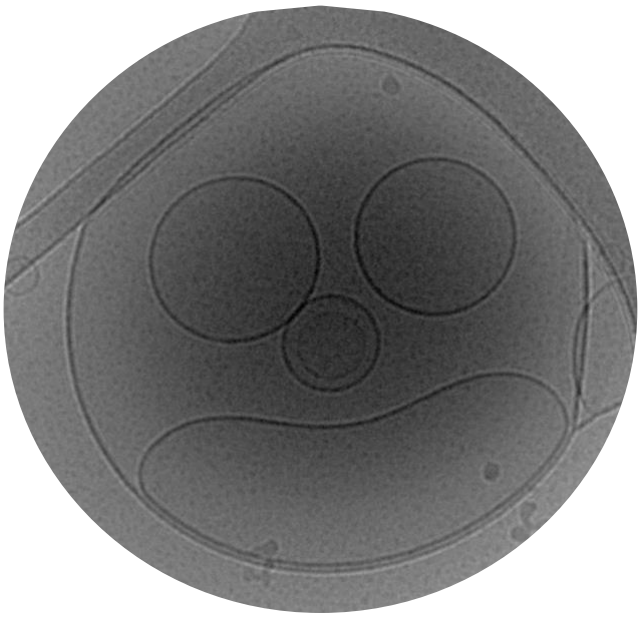
Exploring alternative surfactants to replace PEG within lipid nanoparticles



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Abstract

Over the past decade an increasing amount of studies has demonstrated that PEGylation not only precipitates reductions in drug delivery efficiency but also triggers the immune system to produce antibodies, specifically anti-PEG antibodies. The objective of my thesis is to explore alternative surfactants that could viably replace PEG within lipid nanoparticles. Specifically, Polysorbate 80 (P80), alpha-tomatine, and N-hexadecyl beta-d-maltoside (HDM).

The key findings from this research are that smaller particles have formed with combination of phospholipid DOPC and MQ-water. Upon decreasing total lipid concentration a trend could be observed where the size increased with decreased cholesterol concentration. The most stable formulations, from stability study, are the following: 3 and 10% HDM, 2 and 10% tomatine and 3% P80. When analyzing the samples with Cryo-TEM, it was evident that 30 day old samples exhibited less size variation and appeared smaller. Overall, it could also be seen that tomatine formed more simple vesicles than HDM.

Some further investigations are needed to explore the impact of different buffers on formulation of LNP. It could be done by varying pH and concentrations of these buffers. Incorporation of cationic lipid into the formulation could be the next step in this research.

Acknowledgement

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This thesis would not have been possible without the support and contributions of all those mentioned above. Thank you for being part of this important milestone in my academic journey.

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Abbreviation

Abbreviation	*Definition*
PEG	Polyethelyne glycol
LNP	Lipid nanoparticle
HDM	N-hexadecyl β d-maltoside
T	α-tomatine
Chol	Cholesterol
P80	Polysorbate 80
PB	Phosphate buffer
MQ	MilliQ-water
DLS	Dynamic light scattering
PDI	Polydispersity index

1. Background

Lipid nanoparticles (LNPs) serve as crucial colloidal particles with significant applications in diverse clinical fields like cancer treatment, diagnostics, and vaccine development. In these contexts, LNPs function as carriers for various substances including antibodies, proteins, peptides, and contrast agents (Musielak et al., 2022). Specifically in vaccine delivery, LNPs play a pivotal role in protecting the cargo, such as nucleic acids, shielding it from enzymatic degradation until it reaches its intended target. The formulation of LNPs typically involves a combination of lipids, such as ionizable cationic lipids and helper lipids such as phospholipids, PEG-lipids and cholesterol (Hald Albertsen et al., 2022). Cationic lipids are generally used for several reasons. The main reason is to be able to encapsulate nucleic acids such as DNA and RNA . Since nucleic acids phosphate groups are negatively charged, electrostatic attraction will be formed with cationic lipid. Anionic lipids may obstruct some biochemical reactions leading to cytotoxicity. The focus in this master thesis is on the helper lipids such as cholesterol, phospholipid and an alternative to polyethylene glycol.

Polyethylene glycol (PEG) has a broad spectrum of applications across pharmaceutical formulations and cosmetic products. (Dabaja A, 2023) This master thesis specifically examines its role within LNPs. PEG is used for its stealth properties and biocompatibility. (Verhoef & Anchordoquy, 2013; Yang & Lai, 2015) The mechanism of PEGylation is generally assumed to extend the circulation so called “stealth” behavior (Verhoef & Anchordoquy, 2013; Yang & Lai, 2015), improvement of pharmacokinetics, enhancement of drug efficiency etc (Hong et al., 2020; Yang & Lai, 2015).

However, over the past decade an increasing amount of studies has demonstrated that PEGylation not only precipitates reductions in drug delivery efficiency (Verhoef & Anchordoquy, 2013) but also triggers the immune system to produce antibodies, specifically anti-PEG antibodies. This phenomenon leads to the observed 'accelerated blood clearance' (ABC) of PEGylated drugs (Garay et al., 2012; Verhoef & Anchordoquy, 2013; Yang & Lai, 2015). Which may result in for instance increase the risk and severity of infusion reactions, reduce the efficacy of therapeutic and/or reduce tolerance. (Garay et al., 2012; Hong et al., 2020)
Furthermore, the extensive use of PEG in cosmetics and other consumer products results in pre-existing PEG-specific antibodies. The presence of elevated anti-PEG antibodies can lead to severe allergic reactions. (Hong et al., 2020)

Environmental footprint can also be changed by synthesizing surfactants that are derived from renewable resources making them more sustainable and biodegradable (Larsson, 2021). Here α-tomatine and N-hexadecyl β d-maltoside (HDM) are both made from renewable resources and are biodegradable.

1.1 Purpose of work

The objective of this master thesis is to explore alternative surfactants that could viably replace PEG within lipid nanoparticles. Specifically, the investigation will focus on the surfactants Polysorbate 80 (P80), α-tomatine, and N-hexadecyl β d-maltoside (HDM). Diverse nanoparticle solutions will be formulated, varying in surfactant concentration and total lipid concentration. Through systematic experimentation, the study aims to assess the size and stability of these varied nanoparticle formulations, aiming to identify the most optimal and stable nanoparticle configuration.

In this master thesis I will investigate the following:

- If Tomatine and/or HDM can be employed in the formation of lipid nanoparticles (LNPs) within desired size range of 100-500nm.
- If the resulting particles are stable under relevant storage temperatures, such as room temperature and in the fridge at 4 degrees Celsius.
- The particle formation in phosphate buffer versus pure milliQ-water.
- The morphology of the resulting particles.

2. Theory

2.1 Lipid nanoparticle

As mentioned before, LNP is a type of nanoscale delivery system that is engineered to package, shield and deliver the cargo to the target location within the body. The basic structure of LNPs typically involves a combination of lipids, such as ionizable cationic lipids and helper lipids such as PEG-lipids, phospholipids and cholesterol. (Hald Albertsen et al., 2022). An example of such structure of a lipid nanoparticle can be seen bellow in Figure 1.

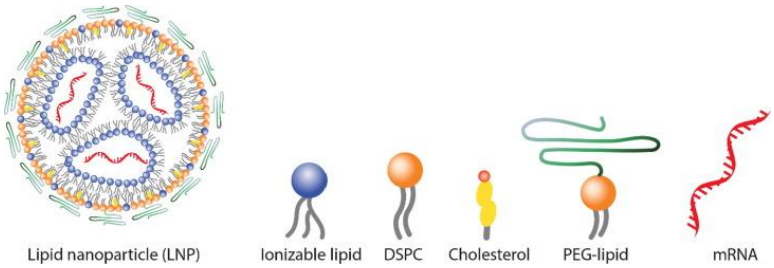

Figure 1. A typical structure of lipid nanoparticle with different components (Hald Albertsen et al., 2022)

The outer layer of LNP consist of PEG-lipids, phospholipid, cholesterol and ionizable lipid. Inside the LNP a reverse micelle is formed that consists of ionizable lipid, its hydrophobic tail is angled toward the hydrophobic tails of helper lipids. More specifically, cationic lipid interacts with nucleic acid with its positively charged head group which leads to encapsulation of nucleic acid. Resulting in for instance protection against degradation. In order for the cationic lipid to be charged, the pH must be below its pKa.

There are also other types of LNPs, one of them are liposomes. (Tenchov et al., 2021) Similarly to LNPs, liposome may consist of an outer layer of PEG-lipids, phospholipid, cholesterol and ionizable lipid. The main difference is that liposomes have a simpler structure, they have a double layer. Inside the liposome a hydrophilic drug can be encapsulated while hydrophobic drugs can be trapped in the hydrophobic tails. As can be seen in Figure 2. Liposomes are divided into two groups depending on the lamellar structure. Unilamellar vesicles such as

small unilamellar vesicles (SUV), large unilamellar vesicles (LUV) and giant unilamellar vesicles (GUV). Multilamellar vesicles such as MLV and multivesicular vesicles (MVV). The size range that is of interest in this master project is 100-500nm which means that SUV and LUV are of interest. (Tenchov et al., 2021)

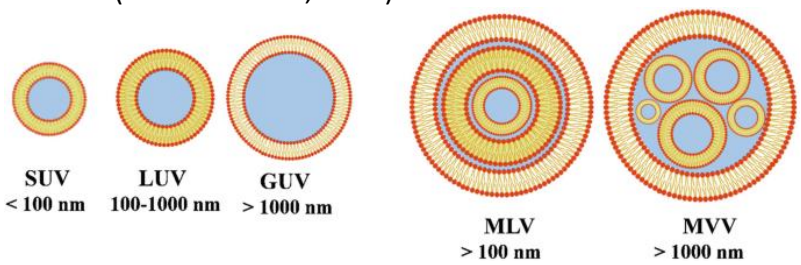

Figure 2. Structure of liposomes with different sizes. (Mahajan et al., 2023)

2.1.1 Phospholipid

Phospholipids play various roles in LNPs, including enhancing encapsulation (as observed with cholesterol) and facilitating cellular delivery. Common phospholipids in LNP formulation are 1,2-Distearoyl-sn-Glycero-3-Phosphatidylcholine (DSPC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE). Saturated phosphatidylcholine (PC) lipids such as DSPC in LNP formulations are derived from small-molecule liposomal delivery systems, which necessitate high Tm lipids for prolonged circulation times and overall stability. (Hald Albertsen et al., 2022) Already commercial LNP systems typically feature only DSPC, likely due to its established stability in commercial liposomes (Hald Albertsen et al., 2022)

However, by substituting DSPC with unsaturated phospholipids, specifically DOPC and SOPC, led to an increase in particle uptake and enhanced intracellular delivery.
(Hald Albertsen et al., 2022)

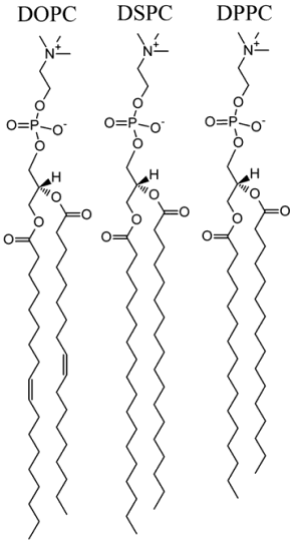

Figure 3. Chemical structure of three phospholipids, DPPC, DSPC and DOPC. (Baykal-Caglar, 2010)

The molecular structure for the three phospholipids mentioned below are illustrated in Figure 3.

2.1.1.1 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine (DOPC)

DOPC, $C_{44}H_{88}NO_8P$, is a phospholipid with two carbon tails that consist of 18 carbons and a double bond. The headgroup consist of phosphatidylcholine (PC). The molecular weight is 786.1 g/mol. (National Center for Biotechnology Information, 2024g) DOPC is soluble in ethanol at a concentration of 25 mg/mL (Cayman Chemical, 2024). Melting point is around -21°C (Beattie et al., 2005).

2.1.1.2 Dipalmitoylphosphatidylcholine (DPPC)

DPPC, $C_{40}H_{80}NO_8P$, is a phospholipid with two carbon tails that consist of 16 carbons. The headgroup consist of phosphatidylcholine (PC). The molecular weight is 734 g/mol. (National Center for Biotechnology Information, 2024c) The phospholipid is in a solid/gel phase at 37°C and has a melting point at 41.3°C (Beattie et al., 2005). DPPC is soluble in ethanol at a concentration of 30mg/mL (Cayman Chemical, 2022).

2.1.1.3 1,2-Distearoyl-sn-Glycero-3-Phosphatidylcholine (DSPC)

DSPC, $C_{44}H_{88}NO_8P$, is a phospholipid with two carbon tails that consist of 18 carbons. The headgroup consist of phosphatidylcholine (PC). The molecular weight is 790.1 g/mol (National Center for Biotechnology Information, 2024e). DSPC is soluble in ethanol at a concentration of 1 mg/mL. The transition temperature is approximately 55°C (Cayman Chemical, 2023) .

2.1.2 Cholesterol

Cholesterol, $C_{27}H_{46}O$, consist of 27 carbons and a specific structure with hydrocarbon tail, a central sterol nucleus that composed of four hydrocarbon rings, and a hydroxyl group. The hydrocarbon tail and central sterol are non-polar (Craig M, 2023). See Figure 4. Cholesterol has a molecular weight of 386.7g/mol and is soluble in ethanol (National Center for Biotechnology Information, 2024b).

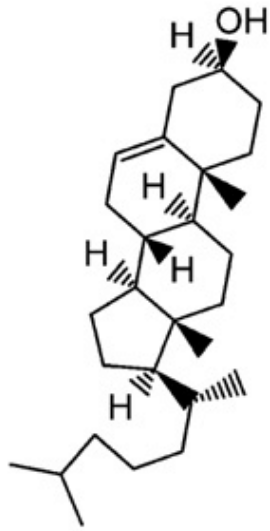

Figure 4. Chemical structure of cholesterol. (Baykal-Caglar, 2010)

The function of cholesterol in a LNP system is not fully clear. One study showed that DSPC-Cholesterol in an empty LNP stays in the outer layers (both monolayer and bilayer) meanwhile in a loaded LNP with siRNA the DSPC-cholesterol complex internalized together with the

cargo. The authors also showed that LNP size decreased upon increasing DSPC-Cholesterol complex in the relation to ionizable cationic lipid. (Kulkarni et al., 2019)

The role of cholesterol in the outer layer of an LNP system is influenced by the type of phospholipid it is paired with. When paired with low-Tm phospholipids, such as DOPC, cholesterol promotes a liquid-ordered phase, which leads to decreased membrane fluidity and increased bilayer thickness (Hald Albertsen et al., 2022). Conversely, when paired with high Tm phospholipids, such as DPPC, cholesterol increases membrane fluidity and narrows the bilayer (Hald Albertsen et al., 2022). In both cases, cholesterol directs lipids towards a liquid-ordered phase.

The incorporation of cholesterol in a loaded LNP system serves two purposes. First, it is a versatile molecule that can accumulate within the liposome during circulation (Hald Albertsen et al., 2022). Second, cholesterol reduces surface-bound protein and increases circulation half-life, stabilizes the particle, and increases membrane rigidity, which leads to reduced drug leakage from the cargo (Hald Albertsen et al., 2022). In order to preserve the integrity of membranes, LNP formulations typically comprise an equimolar ratio of cholesterol to that of endogenous membranes. This arrangement helps prevent both net efflux and influx of molecules across the membrane. It is also worth noting that cholesterol is a major component of biomembranes and is highly biocompatible (Tenchov et al., 2021).

2.1.3 Surfactants

Surfactants play a crucial role in the structure of LNPs, providing stabilization at specific concentrations. If the concentration of surfactants exceeds a particular limit, then micelles are more likely to form instead of surfactants integrating into the LNP system.

Surfactants lower the surface tension and consist of a hydrophobic tail and hydrophilic head helping to connect the water with the lipids. There are different types of surfactants, those that are nonionic and ionic. The classification is made depending on the polar head. If the net charge is negative then the surfactant is anionic. If it's positive, surfactant is cationic. If it has both negatively and positively charged parts then it's amphoteric (Chung, 2017).

A surfactant that is nonionic is of interest for a LNP structure. The compactness in a LNP structure is important because a nonionic surfactant will neither be repulsed or attracted to other lipids in the system. Leading to a compact lipid that can both hold the cargo and release when needed.

2.1.3.1 Tomatine

α-Tomatine, $C_{50}H_{83}NO_{21}$, (see Figure 5) has a molecular weight of 1034,2 g/mol and is soluble in ethanol. It's melting point is between 263-268°C (National Center for Biotechnology Information, 2024d).

Tomatine is a surfactant in this lipid nano structure. Tomatine adjuvant is based on a glycoalkaloid lycopersicin which is derived from the leaves of a wild tomato species Lycopersicon. Several articles have shown that this compound can disturb membrane qualities, similar to immunostimulators. It has also been shown that tomatine have a significant effect as a vaccine adjuvant for infectious diseases and also as cancer

immunotherapy. It is also capable of inducing humoral and cellular immune responses and is well tolerated and non-toxic (Morrow et al., 2004). α-tomatine has different properties, it can reduce plasma LDL cholesterol level, inhibit growth of cancer cells and microorganisms and stimulate the immune system (Yamanaka et al., 2008).

Tomatine can form a complex with cholesterol and other 3beta-hydroxy sterols. They form 1:1 complex and disturb the membrane (Stine et al., 2006).

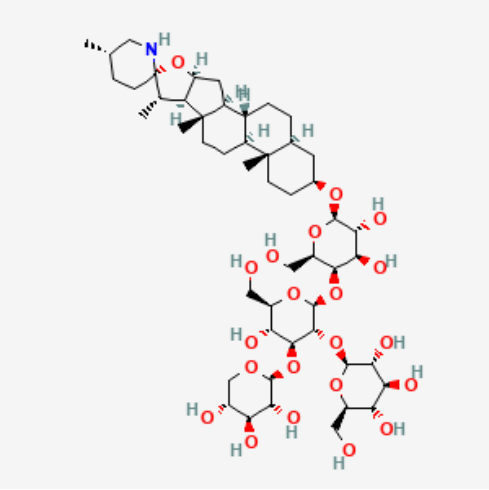

Figure 5. Chemical structure of α-tomatine. (National Center for Biotechnology Information, 2024d)

2.1.3.2 N hexadecyl β d maltoside (HDM)

N hexadecyl β d maltoside ,$C_{28}H_{54}O_{11}$, (see Figure 6) has a molecular weight of 566.7 g/mol,(National Center for Biotechnology Information, 2024f). It is an alkylglycoside meaning that it is a non-ionic surfactant with a carbohydrate headgroup and an alkyl chain tailgroup. The tail of this HDM is connected in the equatorial position to the headgroup making it a β configuration. It is a mild surfactant which forms elongated worm like micells in water. The micelle size are bigger in lower temperatures otherwise the size is independent of temperature (National Center for Biotechnology Information, 2024f). Since HDM is not usually used in lipid nanoparticle formulation, there are no studies found on how HDM would behave with other components in LNPs.

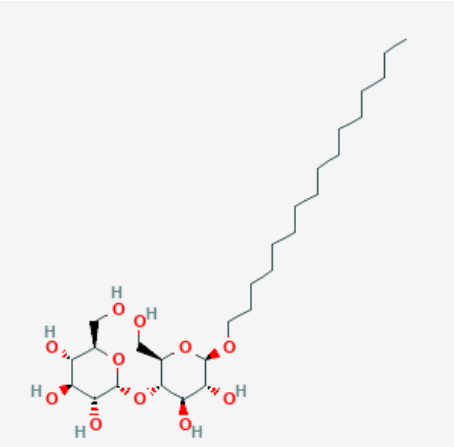

Figure 6. Chemical structure of HDM. (National Center for Biotechnology Information, 2024f)

2.1.3.3 Polysorbate 80 (P80)

Polysorbate 80, P80, has a molecular weight of 1310 g/mol and is in liquid form at 25°C and has a melting point at -20°C. P80 is also soluble in ethanol (National Center for Biotechnology Information, 2024a). It is a biodegradable (López-Machado et al., 2021) non-ionic surfactant that is a food graded additive and emulsifier as well as it is approved by FDA as inactive ingredient in several drug products (The European Commision, 2012; U.S. Food & Drug Administration, 2023).

P80 has been used in different LNP systems as a dispersing agent. The addition to DGMO/GMO-50/water system resulted in an increase in L3 phase from 60% to 60-70% water. Meaning that P80 contributes to the formation of swollen phases (Valldeperas Badell, 2019). The addition of P80 to a liposome resulted in a decrease of surface tension and formation of smaller droplets (López-Machado et al., 2021).

P80 has a 18 carbons in its tail and a double bond between 9-10 carbon (see Figure 7) which is similar to the tail length and the double bond of phospholipid DOPC. The melting point is also similar, however the molecular weight of P80 is almost double the weight of the phospholipids that has been mentioned. Which indicates that the head of P80 is larger than phospholipids, it can also be seen in the Figure 7 and 3.

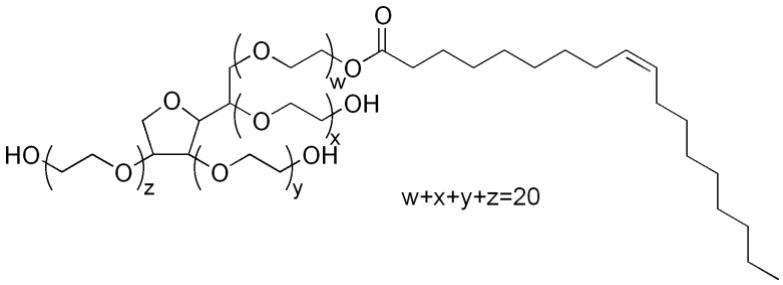

Figure 7. Chemical structure of P80. (Edgar181, 2008)

2.2 Stability study

The purpose of a stability study is to provide data on how the quality of drug product changes with time in different environments such as change in temperature, humidity and light. It also conducted to create a re-test period, shelf life and recommended storage conditions for a drug product (European Medicines Agency, 2003).

In this master thesis a stability study will be conducted by changing the temperature. The samples will be kept in dark to avoid other factors such as light affect the study. The samples will be analyzed after 3, 7 and 30 days. The formulations resulting in particles smaller than 500 nm are suitable for the stability study.

2.3 Preparation methods

There are several different ways to make a lipid nanoparticle. A common method is by using a microfluidic chip. A solution of lipids in ethanol are loaded into two syringes and are forced to flow into the chip where it meets a buffer in a T-crossed channel. Lipid/ethanol solution mixes with buffer inside the chip at a certain flow rate. Upon assembly in the focused alcohol/buffer interface, the lipid is forced to participate and self-assemble into liposomes (Tenchov et al., 2021). Here 50 and 100 uml/ml was used. A suitable amount of the sample is

collected. In order to remove ethanol, approximately 1/3 of the sample is evaporated in vacuum. The weight is noted before and after the evaporation to ensure that 1/3 is removed. In practice it is also possible to smell the sample to ensure that no alcohol is present.

In this project a film hydration method will be used. Lipids are first dissolved in ethanol and then evaporated with speed vac at a certain temperature so that a thin film is obtained at the bottom of a glass tube. An aqueous phase, buffer, is added to hydrate the film and form a liposomal dispersion. In order to shrink and homogenize the particles, the samples are sonicated at a certain amplitude and time (Tenchov et al., 2021). Afterwards the samples are left to rest on a waving board. The size is then analysed with DLS and a stability study were done in darkness at room temperature and in the fridge.

2.3.1 Ultra sonication

Sonication is conducted soon after the samples have been hydrated with a buffer. There are different sonication methods, ultrasonic bath, and ultrasonic probe (sonicator). Ultrasonic frequencies are applied as energy to the samples in order homogenize particles and decrease the size (Chung, 2017; Nasri et al., 2022). Ultra-sonication is also conducted in order to prevent the agglomeration or break them as the technique affect the surface and structure of nanoparticles (Nasri et al., 2022). An appropriate amount of both time and energy is crucial so that the LNPs are not over sonicated or burned.

2.3.2 Extrusion

Extrusion is a method that is used in order to form particles. Lipids are forced through a small membrane which results in small and more homogeneous particles. A solution that contains liposome is passed through a membrane filter of a certain pore size. During the filtration there are several parameters that may influence the size of particles such as pressure, number of cycles and pore size (Ong et al., 2016). An extruder consists of a platform made of metal that holds two glass syringes and a holder for the membrane filter. Figure 8 provides a more detailed illustration of the components in the membrane filter holder.

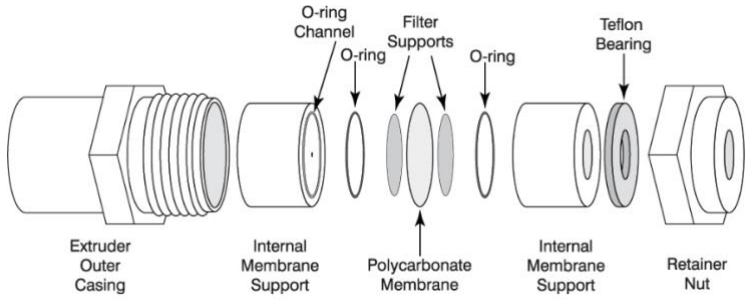

Figure 8. Composition of extruder (Avanti Polar Lipids, 2024).

2.4 Analytical methods

2.4.1 Dynamic light scattering (DLS)

DLS is an analytical tool for measuring the size, polydispersity and Z-potential of small particles (<1um) in a solution. DLS is possible to conduct in wide range of buffers, temperature and concentrations. It is also not an invasive technique that requires low amount of sample. It can be used to measure the homogeneity of proteins, RNA and their complexes (Stetefeld et al., 2016).

By measuring Brownian motion of particles, the size of particles can be obtained. Brownian motion is a random path that particles take when they are being "pushed around" by the solvent molecules that are larger. Smaller particles move faster and more rapidly than large particles. This velocity of Brownian motion is known as translation diffusion coefficient, D. By using Stokes-Einstein equation the hydrodynamic diameter, dH of a particle can be calculated(Malvern Instruments, 2018; Stetefeld et al., 2016).

The following equation is a Stokes-Einstein equation where, dH is the hydrodynamic diameter, k is Boltzmann's constant, T is an absolute temperature, η is viscosity and D is the translational diffusion coefficient (Malvern Instruments, 2018; Stetefeld et al., 2016).

$$dH = \frac{kT}{3\pi\eta D} \quad (1)$$

Worth mentioning is that DLS measures a particles diffusion in a solvent with time. Since diffusion of a particle depends on the type of solvent, it is important that viscosity is constant throughout the measurement, therefore temperature should remain the same (Malvern Instruments, 2018).

The set-up of DLS consists of a cell, laser, attenuator and detector at a certain angle. Laser supplies a light source for illumination of the sample inside the cell. If a sample is diluted, the laser beam passes through it. Nevertheless, some light is scattered by the particles in all directions. Since particles follow Brownian motion, the distance between the scatterers change over time leading to fluctuation. The detector, at a certain angle, measures the intensity of fluctuated scattered light. However, when too much light is detected, the detector becomes oversaturated. Therefore, an attenuator is used to reduce the intensity (Malvern Instruments, 2018).

To define PDI, polydispersity index, an intensity particle size distribution is needed. DLS has calculated the mean and standard deviation of the peaks. PDI is calculated by dividing the standard deviation with the square of the mean (Malvern Panalytical, 2015).

2.4.2 Small angled x-ray scattering (SAXS)

SAXS is a powerful tool that is used to study macromolecules and nanoparticles in a solution by using X-ray (Rumancev et al., 2022). By using SAXS, the lipid nanoparticles shape, structure (for example outer layer) and size can be obtained.(Malvern Panalytical, 2024) As well as the lamellarity of LNPs. (Goel et al., 2022)

2.4.3 Cryogenic transmission electron microscopy (Cryo-TEM)

Cryo-TEM is used in order to determine the morphology of a macromolecule such as shape, size and internal structure. A beam of electrons at high voltage is transmitted through the specimen (grid) in order to create an image from intensity of the transmitted electrons. The sample, that is fixed on the grid, is analyzed at the cryogenic temperature meaning that the sample is frozen at the temperature of liquid nitrogen. (Goel et al., 2022)

3. Material

Lipids:

- Cholesterol from sigma, purity ≥92.5%
- DOPC from avanti, purity >99
- DPPC from sigma, purity >99%
- Tomatine from Tokyo Chemical Industry (TCI), purity >80%
- HDM from anatrace, purity ≥97%
- P80 (super refined polysorbate) from CRODA.

Chemicals:

- Ethanol 99% from solveco
- MilliQ-water from ultrapure water purification system (gradient)
- Disodium phosphate (Na_2HPO_4) from sigma, purity ≥98%
- Monosodium phosphate (NaH_2PO_4) from sigma, purity ≥98%

4. Experimental

4.1 Preparation of stock solutions

Scale was tared with a beaker or a conical flask. Certain amount of lipid or surfactant was added and weighted on the scale. Specific volume of 99% ethanol was then added with a micropipette in order to dissolve the lipid/surfactant.

4.2 Preparation of samples

By using a Matlab script ,see Appendix A1, different volume of components were calculated for transfer using a micropipette into a glass tube. (plastic tubes were tested but were suspected of shedding plastic particles into the sample) Prior to handling, a micropipette was calibrated so that the settings on the pipette matches with the weight of water. For example, if 1 uL of water was measured with micropipette then the water should weight 1 mg on the scale. Otherwise, a smaller volume was measured with micropipette until the amount of water on the scale was 1mg. This has been done with 3 volumes on two micropipettes with different range.

Because of high final volume, the solution was aliquoted so that 1 mL of buffer is needed for hydration to obtain a certain concentration.

In table 1 an example of composition of particles could be seen.

Table 1. Composition of sample 1-3 where the components are in percentage.

Sample	*Phospholipid*	*Cholesterol*	*Surfactant*
1	20	78	2
2	20	70	10
3	20	60	20

Phospholipid concentration were kept constant at 20% throughout the tests while surfactant and cholesterol concentration were varied so that the sum of them would be 80 %. An example of compositions could be seen in table 1. When 2% surfactant is used, there are also 78% of cholesterol, making it 80% in total.

4.2.1 Evaporation

Centrifugal concentrator (miVac) from Genevac was used. Temperature was set to 30°C at a time of approximately 3-5h. More time was needed for samples with higher volume in order to evaporate all ethanol. Evaporation continued until a dry film could be seen at the bottom of tube.

4.2.2 Hydration

In order to hydrate the lipids, phosphate buffer (PB) and later pure milli-Q water was used. Milli-Q water was obtained from ultrapure water purification system. Meanwhile 1L of PB were prepared by dissolving 4.45 g disodium phosphate (Na_2HPO_4) and 2.19 g monosodium phosphate (NaH_2PO_4) in milli-Q water. This resulted in 25mM PB with 7.1 pH.

1 mL of aqueous solution (either PB or MQ) was added to each sample before sonication.

4.2.3 Sonication

Probe sonication from sonics was used. Shortly after hydration the samples were sonicated with the following settings in Table 2.

Table 2. Setting for sonication

	Setting
Amplitude	30 %
Time	6 min
Pulse	1s on/ 1s off

The probe (sonicater) was cleaned with ethanol and milliQ-water and dried with tissue before the procedure and after each sample to reduce the risk of contamination. A circular dish was filled with deionized water, and a test tube rack was positioned inside. The glass tube containing the sample was placed in the rack, ensuring that the solution remained below the water level. This precaution was taken to prevent overheating of the sample. The sonicator was positioned within the tube, and the probe was carefully centered, with its tip placed as far down as possible without getting into contact with the glass.

4.2.4 Extrusion

After sonication or after the samples were mixed for several days on the rocker an extrusion was made. Firstly, a filter of 100nm was placed into a filter-holder that was secured by metal container which was screwed tightly, which was then placed and secured on the metal platform. An empty syringe was placed inside the filter holder. The filled syringe was also placed inside the filter holder. A constant pressure was carefully applied on the plunger of the filled syringe. When the sample has transferred into the second syringe, the same force was applied to the plunger of the now filled syringe. This procedure was repeated 20 times. Afterwards, the syringe with the sample was carefully removed and the sample was transferred to a new glass tube. The sample was left on the rocker to rest for 1 day before analysis. Exactly what syringes and filters that were used are summarized in the Table 3.

Table 3. Type of components used in extruder.

	Type
Syringe	1000 μL Gastight #1001
Filter	Whatman® Nuclepore™ Track-Etched Membranes diam. 13 mm, pore size 0.1 µm, polycarbonate #8035036
Platform	Avanti Polar Lipids, Inc.

4.3 Analytical method

4.3.1 DLS

DLS instrument used was Zetasizer nano series from Malvern. 50 μL of sample was diluted in a VWR cuvette PS macro with 1 mL of buffer (PB or MQ-water). The cuvette was placed inside the cell and the measurement could start. The settings that were used can be seen in Table 4.

Table 4. Settings for DLS.

	Setting
Material	Lipid
Dispersant	Water
Temperature	25°C
Cell	Disposable cuvettes (DTS0012)
Backscattering	173° (NIBS default)
Measurement duration	Automatic
Number of measurements	6
Data processing	General purpose (normal resolution)

The following data was then obtained from DLS: Correlation data, PDI, Z-average, size distribution by number and by intensity.

4.3.2 SAXS

Small amount of sample were transferred into a quartz capillaries 1.5mm. They were then loaded in the magnetic holder which were then placed inside the capillary flow cell that was vacuum-enclosed with controlled temperature. Each sample were then subjected to X-ray for 7h at high resolution.

4.3.3 Cryo-TEM

GloQube was used to clean/prepare TEM grids. Leica EM GP was used to plunge freeze the samples. Firstly, clean TEM grid was placed inside Leica, then small amount of sample were applied to the TEM grid with a micropipette. The excess liquid was removed with blotting paper inside the machine. The grid is then plunged into liquid ethane and transformed to liquid nitrogen by hand. The grid was stored in liquid nitrogen for 1h prior to transferring to microscope. In order to avoid frost a cryo-transfer holder was used. The grid was placed into

the holder and loaded into microscope. Computer was then used to view the particles that were fixed on the grid.

4.4 Stability study

After DLS, the samples were sorted by their size. The samples with Z-average <500nm and PDI < 0.5 were used in stability study. One sample was divided into two glass tubes. By using a plastic cork and parafilm the glass tube was secured. In order to keep the samples in darkness, aluminum foil was used around the glass tube.

5. Result and discussion

5.1 Procedure

5.1.1 Concentration and method variation

The samples were prepared as mentioned above. Samples with S were only sonicated, samples with E were only extruded. However samples with S&E were first sonicated then extruded. In Figure 9, DPPC and buffer solution PB was used.

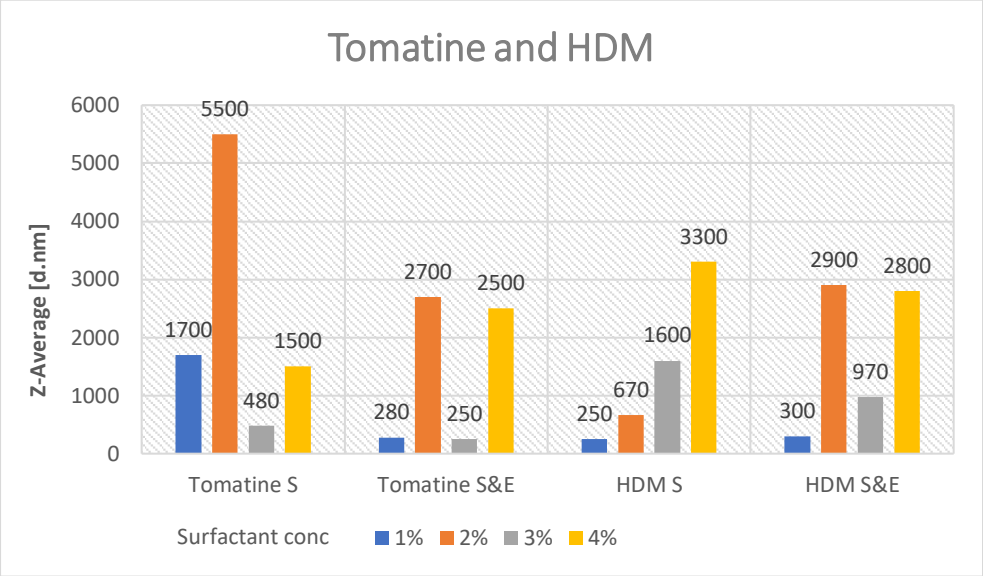

Figure 9. Size of particles for HDM (DPPC:Chol:HDM) and Tomatine (DPPC:Chol:Tom) in PB with four different surfactant (1, 2, 3, and 4 %) and cholesterol (79, 78, 77, 76 %) concentrations and varying particle formation method (S and S&E) .

In initial testing, it was evident that the particles exceeded the nanoscale, appearing notably large. A conclusive trend ,in Figure 9, regarding the superiority between sonication alone and sonication combined with extrusion couldn't be established. When sonicating the particle with HDM, there was a noticeable increase in particle size with higher concentrations. Conversely, this trend wasn't observed in the other cases such as tomatine S, tomatine S&E and HDM S&E. Specifically, when sonicated with 2% tomatine, the particle size reached 5500nm. The larger size observed could potentially result from the formation of a complex between cholesterol and tomatine (Stine et al., 2006).

From Figure 9 it can be seen that 3% tomatine S, S&E and 1 % HDM S, S&E stand out by having particles smaller than 500nm. To assess the formulation's stability, a stability study was conducted specifically on these four samples.

5.1.2 Increase of concentration

It was attempted to achieve smaller particle sizes by increasing the concentration of both tomatine and HDM. To prevent larger particles due to the possibility of cholesterol and tomatine complex formation, the amount of cholesterol was eliminated in tomatine samples but not in HDM samples. In Figure 10, DPPC and buffer solution PB was used.

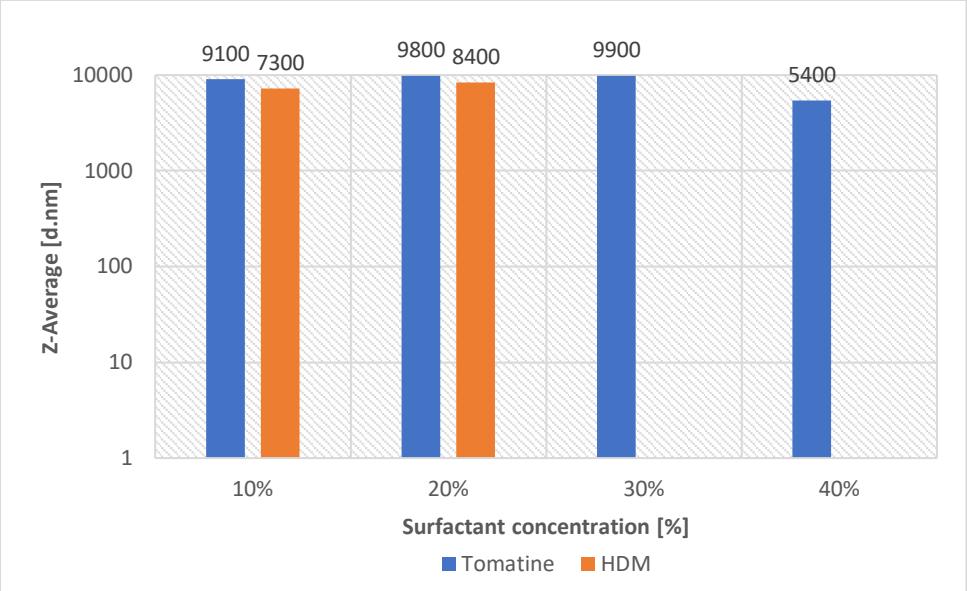

Figure 10. Size of particles for HDM (DPPC:Chol:HDM) and Tomatine (DPPC:Tom) in PB with higher surfactant concentrations 10, 20, 30 and 40 % and lower cholesterol concentration for HDM sample (70 and 60%).

Despite the adjustments, Figure 10 indicates that all concentrations still resulted in larger-sized particles. Notably, among these concentrations, only the particles containing 40% tomatine were smaller compared to the other concentrations. Upon closer inspection of Figure 10, it's evident that particles across all concentrations appear large. However, the smallest particle sizes were observed at 10% for HDM (measuring 7300nm) and 40% for tomatine (measuring 5400nm).

5.1.3 Alteration of lipid concentration

A dilution of lipid concentration was implemented for 40% tomatine and 10% HDM. Specifically, the lipid concentration was decreased from 4mg/ml to 2mg/ml and further to 1mg/ml. In Figure 11, DPPC and buffer solution PB was used.

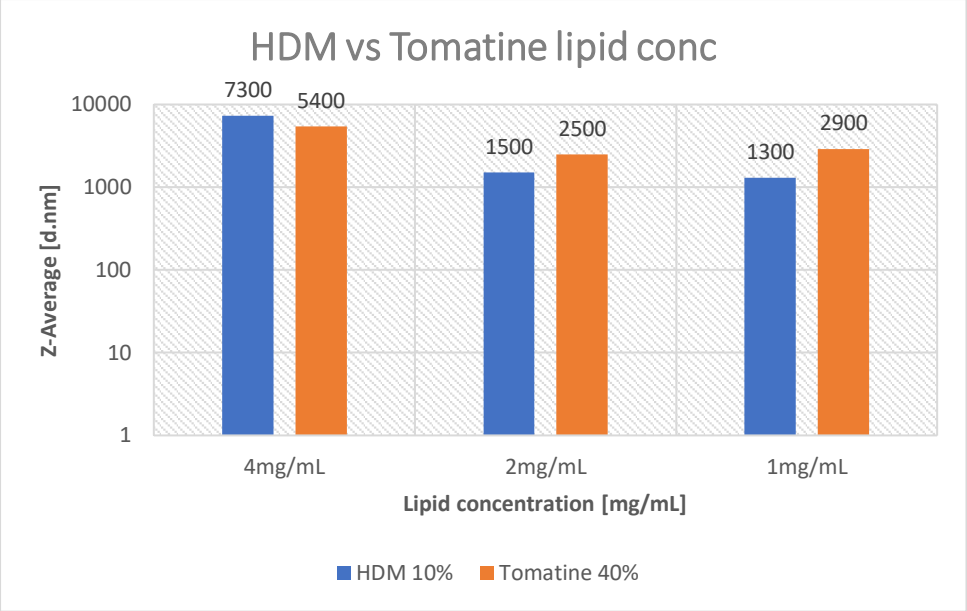

Figure 11. Size of particles for HDM (DPPC:Chol:HDM) and Tomatine (DPPC:Tom) in PB at 10% HDM (cholesterol concentration at 70%) and 40% Tomatine with varying lipid concentration (4, 2 and 1mg/mL).

In the case of the tomatine sample, the particle size demonstrated a reduction from 5400nm at 4mg/ml to 2500nm at 2mg/ml and increase to 2900nm at 1mg/ml (see Figure 11). Similarly, a decrease in particle size was observed for the HDM sample, depicting a trend where particle size decreased from 7300nm at 4mg/ml to 1500nm at 2mg/ml and 1300nm at 1mg/ml. Notably, a trend emerged showing a decrease in particle size corresponding to the reduction in lipid concentration. However, direct comparison between the two series became unfeasible due to the exclusion of cholesterol in the tomatine samples.

5.1.4 Investigation of relation cholesterol and phospholipids

Furthermore, multiple samples were formulated without surfactants to investigate particle formation with DPPC/DOPC and cholesterol. These included variations such as 20% DOPC/DPPC paired with either 76% or 40% cholesterol. Additionally, a different lipid concentration, specifically 4 and 2 mg/ml, was explored. In Figure 12, buffer solution PB was used.

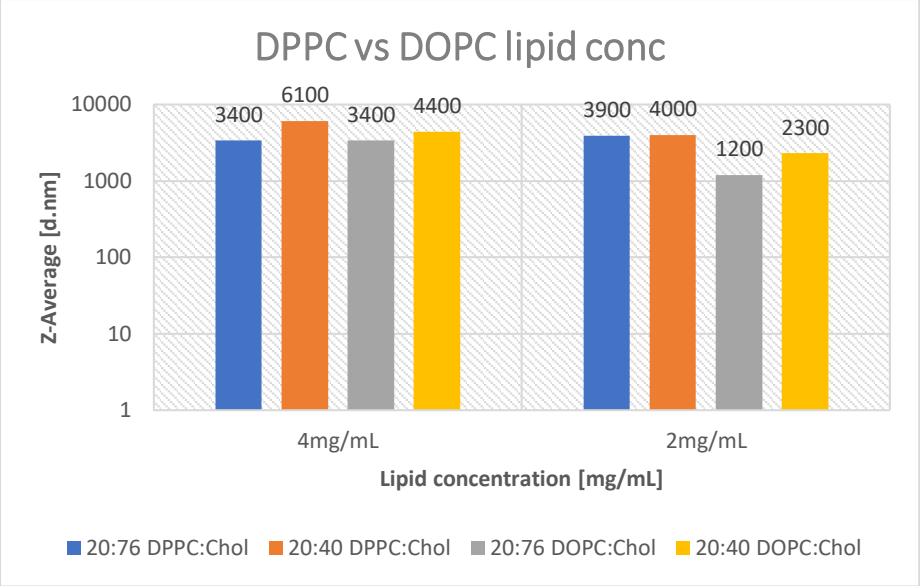

Figure 12. Size of particles for phospholipid and cholesterol complex (DPPC:Chol blue and orange and DOPC:Chol grey and yellow) with varying cholesterol (76 and 40 %) and lipid (4 and 2 mg/mL) concentration.

The data presented in Figure 12 reveals that even in the absence of surfactants, the particle size remains significant. Interestingly, higher concentrations of cholesterol (76%) correlate with smaller particle sizes. Moreover, reducing the lipid concentration appears to result in smaller particles. When comparing the two phospholipids one may see that the particles with DOPC tend to be smaller than those formed with DPPC. Worth mentioning, particle size seem to increase with lower cholesterol concentration.

Even though the structure of the two phospholipids is similar, the Tm differs a lot. Due to low Tm, DOPC (-21°C) exist in a fluid like liquid crystalline state at room temperature. Meanwhile, DPPC (40°C) is in solid-like gel state at room temperature. This difference may affect membrane since the phospholipids exhibit different interactions (Attwood et al., 2013). The fluid state of DOPC may lead to an easier dispersion of cholesterol and DOPC compared to DPPC since it is in its solid state. Worth noting that the difference in solubility for DOPC and DPPC is not significant. Therefore the solubility shouldn't be as decisive in comparison to Tm since the difference is greater.

5.1.5 Alteration in HDM concentration

To explore the correlation between cholesterol and surfactant concentration, samples were prepared using the following ratios: 20:76:4 DOPC:Chol:HDM and 20:70:10 DOPC:Chol:HDM. These were synthesized at both 2mg/ml and 1mg/ml lipid concentrations. In Figure 13, buffer solution PB was used.

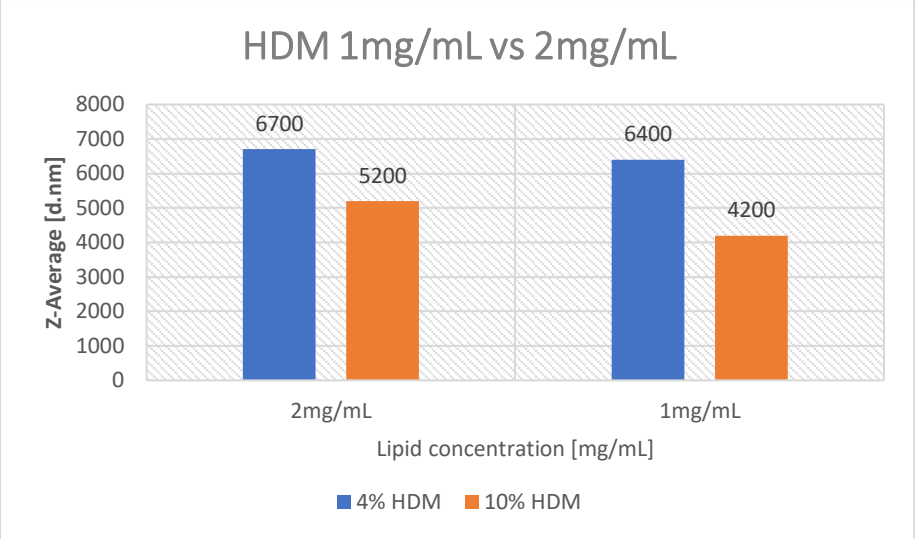

Figure 13. Size of particles formed with the following ratios at 20:76:4 DOPC:Chol:HDM (4% HDM) and 20:70:10 DOPC:Chol:HDM (10% HDM) with varying total lipid concentration (2 and 1 mg/mL).

It can still be observed, in Figure 13, that the particle size decreases as the lipid concentration is reduced. However, despite this trend, the particles remain excessively large, necessitating further investigation. When 10% HDM were prepared using DPPC instead (discussed previously in 5.1.3 Alteration of lipid concentration) the sizes were around 1500 and 1300 nm. This contrasts with the sizes obtained using DOPC, indicating a difference wherein particles appear larger with DOPC compared to DPPC. It also contradicts the previous result where smaller particles were obtained using DOPC and cholesterol.

5.1.5 Change in buffer solution

Due to incoherent results, a comparative study was conducted between PB and MQ water, testing various concentrations of HDM and tomatine alongside a 20% phospholipid composition.

5.1.5.1 Phospholipids

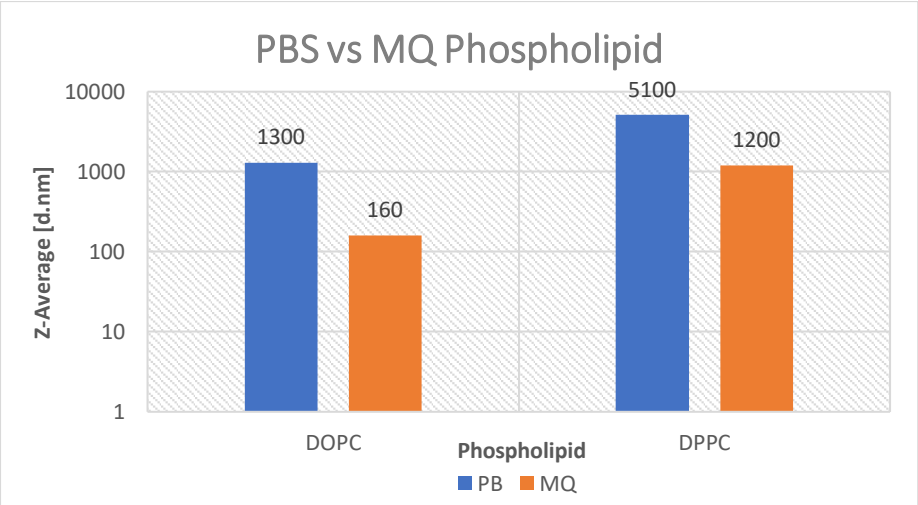

Figure 14. Size of particles for phospholipids DOPC and DPPC in PB and MQ-water.

It can be seen in Figure 14, that overall both phospholipids form bigger particles in PB than in MQ-water. However, DPPC still forms big particles in MQ-water. Meanwhile DOPC has a particle size of 200nm in MQ.

PB is used as a buffer to maintain the physiological pH. When looking at Hoffmeister series, HPO_4^{2-} is on the left side, meaning that it is well hydrated and classified as a kosmotropic ion (strenghthens interaction between water molecules). Phosphate leads to salting out behaviour in proteins, which leads to protein precipitation (Kang et al., 2020). Here, a similar occurrence could occur i.e. affecting the hydrophilic part of the PL leading to lowered amphiphilicity.

5.1.5.2 HDM and Tomatine

The following ratios were used: 20:76:4, 20:70:10 and 20:65:15 DOPC:Chol:Surfactant (either HDM or Tomatine)

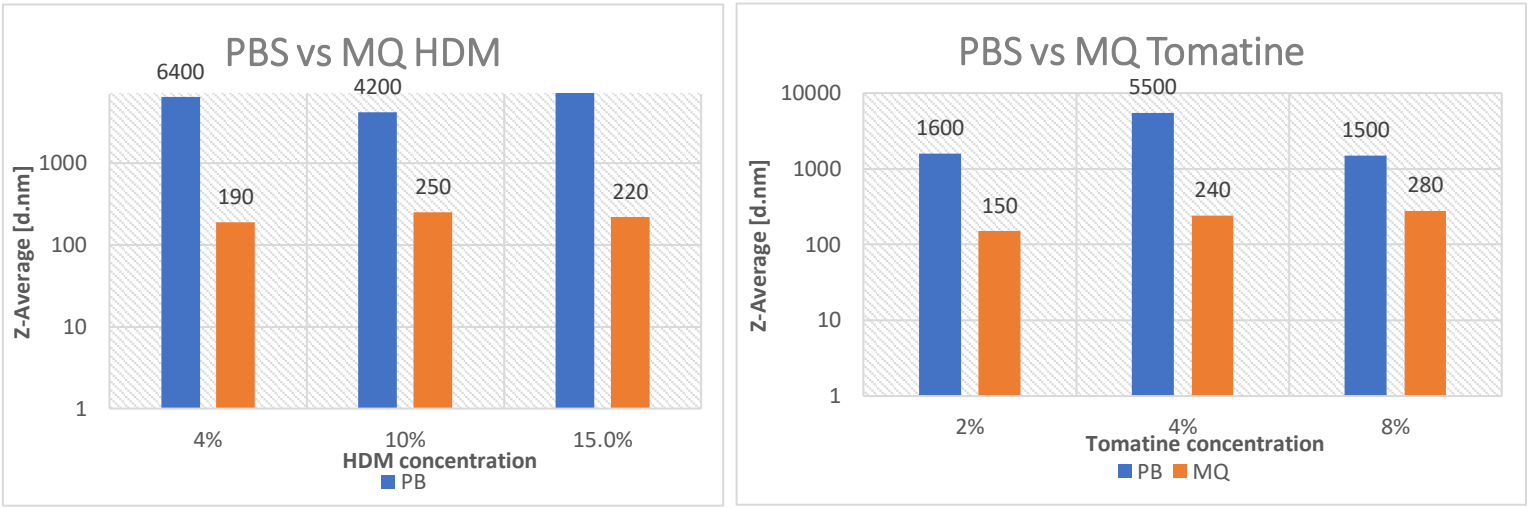

Figure 15. Left. 4-15% HDM in PB and MQ with DOPC. Right. 1-4% Tomatine in PB and MQ with DPPC

The samples in water consistently exhibited smaller sizes compared to those in PB, irrespective of the presence of surfactants or varying concentrations. In general, the particle sizes in water were <500nm. There's a trend in Figure 15 indicating that particle size increased with increasing surfactant concentration. For HDM in water, the size increased from 190nm to 250 and 220nm. Meanwhile for tomatine in water the size increased from 150nm to 240 and 280nm. Simultaneously, a decrease in cholesterol concentration correlated with an increase in particle size. The distinct sizes of HDM and tomatine molecules potentially prompt diverse interactions with the particle surface, thus influencing particle size. However, cholesterol remained a constant factor throughout the experiment, and its evident that decreasing cholesterol concentration corresponds to an increase in particle size.

5.1.6 Altering concentration of surfactant and lipid

Further, 3, 10 and 20% of surfactant were investigated further by increasing the lipid concentration to 2mg/mL. Stability studies were also made with the same concentrations. A maximum concentration was chosen as well as a medium and minimum concentrations with PDI lower than 0.5. 1% weren't chosen due to the concentration being too low and particles

may not have enough material in them. In Figure 16-18, MQ-water and DOPC was used (cholesterol concentration: 78 or 77, 70 and 40 %).

5.1.6.1 HDM

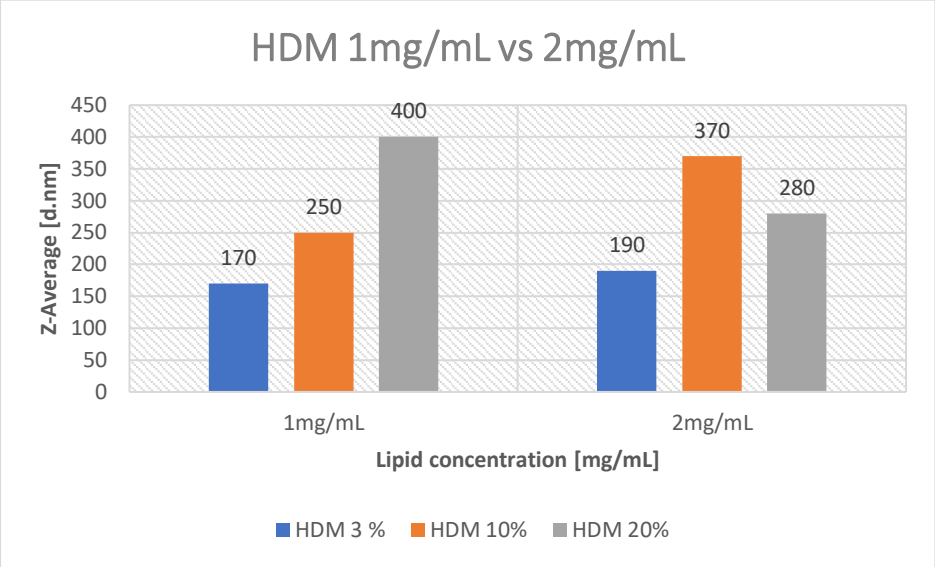

Figure 16. Size of particles for HDM at three concentrations (3%, 10% and 20%) with varying lipid concentration (1mg/ml and 2mg/mL).

In Figure 16, a trend emerges: at a lipid concentration of 1mg/mL, there's a visible increase in particle size corresponding to increased HDM concentration. Conversely, at 2mg/mL lipid concentration, an overall increase in particle size is noticeable.

Interestingly, at 20% HDM, there's a decrease in particle size, which could potentially be attributed to an unstable structure between 10 and 20% HDM. Thus, leading to variation in size. Moreover, it's evident that at 3% and 10% concentrations, particle sizes appear larger when the lipid concentration is elevated to 2mg/mL.

5.1.6.2 P80

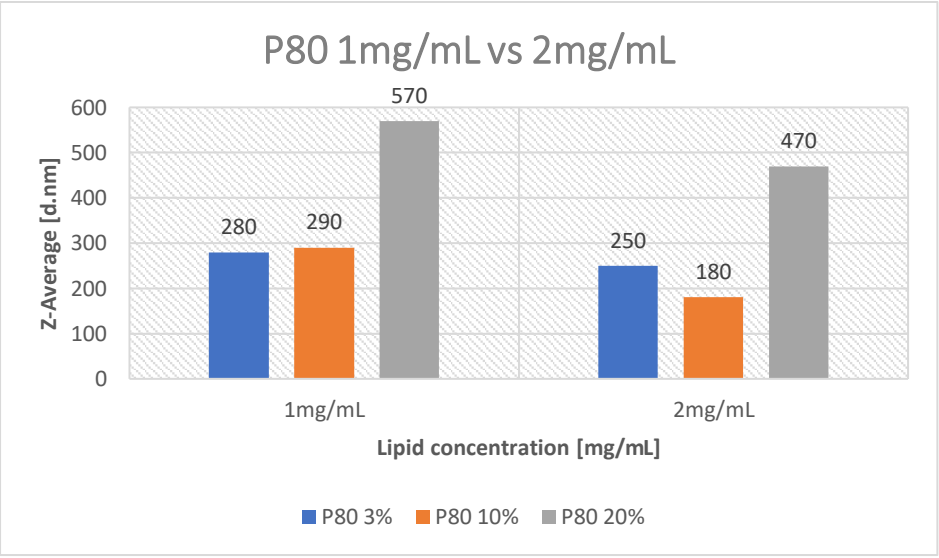

Figure 17. Size of particles for P80 at three concentration (3%, 10% and 20%) with varying lipid concentration (1mg/mL and 2mg/mL).

It could be seen in Figure 17 that at a concentration of 1mg/mL, there's a relatively minimal alteration in particle size despite an increase in P80 concentration from 3% to 10%. However, there's a nearly twofold increase in particle size when the concentration of P80 is raised to 20%. Contrastingly, at a lipid concentration of 2mg/mL, an initial decrease is followed up with an increase in particle size. In general, it can be noted that as the lipid concentration increases, the particle size decreases.

5.1.6.3 Tomatine

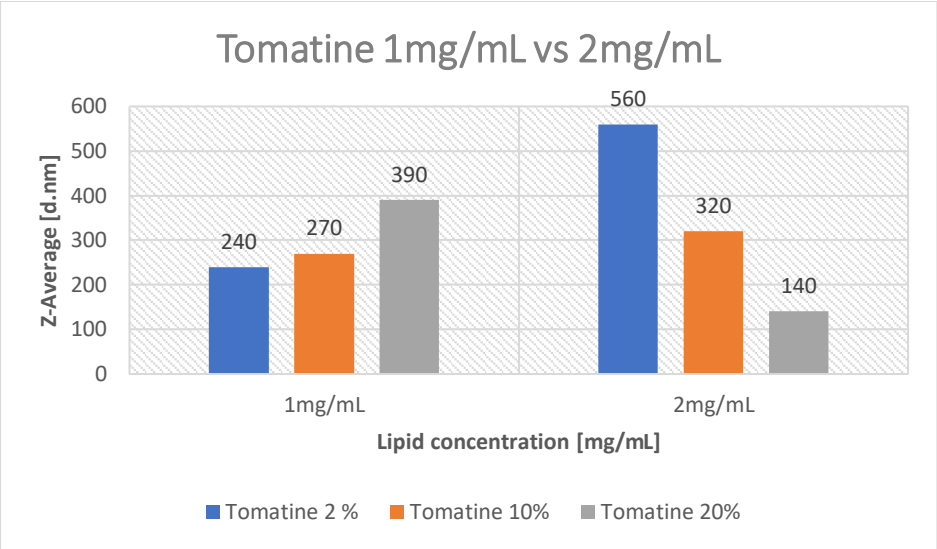

Figure 18. Size of particles for tomatine at three concentrations (2%, 10% and 20%) with varying lipid concentration (1mg/mL and 2mg/mL).

In Figure 18, an increase in size throughout the surfactant concentration is evident at a lipid concentration of 1mg/mL, whereas a reduction is observed at 2 mg/mL lipid concentration. At lower lipid concentration, tomatine tends to behave similarly to P80 and HDM. At 2% tomatine the liposome structure tends to form similar sizes particles with P80 at the same concentration (3%).

Generally, at a lipid concentration of 1mg/mL, an increase in particle size is observed, attributed to the rise in surfactant concentration coupled with a decrease in cholesterol content. Conversely, this trend is not consistent at 2mg/mL lipid concentration, where particle sizes behave differently depending on the surfactant.

5.2 Stability studies

5.2.1 Comparison of sonication and sonication & extruder

The first stability study where different procedure methods were compared. In Figure 19, PB and phospholipid DPPC was used.

5.2.1.1 HDM

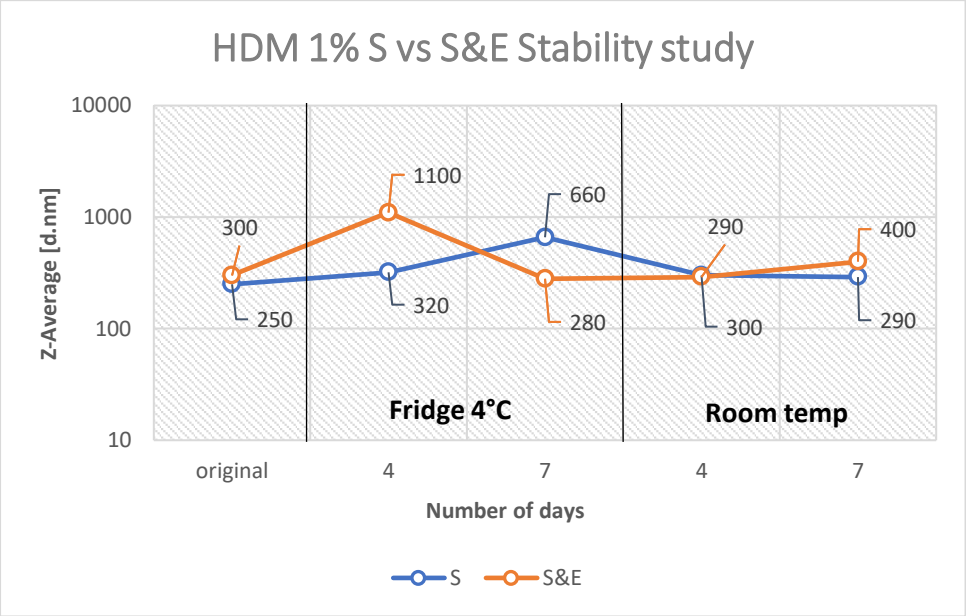

Figure 19. Size of particles for HDM during a stability study. Samples are prepared in PB and particle formation method (S and S&E) varies. S= Only sonicated, S&E= Sonicated then extruded. Samples kept at 4°C in fridge/room temperature for 3, 7 and 30 days after original sample were analyzed.

It can be seen in Figure 19 that the particle sizes for S&E are higher than S after 4 days at 4°C and after 7 days at room temperature. After 7 days at 4°C, the particle size is highest for sonication sample however it has the lowest size for S&E sample. However after 7 days at room temperature, particle size for both S and S&E has increased when compared with original sample. Meaning that the formulation with 1% HDM is not stable due to an increase in particle size.

5.2.1.2 Tomatine

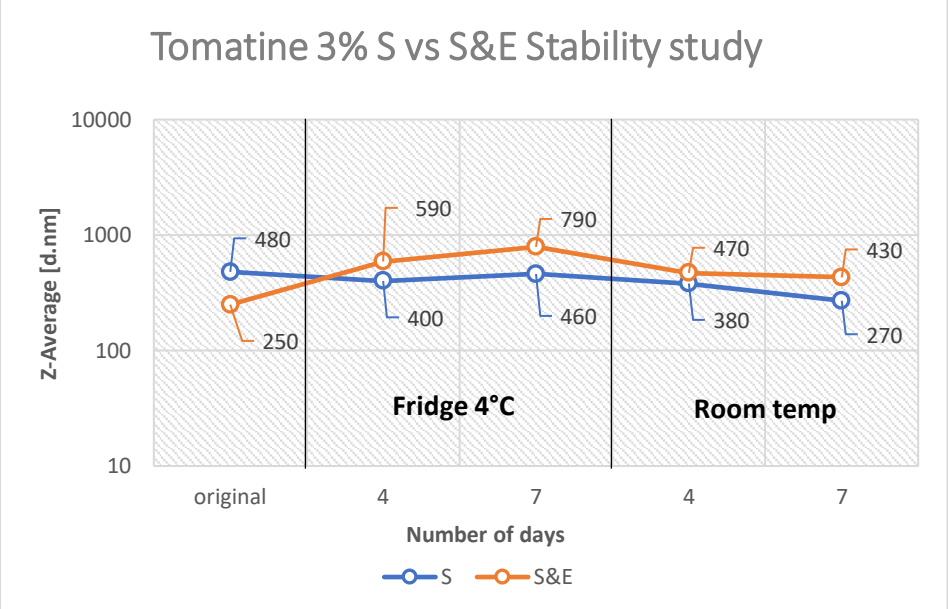

Figure 20. Size of particles for tomatine during a stability study. Samples are prepared in PB and particle formation method (S and S&E) varies. S= Only sonicated, S&E= Sonicated then extruded. Samples kept at 4°C in fridge/room temperature for 3, 7 and 30 days after original sample were analyzed.

It can be seen in Figure 20 that for S&E and overall increase is noted. However, the particle size increased more at 4°C than at room temperature. Meanwhile the opposite could be observed for sonicated samples, where a decrease is first noted followed with a slight increase at 4°C. However at room temperature, the particle size has decreased for sonicated samples.

The overall increase in size for S&E could be due to instability of formulation with 3% tomatine. However, the decrease could be a result of influence from sedimentation of larger particles.

5.2.2 Concentration variation

Since particles were assumed unstable at 2mg/mL, due to formation of larger particles, the stability study was conducted on samples with lipid concentration of 1mg/mL.

5.2.2.1 HDM

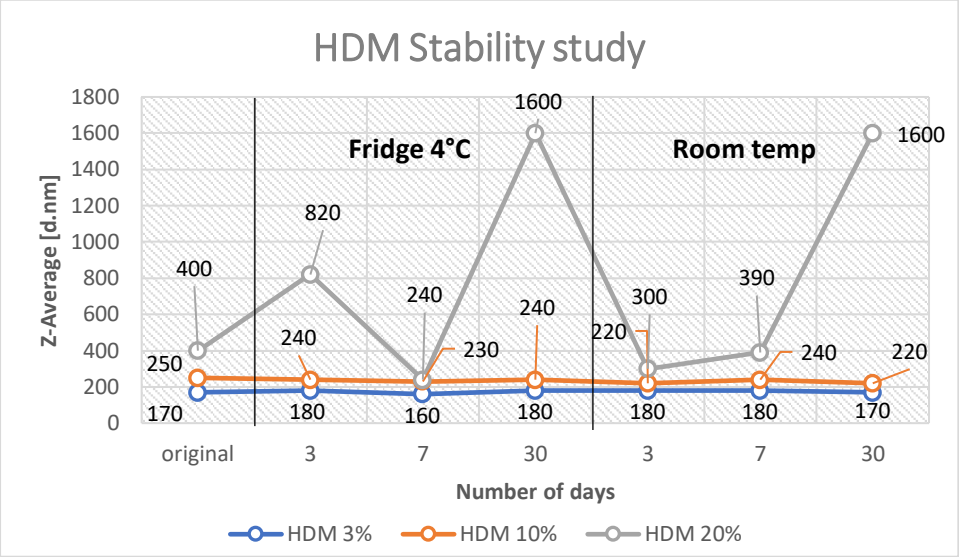

Figure 21. Size of particles for HDM during a stability study. Samples are prepared in MQ-water. Samples kept at 4°C in fridge/room temperature for 3, 7 and 30 days after original sample were analyzed.

A similar trend could be noted in Figure 21 when comparing 3 and 10% of HDM throughout time and temperature. The particle size remains unchanged at both 3 and 10% HDM. When analyzing 3%, the particle size doesn't vary neither throughout time or at different temperature. A small decrease in size could be noted for 10% from 250nm to 220nm (after 30 days at room temp). Meanwhile for 20% HDM the size tends to vary more. After a month, 3 and 10% HDM haven't changed significantly, meanwhile 20% HDM have increased to 1600 nm both at room temperature and 4°C. As mentioned before, the variation in size for 20% HDM could be a result of sedimentation of larger particles. This may indicate instability of samples, the larger difference in size, the more unstable a sample may be due to lower cholesterol concentration.

5.2.2.2 Tomatine

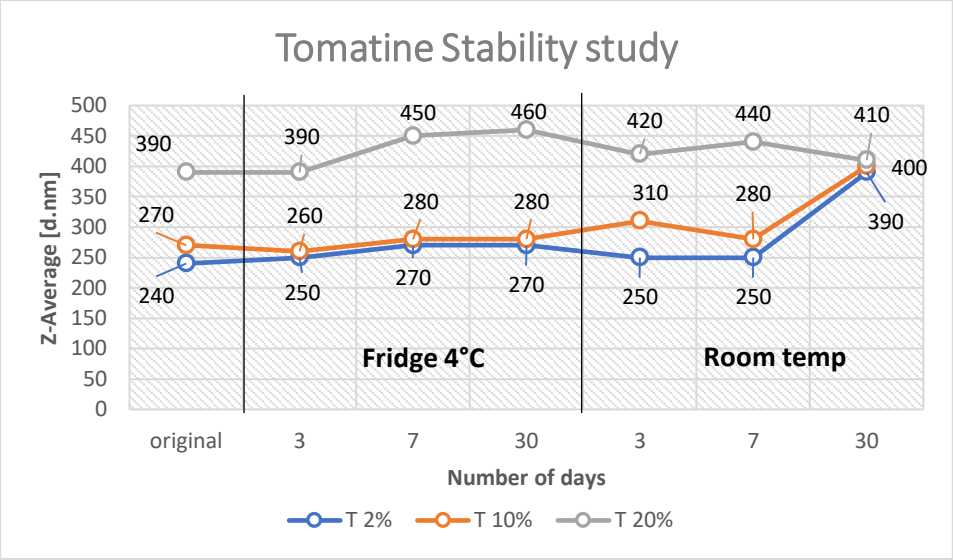

Figure 22. Size of particles for tomatine during a stability study. Samples are prepared in MQ-water. Samples kept at 4°C in fridge/room temperature for 3, 7 and 30 days after original sample were analyzed.

First thing that could be noted, in Figure 22, is that the size of all particles are smaller than 500 nm. The same trend for 2% tomatine could be noted as with 3% HDM where the size of particles are unchanged throughout time and difference in temperature, except after 30 days at room temperature. Meanwhile for 10% tomatine, a slight increase could be noted at room temperature after 3 days followed by a decrease to almost the original size of particle. At 20% tomatine concentration, the largest particle size could be noted. It could be seen that at the 4°C, the particle increases in size after 7 days. However an increase in size could already be noted after 3 days at room temperature. This indicates that 20% tomatine is less stable than 2 and 10% tomatine. After a month, 2 and 10% Tomatine haven't changed significantly at 4°C, however the particle size increased to ≈400nm at room temperature. This indicates that 2 and 10% tomatine are mores table at 4°C than at room temperature.

5.2.2.3 P80

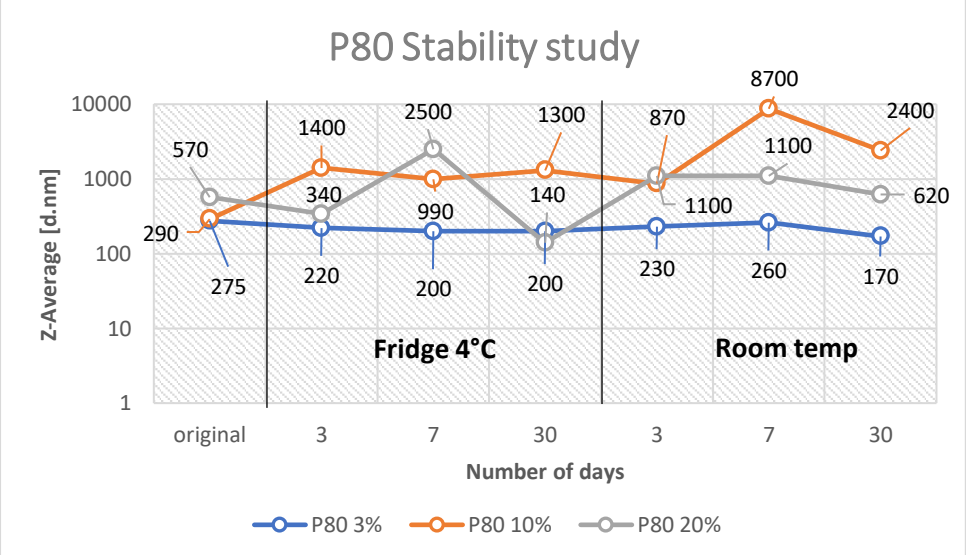

Figure 23. Size of particles for P80 during a stability study. Samples are prepared in MQ-water. Samples kept at 4°C in fridge/room temperature for 3, 7 and 30 days after original sample were analyzed.

Overall, the large size of particles could be observed. The sample with 3% P80 has remain mostly unchanged and follows the pattern discussed previously. Meanwhile, 10% varies a lot from 290nm to 1400 nm followed with a decrease to 990nm at 4°C. After a month, 3% P80 haven't changed significantly at 4°C compared to day 3 and 7, however the particle size decreased to 170nm at room temperature (day 30). The same could be noted at 20% where particle size increases to 2500nm after 7 days and decreases to 140nm after 30 days at 4°C. Meanwhile at room temperature, the particle size increases to 1100nm and decreases to 620nm after 30 days.

One reason for P80 being unstable is that the concentration could be too low. When comparing P80 to other surfactants, P80 is more soluble in water, and could therefore need higher concentration to be incorporated in the LNP structure compared to HDM and tomatine.

5.3 Other analytical methods

5.3.1 SAXS

The result from SAXS could not been interpreted due to the sample being too similar to background (MQ-water) thus nothing could be observed. It can be due to the low concentration (1mg/mL) and/or particle size being too large (>100nm in radius).

5.3.2 Cryo-TEM

New samples were made to compare the structure to the samples from the stability study. New samples could be seen in Figure 24 (A1-4) and samples, from stability study, prepared 1 month ago could be seen in Figure 25 (B1-4).

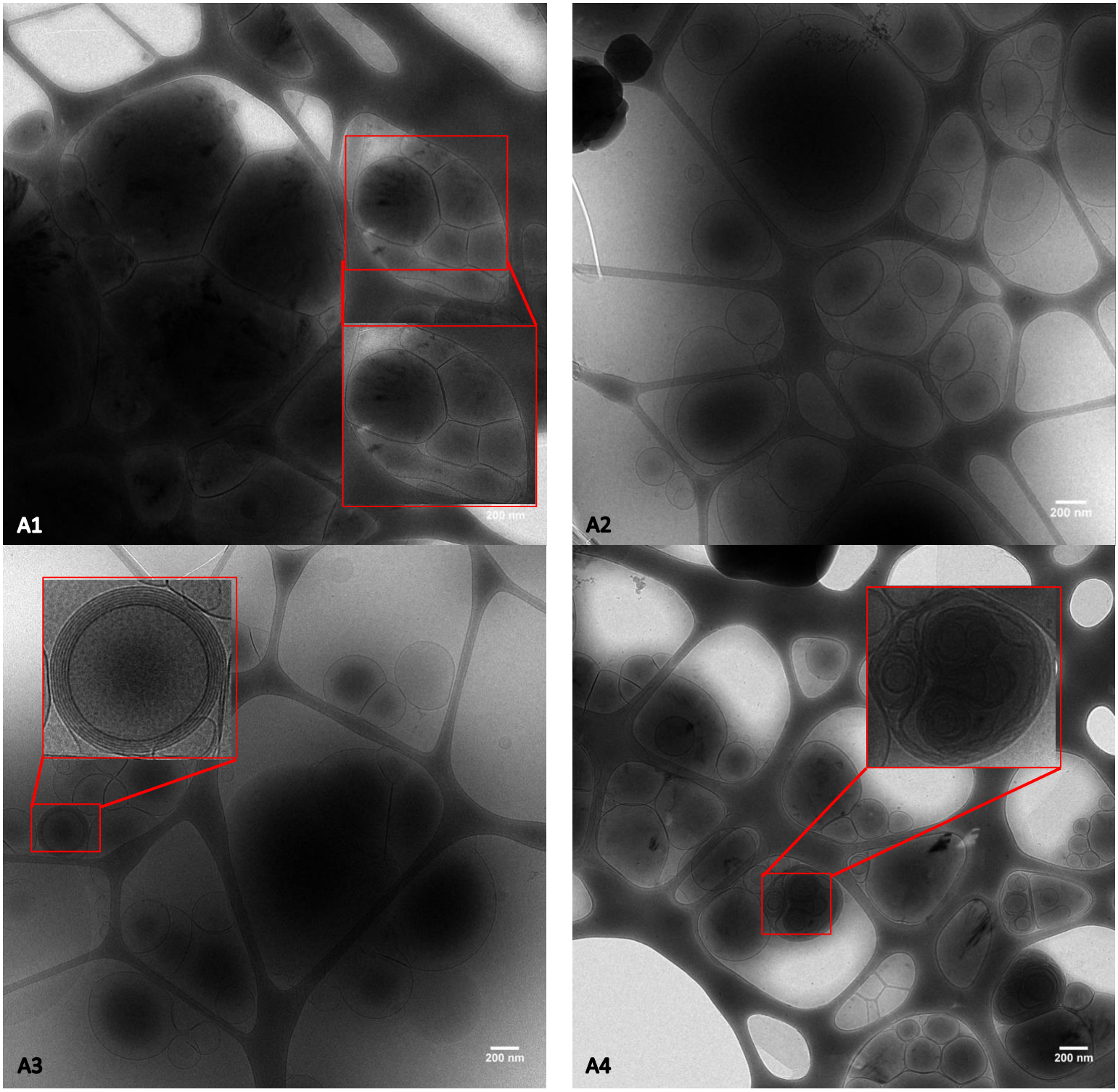

Figure 24. Image obtained from Cryo-TEM of the new samples in MQ-water. A1: DOPC:Chol:T 20:78:2, A2 DOPC:Chol:T 20:70:10, A3 DOPC:Chol:H 20:77:3, A4 DOPC:Chol:H 20:70:10.

In Figure 24, large particles are visible in images A1-4. For tomatine samples (A1-2), both 2% and 10% surfactant concentrations result in the formation of large particles. Some particles exhibit a single outer layer, while others display a double layer. A common characteristic of particles formed with tomatine is the presence of unilamellar vesicles. Conversely, for HDM samples (A3-4), 3% and 10% surfactant concentrations lead to the formation of multilamellar

vesicles (see the red square in A3) and some multivesicular vesicles (see the red square in A4). The grid is not completely filled, and the particles appear smaller compared to those in A1-2.

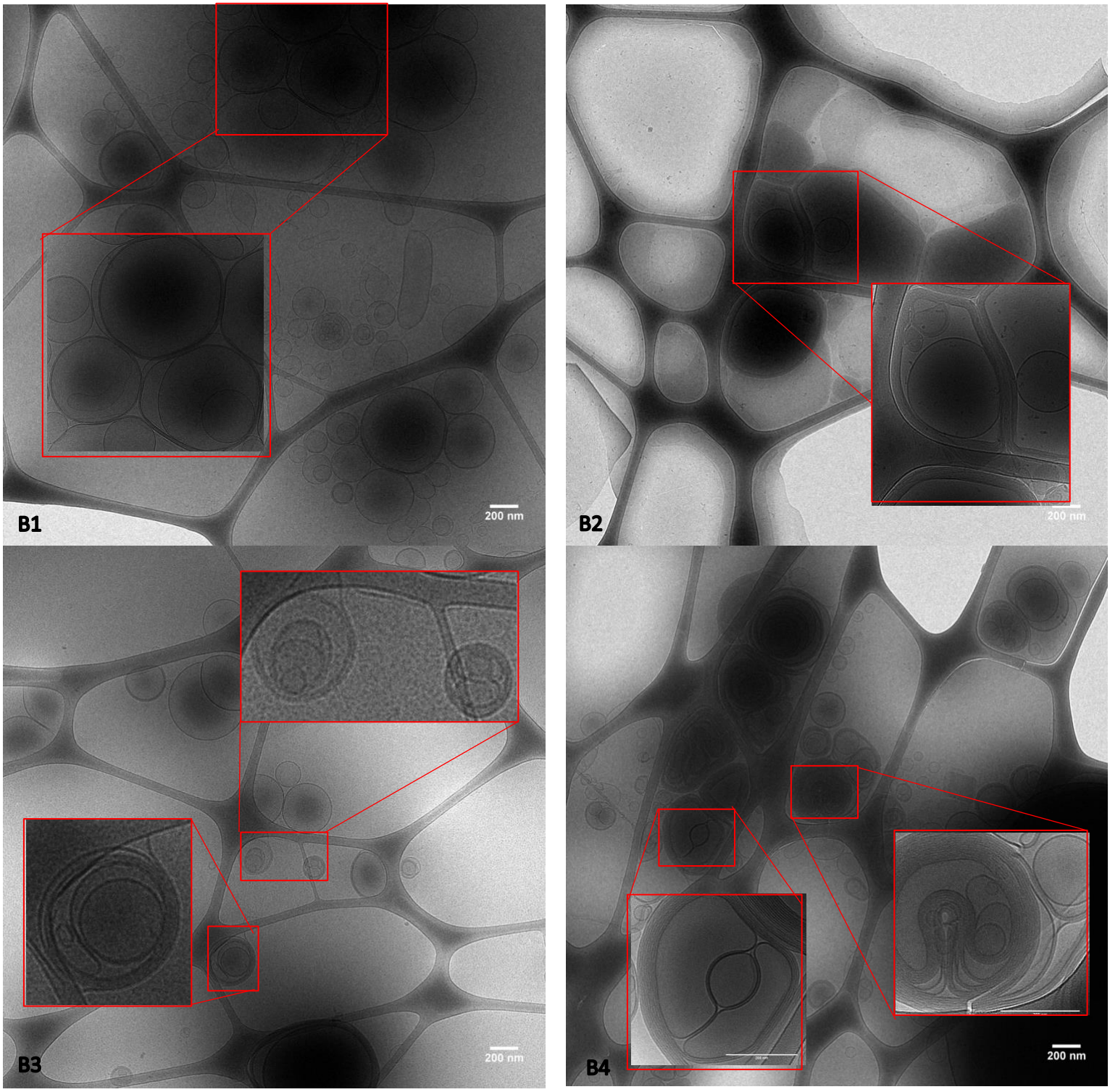

Figure 25. Image obtained from Cryo-TEM of the samples prepared 1 month ago in MQ-water. B1: DOPC:Chol:T 20:78:2, B2 DOPC:Chol:T 20:70:10, B3 DOPC:Chol:H 20:77:3, B4 DOPC:Chol:H 20:70:10.

In general, the particles in Figure 25 appear smaller and exhibit less size variation compared to those in Figure 24. When examining tomatine samples with 2% and 10% surfactant concentrations (referred to as B1 and B2 respectively), a noticeable difference is observed: particles in B2 appear darker than those in B1. This difference might stem from the

multilamellar nature of particles in B2 (see red square), resulting in thicker membranes compared to B1 particles. The darker patches in B1 (see red square) particles could be attributed to their more oval shape.

Furthermore, it is apparent that particles formed using HDM (B3 and B4) as a surfactant tend to contain other particles within them more frequently. It can also be seen that the particles formed are multilamellar and multivesicular vesicles (see two red squares in B3 and B4). Conversely, particles formed with tomatine as a surfactant tend to form empty vesicles.

6. Conclusion

When PB is used, particle size increase. DPPC form bigger particles in both MQ and PB at room temperature. DOPC forms smaller particles in both MQ and PB. However, the particle size is < 500nm when MQ-water is used instead of PB. Only 0.5% HDM and 1.5% tomatine have succeeded to form < 500nm particles in PB.

Upon decreasing total lipid concentration to 1mg/mL a trend could be observed with all three surfactants where particle size increases with surfactant concentration, the same could not be said about 2mg/mL. Since the surfactants are different in sizes, it is more appropriate to say that the particle size increases with a decreased concentration in cholesterol.

Due to inconsistent values where a bigger change in size could be observed, it is important to not only rely on DLS instrument as it is sensitive and can't handle high distribution samples, therefore leading to inconsistent values. It is important to doublecheck the size of particle with other orthogonal method.

The inconsistency of particles discussed indicates the importance of this type of stability study. Even though the particle within the desired size and polydispersity, it is still important to study how it behaves at different temperatures throughout time. The most stable formulation from stability study are 3 and 10% HDM, 2 and 10% tomatine and 3% P80.

Results from Cryo-TEM showed that the older samples appear smaller and exhibit less size variation than new samples. Tomatine, especially 2%, formed more simple vesicles than HDM.

7. Future aspects

For future research, exploring the impact of pH and varying buffer concentrations on particle formulation would be intriguing. Additionally, incorporating cationic lipid into the formulation could be an area of interest. Experimenting with different phospholipids could help identify the most stable formulation. Furthermore, utilizing a phospholipid with a high transition temperature (Tm) by warming up the solution could also be explored.

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Appendix

A1 Matlab script

```
clc

clear all

% Define the molar ratios
molarRatio = [10, 35, 5]; % Phospholipid, cholesterol,Surfactant

% Define the molecular weights (g/mol)
molecularWeights = [786.12, 386.654, 566.72];

% Define the concentration from stock solutions (mg/mL)
Concentration = [50/5, 80/5, 5/5];

% Input: Grams of the first component
WeightOfFirstComponent = 5; %mg

% Calculate the moles of the first component
molesOfFirstComponent = WeightOfFirstComponent / molecularWeights(1);

% Calculate the moles of the other components based on the molar ratios
molesOfOtherComponents = molesOfFirstComponent * (molarRatio / molarRatio(1));

% Calculate the weight of each component (mg)
WeightOfEachComponent = molesOfOtherComponents .* molecularWeights;

% Calculate the volume of each component (mL)
mLOfEachComponent = WeightOfEachComponent./Concentration;
Tot_V=mLOfEachComponent(3)+mLOfEachComponent(1)+mLOfEachComponent(2);

% Display the results in mL
disp('Volume needed of each substance:');
disp(['Phospholipid: ', num2str(mLOfEachComponent(1)), ' mL']);
disp(['Cholesterol: ', num2str(mLOfEachComponent(2)), ' mL']);
disp(['Surf: ', num2str(mLOfEachComponent(3)), ' mL']);
disp(['TotV: ', num2str(Tot_V), ' mL']);

% Calculate the weight of all components combined
TotalWeight = sum(WeightOfEachComponent); %mg

% Define the final concentration after addition of PBS
FinalConcentration = 1; %mg/mL

% Calculate the volume of PBS needed for all aliquotes combined
FinalFullVolume = TotalWeight/FinalConcentration;

% Define number of aliquotes hur många behållare man delar på
nAliquotes = 1;

% Calculate the volume of PBS needed for each aliquote
FinalAliquoteVolume = FinalFullVolume/nAliquotes;

% Display the results in mL
disp('Volume PBS needed for each aliquote')
disp(['PBS: ',num2str(FinalAliquoteVolume),' mL'])
```

A2 Tomatine and HDM sonication and sonication & extrusion

Serie 1-4ab

Sonication (S)

	DPPC %	Cholesterol %	Tomatine %	Z-average n.m	PDI
1a	20	79	1	1648	0.86
5a	20	78	2	5508	0.35
3a	20	77	3	476.7	0.95
6a	20	76	4	1548	1

Sonication & Extruder (S&E)

	DPPC %	Cholesterol %	Tomatine %	Z-average n.m	PDI
1a	20	79	1	271.8	0.89
5a	20	78	2	2692	1
3a	20	77	3	249.7	0.72
6a	20	76	4	2481	0.77

Sonication

	DPPC %	Cholesterol %	HDM%	Z-average n.m	PDI
1b	20	79	1	252.3	0.79
2b	20	78	2	668.9	0.79
3b	20	77	3	1573,	1
4b	20	76	4	3263	0.73

Sonication & extruder

	DPPC %	Cholesterol %	HDM%	Z-average n.m	PDI
1b	20	79	1	296.7	0.93
2b	20	78	2	2859	0.99
3b	20	77	3	964.9	1
4b	20	76	4	2767	0.78

A3 Test with low concentration of surfactants

Serie 5-6

	DPPC %	Cholesterol %	Tomatine %	Z-average n.m	PDI
5a	20	78	2		
6a	20	76	4		

	DPPC %	Cholesterol %	HDM%	Z-average n.m	PDI
5b	20	79	1		

A4 5 and 10 % surfactant concentration

Serie 7-8ab

	DPPC %	Cholesterol %	Tomatine %	HDM %	Z-average n.m	PDI
9a	20	70	10		9068	0.65
10a	20	60	20		9769	0.65
7b	20	70		10	7296	0.38
8b	20	60		20	8390	0.62

A5 Lipid concentration variation

Serie H 1-2 (from 7b)

	DPPC %	Cholesterol %	HDM%	Lipid	Z-average n.m	PDI
H1	20	70	10	2mg/mL 0.2%	1489	0.87
H2	20	70	10	1mg/mL 0.1%	1318	0.85

A6 Test without cholesterol

Serie 9-12a

	DPPC %	Cholesterol %	Tomatine %	Z-average n.m	PDI
9a	20	-	10	9068	0.65
10a	20	-	20	9769	0.65
11a	20	-	30	9910	0.42
12a	20	-	40	5447	0.35

Serie T 1-2 (from 12a)

	DPPC %	Cholesterol %	Tomatine%	Lipid	Z-average n.m	PDI
T1	20	-	40	2mg/mL 0.2%	2475	0.93
T2	20	-	40	1mg/mL 0.1%	2938	0.99

A6 Test with DOPC and DPPC without surfactant

Serie 1-4cd

Furthermore several samples were made without the surfactants in order to see the relation between DPPC/DOPC and cholesterol. In order to see if the change of phospholipid may solve the problem.

	DPPC %	Cholesterol %	Lipid	Z-average n.m	PDI
1c	20	76	4 mg/mL 0.4%	3405	0.69
2c	20	76	2 mg/mL 0.2%	3897	0.84
3c	20	40	4 mg/mL 0.4%	6104	0.51
4c	20	40	2 mg/mL 0.2%	4044	0.34

	DOPC %	Cholesterol %	Lipid	Z-average n.m	PDI
1d	20	76	4 mg/mL 0.4%	3364	0.42
2d	20	76	2 mg/mL 0.2%	1180	0.79
3d	20	40	4 mg/mL 0.4%	4355	0.26

4d	20	40	2 mg/mL 0.2%	2266	0.36

A7 Test with DOPC and surfactant

	DOPC %	Cholesterol %	HDM%	Lipid	Z-average n.m	PDI
5d	20	70	10	1mg/mL 0.1%	4223	0.50
6d	20	70	10	2mg/mL 0.2%	5153	0.37
7d	20	76	4	1mg/mL 0.1%	6434	0.71
8d	20	76	4	2mg/mL 0.2%	6707	0.35

	DOPC %	Cholesterol %	Tomatine %	Z-average n.m	PDI
3a	20	77	3	3811	0.63
	DOPC %	Cholesterol %	HDM %		
1b	20	79	1	6196	0.37

A8 test with only phospholipid

PB

Samples in PB with only DOPC and DPPC were made.

	DOPC %	Cholesterol %	HDM %		
	20	-	-	1372	0.60
	DPPC %				
	20			5110	0.99

A9 HDM series with DOPC

	DOPC %	Cholesterol %	HDM%	Lipid	Z-average n.m	PDI
9d	30	70	10	1mg/mL 0.1%	8490	0.58
10d	15	70	10	1mg/mL 0.1%	1.22*10^4	0.66
11d	20	65	15	1mg/mL 0.1%	2.064*10^4	0.64
12d	20	76	4	1mg/mL 0.1%	1.433*10^4	0.85
	P80 %	Cholesterol %	HDM%	Lipid		
13d	20	70	10	1mg/mL 0.1%	613	0.62

A10 Microfluid

	DOPC %	Cholesterol %	HDM%	Lipid	Flow rate uL/mL	Solution	Z-average n.m	PDI
1micro	20	70	10	1.5 %	50	PB	8112	0.56
2micro	20	70	10	0.75%	50	PB	2642	0.88
3micro	20	70	10	0.38%	50	PB	459	0.52
4micro	20	70	10	0.38%	100	PB	7236	0.56
5micro	20	70	10	0.19%	100	PB	983	0.62
6micro	20	70	10	0.19%	100	MQ	163	0.29

A11 MQ-water series (P80,Tomatine,HDM)

	DOPC %	Cholesterol %	HDM%	Lipid	Z-average n.m	PDI
1MQ	20	70	10	1 mg/mL 0.1%	180	0.32
2MQ	20	76	4	1 mg/mL 0.1%	199	0.51
3MQ	20	79	1	1 mg/mL 0.1%	172	0.48
12MQ	20	78	2	1 mg/mL 0.1%	268	0.50
13MQ	20	77	3	1 mg/mL 0.1%	**172**	0.44
2MQ*	20	76	4	1 mg/mL 0.1%	188	0.43
1MQ*	20	70	10	1 mg/mL 0.1%	**245**	0.43
4MQ	20	65	15	1 mg/mL 0.1%	220	0.39
5MQ	20	60	20	1 mg/mL 0.1%	**402**	0.57

	DOPC %	Cholesterol %	P80%	Lipid		
6MQ	20	79	1	1 mg/mL 0.1%	161	0.43
14MQ	20	78	2	1 mg/mL 0.1%	346	0.57
15MQ	20	77	3	1 mg/mL 0.1%	**275**	0.48
7MQ	20	76	4	1 mg/mL 0.1%	194	0.45
8MQ	20	70	10	1 mg/mL 0.1%	**291**	0.45
9MQ	20	65	15	1 mg/mL 0.1%	289	0.47
10MQ	20	60	20	1 mg/mL 0.1%	**569**	0.65

	DOPC %	Cholesterol %	Tomatine%	Lipid		
21MQ	20	79	1	1 mg/mL 0.1%	151	0.30

20MQ	20	78	2	1 mg/mL 0.1%	**242**	0.62
19MQ	20	76	4	1 mg/mL 0.1%	277	0.47
18MQ	20	70	10	1 mg/mL 0.1%	**274**	0.47
16MQ	20	60	20	1 mg/mL 0.1%	**393**	0.34

	DOPC %	Cholesterol %	Surfactant %	Lipid		
11MQ	20	-	-	1mg/mL 0.1%	158	0.42