Investigations into manufacturing processes for a liposomal parenteral nanoformulation to solubilize poorly soluble drug substance

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Abstract

Background/Objectives: Some pharmaceutical products have poor water solubility thus parenteral formulation strategy is to use liposomal nanomedicine for solubilization. This avoids the use of larger quantities of co-solvents and/or polyoxyethylene based surfactants which are associated with severe side-effects. To reduce such limitations, a poorly soluble drug substance was nanoformulated by packaging into liposomes made from natural phospholipids.
Methods/Statistical analysis: The top-down and bottom-up approach of nanoformulation were investigated with an aim to get the desired properties. Particle size, polydispersity index and zeta potential were determined by photon correlation spectroscopy using a zeta sizer. The effect of process parameters such as homogenization pressure, number of homogenization cycles, lipid concentration and the effect of addition of buffer components were equally investigated.
Findings: The mean particle size for the bottom-up process was 46.0 nm but the encapsulation efficiency was below 30% indicating most of the drug substance is washed away. The top-down approach gave mean particle sizes of about 80.0 nm and the encapsulation efficiency was almost 100% after performing thermodynamic stability. This was evident as crystals were not observed from the polarized light microscope. Increase in homogenization pressure and number of cycles significantly ($p \leq 0.05$) reduced the particle sizes and polydispersity index (PDI). Liposomes with a narrow distribution were obtained when higher lipid concentrated liposome dispersion was progressively diluted to lower concentration followed by addition of buffer components.
Improvements/ Applications: Liposomal formulations composed of natural phospholipids proved to be an interesting tool for solubilization of poorly soluble compounds for parenteral administration.

***Key words*:** Nanomedicine, liposomes, parenteral, phospholipids, lyophilization

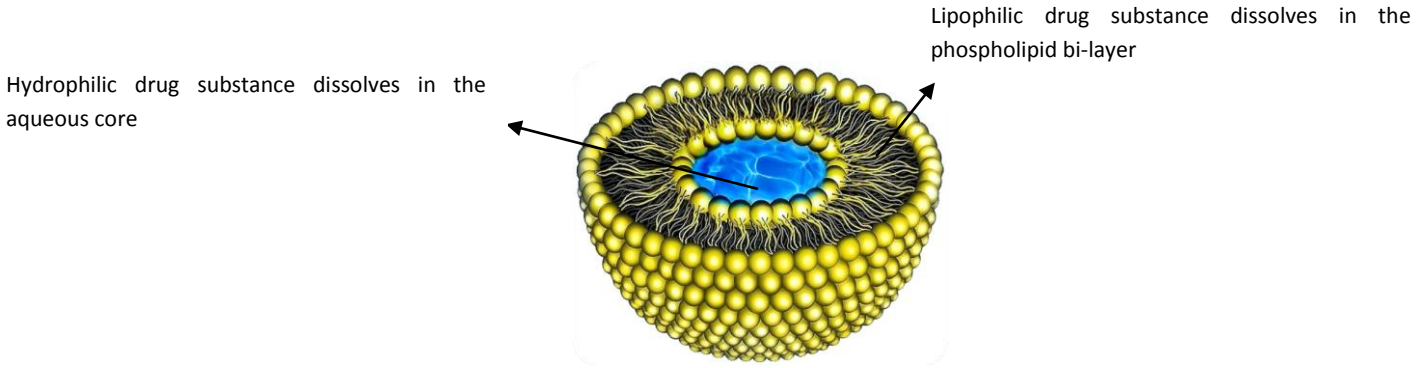
1. Introduction

Drug molecules that nowadays are entering the pharmaceutical development process appear to become increasingly lipophilic [1]. Since many of these potential drugs still need to be administered parenterally, the question often arises as to how to formulate such strongly water-insoluble entities in such a way that they do not precipitate after injection and that the excipients do not add toxicity to the formulation [2]. In simple physicochemical terms, in order to solubilize a lipophilic drug one just has to reduce its chemical potential in solution by choosing a proper solvent [3] or try to design a formulation that will avoid the use of solvents and surfactants [3, 4]. However, upon application in a living organism medicine and pharmacy come into play demanding that only the chemical potential of the drug should be reduced and not simultaneously that of other substances like phospholipids of cell membranes or blood proteins as that might lead to hemolysis [5]. This narrows the number of excipients suitable for this purpose.

Several organic solvents, such as the combination of propylene glycol and ethanol, are used by the average drug developer. Still an acceptable approach to start formulating a lipophilic drug is considered relatively safe although this assumption is more often being questioned [6]. Intrinsically safe solubilizers may be represented by structures equal or similar to some we carry in our own body besides albumins which have disadvantages such as low loading capability [7, 8]. Phospholipids are also abundant in our body and in addition they are already applied as drug

carriers. Phospholipids are by definition biocompatible self associating amphiphiles [7, 8]. In their simplest associated form they are called liposomes (Figure. 1). Liposomes are spherical vesicles composed of one or more phospholipids bilayers with an internal aqueous phase. Liposomes can entrap hydrophilic drugs in their internal aqueous compartment or lipophilic drugs within the lipid bilayer [8].

Figure 1. A representation of liposome showing bi-layer space for solubilizing lipophilic drug



Phospholipids are also being used for decades as emulsifiers, for example, in parenteral total nutrition [9] and as such they are considered to be safe and act also as an agent ameliorating the toxicity of drugs [10]. Phosphatidylcholine (PC) is the most common phospholipid used for the preparation of liposomes for pharmaceutical use and because of the amphiphilic nature of the PC molecules, they self assemble spontaneously in aqueous environment [10, 11]. This self assembly can be facilitated by applying several forms of energy including shear forces, ultrasonic treatment, extrusion, fluidization, and others. As a result, liposomes are formed as closed compartments surrounded by one or more (concentric) bilayers of phospholipid [12]. The higher the energy applied, the smaller the final liposome size and the fewer the number of lamellae (bilayers with a thickness in the order of 4–5 nm) will be [13]. Natural Phospholipids (PL) can be isolated from egg yolk and soy beans and are widely used for parenteral products [9]. They contain multiple unsaturated alkychains which makes them good solubilizers [10]. Simple liposomes like those composed of egg- or soy phospholipids are known to be unstable in blood stream and thus a fast release is expected [7-10]. When preparing formulations for parenteral delivery several excipients are mandatory, for instance, 9% lactose or 9% sucrose is used to strike a balance between the drug loaded liposomes and the red blood cells [11-13]. This balance creates an isotonic state in osmolarity and thus no damage to the red blood cells. An isotonic solution is one in which its effective osmole concentration is the same as the solute concentration of a cell. In this case the cell will neither swell nor shrink since there will be no concentration gradient for water to cross the cell membrane. If the concentration of solutes was to be lower outside than inside the cell, it will bring about hypotonicity. Thus to balance the concentration of solutes inside and outside the cell, water will rush into the cell causing it to swell and possibly burst [12, 13]. On the other hand, a higher concentration of solutes outside than inside creates a hypertonic state and thus there is a tendency of water to flow out of the cell in order to balance the concentration of the solutes causing the cell to shrink [14].

The objective of this study was to investigate using a Top-down and Bottom-up approach for making liposomes loaded with a poorly soluble lipophilic model drug compound with an aim to solubilize the drug in the lipid-bilayer using natural phospholipids. The formulations were characterized for particle size, polydispersity index, zeta potential and stability tests. Further optimization was done to scale down the size of the liposomes.

2. Materials and Methods

All materials, reagents chemicals and lipophilic drug substance used in the research were kindly offered by Novartis Pharmaceutical Company AG, Basel, Switzerland. Excipients from natural phospholipids composed of soy and egg lecithins; S100 and E80S were used. The phospholipids mainly composed Phosphatidylcholine. Sodium Oleate, D-Lactose Monohydrate (DLM) and Ethyl acetate (EtOAc) were purchased from Sigma-Aldrich. All other chemical products were commercially available and of analytical grade.

2.1. Preparation of liposome drug carriers for solubilization of lipophilic drug

Liposomes were prepared with a poorly soluble but active drug substance, natural phospholipids composed of soy and egg lecithin's (S100 and E80S), buffer components (9% lactose and 10 mMol Histidine buffer at pH 6.5). The 10mMol histidine buffer is used in the maintenance of pH at 6.5 while 9% lactose is known to have an osmolarity of 280 mOsmole. The normal human reference range of osmolarity in blood plasma is between 260-290 mOsmoles [13-16]. Sodium oleate was used as a stabilizer when soy lecithin S100 lipid was used which gives the phospholipid-bilayer a negative charge, thus reducing the possibility of liposome aggregation over time by electrostatic repulsion [14]. When egg lecithin E80S lipid type was used no stabilizer was used since it naturally has a negative charge [15]. Lipids, stabilizers and buffer components were used in the preparation of liposomes as indicated in table 1.

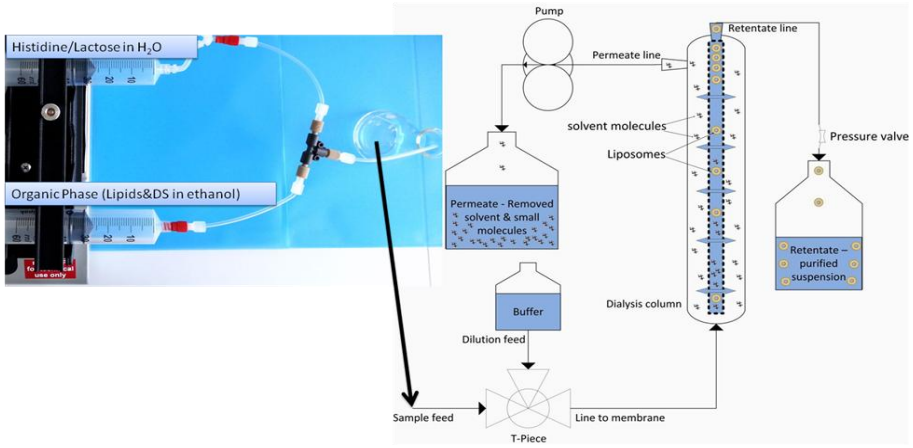
Table 1. Examined lipids, stabilizers and buffer components.

Lipids used	Stabilizers	Buffer components
Soy lecithin S100	Na-Oleate	Histidine (10 mMol)
Egg lecithin E80S		Lactose (9 %)
		pH 6.5

2.1.1. Ethanol injection method

This is a bottom up method for the manufacture of drug loaded liposomes. The set up involves two pumps connected in parallel pushing an organic phase from one end and an aqueous phase from another end (Figure 2). The organic phase contains ethanol as the solvent, egg or soy lecithin (S100 or E80S) and the lipophilic drug substance. The aqueous phase contains the buffer components. The two phases connect in a T-junction where the buffer components and ethanol mix to form drug loaded liposomes. The liposomes are then subjected through tangential flow filtration that removes the ethanol by exchanging it with the buffer by dialysis. The end product is drug loaded liposomes free from ethanol which can be safely used for parenteral delivery. The final emulsion is then sterile filtered and/or lyophilized [17, 18].

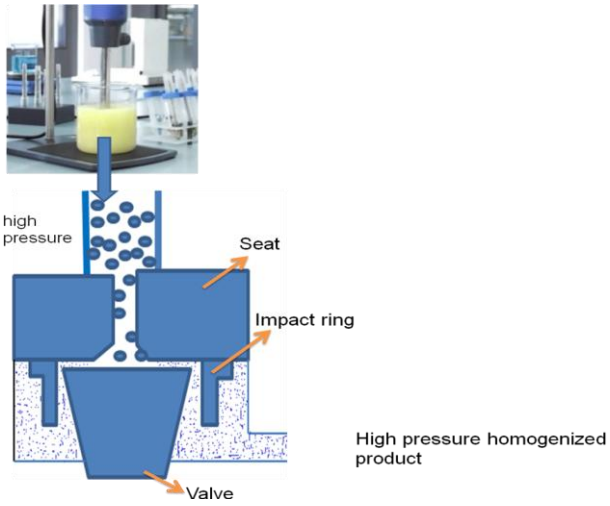
Figure 2. Schematic diagram showing ethanol injection method for manufacturing drug loaded liposomes.



2.1.2. High pressure homogenization method for production of drug loaded liposomes.

Liposomes were also prepared using the high pressure homogenization method (Figure 3). This is a top-down process that involves breaking down of larger particle size liposomes to form smaller ones [7, 13]. The process avoids the use of solvents during the solubilization of the lipophilic drug substance. This involved dispersing the lipids (S100 or E80 S) in water by high shear mixing using an ultra turrax or high speed homogenizer. For further size reduction, the lipids were homogenized under varied high pressures and increased homogenization cycles in the high pressure homogenizer. Other process parameters such as varying lipid concentration, varying when to include drug substance and buffer components were investigated. The homogenized product was then sterile filtered, characterized for particle size, PDI and zeta potential before optimization was done and eventual lyophilization.

Figure 3. Schematic diagram showing the top-down approach of nanoformulation using high shear homogenization followed by high pressure homogenization for preparing drug loaded liposomes.



2.2. Particle size analysis and polydispersity index

Particle size analysis was performed by photon correlation spectroscopy (PCS) with a Malvern Zetasizer Nano ZS (Malvern Instruments, Worcester shire, United Kingdom). PCS yields the mean diameter and the polydispersity index (PDI) which is a measure of the width of the size distribution. The mean diameter and PDI values were obtained at an angle of 90° in 10 mm diameter cells at 25 °C. Prior to the measurements of each sample, a drop of drug loaded liposomes formulation or placebo liposomes formulation were suspended in histidine buffer, then vortexed for a few minutes to produce a suitable scattering intensity and each sample measured in triplicate.

2.3. Zeta potential

The zeta potential, reflecting the electric charge on the particle surface and indicating the physical stability of colloidal systems was measured by determining the electrophoretic mobility using the Malvern Zetasizer Nano ZS (Malvern Instruments, Worcester shire, United Kingdom). The sample was prepared as for particle size measurement but used a different curvet that could conduct current during the measurement.

2.4. Determination of encapsulation efficiency

The quantity of the drug substance encapsulated into the liposomes could be visualized using the polarized light microscope. Since the drug substance is lipophilic, crystals could easily be visualized when the drug substance is not wholly encapsulated into the liposomes. Reverse phase High Performance Liquid Chromatography was also used to determine the amount of drug substance loaded into the liposomes.

3. Results and Discussion

3.1. Particle size and size distribution of liposomes prepared using bottom-up approach

The bottom-up process led to unilamellar liposomes, below 50 nm liposomes with narrow particle size distribution (Table 2). However, during dialysis around 70 % of the drug substance is lost was determined by reverse phase HPLC. This showed that the ethanol injection method can result smaller liposomes which are homogeneous but the removal of the solvent is the major hindrance towards liposome cleansing.

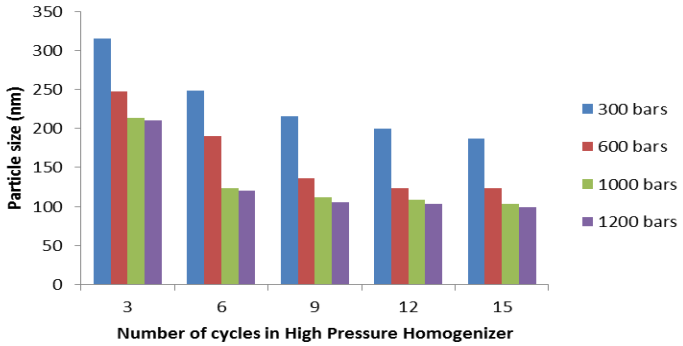
Table 2. Preliminary characteristics of liposomes prepared by ethanol injection method

Lipid (40 mg*mL^{-1})	Size (nm)	PDI	ZP (mV)	EE%
S100+1% NaOleate	46.0	0.01	-22	~30%

3.2. Effect of homogenization pressure and number of cycles on particle size & PDI of liposomes.

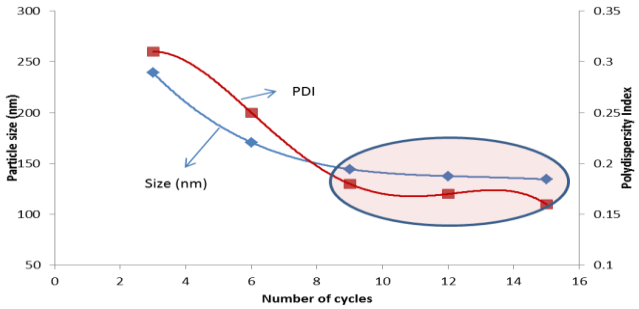
Higher pressures combined with more homogenization cycles lead to significantly ($p \leq 0.05$) smaller particle sizes with narrow size distribution (Figure 4). This was expected as increase in pressure increases the deformation of the bigger particles as they are exposed to tangential stresses [19] through numerous numbers of cycles. For the particle reduction the droplets must be exposed to tangential stress over a period of time [20]. These results were similar to those obtained by Omwoyo *et al*., [20] for the primaquine loaded solid lipid nanoparticles. In this formulation the tangential stress must have been achieved at about 1000 bars with 10 cycles of homogenization for the C3 high pressure homogenizer.

Figure 4. Particle sizes of egg lecithin E80 S based liposomes (40 mg/mL) as a function of homogenization pressure (bars) and the number of homogenization cycles.



The more the number of homogenization cycles, the smaller the particle sizes became and the more narrow the size distribution. It was observed that at homogenization cycles of more than 10 cycles, there was no significant change in particle size or size distribution (Figure 5). This could be attributed to the fact that, after the tangential stress has been obtained and particle deformation has taken place then further increase in stress would only result to particle disruption which does not have any effect to the particle size and size distribution [7, 20, 21]. This can be further explained with regards to the operational nature of the high pressure homogenizers. They consist of an internal channel through which the viscous liposome premix is pumped to a narrower channel formed by the valve seats [13, 17]. A valve that can be controlled by high pressure is positioned downstream behind the seats.

Figure 5. Particle sizes and size distribution of egg lecithin E80 S based liposomes (40 mg/mL) as a function of number of homogenization cycles.



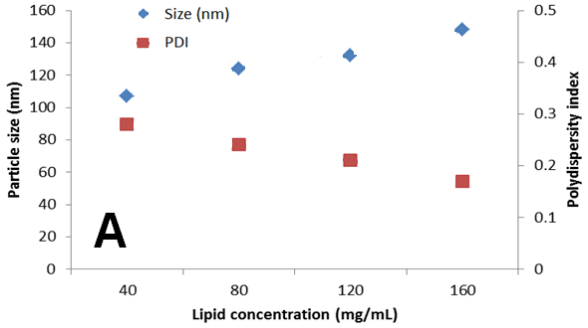
Note: The points circled are not significantly ($P \leq 0.05$) different.

During the homogenizing process, a coarse liposome premix is forced under high pressure through this narrow orifice that is formed between the seats and the valve. Intense disruptive stresses result from impact forces, shear forces, cavitations, and turbulence within the high pressure valve. These disruptive stresses force large micrometer-scale liposomes to break down into smaller size liposomes. The homogenized product can be collected at the outlet and then be recycled back as the feed. In this way, multiple cycles of homogenization process can be performed on the same sample [7, 13, 22]. There are several types of high pressure homogenizers including EmulsiFlex-C5, C3, and C160. The mechanisms of homogenization of the three types of homogenizers are the same, but they differ with respect to their minimum sample volume, capacity and motor types [23, 24].

3.3. Effect of lipid concentration on particle size and PDI of placebo liposomes.

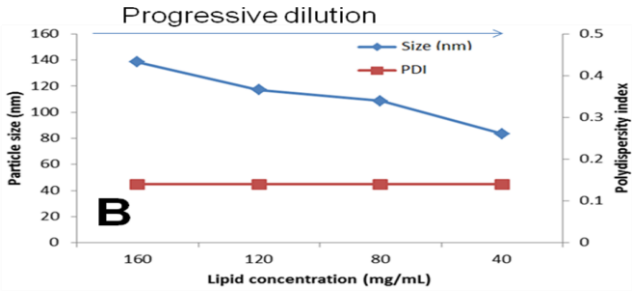
Effects of lipid concentration on particle size and size distribution of placebo liposomes are presented in figure 6. At higher lipid concentration larger particles were obtained but with narrow distribution while with lower lipid concentrations smaller particles were obtained but with a wider size distribution. In order to obtain smaller liposomes with a narrow size distribution, a so-called "progressive high pressure homogenization" (pHPH) method was adopted in which the lipid concentration is serially reduced by dilution with the buffer to strike a balance between particle size and size distribution (Figure 6).

Figure 6. Particle size and size distribution of liposomes as a function of lipid concentration at 1000 bar homogenization pressure using 10 cycles.



This progressive high pressure homogenization started with high lipid concentration which had a narrow size distribution. With continual dilution, the size distribution was maintained at almost 0.1 while the particle size significantly reduced (Figure 7).

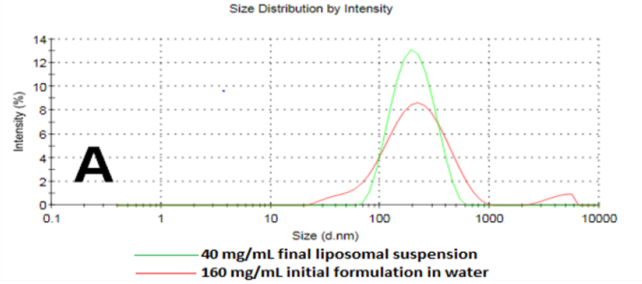
Figure 7. Particle size and size distribution of liposomes as a function of progressive dilution of lipid concentration at 1000 bar homogenization pressure using 10 cycles.



3.4. Effect of addition of drug substance and buffer components before and after the formation of liposomes.

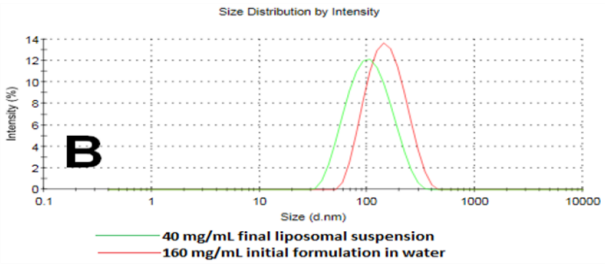
Starting with the mixture containing all components containing lipids, drug substance and buffer components prior to progressive high pressure homogenization yielded liposomes that are relatively large in size with a broad size distribution (Figure 8).

Figure 8. Particle size and size distribution of liposomes starting with drug substance and buffer components at 160 mg/mL in the first homogenization and progressively diluting to 40mg/mL in the final formulation.



Starting with the lipids dispersed in water the later followed by the addition of drug substance and buffer components after formation of placebo liposomes with progressive high pressure homogenization led to smaller particle size with a narrow distribution (Figure 9).

Figure 9. Particle size and size distribution of liposomes starting with only lipid and water at 160 mg/mL in the first homogenization and progressively diluting to 40mg/mL in the final formulation.



4. Conclusion

The ethanol injection method can result in liposomes smaller than 50 nm with very narrow size distribution. However, in this specific case there was a great process related loss of DS. With HPH at lab scale, filterable (0.22 µm) liposomes with a narrow distribution were obtained when higher concentrated liposome dispersion was progressively diluted. The lipophilic model drug substance could readily be incorporated in the pre-prepared placebo liposomes. This demonstrated that small unilamellar liposomes, presenting a large lipid phase in the aqueous formulation, obviously have a very strong solubilization power. The liposomal formulations composed of natural phospholipids may be an interesting tool for solubilization of poorly soluble compounds for parenteral administration.

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