CHAPTER 2

MICROEMULSION EXTRUSION TECHNIQUE: A NEW METHOD TO PRODUCE LIPID NANOPARTICLES

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Abstract. Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) have been intensively investigated for different applications, including their use as drug and gene delivery systems. Different techniques have been employed to produce lipid nanoparticles, of which high pressure homogenization is the standard technique that is adopted nowadays. Although this method has a high efficiency, does not require the use of organic solvents, and allows large-scale production, some limitations impede its application at laboratory scale: the equipment is expensive, there is a need of huge amounts of surfactants and co-surfactants during the preparation, and the operating conditions are energy intensive. Here we present the microemulsion extrusion technique as an alternative method to prepare lipid nanoparticles. The parameters to produce lipid nanoparticles using microemulsion extrusion were established, and the lipid particles produced (SLN, NLC and liposomes) were characterized with regard to size (from 130 to 190 nm), zeta potential, and drug (mitoxantrone) and gene (pDNA) delivery properties. In addition, the particles’ in vitro co-delivery capacity (to carry mitoxantrone plus pDNA encoding the phosphatase and tensin homologue, PTEN) was tested in normal (BALB 3T3 fibroblast) and cancer (PC3 prostate and MCF-7 breast) cell lines. The results show that the microemulsion extrusion technique is fast, inexpensive, reproducible, free of organic solvents, and suitable for small volume preparations of lipid nanoparticles. Its application is particularly interesting when using rare and/or costly drugs
or ingredients (e.g. cationic lipids for gene delivery or labeled lipids for nanoparticle tracking/diagnosis).

2.1 Introduction

Solid lipid nanoparticles (SLN) and their second generation, called nanostructured lipid carriers (NLC), have been firstly described in the 90’s [1]. They have no inner aqueous compartments and their lipid content is higher than that of liposomes, the most well-known lipid-based drug delivery system [2,3]. SLN and NLC have been used as drug delivery systems [4-7], gene delivery systems [8-11], and several of these applications have been subject of patents [12,13]. The use of lipid nanoparticles presents advantages over that of polymeric nanoparticles, since their production is water based (avoiding use of organic solvents) and most lipids used in the formulations are biodegradable, which guarantees an excellent biocompatibility. In addition, the standard method (high pressure homogenization) for SLN and NLC production is already successfully implemented in pharmaceutical industry [14-16].

Several techniques have been applied to produce lipid nanoparticles [12,15,17-19], and new methods are being reported, such as coacervation method and Mumper and Jay method [19-21]. Among those the high pressure homogenization technique (HPH), described by Müller and Lucks [22,23], is the standard method to produce lipid nanoparticles. Through the high pressure (100-2000 bar) and high shear stress generated by the homogenizer particle size can be reduced to the desired sub-micron range [24-26]. HPH is highly efficient, it does not require the use of organic solvents, and it allows lipid nanoparticles production at large-scale. On the other hand, the equipment has a significant capital cost, there is a need of large amounts of surfactants/co-surfactants during the nanoparticles preparation, and the operating conditions are energy intensive [27]. Such pitfalls of HPH are especially critical when only small sample volumes are desirable, for example, in preformulation studies or with rare and/or expensive ingredients.

As an alternative to HPH, we propose the use of the microemulsion extrusion technique to produce lipid nanoparticles. The microemulsion extrusion technique is fast, inexpensive, reproducible, free of organic solvents, and suitable for small volumes [28]. This method is particularly interesting when using rare or costly compounds, such as certain drugs, targeting molecules, and nanoparticle components (e.g. cationic lipids). Since small-volumes extrusion is an established method to prepare liposomal formulations [28] we have included liposomal formulations in our investigation of SLN and NLC particles for the codelivery of drugs and genes. First, we have optimized the production of lipid nanoparticles by extru-
sion, using a mini-extruder apparatus. Second, the lipid particles prepared were characterized by dynamic light scattering (DLS) and tested for their use as drug (mitoxantrone) and gene (pDNA containing the phosphatase and tensin homolog, PTEN construct) delivery systems for cancer therapy. Mitoxantrone inhibits DNA duplication in the S phase of the cell cycle [2] while PTEN is related to tumor suppression and phosphoinositide-3 kinase (PI3K) signal transduction, being deleted or dysfunctional in prostate and breast cancer [4]. The formulations capacity to affect cell viability in vitro was tested in normal (BALB/3T3 fibroblasts) vs. cancer (PC3 prostate, and MCF-7 breast) cells.

2.2 Material and Methods

2.2.1 Materials

Egg phosphatidylcoline (EPC), Pluronic F68 (PLF68), mitoxantrone and stearic acid (SA) were purchased from Sigma-Aldrich (St. Louis, MO). N-[1-(2,3-dioleoyloxy) propyl]-N,N,N-trimethyl-ammonium chloride (DOTAP) was obtained from Avanti Polar Lipids (Alabaster, AL) and glyceryl monostearate (GMS) was kindly donated by Croda (Campinas, Brazil). pTrcHis Topo TA (Invitrogen, Carlsbad, CA) expression vector containing the sequence of the PTEN was kindly provided by M. Peppelenbosch, Erasmus University Rotterdam.

2.2.2 Microemulsion Extrusion method

The composition of SLN, NLC and liposomes are given in Table 2.1. To prepare the formulations, we used an adaption of the microemulsion method followed by extrusion through a 100-nm polycarbonate membrane (Millipore, Darmstadt, Germany) using an Avanti mini-extruder (Avanti Polar Lipids, Inc., Alabaster, AL). Briefly, a solution of Pluronic F68 (surfactant) and DOTAP (co-surfactant) (pre-heated above the melting temperature of the solid lipid used) was added to the melted lipid followed by homogenization using vigorous stirring. For some samples sonication was necessary to produce a homogeneous suspension before extrusion. Then, the hot emulsion was loaded into the donor syringe and extruded 15 times, unless otherwise stated, through the double-syringe extruder (Fig. 2.1), always finishing at the receiver syringe, to avoid contamination. A heating block, pre-heated above the melting temperature of the lipid, warmed the system during the whole process. Finally, the lipid nanoparticles were transferred to an ice bath and then stored at 4 °C. A systematic study was performed to test different production parameters, such as the number of extrusion cycles (0, 5, 10, 15, 20, 25 and 30 times), and temperature (5, 10 or 15 °C above the melting point of the solid lipid, being 58-59 °C for GMS and 69.6
<table>
<thead>
<tr>
<th>Formulations</th>
<th>Stearic Acid (mM)</th>
<th>DOTAP (mM)</th>
<th>Pluronic F68 (mM)</th>
<th>GMS (mM)</th>
<th>EPC (mM)</th>
<th>Size (nm)</th>
<th>Zeta Potential (mV)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPC Lipo-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>113.3 ±4.68</td>
<td>2.90 ±0.43</td>
<td>0.101 ±0.001</td>
</tr>
<tr>
<td>NLC</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>5</td>
<td>3</td>
<td>115.7 ±8.07</td>
<td>1.30 ±0.42</td>
<td>0.131 ±0.001</td>
</tr>
<tr>
<td>DOTAP Lipo-</td>
<td>-</td>
<td>2.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>189.5 ±0.76</td>
<td>57.60 ±3.39</td>
<td>0.127 ±0.009</td>
</tr>
<tr>
<td>SLN</td>
<td>7</td>
<td>2.5</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>135.9 ±1.31</td>
<td>40.65 ±0.49</td>
<td>0.164 ±0.007</td>
</tr>
</tbody>
</table>

Each value represents the mean ±SD (n = 3).
°C for SA, used in the formulation). Of note, to avoid extruder membrane disruption 10 mM was the maximum lipid concentration used in our studies. This concentration may vary when other lipid mixtures are used and also depends on the type of formulation (liposomes vs. SLN vs. NLC) that is produced. Second, to guarantee that the complete system has reached the same temperature, the heating block, the lipid and the aqueous phases should be heated to the desired temperature, at least 15 min. before the extrusion.

2.2.3 Particle size and zeta potential measurements

The hydrodynamic diameters, polydispersity index (PDI) and zeta potentials of the lipid nanoparticles and liposomes were measured using dynamic laser light scattering on a Malvern Zetasizer Nano system (Malvern Instruments Ltd, Worcestershire/UK) at 25 °C. One hundred microliters of the formulations were diluted in 1 ml deionized water prior to the measurement.

2.2.4 Lyophilization studies

Lipid nanoparticles were diluted (75 µL of sample in 1.5 mL of deionized water) and freeze dried in the absence or presence of a cryoprotector (trehalose or sucrose, at a final concentration of 4 wt/v %). The resulting dispersions were frozen in liquid nitrogen, and subsequently lyophilized using a Freezone 4.5 (Labconco, USA) for 48 hours under vacuum. Lyophilized samples were reconstituted in deionized water and their size, zeta potential, and PDI were measured and compared with freshly prepared (non-lyophilized) samples.

2.2.5 Morphology Study using Transmission Electron Microscopy (TEM)

The TEM grid was exposed to a droplet (10 µL) of each formulation for 10 s and let to air-dry. After that, negative staining was performed to improve the contrast: the TEM grid was exposed to a droplet (10 µL) of uranyl acetate solution (1 % wt, pH 4) for 10 s. Then, to avoid over staining, the grid was placed in a droplet of water for a few seconds and the excess of fluid was removed with a filter paper. Subsequently, the sample was analyzed using a JEOL JEM-2010HC transmission electron microscope (JEOL Co. Ltd., Tokyo, Japan).
2.2.6 Lipid nanoparticles entrapment efficiency

Lipid nanoparticles were prepared as previously described with addition of mitoxantrone (MTX, 5 mM) in the lipid phase. For liposomal formulations, an aqueous solution containing MTX was used to suspend the lipids. The entrapment efficiency (% EE) of MTX into lipid nanoparticles was determined by ultrafiltration method using centrifugal filter tubes with a molecular weight cut-off of 10 kDa (Amicon Ultra, Millipore, Ireland) at 3,550 × g for 5 min. The concentration of MTX in lipid nanoparticles and in the filtrate ($f_{\text{free}}$MTX) was analyzed at 672 nm, using a Cary® 50 UV-Vis spectrophotometer (Varian, USA) and quantified using equation for developed analytical curve ($\text{absorbance} = 0.0975 \times \text{concentration (M)} + 0.1525, r = 0.9982$). %EE was calculated according to:

$$\%\text{EE} = \frac{\text{totalMTX} - f_{\text{free}}\text{MTX}}{\text{totalMTX}} \times 100 \quad (2.1)$$

2.2.7 Drug Release assay

MTX release was determined using Franz diffusion cells (1 ml in donor compartment and 4 ml in acceptor compartment). Membranes of 12-14 kDa (Spectrumlab, EUA) molecular weight cut off were used and PBS 5 mM (pH 7.4) was set as the release medium. Sample was diluted to 5 µmoles of MTX (in solution or in lipid nanoparticles) and placed in the donor compartment (1 mL). The concentration of MTX in the acceptor compartment was quantified at 672 nm using a Cary® 50 UV-Vis spectrophotometer (Varian, USA) at specified time points (1, 2, 3, 4, 5, 6, 7, 8, 24, 48 and 72 h). The acceptor compartment was homogenized using a magnetic stirrer. The drug concentration was quantified using the equation of a previously determined analytical curve ($\text{absorbance} = 0.0975 \times \text{concentration (M)} + 0.1525, r = 0.9982$).

2.2.8 Plasmid Purification

The pTrcHis Topo TA (Invitrogen, Carlsbad, CA) expression vector containing the sequence of the PTEN gene was transformed into Escherichia coli. The bacteria were incubated in Luria-Bertani (Bertani, 2004) medium (10 g tryptone, 5 g yeast extract and 10 g NaCl was dissolved in 1 liter of distilled water and the pH was adjusted to 7.0 with 1 M NaOH; then the mixture was autoclaved for 25 min at 120 °C) in presence of the selective antibiotic ampicillin. Later, the plasmid was purified using GenElute™ HP Plasmid Maxiprep Kit (Sigma-Aldrich Corporation) according to manufacturer’s instructions.
2.2.9 DNA binding assay and DNase I protection assay

For DNA binding, complexes of DNA and lipid nanoparticles or liposomal formulations were prepared using 1 µg of pDNA and 2 µL of the carrier (EPC liposomes, SLN, DOTAP liposomes and NLC) in water. After 20 min of incubation at room temperature, the samples were loaded in 10% glycerol on a 0.8% agarose gel containing ethidium bromide (0.5 µg/ml), and immersed in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM ethylenediaminetetraacetic acid, EDTA). The samples were run at 50 V for 2 h. Images were taken using a UV trans illuminator and processed using ImageJ software (http://imagej.nih.gov/ij/). For DNase I protection assay complexes of DNA and lipid carriers were prepared as described above for the DNA binding assay. Then, samples were exposed to DNase I (1 unit DNase/2.5 µg pDNA) at 37 °C for 30 min. After that the samples were treated with 1% sodium dodecyl sulfate (SDS) to release DNA from the lipoplexes, and submitted to agarose gel electrophoresis and analyzed, as described for the DNA binding assay.

2.2.10 Cell culture

BALB/3T3 and MCF-7 cells were maintained in Dulbecco’s Modified Eagle’s Medium, DMEM (Sigma Chemical Co.), while PC3 cells were maintained in RPMI 1640 culture medium. DMEM and RPMI 1640 culture media were supplemented with 10% (v/v) Fetal Bovine Serum and 1% (v/v) Penicillin/streptomycin. Cells were grown at 37 °C under a humidified atmosphere with 5% CO₂ in air and subcultured every 2 days using trypsin/EDTA. Mitoxantrone-resistant MCF-7 cells were cultured in medium containing 100 nM of MTX [8].

2.2.11 Cytotoxicity

The cytotoxicity of the lipid nanoparticles formulations was tested on mouse embryonic fibroblasts (BALB/3T3), prostate cancer cells (PC3) and human mammary cancer cells (MCF-7). The cells were plated at a concentration of 10⁴ cells per well in 96-well plates and incubated (37 °C and 5% CO₂) overnight for adhesion. Then, the cells were treated for 24 h with lipid nanoparticles formulations containing the drug (10 µM of MTX), the plasmid containing PTEN gene, or double-loaded with drug and gene, in RPMI 1640 medium without serum. After that, the medium was replaced with 100 µL of 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT solution – 0.5 mg/ml) for 4 h incubation at 37 °C; following removal of the MTT solution, the formazan crystals formed were dissolved in 100 µL of DMSO. The solutions were homogenized for 5 min. on a plate shaker, and the absorbance was measured at 570 nm on a microplate.
ELX800 spectrophotometer (Biotek, Winooski/EUA). Cell viability was expressed as percentage of the untreated control.

2.2.12 Statistical analysis

All experiments were performed in triplicate and were repeated independently, at least three times. Mean and standard deviations were calculated. ANOVA followed by Tukey’s post-test were performed to test for statistical significance ($p < 0.05$) between the treatment groups.

2.3 Results and discussion

Microemulsion extrusion technique as a new method to produce lipid nanoparticles

Mini-extruder systems are commonplace in bioscience laboratories and have been used to prepare liposomes for many years. Here, we put forward the use of this instrument to prepare also lipid nanoparticles, specifically solid lipid nanoparticles and nanostructured lipid carriers. The formulation compositions tested are shown in Table 2.1. To establish the microemulsion extrusion as a new method to produce lipid nanoparticles, we have analyzed changes in two parameters: extrusion cycles and temperature. We studied the effect of such parameters on the physicochemical characteristics of the SLN and NLC produced and compared them to similar liposomal formulations.

Figure 2.1. Microemulsion extrusion method to prepare lipid nanoparticles. Step 1. The aqueous surfactant–cosurfactant solution (pre-heated above the melting temperature of the solid lipid) was added to the melted lipid followed by homogenization using vigorous stirring and avoiding changes in temperature. Step 2. The hot emulsion was loaded into the donor syringe and extruded several times, always finishing at the receiver syringe to avoid contamination. Extrusion was performed at a temperature above the melting temperature of the lipid, using a heating block. Step 3. The lipid nanoparticles were transferred to an ice bath and stored at 4 °C.
First, we determined the minimum number of extrusion cycles (Fig. 2.2) required to produce a monodisperse and homogeneous population of lipid nanoparticles. The number of extrusion cycles being defined as the number of passages of the hot emulsion from the donor syringe to the receiver syringe and back. As shown in Fig. 2.2A-C, after 10 extrusion cycles no statistical differences were found among the particles, regarding size, zeta potential and PDI, in comparison to particles produced with 30 extrusion cycles. Similar results were observed for the liposomal formulations. Therefore, we determined 15 extrusion cycles as the minimum number of extrusion cycles to guarantee homogeneous production of lipid nanoparticles.

The temperature effect on the production of the lipid nanoparticles regarding size, zeta potential and PDI was determined next. The SLN formulation (Fig. 2.4D) showed a decrease in particle size when prepared at +15 °C above the melting point of stearic acid, while the size of the NLC was not affected by increasing the temperature. Mehnert & Mäder [12] using HPH, have reported lower particle sizes in SLN prepared at higher temperatures, as a result of decreased viscosity in the inner particles phase. Fig. 2.2E also shows that the zeta potential of both formulations was not affected by the increase in temperature. Nevertheless, high temperatures increased SLN heterogeneity (Fig. 2.2F). At +10 °C and +15 °C, the PDI values for SLN were higher than 0.3. Interestingly, the NLC produced at all temperatures kept the PDI values below 0.2. A PDI value lower than 0.3 represents a homogenous and monodisperse particle population [14,16].
Figure 2.2. Optimization of process parameters for the production of lipid nanoparticles by microemulsion extrusion technique. LEFT: the effect of the number of extrusion cycles (0 - 30) on the production of EPC liposomes, NLC, DOTAP liposomes and SLN was evaluated regarding the A) average diameter B) zeta potential and C) PDI. Each value represents the mean ± S.D. (n = 3). The results are expressed as the mean ± SEM of three independent experiments, letters indicate significant differences as determined by one-way ANOVA followed by Tukey’s (p < 0.05) post-test in comparison to: “a” the EPC liposomal formulation after 30 cycles; “b” the NLC formulation after 30 cycles; “c” the DOTAP liposomal formulation after 30 cycles; “d” the SLN formulation after 30 cycles. RIGHT: the effect of temperature (+5 °C, +10 °C and +15 °C above the lipid melting point for SLN and NLC and +5 °C, +10 °C and +15 °C above room temperature for liposomes, which is well above the lipid melting point) used to produce EPC liposomes, NLC, DOTAP liposomes and SLN was also evaluated regarding the D) average diameter E) zeta potential and F) PDI. Each value represents the mean ± S.D. (n = 3). The results are expressed as the mean ± SEM of three independent experiments, letters indicate significant differences as determined by one-way ANOVA followed by Tukey’s (p < 0.05) post-test in comparison to: “a” the EPC liposomal formulation prepared at Tf +5 °C; “b” the DOTAP liposomal formulation prepared at Tf +5 °C; “c” the SLN formulation prepared at Tf +5 °C.
Also for the liposomes increasing the temperature resulted in larger and more dispersed vesicles. Both EPC and DOTAP liposomes presented a significant increase in their average diameter, and their PDI values were higher than 0.3 when prepared at +15 °C. Such instability agrees with previous reports in the literature [17] showing that high temperatures can affect the physicochemical characteristics of lipid vesicles. Taking into account the effects of temperature on particle size, zeta potential and PDI, we have established +5 °C above the lipid melting point as the optimum temperature during extrusion (15 cycles) in order to obtain homogeneous lipid nanoparticles.

In conclusion, it is shown that the microemulsion extrusion technique is a valid method to prepare (small amounts of) lipid nanoparticles.

Morphological and physicochemical comparison between lipid nanoparticles and liposomes produced by the microemulsion extrusion technique

After optimizing the production process of lipid nanoparticles, we have proceeded with the characterization of the formulations presented in Table 2.1. We initiated investigating the morphological differences between liposomes and lipid nanoparticles. It is known that the morphology and architecture of nanoparticles depends on their composition and preparation process [20]. Therefore, the morphology of SLN, NLC and liposomes was investigated using electron microscopy. Preparations of EPC and DOTAP liposomes (Fig. 2.3A and C, respectively) reveal quite homogeneous populations of vesicles, showing the existence of bilayer structures spaced by an internal (aqueous core) compartment. In contrast, the NLC and SLN preparations (Fig. 2.3B and D, respectively) show onion-like layered structures, as previously described for nanoparticles stabilized with saturated phospholipids in the \( \alpha \)-conformation [22]. Thus, while an aqueous core surrounded by a hydrophobic lipid bilayer characterizes liposomal structure, a high-density core characterizes both SLN and NLC lipid nanoparticles.
Figure 2.3. Transmission electron micrographies and long-term stability of lipid nanoparticles: A-D: morphological differences between the ultrastructure of lipid nanoparticles and liposomal formulations. A) EPC liposomes B) NLC, C) DOTAP liposomes, D) SLN (scale bars, 100 nm). Samples used here were prepared using the optimal conditions established: 15 extrusion cycles and +5 °C above the solid lipid melting point. The samples were staining with uranyl acetate to improve the contrast and investigated using a JEOL JEM-2010HC transmission electron microscope (JEOL Co. Ltd., Tokyo, Japan). E-G: the colloidal stability of EPC liposomes, NLC, DOTAP liposomes and SLN stored under refrigeration (4 °C) were followed up to 180 days. Regarding: E) diameter F) zeta potential and G) PDI. Each value represents the mean ±S.D. (n = 3). The measurements were carried using dynamic light scattering on a Malvern Zetasizer Nano system (Malvern Instruments Ltd, Worcestershire/UK), at 25 °C in polystyrene cuvettes with a path length of 10 mm.
Table 2.1 summarizes the physicochemical properties of lipid nanoparticles and liposomal formulations produced. EPC liposomes and NLC showed an average diameter of \(\sim 110\) nm, neutral zeta potential (\(\sim 0\) mV) and homogenous particle size distribution (PDI \(\sim 0.1\)). DOTAP liposomes and SLN had positive zeta potentials (\(> +40\) mV) and also presented a narrow size distribution (\(< 0.2\)). DOTAP liposomes (189.5 nm \(\pm 0.76\) nm) showed slightly bigger particles than SLN (135.9 nm \(\pm 1.31\) nm). Because nanoparticle stability is of remarkable importance for a successful therapeutic application, we investigated the stability of all lipid formulations, regarding size, zeta potential and PDI, over 180 days of storage under refrigeration. Liposomal particles underwent a significant increase in size after 150 days, whereas lipid nanoparticles were stable over the 180 days tested (Fig. 2.3E). The cationic (DOTAP) liposomes showed a decrease in the zeta potential after 90 days (Fig. 2.3F), which could compromise its ability to bind genetic material, because it is mediated by electrostatic interactions. In addition, the homogeneity of the liposomal formulations was compromised after 90 days (Fig 2.3G), as demonstrated by the increase in the PDI. Remarkably, the lipid nanoparticles (SLN and NLC) size, zeta potential and PDI values remained stable over the 180 days of storage.

Subsequently, it was tested whether the lipid nanoparticles and liposomal formulations could keep their physicochemical properties after freeze drying. Lyophilization of nanoparticles brings several pharmaceutical advantages, including the possibility for long-time storage, transportation at room temperature, and increased particle stability [24,26]. Nevertheless, this process exposes nanoparticles to freezing and drying stresses that by itself can cause changes in physical characteristics, e.g. due to water sublimation, eventually leading to particle aggregation. Indeed, as indicated in Table 2.2 the formulations that were lyophillized without cryoprotector showed a significant increase in size compared to freshly prepared lipid formulations (non-lyophillized), i.e. 30-50 fold for the neutral formulations (EPC liposomes and NLC) and 8-10 fold for the cationic formulations (DOTAP liposomes and SLN), the latter in accordance to the report of Das & Chaudhury (2011) showing that SLN lyophilization without cryoprotectant resulted in particle aggregation [3,29]. To prevent the aggregation of particles cryoprotectors can be used, which will also improve the re-dispersion of the dry product. Although the liposomes kept their original sizes following redispersion after lyophilization in the presence of cryoprotectant (trehalose and sucrose), their homogeneity was severely compromised (PDI \(> 0.3\)). Conversely, lipid nanoparticles only slightly increased their average diameter, when freeze dried with sucrose, and kept their size, zeta potential and homogeneity, when lyophilized with trehalose. Therefore, trehalose was demonstrated to be an effective cryoprotectant to avoid aggregation during lyophilization and subsequent reconstitution of these lipid nanoparticles.
Table 2.2. Physicochemical characterization of lipid nanoparticles (liposomes, SLN and NLC) that were freshly prepared (non-lyophilized) or reconstituted following lyophilization in the absence or presence of cryoprotectants.

<table>
<thead>
<tr>
<th></th>
<th>Non lyophilized</th>
<th>Lyophilized without cryoprotector</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean diameter (nm)</td>
<td>Zeta Potential (mV)</td>
</tr>
<tr>
<td>EPC liposomes</td>
<td>147.7 ±11.4</td>
<td>4.62 ±5.82</td>
</tr>
<tr>
<td>NLC</td>
<td>131.5 ±10.4</td>
<td>12.43 ±7.98</td>
</tr>
<tr>
<td>DOTAP liposomes</td>
<td>190.7 ±17.7</td>
<td>55.23 ±7.30</td>
</tr>
<tr>
<td>SLN</td>
<td>140.8 ±15.8</td>
<td>48.32 ±10.32</td>
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<tr>
<td>Lyophilized with threalose</td>
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<tr>
<td></td>
<td>Mean diameter (nm)</td>
<td>Zeta Potential (mV)</td>
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<tr>
<td>EPC liposomes</td>
<td>135.3 ±14.8</td>
<td>4.65 ±5.92</td>
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<tr>
<td>NLC</td>
<td>137.7 ±14.8</td>
<td>5.84 ±4.74</td>
</tr>
<tr>
<td>DOTAP liposomes</td>
<td>304.3 ±10.6*</td>
<td>60.29 ±14.73</td>
</tr>
<tr>
<td>SLN</td>
<td>147.3 ±21.0</td>
<td>58.88 ±7.38</td>
</tr>
</tbody>
</table>

Each value represents the mean ±SD (n = 3). The results are expressed as the mean ±SEM. Differences between non-lyophilized and lyophilized samples were determined by Student's t-test regarding the average diameter (*) or PDI (**).
Lipid nanoparticles produced by microemulsion extrusion as drug delivery system

Next we investigated the lipid nanoparticles produced as drug delivery system, taking mitoxantrone (MTX) as a model drug. MTX is a hydrophobic synthetic anthracenedione chemotherapeutic drug, extensively used for the treatment of several types of cancer, including breast cancer, prostate cancer, lymphoma, and leukemia. Several nanoparticle formulations containing MTX are reported [5-7,30-32]. First, the SLN and NLC capacity to entrap MTX was measured and compared to the entrapment efficiency of the liposomal formulations. Liposomal formulations showed low entrapment efficiency: EPC and DOTAP liposomes entrapment efficiency was 9.94% (±4.6%) and 14.05% (±4.1%), respectively. In contrast, lipid nanoparticles showed higher entrapment efficiency: NLC entrapment efficiency was 81.03% (±2.9%) and SLN entrapment efficiency was 63.95% (±3.8%).

Second, the MTX release profile from the formulations was assessed using a Franz diffusion cell. As depicted in Fig. 2.4, liposomal formulations displayed a release kinetic profile very similar to free MTX. Within the first 8 h, all the MTX was detected in the receptor compartment. Presumably, the drug is loaded into the lipid bilayer in liposomes; therefore fast equilibrium with the medium could be responsible for the fast release profile. Conversely, lipid nanoparticles displayed a significantly different release kinetic profile from free MTX and liposomes formulations. Even after 72 h, little MTX was detected in the receptor compartment (ca. 10.4% from SLN and 12.7% from NLC). In lipid nanoparticles, the drug is loaded in the lipid matrix, which may delay drug release, resulting in a sustained release system. Otherwise, in some cases the payload is expelled in one go from the lipid nanoparticles, this is called “burst effect”, and it is a highly undesirable feature found in some solid lipid nanoparticles formulations [9-11,33]. From a therapeutic perspective, it was interesting to note that lipid nanoparticles prepared here provided a sustained release of MTX, as previously described for lipid nanoparticles incorporation MTX [13,32].
Figure 2.4. Mitoxantrone release assay. Drug release profiles of MTX from EPC liposomes, NLC, DOTAP liposomes and SLN in phosphate-buffered saline pH 7.4 (mean ±SD, n = 3). The MTX release was measured using a Franz diffusion cells (1 ml in donor compartment and 4 ml in acceptor compartment) at specified time points (1, 2, 3, 4, 5, 6, 7, 8, 24, 48 and 72 h). Drug concentration was quantified according to the analytical curve (absorbance $= 0.0975 \times$ concentration (M) + 0.1525, r = 0.9982).

Lipid nanoparticles produced by microemulsion extrusion as gene delivery system

Having determined that lipid nanoparticles properly accommodate MTX and show a sustained release profile, we assessed whether these particles could also act as gene delivery system. First, the DNA binding capacity of the lipid nanoparticles was evaluated and compared with that of liposomal formulations (Fig. 2.5A). As expected, the formulations containing the cationic phospholipid DOTAP, i.e. DOTAP liposomes and SLN, were able to efficiently bind pDNA. On the other hand, formulations without DOTAP, i.e. EPC liposomes and NLC, presented no capacity to bind pDNA. The ability of these formulations to protect pDNA from DNase I action correlated with their DNA binding capacities (Fig. 2.5B). Specifically, the formulations containing DOTAP completely protected pDNA from DNase I, while DOTAP-free formulations could not protect against DNA degradation. Together, these
results support the idea that cationic lipid nanoparticles (SLN) and cationic (DOTAP) liposomes are able to carry pDNA.

Figure 2.5. DNA binding and protection by lipid nanoparticles and liposomal formulations produced by microemulsion extrusion. DNA binding assay and DNase I protection assay evaluated the potential for gene delivery of lipid nanoparticles and liposomal formulations. A) DNA binding assay for EPC liposomes, NLC, DOTAP liposomes and SLN. B) DNase I protection assay for EPC liposomes, NLC, DOTAP liposomes and SLN. Complexes of DNA and lipid nanoparticles or liposomal formulations were prepared using 1 µg of pDNA and 2 µL of carrier in water. After 20 min of incubation at room temperature, the samples were loaded in 10% glycerol on a 0.8% agarose gel containing 0.5 µg/ml ethidium bromide, and immersed in TBE buffer. The samples were run at 50 V for 2 h and images were taken using a UV trans illuminator. For DNase I protection assay, prior to submitting the samples to electrophoresis, complexes of DNA were exposed to DNase I (1 unit DNase/2.5 µg pDNA) for 30 min. at 37 °C.

Co-delivery of drugs and genes by lipid nanoparticles produced by microemulsion extrusion

Recently, co-delivery systems, which are able to carry drugs and genes together, have been described as a new strategy for cancer treatment [15,34,35]. Since the lipid nanoparticles produced here have drug and
gene delivery properties, we tested their co-delivery capacity on cancer cells.

The chemotherapeutic drug MTX and the phosphatase and tensin homolog (PTEN) tumor suppressor gene were chosen for delivery into cancer cells. MTX intercalates into DNA and binds topoisomerase II, thereby compromising cellular replication. Nevertheless, it provides modest survival and palliative benefits in advanced stages of cancer \([12,15,18,19,36]\). With that in mind, we tested MTX in association with the tumor suppressor PTEN, aiming for therapeutic benefits to the treatment of advanced stages of cancer. PTEN is downregulated in cancer cells, which has been shown to play an important role in advanced cancer \([4,19,21]\), amongst others by the development of resistance or reduced sensitivity to radiation and chemotherapy \([23,37]\). The combinatory effect of MTX and pDNA-PTEN in advanced cancer treatment, was tested on PC3 prostate cancer cells that lack PTEN, and MTX-resistant MCF-7 breast cancer cells \([8,25]\).

![Figure 2.6](image)

**Figure 2.6.** *in vitro* antitumor effect of MTX in 3T3 fibroblasts, prostate cancer cells, breast cancer cells and resistant breast cancer cells. BALB/3T3 cells, PC3 cells, MCF-7 and MCF-7 resistant were treated for 24 h with increasing concentrations of MTX and cell viability was assessed by MTT assay. The results are expressed as the mean ±SEM of three independent experiments.

**Delivery of MTX and pDNA-PTEN by liposomes and lipid nanoparticles: effect on cell viability**

To first determine the IC50 of free MTX to induce cell death, the cell viability of BALB/3T3 fibroblasts, PC3 prostate cancer cells, and MCF-7 resistant and non-resistant breast cancer cells in the presence of different concen-
trations of MTX, ranging from 0-100 µM, was assessed (Fig. 2.6). At a concentration of 10 µM a cell viability of 50% was observed for all cell types, except for the MCF-7 resistant cells, that showed an IC50 at a MTX concentration of 55 µM.

Subsequently, the BALB/3T3 cell viability following treatment with the lipid-based formulations containing 10 µM MTX and/or pDNA-PTEN was determined (Fig. 2.7A, B). EPC and DOTAP liposomes containing MTX or containing MTX and pDNA reduced BALB/3T3 cellular viability to a similar extent. Cell viability was 47.5% ±10.6% for cells treated with EPC lip$_{MTX}$, 62.7% ±7.7% with EPC lip$_{MTX+pDNA}$ (Fig. 2.7A), 62.9% ±9.4% with DOTAP lip$_{MTX}$ and 58.8% ±7.1% with DOTAP lip$_{MTX+pDNA}$ (Fig. 2.7B). Liposomal formulations and MTX in solution showed similar effect on BALB/3T3 cell viability, this could be explained by the high percentage of free MTX in the liposomal formulations.

Free plasmid, empty liposomes and lipid nanoparticles showed no effect on BALB/3T3 cell survival. Interestingly, neither lipid nanoparticles containing MTX, nor lipid nanoparticles containing MTX plus pDNA reduced BALB/3T3 cellular viability, as shown in Fig. 2.7A (NLC) and Fig. 2.7B (SLN). Presumably, the slow release of MTX from these formulations prevents a cytotoxic effect on BALB/3T3 cells.

Next, we tested the formulations activity against a prostate cancer cell line (Fig. 2.7C, D). PC3 prostate cancer cells treated with free MTX (10 µM) showed a reduction in cell viability to 52.3% ±5.9%. As observed for BALB/3T3 cells, EPC liposomal formulations reduced PC3 cellular viability to similar extents (57.6% ±10.5% for EPC lip$_{MTX}$ and 52.7% ±10.3%, for EPC lip$_{MTX+pDNA}$, Fig. 2.7C), probably due to the high percentage of free MTX in those formulations. For EPC lip$_{pDNA}$ no effect was noticed on PC3 cell viability (96.2% ±8.6%), most probably because of the lack of pDNA-PTEN binding to such liposomes (see Fig.2.5A).

Treatment of PC3 cells with NLC did not reduce cell viability (Fig. 2.5C), neither with NLC$_{MTX}$ (103.6% ±5.4%) nor with NLC$_{MTX+pDNA}$ (101.6% ±7.1%). This result can possibly be explained by the high entrapment efficiency (81% ±5.9%) but slow release kinetics of NLC, which may prevent release of sufficient amounts of MTX to reach a therapeutic level within the cells. Alternatively, the intracellular fate of this nanoparticle, for instance in degradative lysosomal compartments, could prevent its therapeutic effect [28,38]. pDNA-PTEN (98.1% ±5.1%), EPC Lip. pDNA (92.2% ±7.4%) and NLC$_{pDNA}$ (102.2% ±5.0%) were nontoxic to PC3 cells either, probably because of an inefficient delivery of pDNA-PTEN (Fig. 2.7C).

On the other hand, treatment of PC3 cells with cationic formulations (DOTAP liposomes and SLN) caused reduction in cell viability (Fig. 2.7D). Delivery of pDNA-PTEN with SLN resulted in a more pronounced cytotoxic effect in PC3 cells than with DOTAP liposomes (40.2% ±8.5%), either alone (SLN$_{pDNA}$ 25.8% ±5.4%) or in conjugation with MTX (SLN$_{MTX+pDNA}$
Figure 2.7. Cytotoxic and *in vitro* antitumor effect of MTX co-delivered with PTEN gene using lipid nanoparticles and liposomal formulations. BALB/3T3 cells (A-B), PC3 cells (C-D), and MCF-7 resistant (E-F) cells were treated for 24 h with empty carriers, MTX, naked pDNA-PTEN, MTX-containing carriers, pDNA-PTEN containing carriers or with carriers containing both MTX and pDNA-PTEN. Lefts panels (A, C, E): carriers are EPC liposomes and NLC; Right panels (B, D, F): carriers are DOTAP liposomes and SLN. Cell viability was assessed by MTT assay. The results are expressed as the mean ±SEM of three independent experiments. Different letters indicate significant differences: “a” compared to empty EPC liposomes group; “b” compared to empty DOTAP liposomes group; “c” compared to free pDNA-PTEN group; “d” compared to empty SLN group; “e” compared to DOTAP Lip.MTX group; “f” compared to DOTAP Lip.MTX+pDNA-PTEN group and “g” compared to SLNPpDNA-PTEN group. The significance was determined by one-way ANOVA followed by Tukey’s post-test (n = 3, p < 0.05).
25.1% ±7.1%). PC3 cells viability was not affected after treatment with SLN$_{MTX}$ (101.3% ±7.6%). Similar to EPC liposomes, DOTAP lip$_{MTX}$ (54.5%±8.6%) and DOTAP lip$_{MTX+pDNA}$ (40.7%±7.7%) were as cytotoxic as free MTX to PC3 cells (Fig. 2.7D).

Altogether, the delivery of PTEN with DOTAP liposomes resulted in a slightly higher reduction in cell viability than delivery of MTX with DOTAP liposomes in PC3 cells. Co-delivery of PTEN and MTX with DOTAP liposomes gave a similar reduction in cell viability as delivery of just PTEN. Therefore, MTX seems not to have an additional effect over PTEN in PC3 cells. In addition, SLNs showed an inefficient delivery of MTX into PC3 cells, while delivery of PTEN resulted in a greater reduction in cell viability than delivery of PTEN with DOTAP liposomes. Therefore, gene delivery with SLN seems more efficient than with DOTAP liposomes, but the SLN formulation used here is not yet suitable for co-delivery of MTX. The SLN formulation would need to be improved to obtain better release kinetics for the cytotoxic drug into PC3 cells.

Finally, the formulations were tested on resistant breast cancer cells. Resistant MCF-7 breast cancer cells (see Material and Methods) treated with free MTX (10 µM) showed no reduction in cell viability, as expected from its higher IC50 value (55 µM, Fig. 2.6). Similarly, empty EPC liposomes, NLC$_{MTX}$, NLC$_{pDNA}$, and NLC$_{MTX+pDNA}$ did not reduce MCF-7 cellular viability (Fig. 2.7E). Neither DOTAP-containing MTX formulations (DOTAP lip$_{MTX} =$ 97.5%±8.5% and SLN$_{MTX} =$ 100.1%±4.7%) were cytotoxic to resistant MCF-7 cells (Fig. 2.7F). Interestingly, both DOTAP formulations containing pDNA-PTEN were able to reduce resistant MCF-7 cells viability to similar extents (DOTAP lip$_{pDNA} =$ 60.2%±8.5% and DOTAP lip$_{MTX+pDNA} =$ 62.7%±7.5%, Fig. 2.7F), indicating no synergistic effect between MTX and pDNA in DOTAP liposomes. On the other hand, SLN$_{pDNA}$ reduced MCF-7 viability to 49.6%±4.4% and SLN$_{MTX+pDNA}$ was even more effective, decreasing the cell survival to 23.7%±5.2%. This indicates a synergistic effect for the combination of MTX and pDNA-PTEN in SLN, resulting in reversion of mitoxantrone resistance of MCF-7 cells.

2.4 Conclusions

Mini-extruder systems are commonplace in bioscience laboratories. The results presented here provide a satisfying proof of principle that such extruders can be used to produce lipid nanoparticles at laboratory scale. Additionally, the lipid nanoparticles produced were stable and versatile, being applicable to drug and gene delivery. In fact, the co-delivery of the chemotherapeutic drug MTX and the pDNA-PTEN construct successfully reduced the viability of PC3 prostate cancer cells and reverted the resistance of MCF-7 breast cancer cells to mitoxantrone, with no effect on nor-
normal (3T3) cells viability. Therefore, we conclude that the microemulsion extrusion technique is a very efficient way to screen for new formulations of lipid-based carriers, such as SLN, NLC and liposomes.

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