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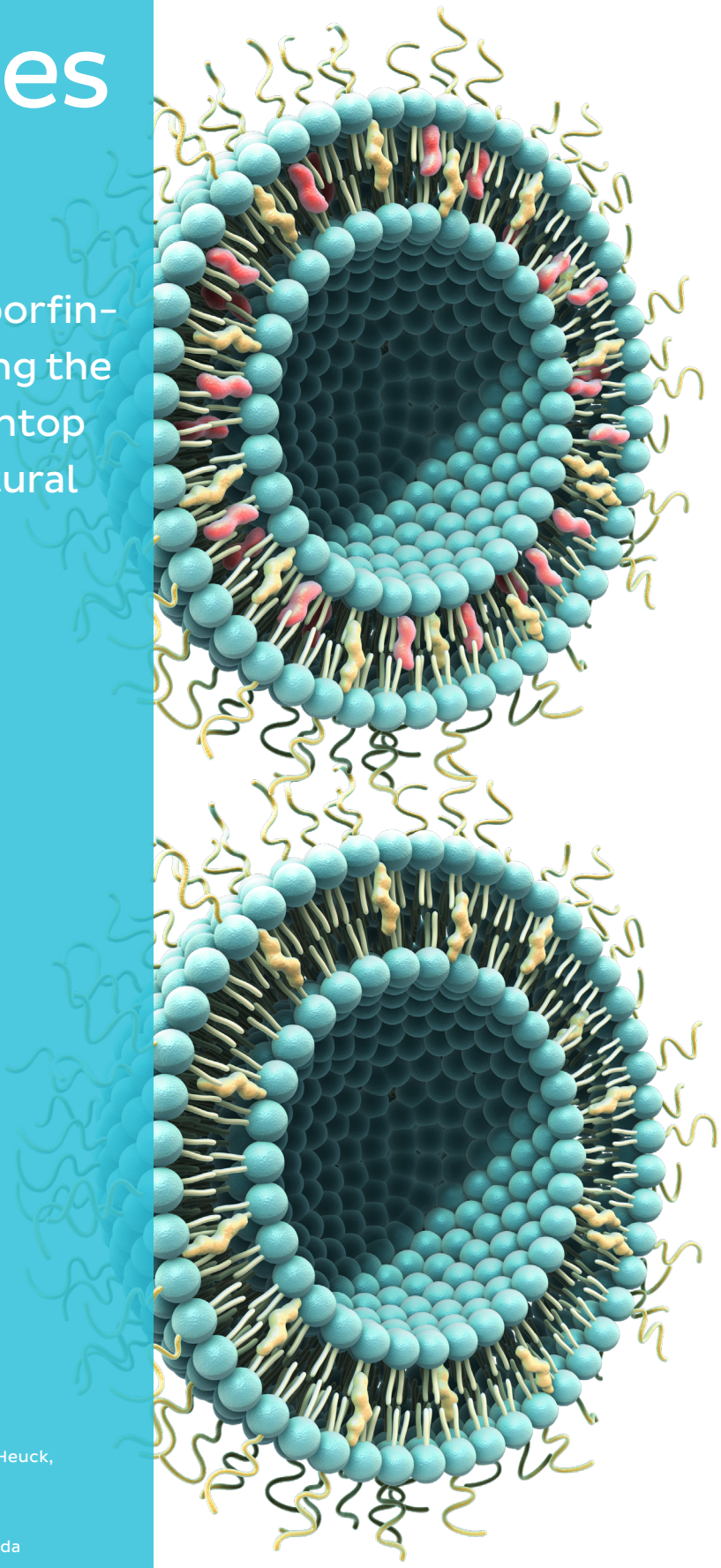
Liposomes

Preparation of Verteporfin-loaded liposomes using the NanoAssemblr® Benchtop and the effects of natural and synthetic lipids



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Abstract

Both, natural and synthetic lipids are utilized in FDA approved liposomal drug products. While synthetic lipids are often favoured for their purity, natural lipids are less costly. In this study, liposomes containing mostly natural lipids with cholesterol a PEGylated lipid were produced using the NanoAssemblr® microfluidic technology. Microfluidic technology was used to achieve exquisite control over liposome size and *in situ* loading of the hydrophobic drug verteporfin. An encapsulation efficiency of 90% and a limit size of 34 ± 1.5 nm was achieved with soy-PC formulations. Egg-PC formulations had >80% encapsulation efficiencies at a size of 39.3 ± 0.9 nm. Interestingly, when substituting a synthetic PC analog for egg-PC, formulations with the synthetic lipid tended to aggregate. A possible explanation considering the differences in molecular organization of saturated and unsaturated PC species is discussed.

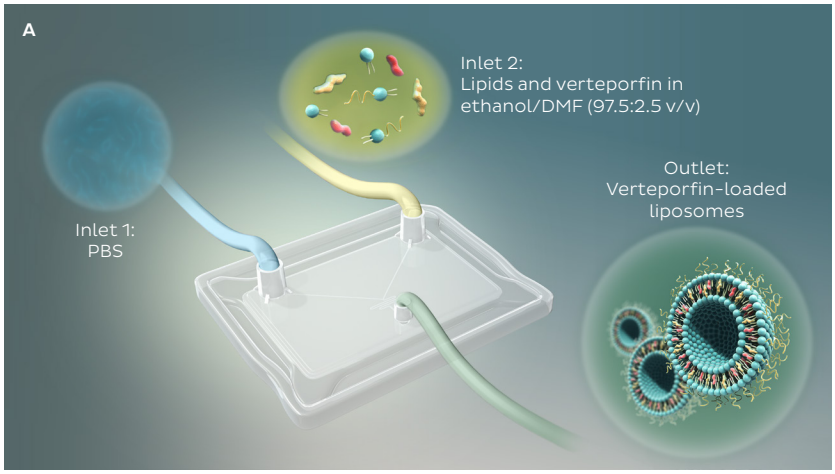
Introduction

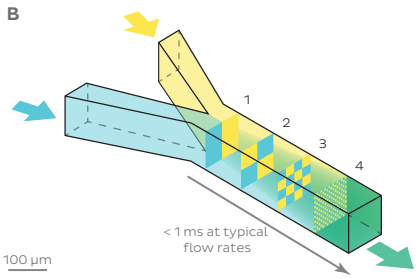
Phosphatidylcholines (PCs) used to form liposomes can be naturally derived or chemically synthesized. Natural phospholipids such as egg-PC and soy-PC contain a mixture of diverse PCs. Synthetic phospholipids are offered as higher purity reagents consisting of one PC species. However, due to the synthesis process, the cost is also significantly higher. Both, natural and synthetic lipids are utilized in FDA approved liposomal drug products[1]. Microfluidic preparation of unilamellar liposomes comprised of synthetic lipids and loaded with hydrophobic and hydrophilic drug molecules has previously been reported using the NanoAssemblr platform [2,3,4]. Herein, we report microfluidic assembly of soy-PC and egg-PC liposomes using the NanoAssemblr Benchtop instrument. For comparison, liposomes were also formulated using a synthetic analog for egg-PC. The photosensitizer verteporfin was used as a model hydrophobic drug molecule, and liposomes were loaded *in situ* during liposome formation. Verteporfin is commercially available in a liposomal formulation (trade name Visudyne®) and approved by the FDA for photodynamic therapy to treat wet macular degeneration[1].

In situ loading of liposomes allowed for encapsulation efficiencies of over 90%, while liposome size and size distribution were controlled by process parameters available on the NanoAssemblr Benchtop instrument. Unexpectedly, verteporfin liposomes prepared with natural phospholipids were significantly more stable than those prepared with synthetic PCs.

Experimental design

Detailed experimental methods and source of materials are available in the materials and methods section. Briefly, the NanoAssemblr process produces unilamellar liposomes by controlled solvent displacement in a microfluidic mixer as illustrated in ***Figure 1.*** A water-miscible solvent containing dissolved lipids is mixed with an aqueous buffer under laminar flow conditions. Upon mixing, solvent displacement increases the polarity of the environment, causing the self-assembly of amphiphilic phospholipid molecules into liposomes. Laminar flow and computer controlled injection enable reproducible fine-tuning of the conditions of liposome formation by specifying in the NanoAssemblr software, the Total Flow Rate (the sum of flow rates between the solvent and aqueous phases) and the Flow Rate Ratio (the volumetric ratio of buffer to solvent being mixed per unit time). The hydrophobic drug verteporfin was loaded in situ by dissolving it in the solvent phase along with the lipids prior to injection into the microfluidic channels. Hydrophilic drug molecules can also be loaded by including them in the aqueous phase. A range of Total Flow Rates and Flow Rate Ratios were explored to optimize the size of verteporfin-loaded liposomes.





1. Two fluids (Buffer and ethanol) entering the channel under laminar flow do not mix.

2. Microscopic features in the channels are engineered to cause the fluid streams to mingle in a controlled, reproducible, and non-turbulent way.

3. Intermingling of the fluids increases as they continue through the mixer.

4. Fluids emerge from the mixer completely mixed. The whole process takes < 1 ms, which is faster than particles can assemble

Figure 1. A) *PBS buffer is injected into the left inlet while an organic solvent with dissolved lipids and the drug Verteporfin is injected into the right inlet of the NanoAssemblr Benchtop microfluidic cartridge. Following controlled mixing in microfluidic channels, liposomes are spontaneously formed. Computer controlled injection allows specification of the Total Flow Rate and the Flow Rate Ratio of aqueous to organic solvents to control liposome size. Hydrophobic verteporfin is integrated into the hydrophobic region of the lipid membrane.* ***B)*** *Diagram depicting two fluids mixing under laminar flow in a microfluidic mixer. The two fluids enter the channel as adjacent streams. Specially engineered microscopic features within the channels cause intermingling of the two fluid streams, effectively increasing the surface area between them, increasing the interface between the two liquids across which diffusion can take place, and reducing the diffusion distance.*

Separate liposome formulations containing egg-PC or soy-PC mixed with cholesterol and synthetic PEG-lipid at molar ratios of 52:45:3 were compared for size, size homogeneity (polydispersity index or PDI) and efficiency of verteporfin encapsulation. To compare natural PCs to synthetic PCs, egg-PC in these formulations was replaced with equimolar quantities of the synthetic phospholipid POPC. POPC represents the main component of Egg-PC and contains one saturated 16-carbon acyl chain and one mono-unsaturated 18-carbon acyl chain. To further investigate the role of acyl chain saturation on drug loading, formulations with DSPC, which has two saturated 18-carbon acyl chains, were also made. Structural formulae for DLPC (the main component of Soy PC), POPC and DSPC are illustrated in ***Figure 2.***

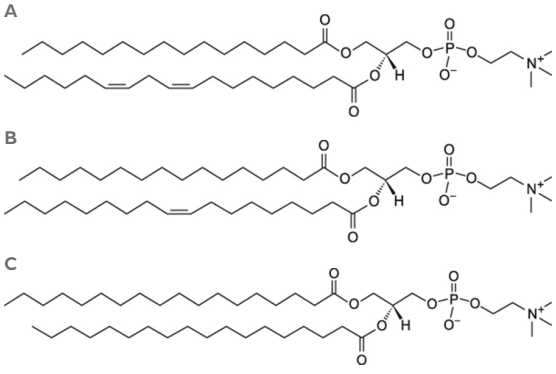
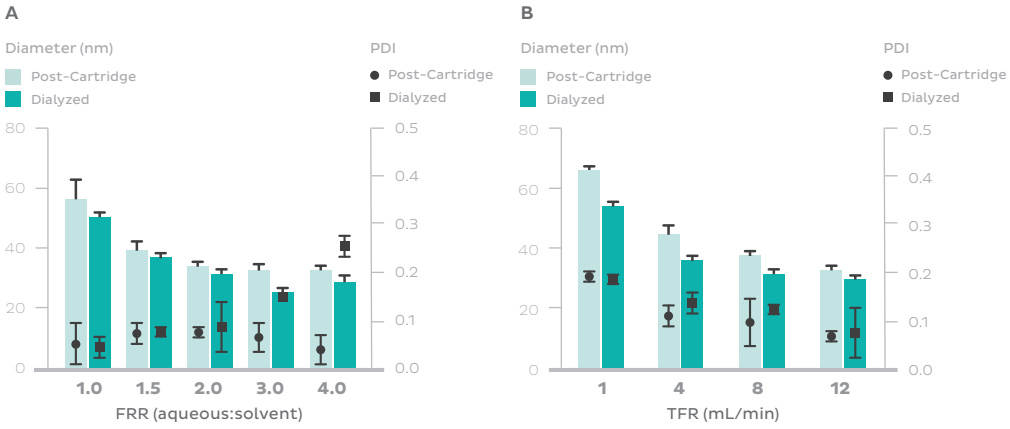


Figure 2. *Structural formulae of* ***A)*** *palmitoyl-linoleoyl-phosphatidylcholine (PLPC), the predominant PC in soy-PC,* ***B)*** *palmitoyl-oleoyl-phosphatidylcholine (POPC), the predominant PC in egg-PC, and* ***C)*** *distearoyl-phosphatidylcholine (DSPC).*

Result

The behavior of empty soy-PC liposomes with respect to the NanoAssemblr formulation parameters TFR and FRR was determined by holding one parameter constant while exploring a range of values for the other parameter. At a constant TFR of 12 ml/min, FRRs of 1:1 through 4:1 were tested. As seen in ***Figure 3A,*** liposome size was found to decrease asymptotically with increasing FRR. Likewise, at a constant FRR of 2:1, the size of soy-PC liposomes was found to decrease asymptotically with increasing TFR as seen in ***Figure 3B.*** This behavior is consistent with previously published trends for synthetic liposome formulations[2], and the asymptote in size has been found to correspond to the so-called "limit size" representing the smallest possible particle size based on ideal packing of constituent molecules[2]. The limit size of 25 nm was achieved at a TFR of 12 mL/min and FRRs greater than 3:1.

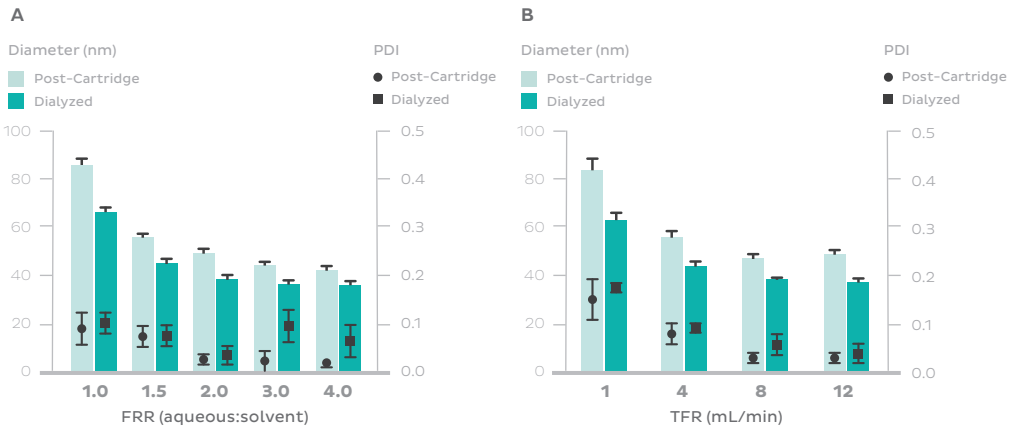


Lipid composition	Soy-PC:Chol:DSPE-PEG (52:45:3 mol%)
Total lipid concentration	10 mg/mL
Organic solvent	Ethanol
Aqueous solvent	PBS pH 7.4
Total Flow Rate	**A)** 12 mL/min **B)** As indicated on horizontal axis
Flow Rate Ratio (aqueous:organic)	**A)** n:1 - n indicated on horizontal axis **B)** 2:1
Solvent Removal	Dialysis

Figure 3. Soy PC liposome size decreases asymptotically with increasing FRR and TFR. *Hydrodynamic size and particle size distribution (expressed as polydispersity index, PDI) (both determined by dynamic light scattering) of soy-PC liposomes formulated at various flow rate ratios (A) and various total flow rates (B) on the NanoAssemblr Benchtop instrument. Samples were prepared in triplicate and values represent mean and error bars represent standard deviation of the mean.*

Compared to soy-PC, egg-PC formulations followed the same general trends, as shown in ***Figure 4.*** Egg-PC liposomes, however, were found to be larger than soy-PC liposomes at the same TFR and FRR. The limit size of egg-PC formulations was approximately 40 nm.

In situ loading of soy-PC and egg-PC liposomes was achieved by dissolving verteporfin and the lipid mixture at a 0.09 (mol/mol) ratio in the organic phase. 2.5% (v/v) DMF was added to the organic phase as a co-solvent, to ensure complete dissolution of verteporfin . The sizes of soy- and egg-PC liposomes were not affected by drug loading ***(Figure 5).*** In contrast, synthetic lipid formulations containing either POPC or DSPC were observed to aggregate in the presence of verteporfin, even though these formulations were stable without the drug. This aggregation is manifested in the larger observed size for the loaded POPC and DSPC liposomes when compared to the empty liposome case.



Lipid composition	Egg-PC:Chol:DSPE-PEG (52:45:3 mol%)
Total lipid concentration	10 mg/mL
Organic solvent	Ethanol
Aqueous solvent	PBS pH 7.4
Total Flow Rate	**A)** 12 mL/min **B)** As indicated on horizontal axis
Flow Rate Ratio (aqueous:organic)	**A)** n:1 - n indicated on horizontal axis **B)** 2:1
Solvent Removal	Dialysis

Figure 4. Egg-PC liposome size decreases asymptotically with increasing FRR and TFR. *Hydrodynamic size and particle size distribution (expressed as polydispersity index, PDI) (both determined by dynamic light scattering) of egg-PC liposomes when formulated at various flow rate ratios (A) and various total flow rates (B) on the NanoAssemblr Benchtop instrument. Samples were prepared in triplicate and values represent mean and error bars represent standard deviation of the mean.*

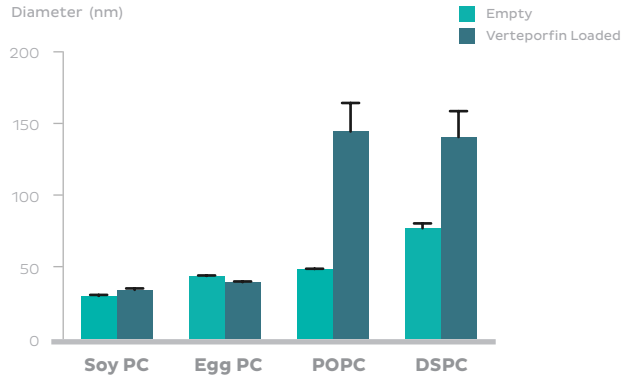


Figure 5. Verteporfin loading did not affect size of natural-PC liposomes. *hydrodynamic size (determined by dynamic light scattering) of empty and verteporfin loaded liposomes prepared with either natural (Egg PC, Soy PC) or synthetic (POPC, DSPC) phospholipids. Synthetic lipids were found to aggregate in the presence of verteporfin. Samples were prepared in triplicate and values represent mean and error bars represent standard deviation of the mean.*

Lipid composition	PC*:Chol:DSPE-PEG (52:45:3 mol%) *PC=EGG PC, Soy PC, POPC, DSPC
Total lipid concentration	10 mg/mL
Verteporfin concentration in lipid mix	1 mg/mL
Drug/Lipid Ratio	0.1 (w/w), 0.09 (mol/mol)
Organic solvent	Ethanol/DMV (97.5:2.5 v/v)
Aqueous solvent	PBS pH 7.4
Total Flow Rate	12 mL/min
Flow Rate Ratio (aqueous:organic)	2:1
Solvent Removal	Dialysis

Verteporfin encapsulation was assessed by fluorescence spectroscopy, and encapsulation efficiencies of over 90% and over 80% were observed for soy-PC and egg-PC formulations, respectively. Significantly lower encapsulation efficiency was observed in DSPC based liposomes, possibly due to the occurrence of large aggregates in presence of verteporfin ***(Figure 6).*** Furthermore, the aggregation was found to result in approximately a 70% loss of material.

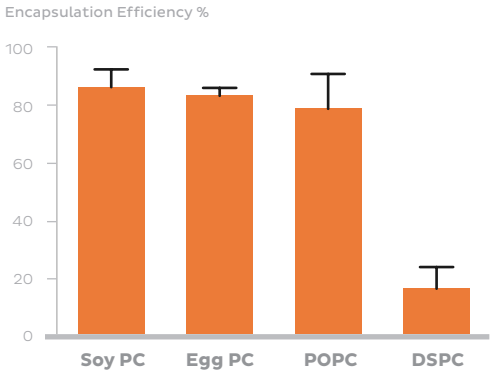


Figure 6. **90% encapsulation efficiency of verteporfin in soy-PC liposomes.** *Encapsulation efficiency of verteporfin loaded liposomes containing natural (egg-PC, soy-PC) or synthetic phospholipids (POPC, DSPC) as the primary lipid component. Aggregation with ~70% loss of material was observed in POPC and DSPC formulations. Samples were prepared in triplicate and values represent mean and error bars represent standard deviation of the mean.*

Lipid composition	PC*:Chol:DSPE-PEG (52:45:3 mol%) *PC=EGG PC, Soy PC, POPC, DSPC
Total lipid concentration	10 mg/mL
Verteporfin concentration in lipid mix	1 mg/mL
Drug/Lipid Ratio	0.1 (w/w), 0.09 (mol/mol)
Organic solvent	Ethanol/DMV (97.5:2.5 v/v)
Aqueous solvent	PBS pH 7.4
Total Flow Rate	12 mL/min
Flow Rate Ratio (aqueous:organic)	2:1
Solvent Removal	Dialysis

Discussion

Soy-PC liposomes were found to have a smaller limit-size than egg-PC liposomes, all other parameters being equal. This is consistent with previous reports that confirm that more saturated lipid species tend to form larger liposomes. This can be understood by considering the flexibility (inversely proportional to the bending modulus) of the lipid bilayer. Smaller liposomes require the bilayer to bend more than larger liposomes, and hence more flexible bilayers will form smaller liposomes, and stiffer bilayers will form larger liposomes. Because unsaturated lipids do not pack as densely and have less intermolecular interactions than saturated lipids, greater degrees of unsaturation lead to more flexible lipid bilayers.

Interestingly, liposomes containing the less saturated soy-PC had greater encapsulation efficiency than the more saturated egg-PC. Given that verteporfin is a hydrophobic drug, it is understood that verteporfin would partition into the lipophilic portion of the bilayer. Hence, one might posit that the less densely packed soy-PC bilayer would have more intermolecular spaces in which the verteporfin molecules could reside.

In comparing natural lipid formulations to synthetic lipid formulations, egg-PC and POPC are directly comparable since POPC is the most abundant PC in egg-PC. It is therefore surprising that these two formulations behaved so differently from each other. While egg-PC formulations remained stable with the addition of verteporfin, POPC formulations, in contrast, aggregated. The reason for this is unclear from the present data. One hypothesis is that at least one lipid component of egg-PC (and likely soy-PC) is significantly unsaturated, and a small quantity of this lipid is sufficient to create much of the intermolecular spaces necessary to accommodate the verteporfin in the lipid bilayer. The observation, that formulations containing DSPC, a fully saturated phospholipid, achieved the lowest encapsulation efficiency, lends to the interpretation that greater intermolecular organization in the bilayer leads to lower solubility of the drug molecule in the lipid portion of the bilayer. Further tests of this hypothesis with a better understanding of the distribution of different PCs in natural lipids and with a variety of different drug molecules are warranted. Notwithstanding, these observations are powerful in directing the choice of lipids in liposomal formulations of hydrophobic small molecule drugs.

Conclusion

Liposomes containing natural lipids, cholesterol, and a DSPE-PEG_{2000} were formulated and *in situ*-loaded with verteporfin using the NanoAssemblr Benchtop instrument. Liposome size was optimized using instrument process parameters to achieve 34 ± 1.5 nm and 39.3 ± 0.9 nm diameter verteporfin-loaded liposomes with soy-PC and egg-PC respectively. Verteporfin encapsulation efficiencies of > 90% for soy-PC and > 80% with egg-PC were obtained. Substituting egg-PC with a synthetic PC representing its most abundant PC, namely POPC, resulted in aggregation and loss of 70% of the material. In the absence of verteporfin however, this POPC formulation assembles into stable 49 nm liposomes. Verteporfin-loaded liposomes featuring the more saturated DSPC resulted in much lower encapsulation efficiency (~15%) and similar aggregation and loss of material as POPC formulations. This suggests that the mixture of different PC species in natural lipids is favourable for the stability and loading of verteporfin. Furthermore, a pattern of lower encapsulation efficiency with higher degrees of lipid tail saturation was observed. This suggests verteporfin may intercalate more effectively between loosely packed unsaturated lipid tails than into more ordered arrangements of saturated lipid tails, but further research is needed to understand these observations. These findings demonstrate that naturally derived phospholipid formulations can be produced with the NanoAssemblr Benchtop and that natural phospholipids can be advantageous for encapsulating hydrophobic small molecules.

Materials & Methods

Liposomes were composed of one of either soy-PC (Lipoid, Germany), egg-PC (Lipoid, Germany), POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) or DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine), cholesterol and DSPE-PEG (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]). Phospholipids, cholesterol and PEGylated lipid a 52:45:3 molar ratio were dissolved in absolute ethanol. Calcium- (Ca^{2+}) and magnesium- (Mg^{2+}) free PBS buffer at neutral pH was used as the aqueous phase. The organic and aqueous phases were rapidly mixed using the NanoAssemblr Benchtop microfluidic instrument at various Flow Rate Ratios (1:1 - 4:1) and Total Flow Rates (1 mL/min – 12 mL/min) to form unilamellar liposomes of various size. Verteporfin was loaded by dissolving it in the lipid solution at a concentration of 1 mg/mL (0.09 molar ratio to total lipids). Here, 2.5% by volume DMF was added to ethanol as a co-solvent. Formulations with high DSPC content were heated at 65 °C during the mixing process using the NanoAssemblr Heating Controller accessory. The Heating Controller accessory for the NanoAssemblr Benchtop can adjust the formulation process temperature with an accuracy of ±1 °C. Formulations were then dialyzed against PBS for ethanol removal. At Flow Rate Ratios 1 and 1.5, formulations were diluted down to 10% ethanol immediately following the mixing process and before dialysis, since high amounts of ethanol can destabilize liposomes. Particle size and integrity were measured before and after dialysis using Dynamic Light Scattering (Zetasizer, Malvern Instruments, UK). Unless otherwise indicated, formulations were prepared in triplicate. Size and polydispersity index (PDI) were represented as the mean of 3 samples, and error bars represent standard deviation of the mean. Verteporfin content was quantified by fluorescence spectroscopy.

Related Material

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