol with an aqueous phase employing a microfluidic micromixer as described earlier (Zhigaltsev et al., 2012). Briefly, the LNPs were formed by injecting the ethanol-lipid solution (10 mg/ml) into the first inlet and an aqueous buffer (300 mM ammonium sulfate) into the second inlet of the SHM micromixer using syringe pumps (KD200; KD Scientific, Holliston, MA). The appropriate flow rate ratios (FRR, ratio of aqueous stream volumetric flow rate to ethanolic volumetric flow rate) were set by maintaining a constant flow rate in the ethanolic channel (typically 1 ml/min) and varying the flow rates of the aqueous channel (typically 3 ml/min). For formulations composed of saturated phospholipids (DPPC and HSPC), a syringe heater (New Era Pump Systems Inc., Farmingdale, NY) was used to maintain the temperature in both ethanolic and aqueous solutions approximately ten degrees above the gel-to-liquid-crystalline phase transition temperature (T_c) of the phospholipid (50 °C for DPPC and 65 °C for HSPC). For radiolabeled formulations, the non-exchangeable, non-metabolized lipid marker [^{3}H]CHE (Charrois & Allen, 2004) was added to the ethanolic lipid mixture (2 μCi/μmol lipid). Aqueous dispersions of LNPs formed this way were collected from the outlet stream and dialyzed against 300 mM ammonium sulfate to remove the residual ethanol; then concentrated to 10 mg/ml lipid with the use of the Amicon Ultra-15 centrifugal filter units (Millipore, Billerica, MA). Prior to and after ethanol removal, LNPs were sized by dynamic light scattering (DLS) using Malvern Zetasizer Nano ZS (Malvern, UK) following diluting of the sample aliquot with phosphate-buffered saline (PBS). An ammonium sulfate gradient required for the drug loading was generated by exchanging the extravesicular solution with PBS (154 mM NaCl, pH 7.4) by dialysis.

Loading of doxorubicin into LNP

Doxorubicin hydrochloride was dissolved in saline at 5 mg/ml (trace amounts of [14-^{14}C]doxorubicin were added for radiolabeled formulations) and mixed with the ammonium sulfate-containing LNP dispersion to give the desired drug-to-lipid molar ratios. The samples were then incubated at 60 °C for 30 min to provide optimal loading conditions. Untrapped doxorubicin was removed by running the samples over Sephadex G-50 spin columns prior to detection of entrapped drug.

Doxorubicin was assayed by fluorescence intensity (excitation and emission wavelengths 480 and 590 nm, respectively) with a Perkin-Elmer LS50 fluorimeter (Perkin-Elmer, Norwalk, CT), the value for 100% release was obtained by the addition of 10% Triton X-100 to a final concentration of 0.5%. Phospholipid and cholesterol concentrations were determined by an enzymatic colorimetric method employing the standard assay kits (Wako Chemicals, Richmond, VA). Loading efficiencies were determined by quantitating both drug and lipid levels before and after separation of external drug from LNP encapsulated drug by size exclusion chromatography using Sephadex G-50 spin columns and comparing the respective drug/lipid ratios.

Cryo-TEM

Cyro-TEM samples were prepared by applying 3 μl LNP at 10−20 mg/ml total lipid to a standard electron microscopy grid with a perforated carbon film. Excess liquid was removed

from the grid by blotting and then the grid was plunge-frozen in liquid ethane to rapidly freeze the sample using a Vitrobot system (FEI, Hillsboro, OR). Images were taken under cryogenic conditions at a magnification of 50 000 × with an AMT HR CCD side mount camera. Samples were loaded with a Gatan 70 degree cryo-transfer holder in an FEI G20 Lab6 200 kV TEM (FEI, Hillsboro, OR) under low-dose conditions with an under-focus of 4–6 μm to enhance image contrast. Experiments were performed at the University of British Columbia Bioimaging Centre (Vancouver, BC). Mean particle diameters were determined by measuring 120 LNPs for each sample.

In vitro assay for drug retention

In vitro release experiments were carried out by diluting LNP formulations with release buffer containing ammonium chloride (50 mM NH_4Cl, 154 mM NaCl), the diluted samples were then placed into dialysis tubing (12–14 K MW cut off) and dialyzed against release buffer at 37 °C. Aliquots at various time points were taken and concentrations of entrapped drug were determined following removal of released drug by running the aliquots over Sephadex G-50 spin columns. The % retention was defined as the drug-to-lipid ratio at time *t* divided by the initial drug-to-lipid ratio.

In vivo drug release

About 6- to 8-week old female CD1 outbred mice were obtained from Charles River Laboratories (Wilmington, MA) and acclimated for 1 week prior to use. Mice were injected with doxorubicin (containing trace amounts of ^{14}C-labeled doxorubicin) encapsulated in [^3H]CHE-labeled LNPs via the lateral tail vein (50 mg/kg lipids). At 1, 4, and 24 h post-injection, mice were euthanized (four mice per time point). Blood was collected via intracardiac sampling in Vacutainer (BD Biosciences, Mississauga, Canada) tubes containing EDTA. Plasma was separated from the whole blood by centrifugation at 1000*g* for 10 min; 50 μL of plasma was then added to scintillation fluid (PicoFluor 40, Perkin-Elmer, Boston, MA) and the drug and lipid concentrations were determined by dual label scintillation counting using a Beckman LS6500 counter (Perkin-Elmer, Boston, MA). The results (normalized drug to lipid ratios) were plotted as log percent versus time, and the $t_{1/2}$ was calculated using the regression line from the linear portion of the curve.

All procedures involving animals were approved by the Animal Care Committee at the University of British Columbia and performed in accordance with the guidelines established by the Canadian Council on Animal Care.

Results

In a previous work (Zhigaltsev et al., 2012), we demonstrated that addition of cholesterol results in a size increase for POPC-based non-PEGylated limit-size systems. As the presence of cholesterol is an essential factor in decreasing the permeability of liquid-crystalline bilayers, it was of interest to determine whether PEGylated lipids such as DSPE-PEG2000 can be used to control the size of POPC/Chol systems produced by microfluidic mixing, aiming at a 30–40 nm size range. Also, it was clearly of interest to determine whether this technique can be extended to systems based on saturated phosphatidylcholines, which also require incorporation of cholesterol and PEGylated components in order to achieve long circulatory lifetimes (Allen et al., 1991; Semple et al., 1996). The limit-size LNP formulations resulting were characterized in terms of their immediate and long-term stability, correspondence to the ''ultra-small'' size criteria, and ability to load and retain the drug cargo.

Generation of limit-size vesicular LNPs of various sizes and lipid compositions

Size characteristics (means of three repeat measurements; standard deviations in brackets) of LNP systems formed by microfluidic mixing are presented in Table 1. It may be noted that, as observed elsewhere (Chen et al., 2014; Belliveau et al., 2012; Zhigaltsev et al., 2012), when sizing LNP dispersions, number-weighted DLS values usually show a closer correspondence to sizing data obtained from cryo-TEM micrographs than *Z*-average means. These observations are consistent with the data presented here (see ''Cryo-TEM'' section); for that reason, number-weighted DLS size determinations are used throughout this report.

As shown in Table 1, the smallest systems obtained (~22 nm diameter) were composed of an unsaturated lipid (POPC or SOPC), Chol, and DSPE-PEG2000 at a molar ratio of 65/35/3. Increasing the cholesterol content above 40% mol resulted in a noticeable increase in size and polydispersity. There was no difference between sizing data obtained before and after the removal of ethanol. However, that was not the case when saturated phospholipids were used to replace POPC and SOPC. Although apparently small systems were formed as a result of rapid mixing at temperatures above the gel-to-liquid-crystalline phase transition, initially optically clear dispersions of DPPC/Chol/DSPE-PEG2000 and HSPC/Chol/DSPE-PEG2000 turned turbid within minutes after formation, indicating temperature-triggered aggregation and fusion into larger vesicles. Interestingly, resulting HSPC-based systems were smaller and less heterogeneous than DPPC-based ones, although still exceeding the 40 nm boundary set as an upper limit for the ''ultra-small'' systems developed in this work. However, partial replacement of POPC with DPPC (up to 20 mol%) resulted in somewhat larger (~33 nm) but still relatively homogeneous (PDI 0.13) and stable systems.

All formulations described above were formed at a total flow rate of 4 ml/min and FRR 3/1 (aqueous to ethanol). Further increases in the flow rate and/or FRR did not result in the formation of smaller particles (results not shown), indicating that ''limit-size'' conditions have been achieved.

Drug loading and *in vitro* release studies

The next set of experiments was aimed to see whether drugs can be loaded and stably retained in these extremely small LNP systems. The following studies employed systems containing unsaturated lipid (POPC) in the absence and the presence of DPPC; as DPPC and HSPC-based systems were unstable, they were excluded from further studies. Doxorubicin, a widely used antineoplastic agent, was chosen

Table 1. Sizes of LNP formulations prepared in 300 mM ammonium sulfate as evaluated by DLS. Measurements were taken after ethanol removal.

Lipid composition	Mean diameter, number-weighted (nm)	*Z*-average means (nm)	Polydispersity index (PDI)
POPC/Chol/DSPE-PEG2000 (65/35/3)	22 (1.8)	34 (2.2)	0.13 (0.02)
SOPC/Chol/DSPE-PEG2000 (65/35/3)	23 (2.0)	35 (2.2)	0.13 (0.02)
POPC/Chol/DSPE-PEG2000 (55/45/3)	35 (3.3)	78 (6.0)	0.25 (0.06)
DPPC/Chol/DSPE-PEG2000 (65/35/3)	>100	>200	>0.3
HSPC/Chol/DSPE-PEG2000 (65/35/3)	57 (4.0)	92 (5.5)	0.15 (0.02)
POPC/DPPC/Chol/DSPE-PEG2000 (55/10/35/3)	29 (2.1)	40 (3.1)	0.11 (0.04)
POPC/DPPC/Chol/DSPE-PEG2000 (45/20/35/3)	33 (2.2)	44 (3.0)	0.135 (0.03)

The standard deviation values from the repeat measurements are shown in brackets.

as a model drug as it has been shown to be readily accumulated into bilayer liposomal systems (80–100 nm size range) in response to an ammonium sulfate gradient (Haran et al., 1993). In an earlier report, it was shown that 20-nm diameter POPC systems could be loaded with doxorubicin with drug/lipid ratios up to 0.2 mol/mol (Zhigaltsev et al., 2012); however, subsequent experiments (data not shown) demonstrated that they lost their cargo within minutes of entering the bloodstream. This behavior was attributed to the lack of cholesterol which is required to retain drug in LNP systems (Semple et al., 1996). Thus, it was of interest to load doxorubicin into cholesterol-containing limit-size LNPs, with ensuing release studies to identify compositions that led to adequate drug retention.

A standard loading procedure (see ''Materials and methods'' section) was applied to POPC/Chol/DSPE-PEG2000 (65/35/3), SOPC/Chol/DSPE-PEG2000 (65/35/3), POPC/Chol/DSPE-PEG2000 (55/45/3), POPC/DPPC/Chol/DSPE-PEG2000 (55/10/35/3), and POPC/DPPC/Chol/DSPE-PEG2000 (45/20/35/3) systems exhibiting an ammonium sulfate gradient. Approximately 100% drug uptake was observed at a drug/lipid ratio of 0.1 mol/mol, with no significant size increase. The retention properties of the loaded formulations were then assayed using an *in vitro* release test employing incubation of a sample aliquot placed in ammonium chloride-containing buffer (Johnston et al., 2008; Zhigaltsev et al., 2006). Ammonium chloride acts to dissipate the pH gradient across the liposome membrane, thus raising the interior pH which results in more of the entrapped drug adopting the deprotonated (neutral) form, which is the more membrane permeable form. This increases the rate of drug release to an experimentally convenient time scale. As shown in Figure 1, the fastest rate of release was observed for the smallest systems (22 nm POPC/Chol/DSPE-PEG2000), substitution of SOPC for POPC did not lead to improved retention (results not shown). POPC/Chol/DSPE-PEG2000 (55/45/3) and POPC/DPPC/Chol/DSPE-PEG2000 (45/20/35/3) systems demonstrated comparable retention, however, the much larger size heterogeneity of the system that did not contain DPPC (as evidenced by *Z*-average and PDI values) makes it less preferable.

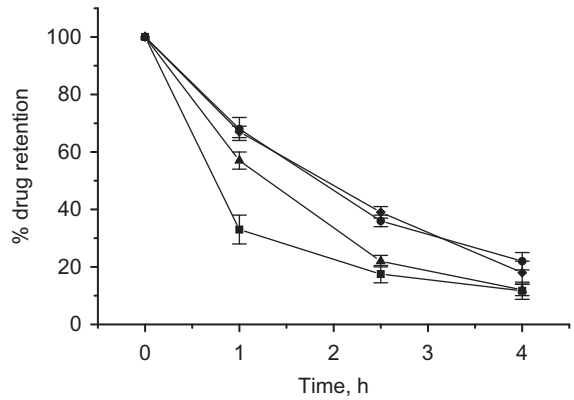

Figure 1. *In vitro* release of doxorubicin loaded into 22 nm POPC/Chol/DSPE-PEG2000 65/35/3 (squares), 35 nm POPC/Chol/DSPE-PEG2000 55/45/3 (circles), 29 nm POPC/DPPC/Chol/DSPE-PEG2000 55/10/35/3 (triangles), and 33 nm POPC/DPPC/Chol/DSPE-PEG2000 45/20/35/3 (diamonds) systems using ammonium sulfate gradient at drug-to-lipid ratio 0.1 mol/mol. Release was measured in the presence of 50 mM ammonium chloride at an incubation temperature 37 °C. Aliquots were taken at different time points for the determination of the lipid and drug concentrations as described in ''Materials and methods'' section. The % of retention was defined as the drug-to-lipid ratio at time *t* divided by the initial drug-to-lipid ratio. The results represent the mean values ± SD.

In vivo release studies

Having identified the most retentive formulations *in vitro*, *in vivo* release studies were undertaken in order to compare the retention properties of 33 nm LNP composed of POPC/DPPC/Chol/DSPE-PEG2000 (45/20/35/3) and 22 nm LNP composed of POPC/Chol/DSPE-PEG2000 (65/35/3). CD1 mice were injected intravenously with doxorubicin-loaded formulations containing trace amounts ^{14}C-labeled doxorubicin (drug marker) and [^{3}H]CHE (non-exchangeable lipid marker). The percentage of drug remaining in the LNP (drug retention) was calculated by dividing the drug/lipid ratios found in plasma samples by those of the injected LNP formulations, taken as 100%. The resulting *in vivo* drug retention profiles are shown in Figure 2, where POPC/DPPC/Chol/DSPE-PEG2000 systems released the drug with a halftime of about 12 h with ~30% of the drug remaining entrapped 24 h post-injection. In contrast, POPC/Chol/DSPE-PEG2000 vesicles displayed substantially faster release over the 24 h time course ($t_{1/2} \sim 1$ h). Quantitation of LNP lipid recovered in plasma demonstrates that the rates of elimination were similar for both systems (Figure 3), indicating that the difference in their ability to retain drug cannot be attributed to the differences in their clearance rates. These data are in a good agreement with the results of the *in vitro* release data and demonstrate that the drug can be efficiently retained in sub-40 nm vesicular LNPs *in vivo*.

J Liposome Res, 2016; 26(2): 96–102

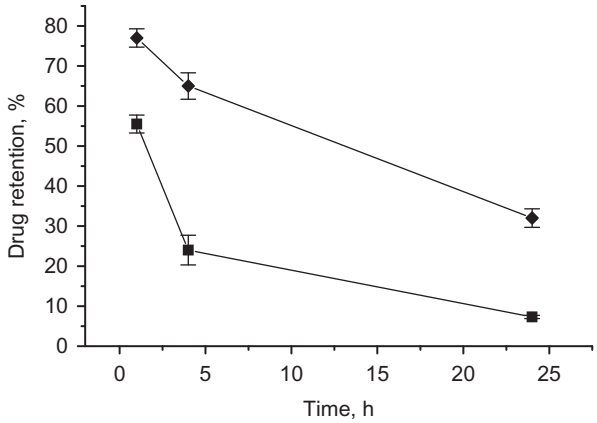

Figure 2. Drug retention in 22 nm POPC/Chol/DSPE-PEG2000 65/35/3 (squares) and 33 nm POPC/DPPC/Chol/DSPE-PEG2000 45/20/35/3 (diamonds) systems determined *in vivo*. LNP formulations containing trace amounts of the tritiated lipid [^{3}H]-CHE were loaded with ^{14}C-labeled doxorubicin at a drug-to-lipid ratio 0.1 mol/mol and then injected intravenously into CD1 mice at a lipid dose of 50 mg/kg. Plasma samples taken at the indicated time points were analyzed for lipid and drug content by liquid scintillation counting as described in ''Materials and methods'' section. Each data point represents mean values ± SD from each group of mice ($n = 4$).

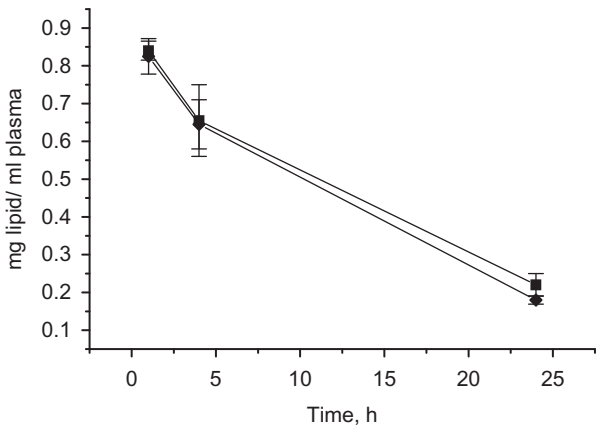

Figure 3. Liposomal lipid levels obtained in plasma of CD1 mice injected with 22 nm POPC/Chol/DSPE-PEG2000 65/35/3 (squares) and 33 nm POPC/DPPC/Chol/DSPE-PEG2000 45/20/35/3 (diamonds) systems. Lipids were quantified as indicated in Figure 2 and ''Materials and methods'' section. Each point represents mean values ± SD from each group of mice ($n = 4$).

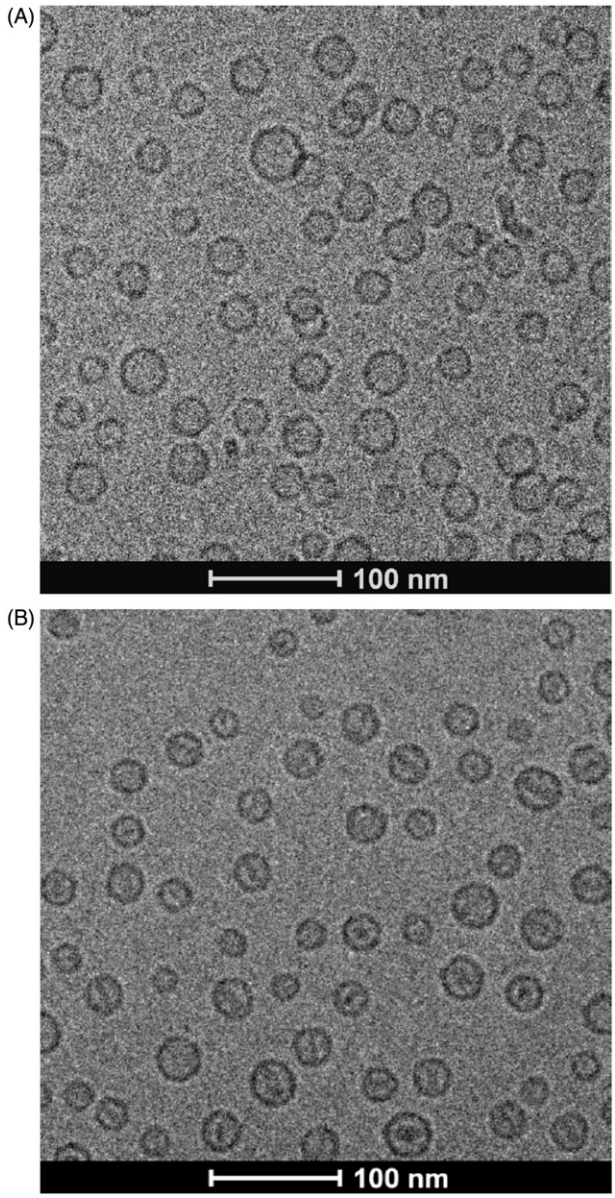

Figure 4. Cryo-TEM micrographs of LNP composed of POPC/DPPC/Chol/DSPE-PEG2000 (45/20/35/3) prior to (A) and after (B) loading with doxorubicin at a drug-to-lipid ratio 0.1 mol/mol. The bar represents 100 nm. For details of sample preparation and cryo-TEM protocols, see ''Materials and methods'' section.

Cryo-TEM study

Figure 4 shows representative images of ''empty'' (A) and doxorubicin-loaded (B) POPC/DPPC/Chol/DSPE-PEG2000 (45/20/35/3) LNPs. As seen, doxorubicin loading at a 0.1 mol/mol ratio did not affect the predominantly spherical shape of the pre-loaded vesicles; a characteristic ''coffee-bean'' appearance of the internally precipitated drug can be observed that is similar to the appearance of larger liposomal doxorubicin formulations such as Doxil®. The cryo-TEM micrographs also provide size information that can be used to validate the sizes determined by the light scattering technique. A size analysis based on a sample of ~120 particles indicated mean diameters of 33 ± 4 nm (mean ± SD) for both empty and loaded LNPs, in good agreement with the number-weighted size values determined by DLS.

Long-term stability

In a final area of investigation, a long-term (up to 6 months) stability of doxorubicin-loaded POPC/DPPC/Chol/DSPE-PEG2000 (45/20/35/3) systems stored at 4 °C has been studied. LNPs were loaded with drug at 0.1 mol/mol drug-to-lipid ratio and concentrated to 10 mg/ml total lipid. No significant changes in mean LNP size and drug entrapment were observed during the course of the study (results not shown).

Discussion

As pointed out in the ''Introduction'' section, two approaches to formation of ultra-small vesicular LNP systems can be taken, namely ''top down'' size reduction methods which usually are harsh procedures requiring high energy input such

as sonication, and more gentle ''bottom up'' approaches that achieve self-assembly of the particles from the solubilized lipids by increasing the polarity of the organic solvent containing solubilized lipid. The SHM-aided microfluidic formulation procedure described here offers several advantages not only over ''top down'' methods but also over previously developed ''bottom up'' techniques employing mixing at macro- and micro scales, such as hydrodynamic flow focusing (Jahn et al., 2007, 2010). The results presented here demonstrate the ability of the SHM to produce limit size LNP systems where the size can be controlled over the 20–40 nm size range by variation of the lipid composition and that can exhibit therapeutically relevant levels of drug retention.

This study was performed to identify lipid compositions that lead to adequate retention of amino containing drug in sub-40 nm vesicular LNP systems. In this regard, three major factors that influence retention in bilayer LNP systems are size, cholesterol content, and the presence of a saturated lipid such as DPPC. The high membrane curvature of smaller systems may be expected to lead to greater membrane permeability. Second, it is known that cholesterol is required to stabilize bilayer vesicles *in vivo*, particularly smaller systems (Semple et al., 1996). Finally, the presence of more saturated, long-chain phospholipids, in the presence of cholesterol, is associated with increased stability and reduced membrane permeability (Bally et al., 1990; Mayer et al., 1989; Semple et al., 1996). All those factors may contribute to the relatively stable *in vivo* drug retention displayed by the POPC/DPPC/Chol/DSPE-PEG2000 (45/20/35/3) system. The ability of this system to retain drug cargo while exhibiting sufficiently long blood circulation residence times to enable accumulation at disease sites such as sites of inflammation, infection, and tumors due to the enhanced permeation and retention (EPR) effect (Maeda et al., 2000) makes it well suited for use in therapeutic applications. Further, the very small size of these systems may be expected to enhance penetration into target tissue such as tumors.

Conclusion

We have shown that the microfluidic-based procedure for generating limit-size bilayer LNP drug carriers with diameters less than 50 nm satisfy basic criteria necessary to establish them as pharmaceuticals (ease and scalability of formulation, ability to load and stably retain drug cargo, reasonable payout rates *in vivo*, apparent long-term stability). Further the small size of these limit-size systems should enhance drug potency due to deeper penetration into target tissue.

Declaration of interest

P. R. C. has an ownership position in Precision NanoSystems. This research work was supported by the Cancer Research Society and Canadian Institutes for Health Research (CIHR FRN 111627).

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