Lipid Encapsulation of Curcumin and Ibuprofen for Improving Drug Delivery

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DECLARATION OF ACADEMIC INTEGRITY

I confirm that the presented work is completely my own. I have not colluded with others in the preparation of this work. This report explicitly illustrates the contribution of my work and other literature authors publications related to this work. I confirmed that all the quotes and writings of others who have researched in this field are mentioned as references and their rights are protected.

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ABSTRACT

The main motivation of this project is to combine ibuprofen (chemical origin) and curcumin (natural-based origin) in liposomal formulation of an herbal-chemical anti-inflammatory and painkiller drug with reduced side effects.

Improving bioavailability of this product is vital, for instance curcumin has poor absorption and can bind to proteins, leading chemical instability, rapid elimination, and fast liver metabolism, while ibuprofen has poor solubility and absorption in small intestine. For this purpose, ibuprofen and curcumin have been co-encapsulated inside liposome.

DPPC (1,2-Dipalmitoyl-sn-glycero-3-phosphocholine) has been used as a polar liposome film, produced by a rota evaporator. Dihexadecyl Phosphate (DCP) which is a mixture of diesters of cetyl alcohol and phosphoric acid, was used to increase the liposome stability by inducing a negative charge.

Phosphate buffer solution (PBS) were used for hydration while chloroform and acetone were used as solvents that can mix curcumin with ibuprofen without any chemical reactions, while enabling encapsulation into liposomes. The prepared film which has co-encapsulated curcumin and ibuprofen, has been isolated and analysed using a variety of techniques to verify its physical and chemical characteristics. HPLC-UV analysis, H and C -NMRs were used for determination of curcumin and ibuprofen inside as co-encapsulated materials. Following using rota-evaporation, freeze-drying (thawing) cycles and sonications were implemented for size control with different times. Sample 1 was prepared using DPPC, cholesterol, DCP, ibuprofen, curcumin, and chloroform. In Sample 2, the amounts of DPPC, curcumin, and ibuprofen were kept constant; however, the amount of cholesterol was slightly increased, DCP was omitted, and mixture of chloroform (4 ml) and of acetone (1 ml) was used as the solvent. Furthermore, liposome encapsulation efficiency in Sample 1 was notably higher, with 92.01% of curcumin and 61.86% of ibuprofen retained, compared to Sample 2, where 88.38% of curcumin and only 38.37% of ibuprofen remained encapsulated. Co-encapsulating ibuprofen alongside curcumin within liposomes as a drug delivery system represents a novel approach that holds promise for the future

development and formulation of hybrid phytochemical-synthetic combination drugs. This strategy may significantly enhance the bioavailability and therapeutic efficacy of such compounds.

Key words: Ibuprofen, Curcumin, Liposome, Lipid Encapsulation, DPPC, PBS, DCP, Cholesterol, Thawing.

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Table of Abbreviation

C18-PEG-2000	1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000]
$CDCl_3$	Deuterated Chloroform
COX	Cyclo-oxygenase isozymes
Cryo-SEM	Scanning Electron Microscope
cyt-C	Protein cytochrome C
D.water	Deionised water
DCP	Dihexadecyl Phosphate
DLS	Dynamic light scattering
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
DMPG	1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol)
DMSO	Dimethyl sulfoxide
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
DSC	Differential Scanning Calorimetry
FT-IR	Fourier transform infrared spectroscopy

HPLC-UV	High-performance liquid chromatography
NMR	Nuclear magnetic resonance machine
PBS	Phosphate buffer solution
P-I	Phospho-ibuprofen
Sample 1	Co-encapsulated product using DCP and without acetone
Sample2	Co-encapsulated product without DCP, and additional cholesterol with acetone
SEM	Scanning electron microscope
soy-PC	soy-phosphatidylcholine
TEM	Transmission electron microscopy
TFA	trifluoroacetic acid
Vit-E	Vitamin E

Chapter 1: Introduction

1.1. Research Background and Motivation

Herbal medicines have been used for a long time in Asia. They are made from natural ingredients which can be found in fruits and various parts of plants such as leaves, roots, fruits, and stems. They can be extracted in different forms, for instance as liquids, solids, and volatile (gaseous) substances.

Nowadays, the costly production and adverse effects of therapeutic agents have encouraged the researchers to gain advantages of natural medicines [1]. Evidence shows that natural substances obtained from herbs can be used instead of chemicals to produce drugs. This is an excellent replacement for chemical medicines which have many side effects. Also, by enhancing their bioavailability, their drug efficacy can be improved [2-5]. Curcumin has a natural origin and is obtained from turmeric powder. Although, curcumin has anti-inflammatory properties, but its poor bioavailability makes it seldom useful as a therapeutic agent.

In addition, ibuprofen blocks cyclo-oxygenase isozymes (COX-1) and COX-2 enzymes which are responsible for the conversion of arachidonic acid into prostaglandin that includes thromboxane and prostacyclin [6]. Similarly, ibuprofen has poor bioavailability and low water solubility; also, can cause stomach and skin irritation as common side effect.

The co-encapsulation of ibuprofen and curcumin in liposome for application as an anti-inflammatory agent have not been reported. lipid bilayer has specific advantages which can make physicochemical barrier for trapped molecules against pro-oxidant elements and make them water-soluble also possibly able to be dispersed in aqueous food combinations and increase their bioavailability [7]. In consideration of the knowledge gap in the literature, the aim is not only to tackle with the challenges above but also generate a therapeutic chemical-herbal agent with significant efficacy with limited toxicity.

1.2. Research Aim

The aim of this study is to improve the delivery of ibuprofen and curcumin by co-encapsulation in liposome to boost their bioavailability.

The specific objectives are to identify suitable solvents for both materials (curcumin-ibuprofen), that does not dissolve or degrade the liposome structure also dissolve curcumin and ibuprofen properly. Furthermore, to co-encapsulate ibuprofen and curcumin in lipid formulation and to recognise ideal post-treatment method of encapsulated product and fully characterise their physical and chemical properties. The process of co-encapsulation is schematically shown in Figure 1.

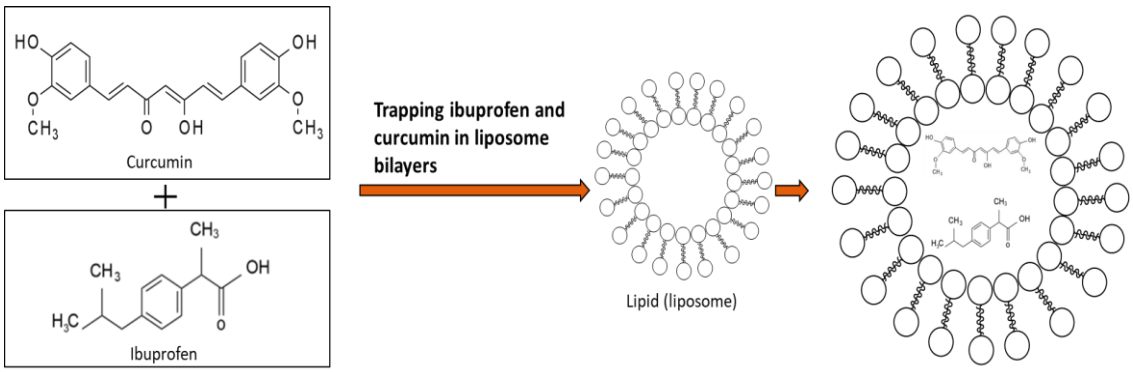


Figure 1. Research aims as schematic.

Chapter 2: Literature Review

2.1. Liposome and Encapsulation Methods

DPPC (1,2-Dipalmitoyl-sn-glycero-3-phosphocholine ($C_{40}H_{80}NO_8P$)) is a commonly used lipid and key component in the lipid encapsulation process. It has glycerol backbone with two fatty acid chains (palmitic acid and fully saturated) and a phosphate group attached to a choline headgroup (Figure 2).

With phase transition temperature 41°C, it commonly used in model membranes, liposome formulations. Furthermore, the conditions and preparation method of manufacturing liposomes are very influential in the leakage of the trapped materials [8, 9]. Liposomes are composed of a lipid bilayer with the hydrophobic chains and the polar head groups of the lipids oriented toward the outer solution and inner cavity [10] and make a liposome shape (Figure 3). In addition, studies show it can improve drug delivery and bioavailability of curcumin which has poor bioavailability [11].

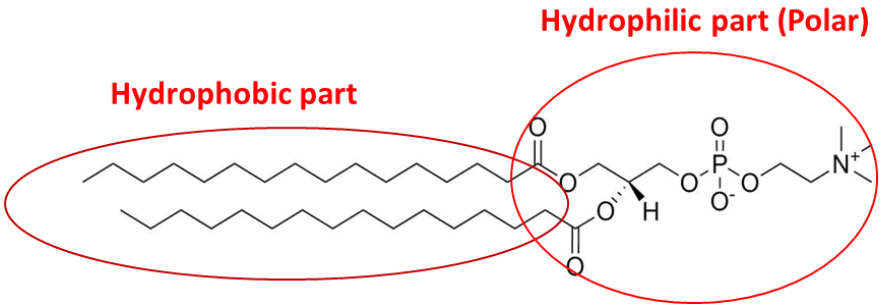


Figure 2. Structure of hydrophobic and hydrophilic parts of DPPC

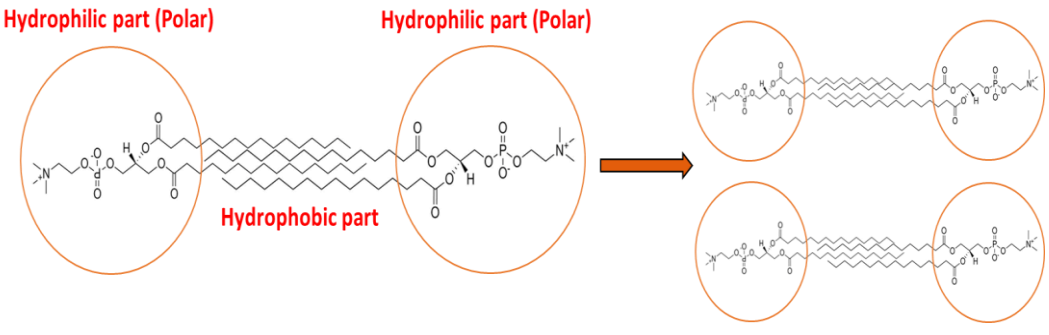


Figure 3. Chemistry definition of liposome structure and how it made from lipid

Fatma et al. [12] investigated the effect of cholesterol on liposome preparation. Moreover, trapping descriptions of hydrophobic vitamin E (Vit-E) and hydrophilic protein cytochrome-C (cyt-C). They also examined the difference between sonication and extrusion on liposomes sizes. According to this study, thin film evaporation and hydration as further step used for lipid preparation. They realized in the formation of liposome; cholesterol promotes the rigidity and increases the particle size in the final suspension. Extruded lipids were generally larger than the sonicated ones and had a bigger vesicle volume. Both preparation methods resulted in liposomes with an equal capacity for Vitamin E. They reported sonication can disrupt the bilayer, but the entrapped Vit-E are still aligned with the hydrophobic region of the liposome. They found encapsulation of hydrophilic protein cyt-C, was only possible when the bilayer contained cholesterol. Furthermore, short sonication times create larger liposomes, while extended sonication leads to smaller liposomes [12] (data are summarised in Table 1).

The effect of freeze/drying (thawing) and sonication on DPPC lipids was investigated by Sook Heun Kim et al. [13]. Effects were studied by examining the particle morphology and sizes with Cryo-TEM, characterising the particle sizes with DLS and spectro turbidimetry. Two samples with two protocols were prepared using heating and stirring method. Protocol 1 without sonication and protocol 2 with sonication [13]. Samples prepared with protocol 1 in water or buffer were turbid and unstable while sample with protocol 2 had stable and transparent solution.

Study by Tan et al. [14] was conducted to understand how carotenoids (carotenoids are naturally occurring pigments that can be observed in plants, fruits, and vegetables) exerted antioxidant activity after encapsulation in a liposome delivery system, for food application. The results showed carotenoid encapsulation in liposomal capsules can enhance the antioxidant activity. They used rota-evaporator as thin film evaporation method following by hydration using PBS (Phosphate buffer solution). Tan et al. concluded that liposomal membrane can strongly keep β-carotene and lutein during storage, whereas this effect was less significant for other carotenoids [14].

In a study conducted by Ong et al. [15], the researchers aimed to examine how liposome sizes and encapsulation efficiency, influence the oral bioavailability of griseofulvin. To achieve this, griseofulvin-loaded liposomes were developed from pro-liposome formulations using different preparation methods. The *in vivo* evaluation involved three types of griseofulvin formulations: an aqueous suspension and two liposomal preparations containing different amounts of encapsulated drug. “Non-shaken”, “moderate stirring” and “freeze-thaw sonication” methods were used to produce different size liposomes [15].

Study by Tomohiro et al. [16] found that ratio between lipid, ingredients (ceramide 3), dicetyl phosphate and chloroform as solvent are as crucial as conditions and preparing method for size and administration. In their earlier research, the investigators examined how ceramide 3 interacts with phospholipids within mixed monolayers using atomic force microscopy, Their findings revealed that ceramide 3 associates with phospholipids in the liquid-expanded phase, resulting in noticeable phase separation [16]. To further purify the liposomal preparation, column chromatography was conducted using Sepharose CL-6B resin, with phosphate-buffered saline (PBS) serving as the mobile phase. Liposomes were detected by measuring the turbidity (The amount of light scattering by particles suspended in a liquid) at 650 nm (wavelength of light) . Lipid preparation was done by blowing nitrogen gas into lipid mixture and removing solvent from it [16].

The key findings of the above literature [12-16] are all summarised in Table 1.

Table 1. Summary of liposome literature review

Topic	Application	Preparation method	Analysis technique	Administration	Status	Findings	Ref
Lipid mixture of Phospholipids DMPC and DMPG (entrapment of hydrophilic protein cytochrome-C and Hydrophobic Vitamin E)	Efficient drug encapsulations	Thin film evaporation method and hydration Phospholipids DMPC and DMPG, cyt-C, cholesterol, Vit-E chloroform, potassium dihydrogen phosphate, cholic acid, sodium salt, acetonitrile and methanol DMPC and DMPG mixed with a molar ratio of 9.45:1. Liposomes prepared with a molar ratio of 9.45:1:3.1 DMPC: DMPG: cholesterol, respectively.	UV-visibility double beam spectrophotometer and HPLC	Better understanding about Liposome release and method effects N/A	*In Vitro*	Cholesterol inserted into the bilayer, caused the liposomes size to increase. Cholesterol stabilised the bilayer, while it can hinder the transfer of hydrophilic molecules in between the inner and outer aqueous regions. Extrusion method prepared particles were larger than the sonicated liposomes. The encapsulated of protein cyt-C was 10	[12]

		Particles downsized through a polycarbonate filter with a defined pore size by mini extruder. Loading to multilamellar vesicles suspensions subjected to 10 cycles of freezing and thawing (37°C water bath) and extrusion was performed about 10°C above the T_c of the lipids. Large unilamellar liposomes by extrusion techniques generated by pressing the lipid dispersion through the 100 nm polycarbonate filters mounted in the mini extruder, fitted with 0.5 mL syringes.				times higher with extrusion method. Encapsulation cyt-C (Hydrophilic) just possible when liposome contained cholesterol. Sonication can disrupt the bilayers and cause the leakage of cyt-C from the inner core. If sonicator disrupts the layer, it has no effect on the entrapment of vit- E because both hydrophobic parts of liposome and vit- E are linked to each other.	

		The samples were subjected to 11 passes through the filter. Sonicated liposomes were obtained after thin film hydration and freeze and thaw cycles, 15 min in a sonic bath.				Trapped molecules, hydrophilic or hydrophobic, they released from the liposomes, without losing molecular integrity. Vit-E can be encapsulated independently without cholesterol. Short sonication results in larger liposomes, and long sonication results smaller liposomes.	
1000 ppm DPPC dispersions	Heating and stirring	In protocol 1, DPPC dispersion heated 30 min, magnetic stirrer for 1 h at 55–60 °C, above Tc=41°C	Surface tension measurements	N/A	*In vitro*	The DPPC dispersions prepared with protocol 1 (stirring at 55 °C but without sonication) in	[13]

		and cooled to room temperature to yield “frozen liposomes”. In protocol 2, DPPC heated and stirred as in Protocol1, sonicated extensively 7 h until they appeared. dispersions were finally cooled to 25 °C and Sonication done at 55.	Dynamic light scattering (DLS) measurements UV–visibility Spectro turbidimetry TEM			water or buffer were turbid and unstable. The DPPC dispersions with protocol 2 in water or in buffer were clear and transparent, with no particles visible by light microscope. After a freeze–thaw cycle, the DPPC dispersion (Protocol 2) becomes turbid, and large particles precipitate. These particles are coagulated vesicles, which retain their structure. They do not coalesce, apparently because the solubility	

						of DPPC is nearly zero. When a frozen/thawed dispersion is subjected to a second sonication procedure at 55 °C, it becomes clear again, the smaller vesicle sizes are restored, and the surface tension becomes lower, as before the freeze–thaw cycle.	
Lipid for encapsulating carotenoids	Food application	thin-film evaporation method and hydration Carotenoid+ egg Yolk Phosphatidylcholine + Tween 80 dissolved in chloroform.	Raman spectroscopy, fluorescence polarization, and electron paramagnetic resonance	N/A	*In Vitro*	The carotenoid encapsulation in liposomes enhanced antioxidant activity.	[14]

		the liposomal system brought to dryness by rota-evap at 55 °C. The sample dried by vacuum oven (50 °C) Hydration with 0.01 M PBS, 1M NaCl, under Vortexing for 60 min, 55 °C. The liposomal suspension was probe sonicated in an ice bath (10 min) with sequence of 5 s of sonication and 5 s of rest using a sonicator. The final sample was transferred in vials under nitrogen bed and stored in the refrigerator (4 °C in dark).					

Influence of the encapsulation efficiency and size of liposome on the oral bioavailability of griseofulvin-loaded liposomes	N/A	(First product): Amount of griseofulvin was added to 2 mg/g pro-liposomes. Mixed thoroughly for 24 h and hydrated with D.Water. The amount of purified water added was 2 parts to 1 part of the pro-liposome. The mixture stirred at moderate speed for 30 min at room temperature. Suspension of griseofulvin-loaded liposome produced diluted with 5 parts of purified water. (Second product): Like before except that	The liposome size and its distribution determined by photon correlation spectroscopy.	Oral administration.	Animal testing	Results indicated, extent of bioavailability of griseofulvin was improved times when given in the form of liposomes first product compared to griseofulvin suspension. there was an approximately two-fold enhancement of the extent of bioavailability following administration of griseofulvin-loaded liposomes with higher encapsulation efficiency (Second product), compared to those of First product.	[15]

		griseofulvin used dissolved in chloroform. The organic solution containing all materials dried down by flushing with nitrogen gas, (40 °C) prior to hydration with 2 parts of Milli-Q water to 1 part of the pro-liposomes. Griseofulvin formed by flushing the organic solution containing all the materials (in a chloroform solution) with nitrogen gas at 40 °C. The subsequent steps depended on the mechanical dispersion method used and summarised as follows:					

		Non-shaken: The dried lipid film hydrated by addition 7 parts of Milli-Q water. Then, the flask flushed with nitrogen, sealed, and allowed to stand for 3 h at (25 °C). The vesicles were harvested by swirling.					
Preparation of liposomes containing ceramide 3 and their membrane characteristics	Skin administration	<u>Blowing nitrogen gas</u> The composition of liposomes containing ceramide 3: DPPC: ceramide 3: dicetyl phosphate = 10: (0~1.5):1	DLS Measurement of micro viscosity of liposomal bilayer membranes. Column chromatography and thin-layer	N/A	*In Vitro*	Agglutination of ceramide 3 also visually observed for liposome solutions containing more than 0.166 mol fractions of ceramide 3. It has been generally known that more than 10 mol% of lipophilic	[16]

		DPPC, ceramide 3 and dicetyl phosphate were dissolved in chloroform. Solvent then removed by blowing nitrogen gas into tube, and the residual solvent dried overnight at room temperature in a desiccator under vacuum. PBS added to lipid film warmed at 60°C above the phase transition temperature (41°C) of DPPC for 10 min. The test tube shaken by vortex, and the multilamellar vesicles obtained. The multilamellar vesicles sonicated (125 W) for 1 h	chromatography of liposome solution Surface tension measurement.			additives bring about a destruction of liposomal structure[17].	

		and subsequently extracted through a polycarbonate membrane filter.					

2.2. Curcumin

Curcumin is the naturally occurring compound within the *curcuma longa* plant roots (an east Indian plant) that gives it a bright yellow colour and is known as curcuminoids (a group of natural compounds that include curcumin and other related compounds). Curcuminoids are pigments that give turmeric a yellow/orange colour [18]. Turmeric derived from the rhizomes of *curcuma longa*, which is a bioactive phytochemical compound and has a long history of therapeutic application in traditional Asian medicine [19].

2.2.1 Chemistry of Curcumin

The chemical name for curcumin is diferuloylmethane ($C_{21}H_{20}O_6$) and the IUPAC name is (1E-6E)-1,7-bis (4-hydroxy-3-methoxy phenyl)-1,6-heptadiene-3,5-dione with a molecular weight of 368.37 g/mol and melting point of 183 °C. The two aryl rings in this substance are symmetrically connected to a β-diketone moiety by ortho methoxy phenolic groups [20, 21] (Figure 4).

Curcumin exhibits pH-sensitive keto–enol tautomerism, with the enol form being more stable and dominant under alkaline conditions, while the keto form prevails in neutral to acidic environments [21, 22]. Its visible colour also shifts with pH variation—appearing bright yellow in solutions with a pH between 2.5 and 7.0 and transforming into a deep red when exposed to alkaline environments (pH above 7.0) [21, 22]. Numerous clinical studies have confirmed curcumin's excellent safety summary, tolerability, and therapeutic potential even at high oral doses, leading to its approval and commercialisation as a dietary supplement in many countries worldwide [23].

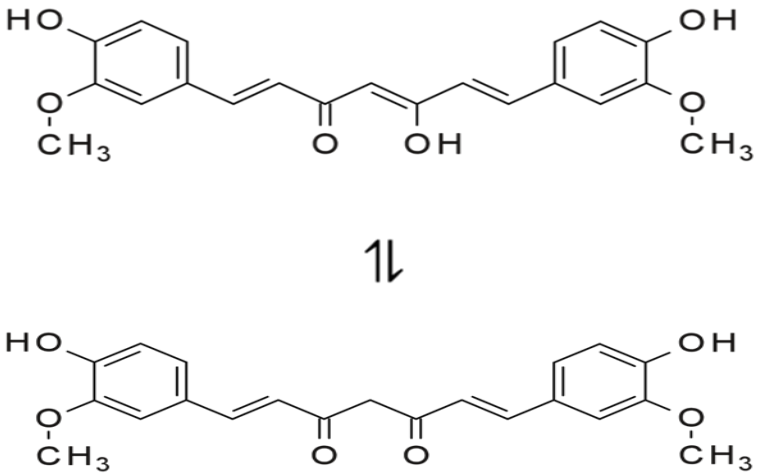


Figure 4. Curcumin keto and enol forms structures

2.2.2. Drug Delivery and Formulation for Curcumin

Various formulations have been reported for the delivery of curcumin and enhance its bioavailability. Diverse research studies focused on the enhancement of curcumin bioavailability, for examples using colloidal systems for improving its delivery for the application of functional foods [24].

Several methods are implemented for encapsulating curcumin with liposome including: pH adjustment [25], thin film evaporation [14, 26], thin film hydration [27], microfluidics [28], ethanol injection [29] and micro-emulsification [30]. In the following, some related studies on the encapsualtion of curcumin are summarised.

2.2.3. Curcumin Encapsulation

Different encapsulation materials have been reported for curcumin with pH adjustment method. For example, sodium caseinate (Na-Cas), a protein in milk, has been used to encapsulate this material. Self-assembly of curcumin within casein nanoparticles has been explored as an effective strategy to enhance its water dispersibility and biological activity [25]. The process was driven by

adjusting the pH. Increase in pH has been associated with enhanced stability, improved water dispersibility, and greater bioavailability of curcumin [25].

In another study, Lan et al. [26] used “liposomal curcumin” as anti-tumour agent. Curcumin has is inhibitory activity against potent NF-κB. Potent NF-κB is a nuclear factor kappa-light-chain-enhancer of activated B cells, a protein complex that plays a crucial role in regulating immune response, inflammation, cell growth, and survival. Despite its proven pharmacological safety, curcumin exhibits limited bioavailability when administered orally. This limitation can potentially be addressed through lipid-based encapsulation strategies, which may improve its solubility and systemic absorption[26].

In a study by Huang et al. [27], liposomal nano-encapsulation was employed to enhance the antioxidant efficacy of co-delivered curcumin and resveratrol. Liposomes prepared at a 5:1 ratio of curcumin to resveratrol demonstrated the smallest particle size (77.50 nm), lowest polydispersity index (0.193), highest encapsulation efficiency, and strongest 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity and lipid peroxidation inhibition [27].

Microfluidic synthesis method were used by Seon Tae et al. [28] to encapsulate curcumin (Figure 5).

In this study, a microfluidic system was employed with precise control over temperature to achieve high encapsulation efficiency and reproducibility. The successful incorporation of curcumin into the formulation confirmed by using FT-IR and DSC analyses. However, during the stability assessment, a particle size increase of 58.3 nm was observed after a few weeks, which was attributed to the presence of cetyl palmitate —a waxy ester lipid— used during synthesis. This synthesis method can enable the loading of many poorly soluble drug and it also can produce stable nanoparticles by choosing the right lipid and surfactant [28].

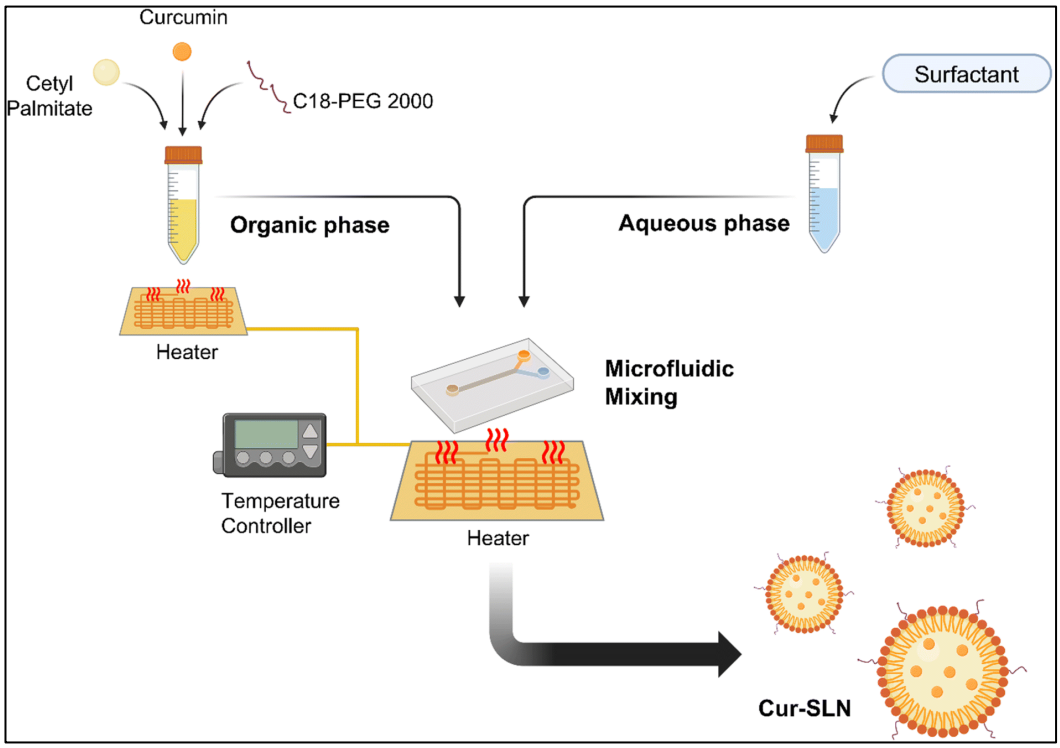


Figure 5. Microfluidic lipid preparation method explanation. Study/picture by Seon Tae et al. [28].

In another study by Vandana et al. [31] curcumin and quercetin were co-encapsulated in liposome using a droplet-based microfluidic device. Liposome were made via phosphatidylcholine and cholesterol with different flow rate ratios of 9:1, 6:1, 3:1 and 1:1 of the aqueous to organic phase (100 to 160 µl/min) where the highest encapsulation efficiency achieved for curcumin and quercetin, with 6:1 flow rate ratio of droplet-based microfluidic device. Microfluidic method benefit is that hydration can be more controllable for average particle size, polydispersity index and hydration process [31-33]. Organic (lipids + ethanol) and aqueous (PBS used) phases, lipid and drug concentration, flow rate ratio of aqueous to organic phase streams, and total flow rate are all crucial in preparation of liposomes using microfluidic device. Mean diameter of capsules is related to lipid concentration and inversely related to device flow ratio rate [34]. Microfluidic technique reduces the variability between batches. Increasing aqueous flow results on narrow lipid stream and formation of liposomes with smaller particle size [31].

The key findings of the above literatures [25-28, 31] are summarised in Table 2.

Table 2. Summary of curcumin literature review

Topic	Application	Encapsulation approach	Preparation method	Analysis technique	Administration	Status	Findings	Ref
Na-Casein-Curcumin	Nutraceutical and functional food	pH adjustment	Hydration of Na-Cas by D. water. pH adjustment to 12 by NaOH to facilities disassembly of casein. Curcumin addition (pH 12, 30 minutes stir). Neutralization by addition of HCl to reassemble casein and encapsulate curcumin. Centrifugation to remove larger particulates.	DLS, zeta potential, TEM, UV-visibility, H-NMR, florescence spectroscopy and *vitro* bioactivity tests.	As an anti-cancer agent in functional food and pharmaceutical product	*In vitro* testing	The increase of the pH increased stability, dispersity, and bioavailability.	[25]

			Lyophilization of the product (Freeze drying)					
Lipo-Curcumin	Liposome-encapsulated curcumin *In vitro* and *in vivo* effects on proliferation, apoptosis, signalling, and angiogenesis.	Thin-film evaporation	Curcumin into DMSO Lipid (DMPC) dissolved in tert-butanol. Use 0.22micro M filter then transfer into freeze dry vials. 24h in dry ice acetone(-22dgree) to remove DMSO (dimethyl sulfoxide) and tert-butanol.	Animal and cell test	Intravenous as anti-tumour agent	*In vivo testing*	The activity of liposomal curcumin was equal to or better than that of free curcumin at equimolar concentrations. *In vivo*, curcumin suppressed pancreatic carcinoma growth in murine xenograft models and inhibited tumour angiogenesis.	[26]

Lipo-curcumin-resveratrol	Enhancing stability and bioavailability of curcumin and resveratrol as an antioxidant	Thin-film hydration and rota-evaporator	Amount of curcumin and/or resveratrol was dissolved in ethanol together with the lipids. Composed of egg Yolk Phosphatidylcholine and Tween 80 (a surfactant) at a fixed mass ratio under magnetic stirring for 30 min. The total concentration of polyphenol was adjusted to 20 μmol mL^{-1}, which was expressed. -Mixture with the ratio of curcumin to resveratrol 1: 5, 1: 1, 5: 1).	DLS, Zeta Potential, UV-visible, FT-IR, and fluorescence spectroscopy	Oral or topical delivery in food/pharmaceuticals	*In vitro* testing	Co encapsulation improved the physical stability, antioxidant capacity, and encapsulation efficiency (up to 80.42%). It also enhanced the protective effects of the compounds during storage and heating conditions.	[27]

			After dissolution, the liposomal system dried by reduced pressure using a rota-evaporator, followed by vacuum-drying overnight. to remove the solvent. The achieved thin film hydrated with 0.01 M phosphate-buffered saline (150 mM NaCl, pH 7.4) under stirring for 2 h at 55 °C. To achieve homogeneous liposomes, the liposomal suspensions subjected to probe sonication processing in an ice bath for 10					

			min with a sequence of sonication for 5 s and rest for 5 s using a sonicator.					
Microfluidic synthesis of curcumin	Research	Microfluidic	Organic phase contains: cetyl palmitate and C18-PEG-2000 or curcumin dissolved in ethanol. The aqueous phase was prepared by dissolving Tween 20, Tween 80, and polyvinyl Alcohol in D.Water. Microfluidic machine with heater and temperature control	DLS Cs-corrector TEM Encapsulation efficiency Release test DSC FT-IR	N/A	*In Vitro testing*	Curcumin was successfully encapsulated. High encapsulation efficiency for curcumin were obtained. heat and temperature in microfluidic synthesis machine can provide high reproducibility and	[28]

			used for organic and aqueous phases. They mixed at ratios of 1: 3 and 1: 5 and after it, centrifuge used.				encapsulation efficiency	
Preparation of curcumin and quercetin co-encapsulated liposomes	N/A	Microfluidic	Aqueous phase (PBS + 0.05% w/v Tween 80) and organic phase (lipids + ethanol) prepared. The organic phase passes through one of the capillaries. Pressure of up to 2 bar used to set the flow rate (at least 1.5 ml of the aqueous phase is required for synthesis of 1 ml of formulation.)	DLS UV-visible spectroscopy Entrapment efficiency Fluorescence spectroscopy TEM SEM	Oral carcinoma	*In Vitro* drug release	Concentrations of lipids, active compounds and surfactants affected the liposomal characteristics. Addition of Tween 80 resulted in decreased particle size indicating reduced aggregation of liposomes.	[31]

			Mixing the aqueous phase and the lipid phase at a specific flow rate ratio of device				It observed that curcumin and quercetin precipitated after microfluidic mixing. An edge activator (charger) enhances lipid bilayer flexibility and stability [35]. The greatest efficiency was 20% and 9% for curcumin and quercetin loading in liposomes.	

							Curcumin had greater loading efficiency compared to quercetin (polar structure). Low flow rate causes higher encapsulation efficiency. The stability study at 2–8°C up to 2 months showed no changes. Curcumin and quercetin co-encapsulated liposomes can be synthesised by the	

							microfluidic technique that show effectiveness against oral cancer.	

2.3. Ibuprofen

Ibuprofen is a nonsteroidal anti-inflammatory drug that is used to relieve pain, fever, and inflammation. This includes painful headaches, and, in some cases, it may be used to treat Patent Ductus Arteriosus (PDA) in infants. Administration can be done orally (by mouth) or intravenously plus typically begins working within an hour [36], absorbed in small intestine .

2.3.1. Chemistry of Ibuprofen

Ibuprofen ((2-(4-isobutylphenyl) propionic acid is an unselective end-product inhibitor of the cyclo-oxygenase isozymes (Cyclooxygenase-1) and cyclooxygenase-2, which these isozymes are in control of the transformation of arachidonic acid into prostaglandins (hormone) [6]. Prostaglandin plays a crucial role in fever, inflammation and pain, and both the analgesic and antipyretic effects of ibuprofen are attributed to inhibition of Prostaglandin E2 and I2 [37].

The drug ability to enter the central nervous system of the body, can lead to local accumulation and central analgesia, contributing to the effectiveness [38]. The pace of dissolution from existing solid dosage forms is limited, resulting in insufficient bioavailability from elevated oral doses [39].

Ibuprofen is a non-steroidal anti-inflammatory medication with low water solubility. It features a large hydrophobic portion made up of an aromatic ring and a carbon tail, along with a small hydrophilic segment created by a carboxylic group (Figure 6). The molecule can exist in either a neutral or anionic form, depending on the solution's pH [40].

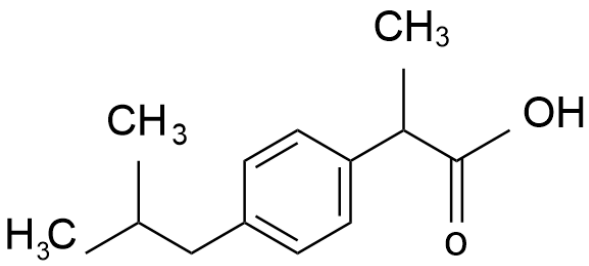


Figure 6. Ibuprofen structure

2.3.2. Ibuprofen Side Effects

Typical side effects include heartburn and skin rash. Compared to other nonhormonal anti-inflammatory drugs, it may have additional side effects, such as gastrointestinal bleeding. Prolonged use has been linked to kidney failure and, on rare occasions, liver failure. Additionally, it can worsen the health of individuals with heart failure [41]. At minimal dose, it seems to pose no raised possibility of heart attack; however, higher doses may elevate that risk. Furthermore, ibuprofen can aggravate asthma symptoms [42].

2.3.3. Ibuprofen Encapsulation

Many formulations and methods have been reported for the delivery of ibuprofen and enhance its bioavailability and few researchers have worked on ibuprofen encapsulation with liposome. The following methods have been used for the enhancement of ibuprofen bioavailability using lipids: ibuprofen loaded microemulsion of liposome using ethanol injection [43-45], encapsulation thin lipid film using hydration method and evaporated inert gas [46, 47] and other methods like reverse phase evaporation [48] .

The study by Gai et al. [43] was focused on developing an optimal drug delivery system using cationic liposomes, ibuprofen eye drops liposomes and ibuprofen natural liposomes to enhance the delivery of ibuprofen. A novel bio adhesive colloidal system is designed to improve precorneal residence time (the time a drug or other substance spends in the precorneal area of the eye), ocular permeation (the process by which drugs pass through the eye) to reach the aqueous humor or other parts of the eye, and bioavailability as well. The formulation of these liposomes, made via the ethanol injection method, was validated using an orthogonal L9 (Taguchi method that is used to optimise multiple factors in experiments) experimental design [43].

Study by Xu et al. [45] was aimed to compare the efficacy of liposomes and microemulsions in the transdermal delivery of ibuprofen and to investigate the underlying mechanisms. Ibuprofen-liposome and ibuprofen microemulsion were prepared using the ethanol injection method, resulting in particle sizes of 228.00 ± 8.60 nm and 56.74 ± 7.11 nm, respectively, with encapsulation efficiencies of 86.68 ± 1.43% (w/w) for liposomes and 91.08 ± 3.27% (w/w) for microemulsions. Percutaneous studies demonstrated that both formulations improved drug permeation and retention in the skin. Fourier Transform Infrared Spectroscopy (FT-IR) and Differential Scanning Calorimetry (DSC) analyses indicated that the enhanced permeation was due to interactions between the formulations and the skin's lipid bilayer and proteins [45].

The study by T Nie et al. [46], successfully achieved the encapsulation of phospho-ibuprofen (P-I) within “poly (ethylene oxide)-b-poly (lactic acid)” co-polymer micelles and liposomes composed of soy-phosphatidylcholine (soy-PC), which were stabilised by a PEGylated lipid (lipid connected to ethylene glycol). The study conducted a comprehensive assessment of the stability, cellular uptake, and cytotoxic effects of phospho-ibuprofen (P-I) when formulated with polymeric micelles and liposomes. Results indicated that liposomal P-I exhibited significant resistance to hydrolysis by esterases in both *in vitro* and *in vivo* environments. Furthermore, the encapsulation of P-I in liposomes not only increased its cytotoxicity and bioavailability but also enhanced its efficacy in a colon cancer xenograft model in mice. The study included assessments of cellular uptake and cytotoxicity of P-I within liposomal and micellar formulations, as well as its *in vitro* metabolic stability in human colon adenocarcinoma cell cultures. Additionally, the performance of liposomal P-I was further investigated through pharmacokinetic studies in mice and in a colon cancer xenograft model [46].

In study by Megan et al. [47], droplet interface bilayer, serving as a model cell membrane, was employed to explore the influence of ibuprofen on membrane behaviour. The results demonstrated that at acidic pH, neutral ibuprofen notably enhanced the water permeability of liposomal lipid membranes. Ibuprofen was shown to cause notable structural changes in DOPC bilayers. Meanwhile, the

absence of cholesterol can enhance this phenomenon. The study concluded that the physicochemical properties and concentration of ibuprofen can influence its interaction with lipid membranes, and different drugs structures may apply distinct effects on liposome membrane permeability [47].

The key findings of the above literatures [43-47] are summarised in table 3.

Table 3. Summary of ibuprofen literature review

Topic	Encapsulation approach	Application	Preparation method	Analysis technique	Administration	Status	Findings	Ref
Ibuprofen neutral liposomes Ibuprofen cationic liposomes Ibuprofen eye drops liposomes	Ethanol injection method	*In vitro* and *in vivo* Studies on a novel Bio adhesive colloidal system: cationic liposomes of loaded ibuprofen	ethanol injection method[44]. Cationic liposomes: amount of soybean phospholipids, cholesterol, octadecyl amine, and ibuprofen dissolved in anhydrous ethanol. Slowly injected in D.water at 50 °C with stirring and then ethanol removed from the preparation by a further stirring. Ibuprofen cationic liposomes were picked by 0.8-µm polycarbonate fiber	The particle size distribution was analysed by a laser particle size analyser. Zeta potential SEM	Topical ocular administration	*In vitro* and *in vivo* testing	Formulation selected according to the drug entrapment efficiency, which showed an appropriate particle size and zeta potential and elevated the corneal permeability, ophthalmic retention capacity, and bioavailability.	[43]

			membrane extruded filter. Neutral Liposomes: Like the first method but octadecyl amine removed Eye drops liposomes: By dissolving ibuprofen in pH 7.4 PBS, the suspension collected.				Consequently, for poor water-soluble drugs, cationic liposome system was a potential approach to improve ocular bioavailability and patient compliance.	
Ibuprofen - liposome ibuprofen microemulsion	Ethanol injection method for liposome and micro emulsions by spontaneous emulsification	Liposome and microemulsion loaded with Ibuprofen: from preparation to mechanism of drug transport	-The ibuprofen liposome prepared by ethanol injection method: The lipid phase consisting of phospholipids,	FT-IR DSC HPLC	Percutaneous Trans dermally	*In vitro* and *in vivo* testing	Liposomes were more suitable carrier systems for ibuprofen than microemulsions.	[45]

			cholesterol, octadecyl amine, and ibuprofen dissolved in ethanol. The aqueous phase consisted of D. water. Both the two phases heated to 50 °C for 20 min. The aqueous phase added to the lipid phase and stirred for 20 min at 600 rpm in a magnetic stirrer. Liposomes stored in a sealed container at 4 °C. The microemulsions prepared by the spontaneous emulsification method:				The *in vitro* release of ibuprofen liposome was slightly better than that of ibuprofen microemulsion . *In vitro*, skin penetration study has shown that ibuprofen liposome better facilitated transdermal drug delivery compared to ibuprofen microemulsion .	

			Oleic acid, Tween-80 (surfactant), and glycerol (co-surfactant) combined into lipid phase, and ibuprofen dissolved in lipid phase. D. water added to the lipid phase until the dispersion was clear. Stirring continued for half an hour until equilibration to obtain microemulsion.				Ibuprofen liposome and ibuprofen microemulsion could improve the permeability of the skin barrier and increasing lipid mobility, furthermore, this destructive effect of ibuprofen liposome was stronger	
Phospho-ibuprofen incorporated in nanocarriers.	Thin lipid film evaporation and hydration method	investigated the use of nanocarriers to improve its pharmacokinetics and anti-tumour efficacy.	Soy-PC, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [amino (polyethylene glycol)-2000] ammonium salt)	HPLC NMR TEM with negative staining DLS	Injection	*In vitro* and *in Vivo* testing	P-I also demonstrated increased cytotoxicity *in vitro*.	[46]

			and P-I were dissolved in chloroform evaporated to a thin film in rotary evaporator, rehydrated with PBS and extruded through double polycarbonate membranes of 0.08 or 0.02 μm pore size using an extruder device. The non-encapsulated drug, separated from liposomes by extensive dialysis against saline. An identical process that omitted P-I used to prepare empty	Zeta potential			Free P-I metabolised quickly to ibuprofen in the presence of purified esterases. In contrast, liposomal P-I, and to a lesser extent micellar P-I, was resistant to esterase-mediated hydrolysis(reaction).	

Ibuprofen and the phosphatidylcholine bilayer	Thin film evaporated under inert gas and hydration	Influence of ibuprofen and cholesterol on lipid bilayers.	DOPC used as mixture with chloroform and dried overnight to remove chloroform from it. Ibuprofen co-dissolved with lipid and cholesterol in chloroform. Two different ratios employed, namely, 4:1 and 1:1 mol ratio for DOPC: Cholesterol. Dried film rehydrated with an aqueous solution of pH 3 and vortex device for couple of minutes	Water permeability vibrational spectroscopy	Research N/A	*In Vitro* testing	DOPC membranes results in an enhancement of the osmotic water permeability with increased concentration of drug. Findings showed ibuprofen can distrupt the membrane and imparting increased fluidity on the lipid bilayer.	[47]

			with 7 times freeze–thaw cycles using N_2 to claim suspension of multilamellar liposomes.					

2.4. Summary of Knowledge Gap

According to the information obtained, it has been seen that curcumin and ibuprofen have not been presented together as a product using liposome co-encapsulation so far. To increase the bioavailability of curcumin, many ways have been proposed, for instance using nano-carriers, cyclodextrin complexes or piperine [49].

liposome has been chosen for this purpose to combine curcumin with ibuprofen and trap them into its bilayers to build a new long-term release pain killer medicine. Obtained information demonstrates that encapsulating curcumin and encapsulating ibuprofen separately was successful; thus, it was decided to combine these two substances and co-encapsulate them with liposome to obtain the drug with less side effects, and the ability to reduce pain in the long term.

Chapter 3: Materials, Experimental and Methodology

3.1. Materials

Curcumin (95%), chloroform (99%) and High-pressure liquid chromatography waster (HPLC-Water) were purchased from Thermo scientific. Ibuprofen (98%), acetone, PBS and cholesterol were obtained from Fluorochem. DPPC (99%) was purchased from Merck. DCP (Dihexadecyl Phosphate) was attained from Cambridge bioscience.

3.2. Experimental and Methodology

3.2.1 Preparation of Lipid

Step 1. Preparation of Lipid Mixture

Two samples were prepared adjusting the parameters slightly as follow.
Sample 1: Chloroform (5 ml) was transferred into a flask. Gradually ibuprofen (0.0020 g) and curcumin (0.0010 g) were added to the flask. Cholesterol (0.0081 g) DPPC (1.25 ml or 0.0312g) and DCP (0.0020 g) were introduced slowly to the flask. DPPC: cholesterol: ibuprofen: curcumin: DCP ≈ 31: 8: 2: 1: 2.

Sample 2: Chloroform (4 ml) and acetone (1ml) was transferred into a flask. Slowly ibuprofen (0.0020 g) and curcumin (0.0010 g) were added to the flask. Cholesterol (0.0107 g) and DPPC (1.25 ml or 0.0312g) were introduced slowly to the flask.
The flask was transferred on the rota-evaporator and heated to 45°C and pressure of 267 mbar to form thin lipid films. DPPC: cholesterol: ibuprofen: curcumin ≈ 31: 11: 2: 1 (Figure 7 demonstrates the preparation steps).

Step 2. Hydration and freeze thawing.

PBS (45 °C, 5ml) was added to the lipid film. Then the flask was agitated by sonicator and vortex, followed by submerging the flask into the liquid N_2. The process was repeated three times for sample 1 and four times for sample 2 (Figure 8).

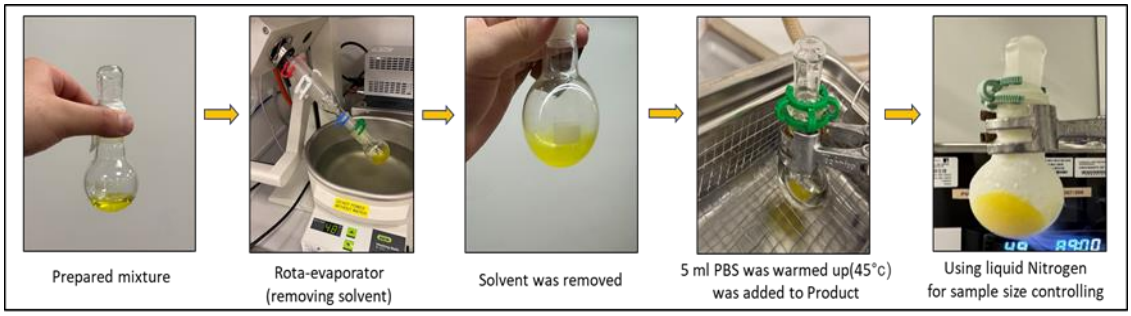


Figure 7. Preparation process and thawing cycle

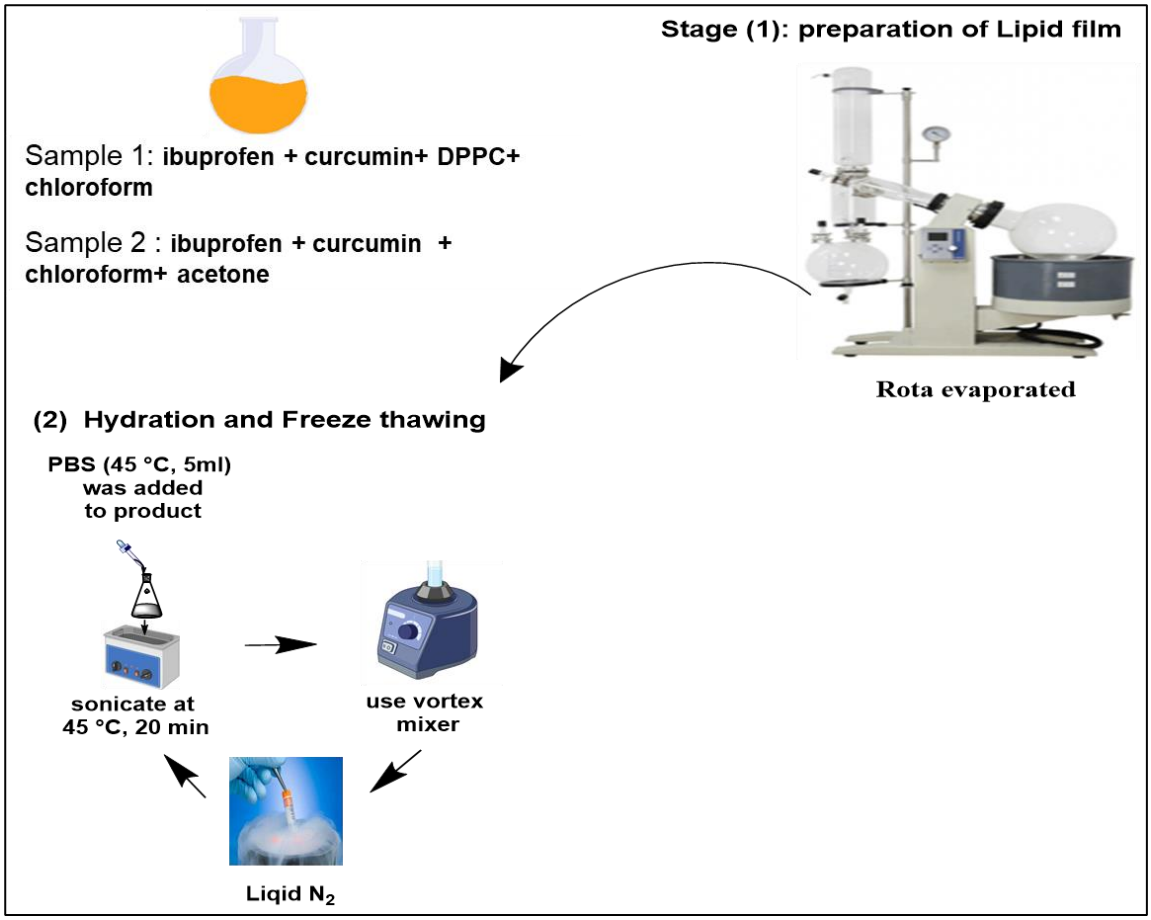


Figure 8. Lipid film preparation using rotary-evaporator, hydration, and thawing cycle.

Step 3. Rinsing and Centrifugation

The samples were rinsed with HPLC-Water three and two times, respectively for Sample 1 and Sample 2 and the waste were taken after centrifuge for HPLC-UV analysis to determine the remaining amount of curcumin and ibuprofen that were not trapped in the capsules (please see Figure 13 and 14). After centrifuge, two phases were obtained: supernatant and pellet (Figure 9 and 10).

Supernatant includes removed ibuprofen and curcumin which they are not trapped in liposomes, or they are removed from it; furthermore, pellet includes liposomes containing curcumin and ibuprofen.

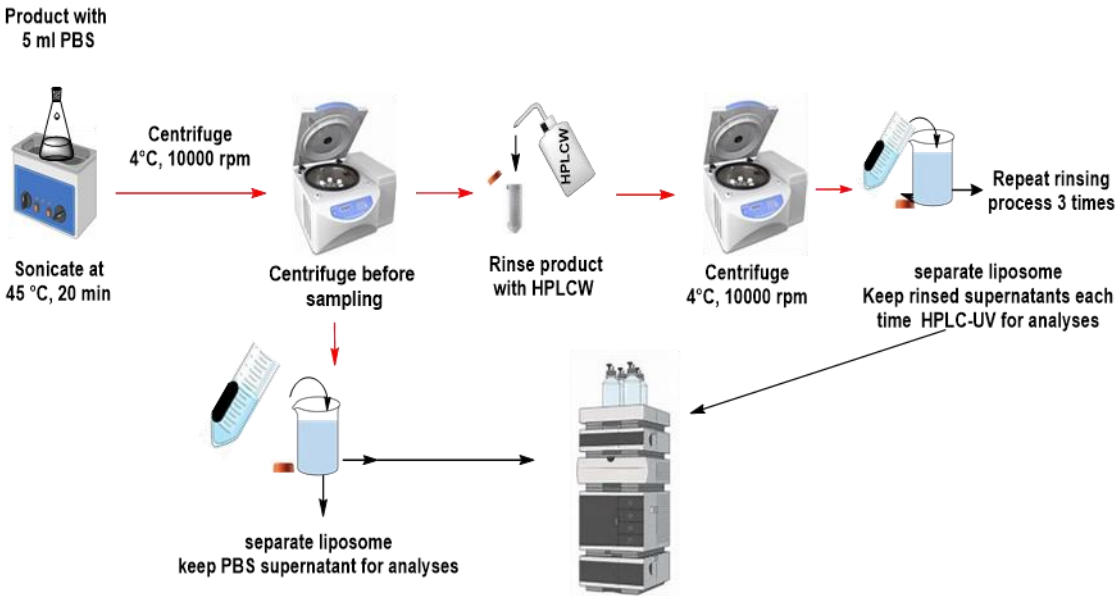


Figure 9. Taking PBS supernatant and rinsing supernatants using centrifuge

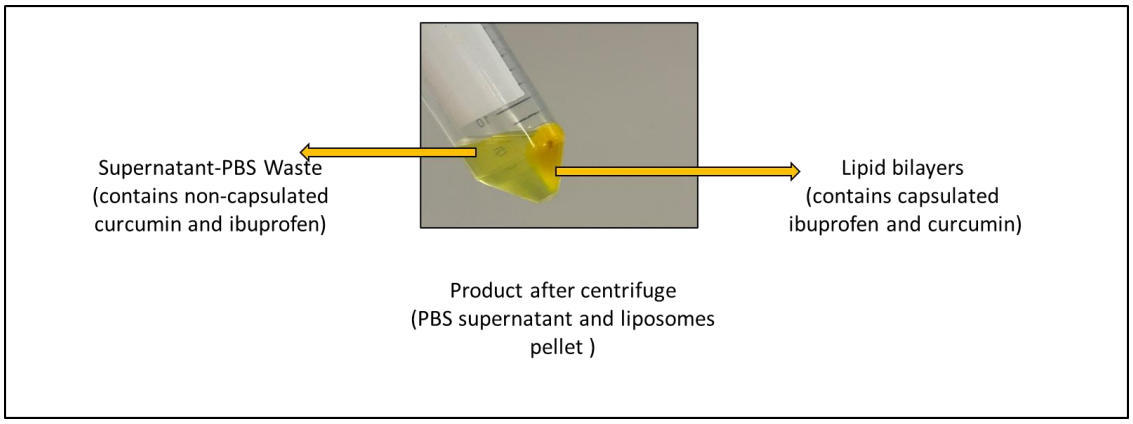


Figure 10. Sample 2 after centrifuge. Pellet and supernatants are showed.

3.3. Characterisation Methods

The encapsulation percentage of curcumin and ibuprofen was quantified using an Agilent 1290 Infinity II HPLC system (Agilent, Santa Clara, CA, United States), supplied with a diode array detector. Chromatographic separations were carried out applying an Agilent Infinity Lab Poroshell 120 EC-C18 (2.1 x 50 mm, 1.9 um) at a column temperature of 40 ºC. The mobile phase used was 0.1 % trifluoroacetic acid (TFA) in water (95 %) and 0.1 % TFA in acetonitrile (5%) and the gradient running to 5 % water over 5 minutes at a flow rate of 0.5 mL/min. The diode array detector monitored the chromatogram at a 254 nm.Cryo SEM (morphology and structure) system is a Quorum Technologies with PP3010 cryo sample preparation system. The sample was kept at -140°C and the SEM that

used was a ThermoFisher scientific Helios (Waltham, Massachusetts, USA). Microscope imaging using Leica microscope, DMIL LED (Wetzlar, Germany). H and C-NMR Bruker AV-NEO (Karlsruhe, Germany) Nuclear Magnetic Resonance (NMR) spectroscopy was recorded applying a four channel Bruker AV-NEO NMR spectrometer operating at a magnetic field strength of 11.7 T, corresponding to a 1H resonance frequency of 500 MHz. the instrument was facilitated with two 5mm probes: a TBO probe applicable for 1H/19F and broadband nuclei and a TXI probe optimised for 1H/13C/15N probes, providing high-resolution multi-nuclear detection. Spectra were obtained applying standard pulse sequences (e.g., zg30) at room temperature and the chemical shift was reported in parts per million (ppm), with a typical 16 scans and exponential line broadening of 0.3 Hz to improve the signal-to-noise ratio.

Chapter 4: Results and Discussion

4.1. HPLC-UV Analysis (quantification of curcumin and ibuprofen)

The HPLC-UV was used for the quantification of ibuprofen and curcumin at post synthesis treatment stages. After synthesis the sample was centrifuged and the supernatant containing PBS (0.2 ml) was taken and diluted with HPLC water (0.5 ml) then homogenised with a vortex mixer (mentioned in Figure 11).

The separated samples were then rinsed different times using HPLC water. The rinsing supernatants were taken for the HPLC-UV analysis without further dilution.

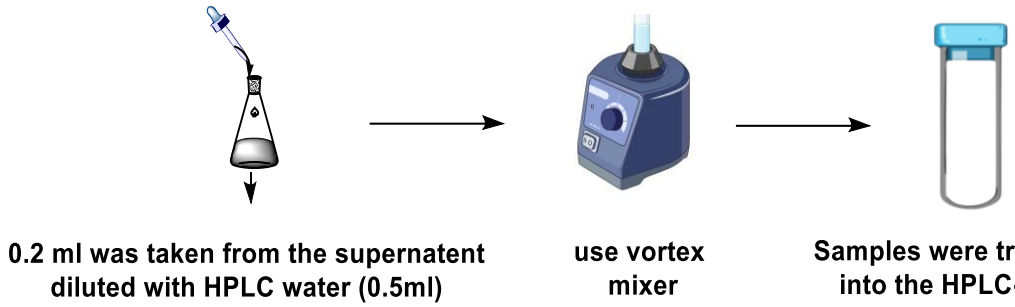
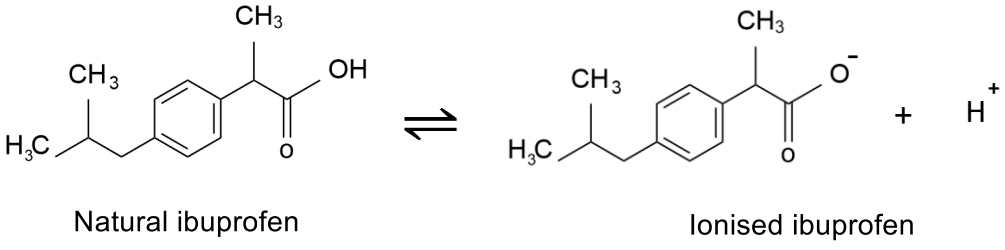


Figure 11. Preparation of PBS supernatant for HPLC-UV analysis

The results for supernatant and rinsing process for sample 1 and sample 2 are shown in Table 4 and Table 5, respectively.

HPLC-UV analysis showed different concentrations of curcumin and ibuprofen within the supernatants, at different stages (Table 4 and Table 5).

In both samples, the amount of ibuprofen remaining in the synthetised supernatant was higher, compared to curcumin. This could be due to the effect of PBS, which caused osmotic pressure, leading to low encapsulation of ibuprofen within the liposome. As ibuprofen is a weak acid with a pKa : 4.5, a large portion of ibuprofen can be ionised at pH 7.4 [50] which has less tendency to remain in/on lipid bilayers, thus it is easier to be separated from the lipid film as PBS were used for hydration steps at 45°c. Furthermore, ibuprofen amount was higher than curcumin in both mixtures which make ibuprofen to leak more than curcumin in PBS supernatant.



$$pH = pKa + \log\left(\frac{[A^-]}{[HA]}\right)$$

(COO^-) : $[A^-]$

$(COOH)$: $[HA]$

pH=7.4 pKa=4.5

Figure 12. Ionization of ibuprofen

Ibuprofen does not tend to ionise in acidic media. PBS with a pH of 7.4 can result in 794 ionised molecules for every 1 non-ionised molecule as calculated with

formula above (Figure 12). Compared to HPLC water with pH 7, PBS has sodium ions (Na^+) and (HPO_4^{2-}) these ions can make ibuprofen more soluble in PBS than water. The solubility of ibuprofen in water (25°C) is above 0.025 mg/ml and in PBS, the solubility of ibuprofen enhanced to about 6.020 mg/ml [51]. With high “osmotic pressure”, pressure can cause more ibuprofen to dissolve in the aqueous phase, thereby accelerating its release from the system.

While DPPC is a hydrophobic lipid, it is typically and expectedly able to encapsulate hydrophobic materials better than hydrophilic ingredients (hydrophobic ingredients encapsulation can be improved by presence of cholesterol as reported before [12]).

Curcumin would have better stability in PBS and lipid bilayers with higher encapsulation within the liposome and low solubility in water.

During the rinsing process, addition of water led to the diffusion of curcumin through the liposomal bilayer .If a lot of washing is done continuously with water, the lipid structure may begin to degrade, resulting in curcumin and ibuprofen leaking from the system after second and third rinse. Also, the presence or absence of DCP seems vital. Removed ibuprofen for Sample 1 with 38.14% and for Sample 2 with 61.63% might be cause of additional cholesterol and lack of DCP in Sample 2; also, curcumin with 7.99 % and 11.62% respectively, can prove it as well.

Cholesterol can cause increased lipid sizes, stabilised the bilayers, helping transportation of hydrophilic molecules in between the inner and outer aqueous regions; furthermore, encapsulation hydrophilic ingredients only possible when liposome contained cholesterol [12] . It can be seen that cholesterol and DCP together, can assistance ibuprofen and curcumin to be trapped in bilayer and can help both of them be more stabilised in lipid capsules.

The data for HPLC-UV for sample 1 and sample 2 are collected in Table 4 and Table 5, respective.

4.1.1 Encapsulation efficiency

After the lost percentages were calculated with HPLC analysis, in Sample 1 92.01% curcumin, 61.86% ibuprofen and for Sample 2 ,88.38% curcumin and 38.37% ibuprofen remained in liposome capsules (Figure 13 and 14).

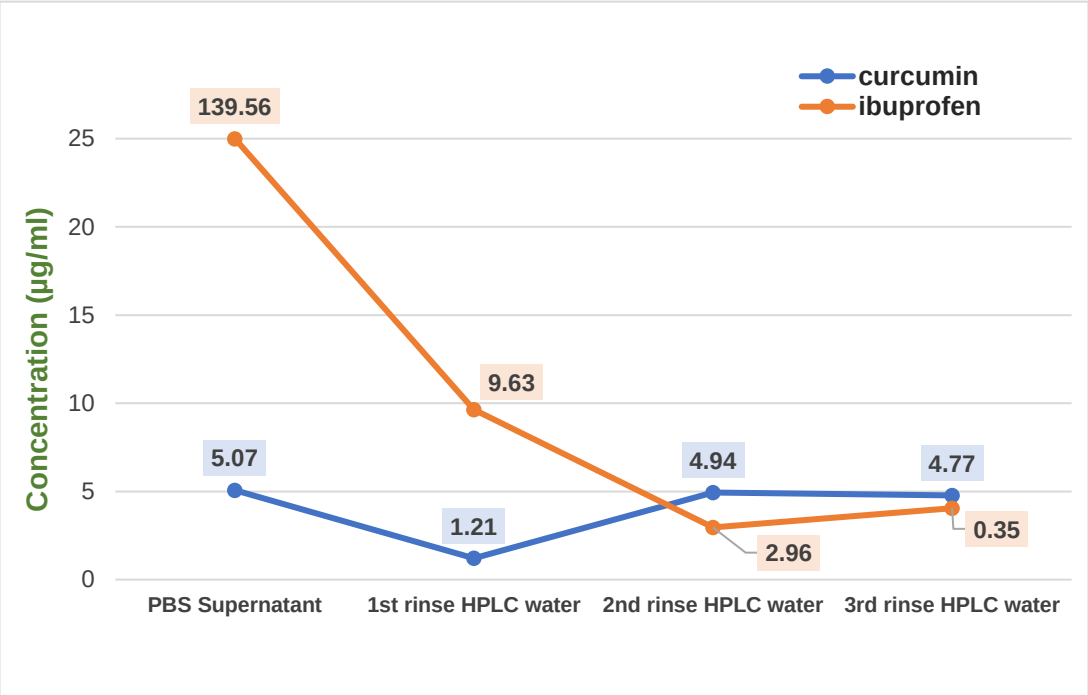


Figure 14. HPLC-UV results for Sample 1

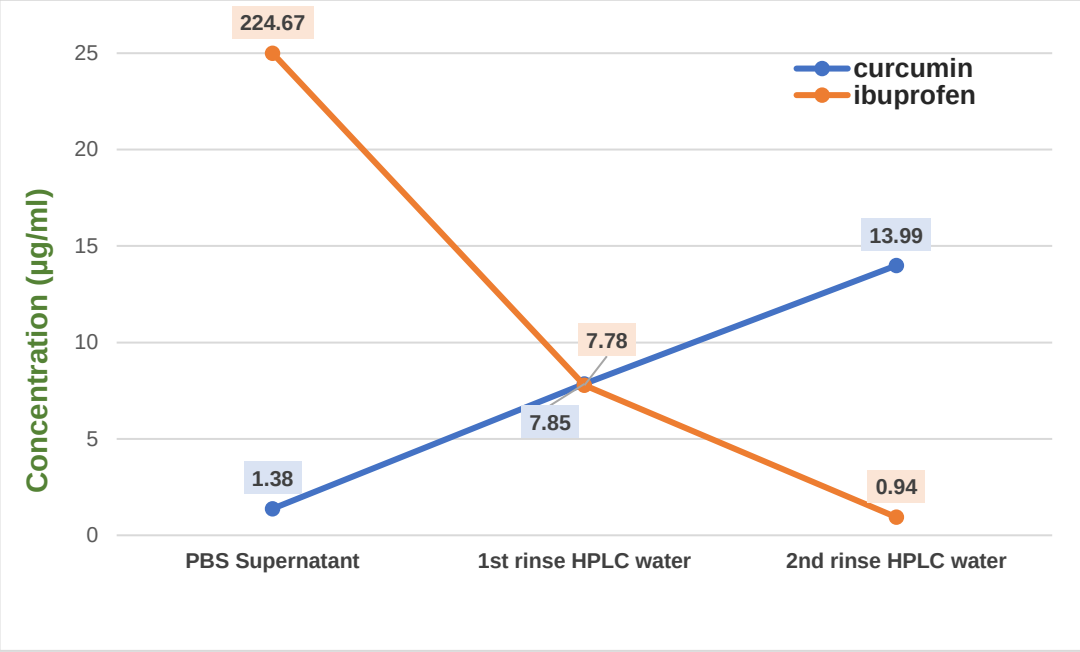


Figure 13. HPLC-UV results for Sample 2

Table 4. HPLC-UV data for quantification of removed ibuprofen and curcumin in Sample 1

Sample 1	Curcumin		Ibuprofen	
	Concentration (µg/ml)	% lost from Sample	Concentration (µg/ml)	% lost from Sample
PBS Supernatant	5.07	2.54 %	139.56	34.90%
1st rinse HPLC water	1.21	0.61%	9.63	2.41%
2nd rinse HPLC water	4.94	2.47%	2.96	0.74%
3rd rinse HPLC water	4.77	2.38%	0.35	0.01%
-	-	Total: 7.99%	-	Total: 38.14%

Table 5. HPLC -UV data for quantification of removed ibuprofen and curcumin in Sample 2.

Sample 2	Curcumin		Ibuprofen	
	Concentration (µg/ml)	% lost from Sample	Concentration (µg/ml)	% lost from Sample
PBS Supernatant	1.38	0.70 %	224.67	56.17%
1st rinse HPLC water	7.84	3.92%	7.78	1.96%
2nd rinse HPLC water	14.00	7%	0.94	3.50%
-	-	Total: 11.62%	-	Total: 61.63%

4.2. Microscope Imaging

Tabletop microscopic imaging of the Sample 1 and Sample 2 was done by taking the wet past (0.023 g) and dispersing in D. water (Figure 15).

According to the images both samples demonstrate liposomal structure with round shape. In sample 1 (Figure 15 (a)) the liposomes showed less aggregation. While in Sample 2 the extent of aggregation was higher, and the particles were in cluster forms. This could be due to the effect of DCP in Sample 1 since it is a negatively charged surfactant which decreased the aggregation of the particles [46]. has also reported that adding DCP to liposomes can increase their surface negative charge, which prevents their aggregation [52]. It can be seen in the Figure 15, overall, the measurable capsule sizes for Sample 2 are larger than Sample 1.

The main colour of these images is yellow due to curcumin, which has a yellowish orange colour as a natural pigment. The colour changed to green or is due to a changing the contrast to better identify the layers of the capsules. The microscopic image is actually a two-dimensional image of spherical liposomes. The limitations of optical sectioning cause them to be like circle.

Particle size distribution were done for optical microscope images (Figure 16 and 17).

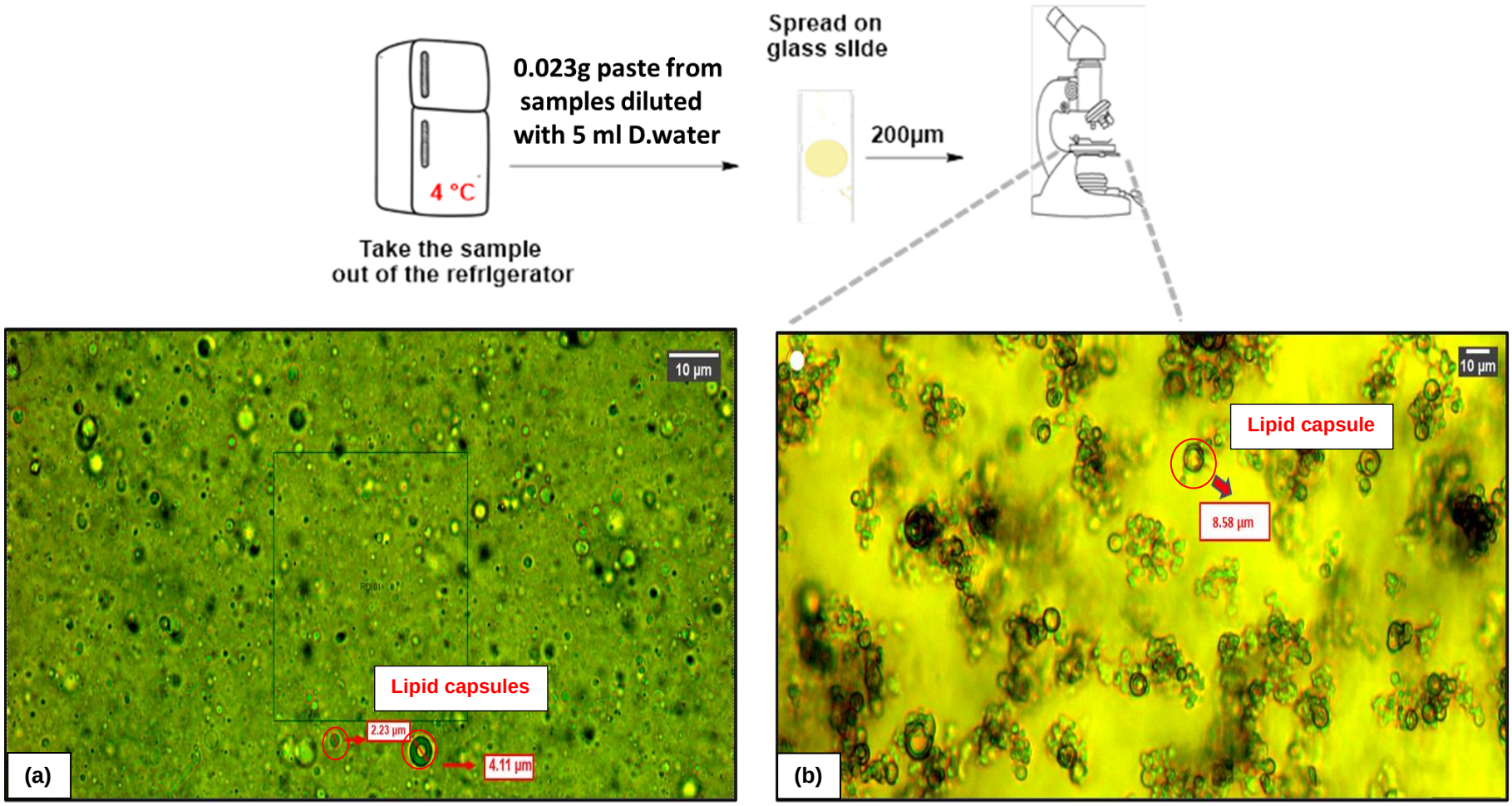


Figure 15. (a and b) Microscope images for Sample 1 and Sample 2

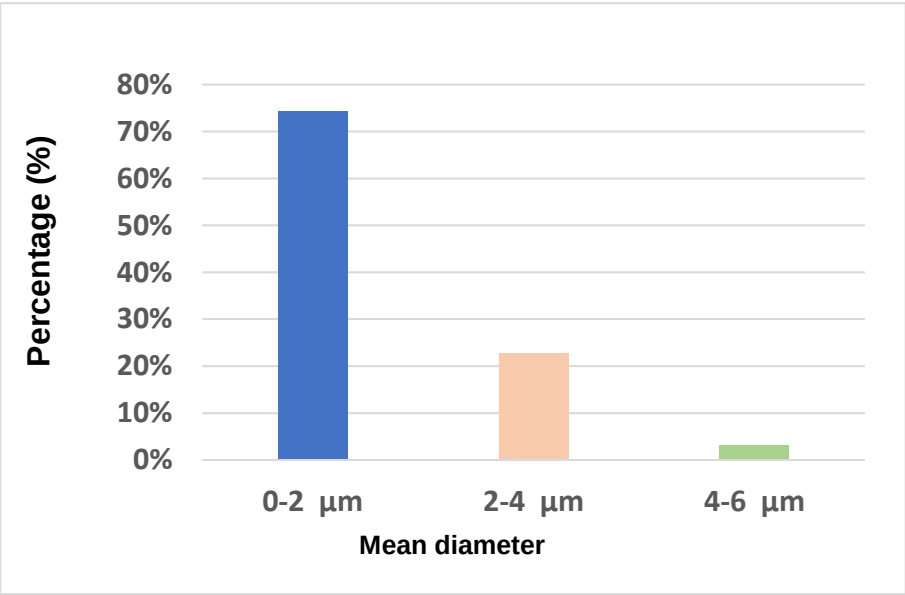


Figure 16. Particle size for Sample 1, measured by microscope imaging.

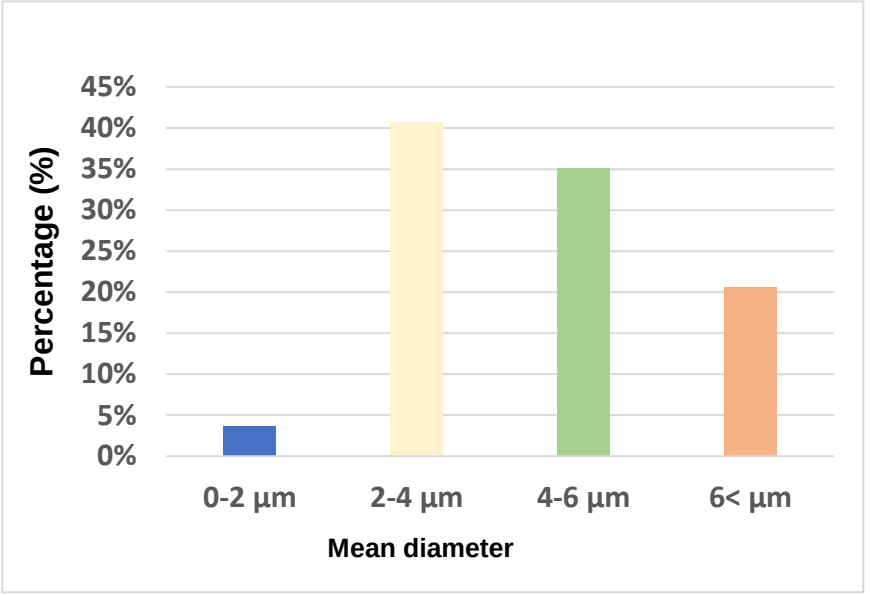


Figure 17. Particle size for Sample 2, measured by microscope imaging.

4.3. SEM Imaging

The Cryo-SEM images in Figures 18 and 19 show the particle morphology, size, internal structure, and potential aggregation. Numerous capsules with a diameter of less than 1 micron can be seen which were not easily observable from optical imaging (section 4.2). In Figures 18 and 19, the cross-section method was used to display the interior of the capsule and measurement of dimensions.

As can be seen in the images, capsules for both samples (1 and 2) are spherical and mostly multi-layered. These layers are many concentric lipid bilayers, and between these layers there are usually spaces which may contain drugs, water, or PBS.

Aggregated particles in Figure 19 (Sample 2 without DCP) can be observed. Overall, Sample 2 results in larger particles sizes but there are more frequent aggregates. Generally, thawing cycle can lead to a reduction in capsule size, reduced agglomeration, increased homogeneity, and improved physical stability of liposomes; in addition, extended sonication leads to smaller liposomes [12, 13, 53]. However, these effects on sizes cannot be compared between samples, due to absence of DCP in Sample 2, and different amount of cholesterol in Sample 2.

Figure 19 demonstrates specific points. In picture F it can be seen liposome bilayer has cracked that might be the effect of additional cholesterol which causes hardness and brittleness. It is also believed that the root-like shapes (they are directional and spread from the centre to the edge structures) are formed in picture C may be due to the crystallization of curcumin and ibuprofen or it is possible that they are a result of freezing during the sample preparation for Cryo-SEM (sample should be froze before imaging). Additionally, DCP influences the aggregation observed with both microscope and Cryo-SEM imaging. Figure 19 pictures (E, F and D) seem more aggregated compared to Figure 18 (A and D).

Meanwhile curcumin can win the competition with ibuprofen to settle in between the layers, near the hydrophobic tails because curcumin is more nonpolar than ibuprofen. The lipid interior results that the circle structure in the middle of the capsule may belong to ibuprofen (Figure 18 C and Figure 19 B).

Furthermore, Sample 2 has bigger capsules compare to Sample 1 which confirms the microscope imaging size distribution (section 4.2).

It should be noted that the Iridium ($_{77}$Ir) used in the coating makes the boundary between the lipid and crystalline phases clearly visible.

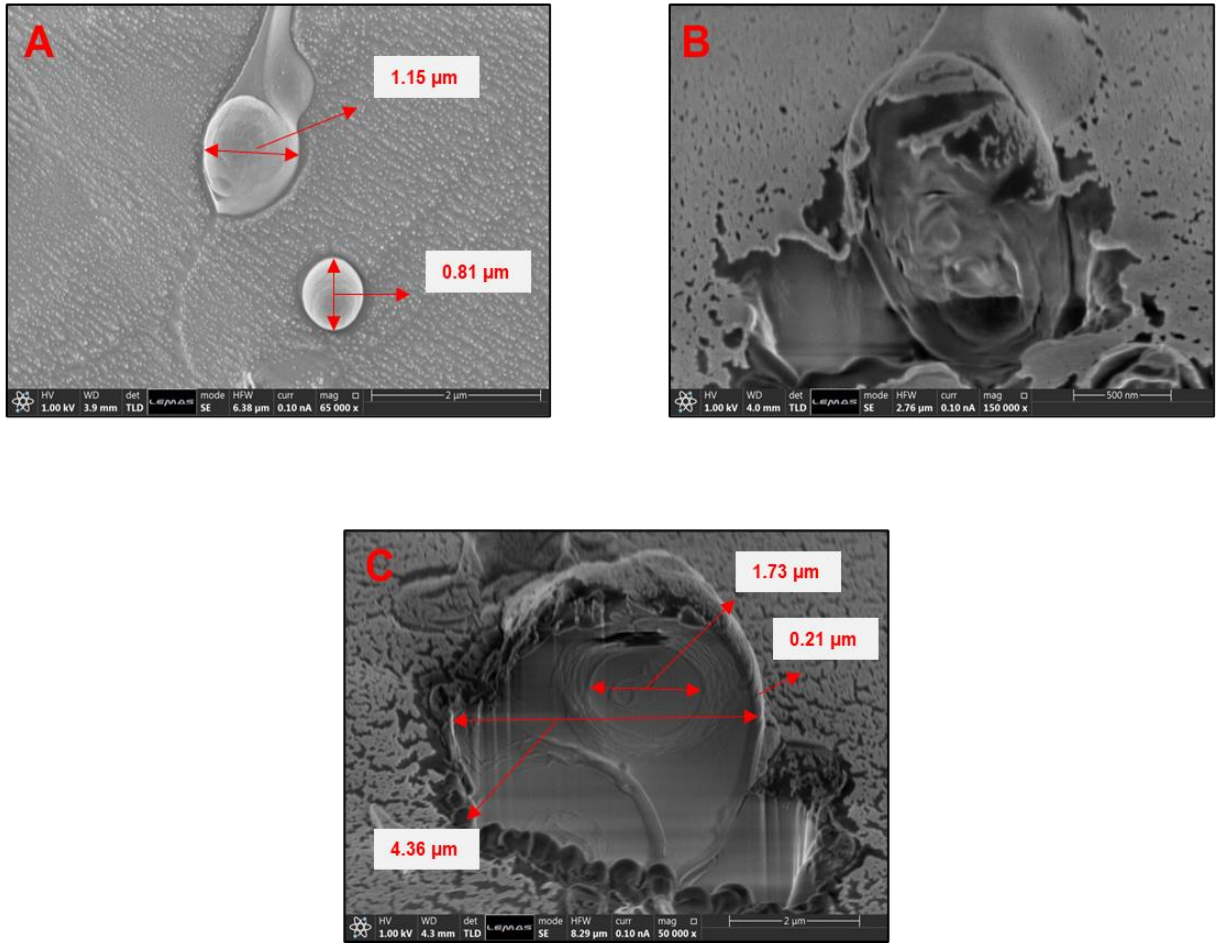


Figure 18. Cryo-SEM images for Sample 1 with DCP with available average size 0.23 µm

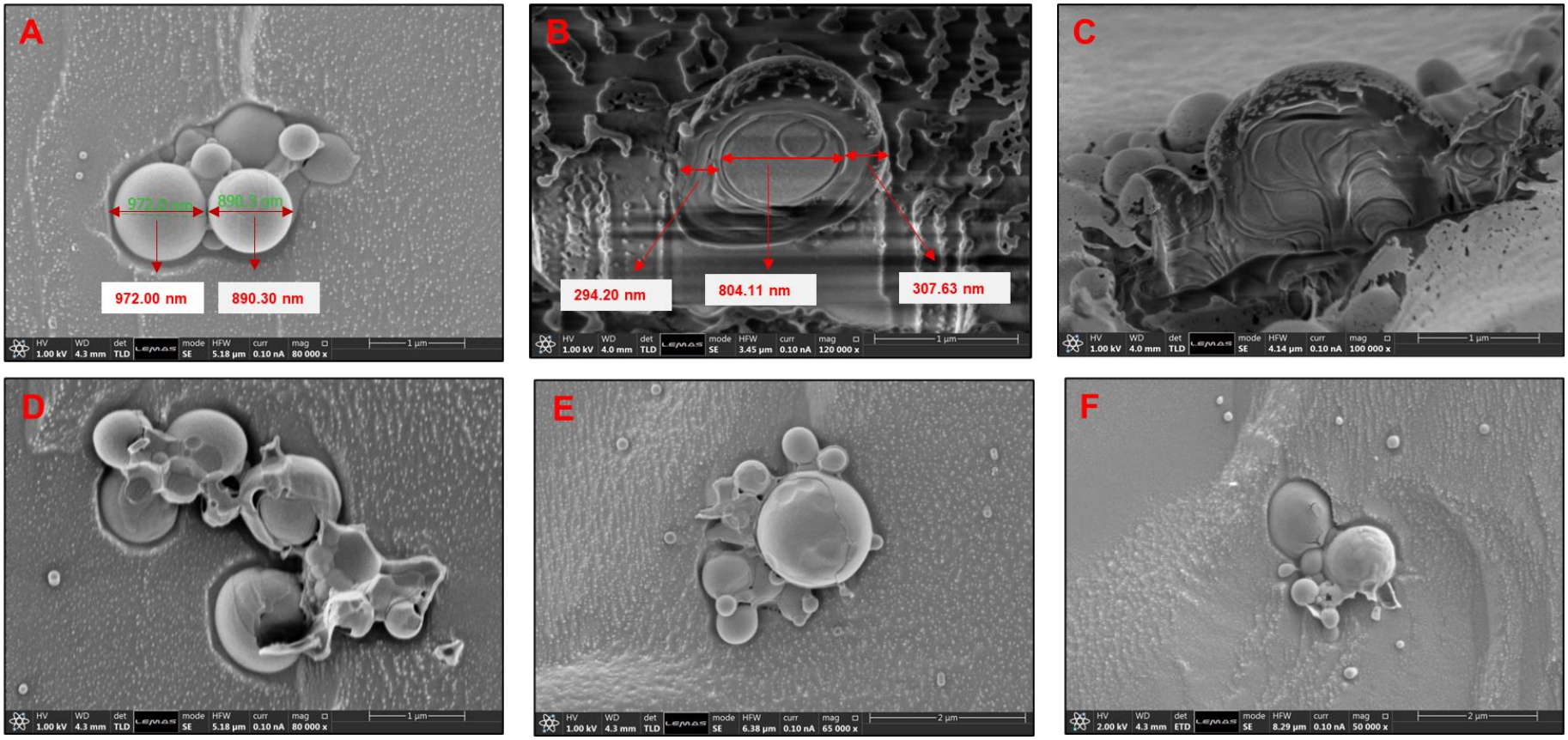


Figure 19. Cryo-SEM images for Sample 2 without DCP with available average size 0.57 μm

4.4. Nuclear Magnetic Resonance Spectroscopy

Nuclear Magnetic Resonance (NMR) spectroscopy was recorded applying a four channel Bruker AV-NEO NMR spectrometer operating at a magnetic field strength of 11.7 T, corresponding to a 1H resonance frequency of 500 MHz. the instrument was facilitated with two 5mm probes: a TBO probe applicable for $^{1}H/^{19}F$ and broadband nuclei and a TXI probe optimised for $^{1}H/^{13}C/^{15}N$ probes, providing high-resolution multi-nuclear detection. Spectra were obtained applying standard pulse sequences (e.g., zg30) at room temperature. with a typical 16 scans and exponential line broadening of 0.3 Hz to improve the signal-to-noise ratio.

Curcumin, ibuprofen, Sample 1 and sample 2 were separately dissolved in $CDCl_3$ (Deuterated Chloroform) for ^{1}H, ^{13}C and ^{35}P NMRs. The samples were also prepared in D_2O; however, the data were poor Upon the preparation of the samples in $CDCl_3$, as it was expected commercial curcumin dissolved immediately in non-polar $CDCl_3$ solvent. This is attributed to hydrophobicity, conjugated double bonds and aromatic rings in curcumin. $CDCl_3$ can interact via van der Waals forces and dipole -induced dipole interactions. In a study by Aghajanpour et al. [54] reported curcumin has a high oil partition coefficient of $\log(K_{ow}) = 3.29$, which explains why it has a high preference for non-polar solvent and poor solubility in water [54] and in D_2O. Similarly, ibuprofen was dissolved rapidly in $CDCl_3$ solvent, however, not in D_2O. As it was explained earlier ibuprofen is non-polar with a slight polar characteristic because of its hydrophobic aromatic rings and carboxylic acid groups, respectively. Hence, this too prefers a dissolution in non-polar solvent such as $CDCl_3$. Although, D_2O is highly polar and capable of hydrogen bonding formation, and the carboxylic groups in ibuprofen can form hydrogen bonding, the ibuprofen's hydrophobic nature is dominant which decreases its solubility in D_2O due to the favourable thermodynamic interactions [55].

As it was expected, the dispersion of Sample 1 in $CDCl_3$ yielded a cloudy solution. This turbidity appearance is consistent with physico-chemical property of the

amphiphilic liposomes. It can emphasise that the presence of DCP retained the liposomal integrity in $CDCl_3$. However, Sample 2 had a contrast behaviour, it was completely dissolved in the solvent and appeared clear. As this sample lack DPC, may have been less capable of maintaining its liposomal integrity in the non-aqueous solvent of $CDCl_3$. Additionally, while the presence of cholesterol is critical for controlling fluidity and stability of membrane in aqueous systems, can also increase the solubility of lipid in organic solvents when the amount is increased. Cholesterol's hydrophobic structure allows stabilisation in hydrophobic conditions via van der Waals interactions [56]. Figure 20 shows the dispersion of curcumin, ibuprofen, Sample 1 and Sample 2 dispersion in $CDCl_3$.

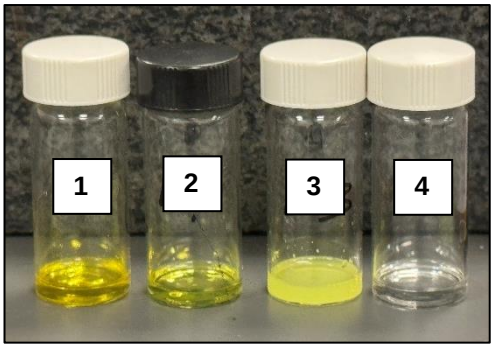


Figure 20. The dispersion of curcumin (1), Sample 2 (2), Sample 1 (3) and ibuprofen (4) in $CDCl_3$

^{1}H NMR Spectroscopic Analysis of Sample 1 and Sample 2

Proton NMR analysis (Figure 21 (a)) verified the simultaneous encapsulation of ibuprofen and curcumin within the liposomal bilayer. Characteristics signals for curcumin appeared at δ 3.88 ppm, δ 7.56, 7.16, 6.93, 6.91, 6.45 ppm which corresponded to methoxy (-OCH) and aromatic carbons, respectively. The peaks at δ 0.87 ppm (terminal methyl protons of isobutyl side chain), δ 1.82 ppm (multiplet, δ 1.80-1.88 ppm, methine proton on the isobutyl side chain), δ 3.74 ppm (multiplet, methine proton α to the carboxylic acid) and δ 7.10, 7.22 ppm (aromatic carbons) confirmed the presence of ibuprofen. Lipophilic contents of the formulation, DPPC, DCP, and cholesterol generated broad peaks between δ 0.60-2.68 ppm, corresponding to the hydrophobic alkyl chains. This broadening arise due to the slow molecular motion and aggregation of lipid assemblies, resulting reduced transverse relaxation time (T2) and enhanced anisotropy [57]. A prominent singlet at δ 3.21 ppm was assigned to the trimethylammonium moiety ($N^+(CH_3)_3$ headgroup) of phosphatidylcholine in DPPC. The resonance at

δ 5.17 ppm was attributed to the C2 of glycerol backbone of DPPC. Furthermore, DCP's aliphatic chains overlaps with DPPC and cholesterol, at δ 0.85-1.85 ppm (CH_3, CH_2, β-CH_2). Compared to DPPC, DPC shows up further downfield due to its anionic, non-quaternary phosphate headgroup with a broad distinct peak at δ 1.26 ppm. Although cholesterol's polycyclic sterol structure give rise to complex and broad NMR spectrum, some key regions are clearly identifiable. Its angular C18 methyl group was observed as a singlet at δ 0. 67 ppm. While the C3 proton near the hydroxyl group and olefinic proton at C6 appeared at δ 3.51 ppm (a multiplet) and at δ 5.36 ppm, respectively. The characteristic peaks match well with the data published on Chemical Book database [58] and the literature values, supporting cholesterol presence in the liposomal bilayer [59-62].

The resulting ^{1}H NMR spectrum of Sample 2 (with no DCP) is shown in Figure 21. (b). The dominant singlet peak observed at δ 7.26 ppm, assigned to the residual proton signal of $CDCl_3$ solvent. The multiplet peaks between δ 6.45-7.65 ppm arise from the aromatic protons of curcumin and ibuprofen. In comparison to the ^{1}H NMR spectrum of Sample 1, no significant broadening was observed in Sample 2. The aliphatic region (δ 0.67-2.28 ppm) showed minimal shifts, suggesting that DCP did not significantly influence the hydrophobic tail region. Additionally, the methylene and methyl peaks displayed little to no variation across both samples. Ibuprofen being partially polar, can associate with the polar headgroup region of the lipid bilayer. In Sample 2, with no DCP, the absence of the electrostatic repulsion likely promotes ibuprofen to stay closer to the bilayer surface rather than penetrating deeper into the membrane. Curcumin being more hydrophobic, preferentially tends to integrate into the nonpolar core of the lipid bilayer. The ^{1}H NMR data for both samples exhibited minimal chemical shift changes for ibuprofen, suggesting that DCP had limited influence on ibuprofen's encapsulation, since this drug embedded in the bilayer.

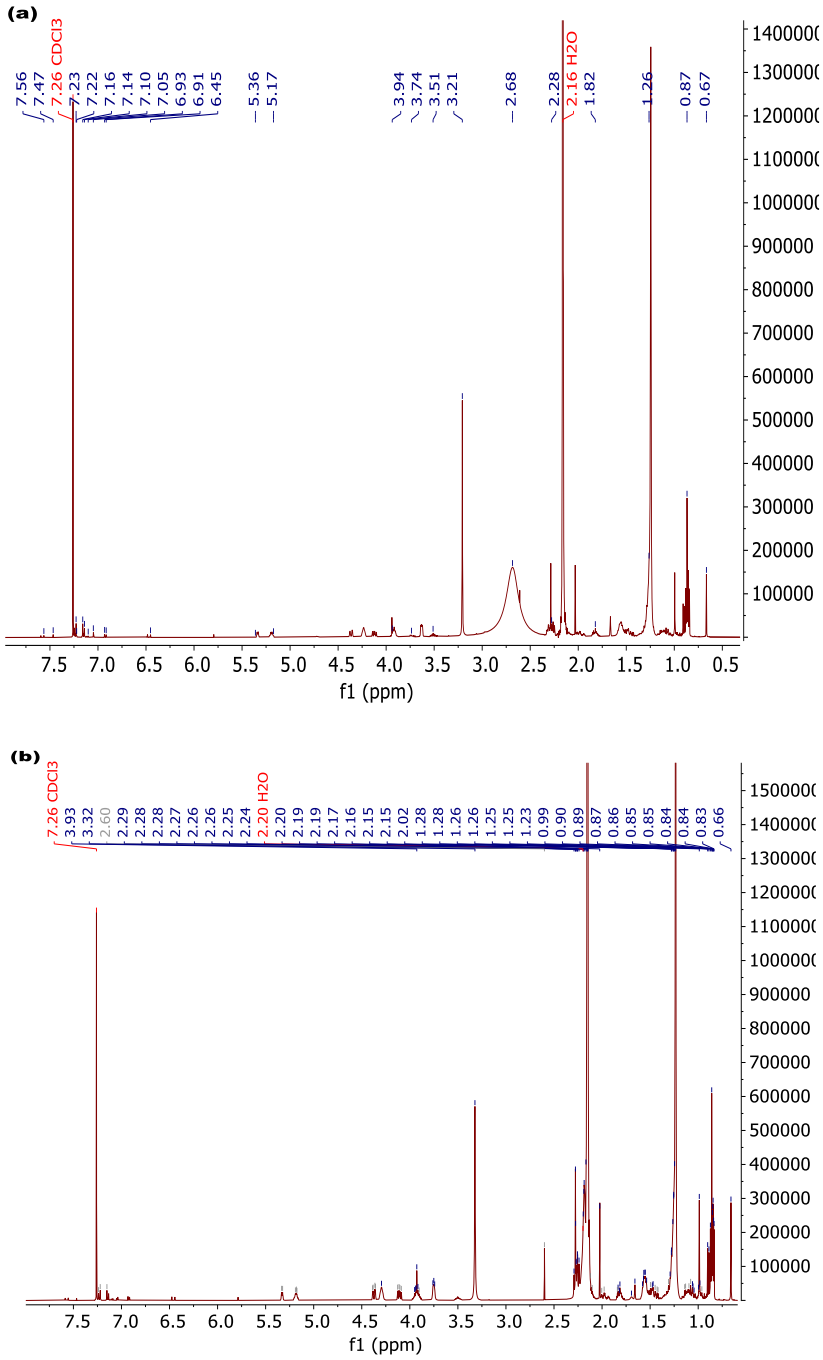


Figure 21. The ^{1}H NMR Spectroscopy of (a) Sample 1 and (b) Sample 2 in $CDCl_3$

^{13}C NMR spectroscopy of Sample 1 and Sample 2

The ^{13}C NMR analysis complemented the ^{1}H NMR results by providing a comprehensive information on the carbon environments of the encapsulated ibuprofen and curcumin and validated their successful incorporation into the liposomal formulation. The spectra for Sample 1 (with DCP) and Sample 2 (without DCP) are shown in Figure 22(a) and Figure 22(b), respectively. A comparison of characteristic ^{13}C NMR chemical shifts from various functional

groups in Sample 1 and Sample 2 are summarised in Table 6 In both samples, the ^{13}C NMR spectrum displayed characteristic resonance attributable to ibuprofen, curcumin, cholesterol, DPPC, and $CDCl_3$ solvent. As expected, the $CDCl_3$ solvent signal appeared at ~ δ 77 ppm, as a triplet due to ^{13}C-^{1}H coupling.

In sample 1 (Figure 22(a)), the aromatic C-H carbon signals of curcumin were observed at δ 107.54 and δ 111.56 ppm, which were slightly up field (i.e., at lower ppm values) region of the spectrum compared to the spectrum of pure curcumin [63]. This up field shift suggests increased shielding, likely due to curcumin being repelled by the presence of negatively charged DCP headgroups and becoming more deeply embedded within the hydrophobic core of the bilayer. By contrast, in Sample 2 (Figure 22(b)), the same corresponding aromatic carbon signals appeared at δ 110.36 ppm and δ 115.06 ppm, which were shifted slightly downfield (higher ppm). This implied decreased shielding, consistent with curcumin stays closer to the bilayer surface, where it likely interacted with the polar headgroups of DPPC, without facing the electrostatic repulsion from DCP.

The aromatic carbon signals of ibuprofen displayed slight up field shift in Sample 1 compared to Sample 2, the chemical shifts were minimal and stayed relatively consistent across both samples. This stability implied that curcumin's embedded within the hydrophobic core of the bilayer and was unaffected by the presence or absence of DCP. This reinforces the idea that curcumin integrated into the nonpolar interior of the membrane and was not significantly affected by surface charge modifications.

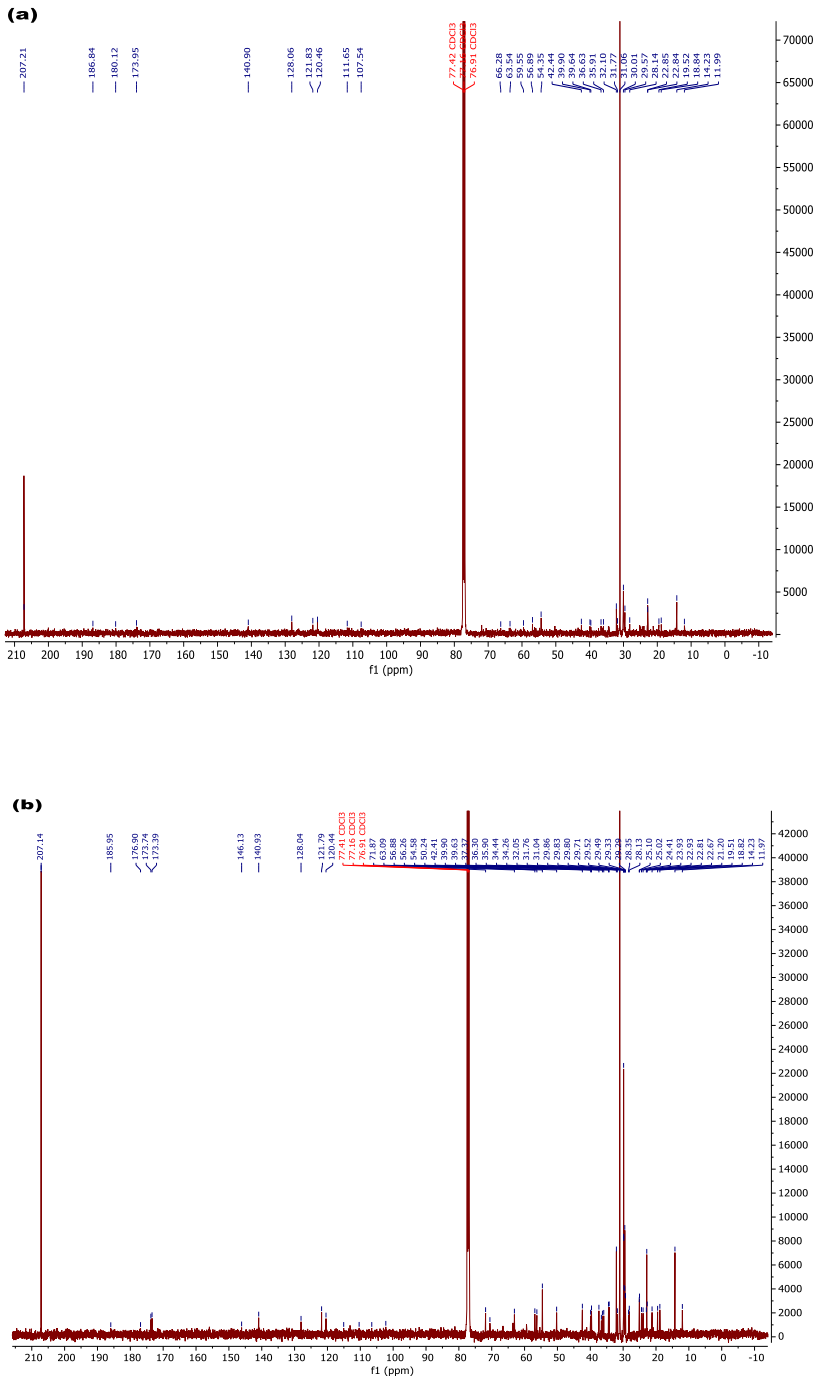


Figure 22. The ^{13}C NMR Spectroscopy of (a) Sample 1 and (b) Sample 2 in $CDCl_3$

Table 6. ^{13}C NMR analysis summery

A comparison of characteristic ^{13}C NMR chemical shifts from various functional groups in Sample 1 and Sample 2.			
Chemical Environment	**Sample 1 (with DCP) Chemical shift (ppm)**	**Sample 2 (without DCP) Chemical shift (ppm)**	**Interpretation**
Keto-carbon C=O	207.21	207.14	Curcumin remained intact in both samples
Carboxylic acid (-COOH) from ibuprofen	180.12, Slightly downfield (less shielded)	176.90 Slightly up field (more shielded)	δ 180.12 ppm due to moderate deshielding by DPPC (via H-bonding) and electrostatic deshielding from DCP pulled ibuprofen closer to surface. δ 176.90 due to hydrophobic interactions and van der Waals forces with cholesterol, ibuprofen embedded deeper in a more shielded environment.
ester (C=O) from DPPC	173.95	173.74 173.39 (With no DCP, can lead to two distinct but close signals)	Consistent with DPPC ester (sn1 or sn2) in $CDCl_3$
Aromatic C-H carbons	107.54, 111.65 from curcumin (up field, lower ppm), 128.06 from ibuprofen	110.36, 115.06 from curcumin (downfield shift, higher ppm) 128.04 from ibuprofen	Peaks in pure curcumin (109.78, 114.99 ppm ppm), ibuprofen (127.40, 129.56 ppm). DCP affected curcumin loading.
Aromatic quaternary carbons	Not present	146.13 from curcumin	DCP caused line broadening or signal loss
Glycerol carbons (C-O) from DPPC,	71.92 from glycerol	71.87 from glycerol, 70.55 from cholesterol	Indicates DCP interaction near polar headgroup

cholesterol OH bearing carbon			
Methoxy carbon (curcumin)	56.89	56.87	Minor differences
Methylene -CH_2- in lipid chain	39.90-30.01	39.90-29.86	Similar pattern, no disruption of lipoid tail confirmation
Methyl -CH_3 in isobutyl of ibuprofen	22.84	22.81	Similar pattern, remained intact
Terminal -CH_3	18.84 (ibuprofen)	18.82 (ibuprofen)	Remained intact

Chapter 5: Conclusion and Contribution

5.1. Conclusion

In this work, the aim was to prepare a co-encapsulated curcumin and ibuprofen in liposomes to improve bioavailability and make a phytochemical painkiller drug with less side effects and less ibuprofen amount in final product. Encapsulation were done using DPPC lipid as liposomes at different conditions (formulation and thawing) using rota-evaporator. Hydration and thawing were done, and results were taken. It has been shown that DCP and cholesterol which were used for stability and bioavailability have crucial role in encapsulating curcumin and ibuprofen. Preparing lipid mixture without DCP is less stable and more aggregated than lipids contain DCP. High amount of cholesterol may cause rigidity for membrane and can make bigger capsules as well.

Curcumin tends to substitute in hydrophobic tails between the bilayers, and this substitution decreases by enhancing cholesterol because cholesterol can replace curcumin (cholesterol is more hydrophobic than curcumin and ibuprofen) and making curcumin more accessible to be removed by rinsing lipid capsules. Since curcumin is more hydrophobic than ibuprofen, it competes with ibuprofen to be more present in the bilayers or in the space between hydrophobic tail and hydrophilic heads (hydrophobic-hydrophilic boundary). It is forcing the ibuprofen particles to settle on the surface or near lipid core (Inner/outer aqueous phase).

Cryo-SEM demonstrates the multi layers of prepared liposome and how some of them cracked in Sample 2 which may be owing to the absence of DCP or higher amount of cholesterol or even freezing the samples during Cryo-SEM.

Visible capsules diameters for Cryo-SEM images were measured and the result also confirmed the size distribution by macroscope imaging and the fact that increasing cholesterol can be helpful to make bigger capsules and how DCP can disaggregate the capsules. Although Sample 2 had one more thawing step than Sample 1, the average diameter of the liposomes in the sample without DCP was larger due to enhancement of cholesterol and absence of DCP. As shown by the microscope and Cryo-SEM images, HPLC and NMRs results, the encapsulation

was successful, and a tolerable percentage of the active ingredients were encapsulated., i.e., 92.01% curcumin, 61.86% ibuprofen in **Sample 1** and 88.38% curcumin and 38.37% ibuprofen remained in **Sample 2**. These results can be helpful for preparing a drug with different encapsulation efficiency and making an anti-inflammatory and painkiller drug with more curcumin and less ibuprofen for reducing ibuprofen side effect. DPPC for oral use must be of detectable purity and pharmaceutical/food grade. Future tests should be conducted on its purity and other ingredients in this drug.

5.2. Research Contribution

In this study, curcumin has been co-encapsulated alongside ibuprofen for the first time. This research demonstrates how effective, combination of phytochemicals can be. Also, by encapsulating these substances within lipids, their bioavailability increases, which could have a significant impact in the field of drug delivery. These medicines can be a magnificent alternative for chemical drugs, with their co-release function in body. It can also be noted that the competition between curcumin, ibuprofen and cholesterol in substitution is very interesting, and the behaviour of the molecules against each other is fascinating.

Chapter 6: Future Recommendation

For future experiments, designs are proposed that can help to further understand the behaviour, structure, and three-dimensional positioning of liposomes alongside a combination of chemical and herbal materials; also, changing amount of DCP and cholesterol or using another ingredient for providing negative or positive charges.

- **Changing the preparation method**

  In this study, thin film evaporation method and thawing were used which can be changed for future studies. I recommend preparing liposomes with pH adjustment or microfluidic methods. Meanwhile, different ways, such as using filters (extrusion), can be helpful for size control.

- **Changing the ratio**

  Amount of active ingredients, surface charger, cholesterol and solvents can be changed for future research to investigate liposomes behaviour and the balance between encapsulation efficiency and materials ratio.

- **Coating the capsules**

  Because of relative durability, absorbent properties, and controlling leakage and osmotic pressure, coating should be done for further research. Coating can be done by lipids and polymers.

- **Changing charges**

  Using positive charger substance instead of DCP as a negative charger. We highly offer researchers to use other surface chargers and compare it with similar studies.

- **Using different lipids as liposome**

  DPPC were used as liposome, but many other lipids (other phosphatidyl choline lipids, cation or anionic lipids or some egg phospholipids) and micelles with different behaviour are available for encapsulation and we encourage researchers to use different lipids as a future works.

- **Drug release in acidic and basic environment**

  This study can encourage researchers to work on behaviour of these capsules with drug release tests on different pH environments and conditions.

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Chapter 8: Appendix

8.1.SEM images

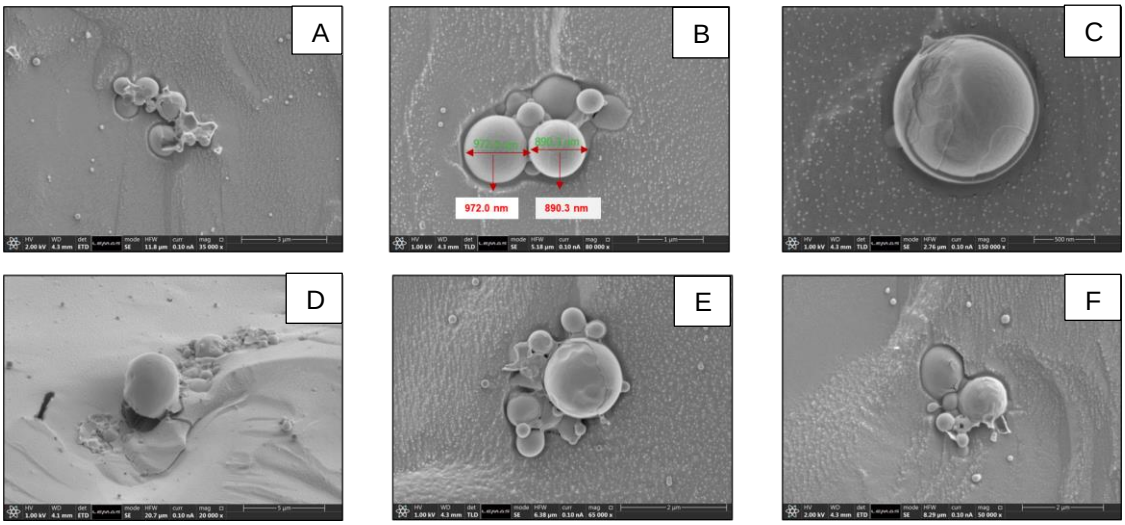

Figure 23. Cryo-SEM images for Sample 2

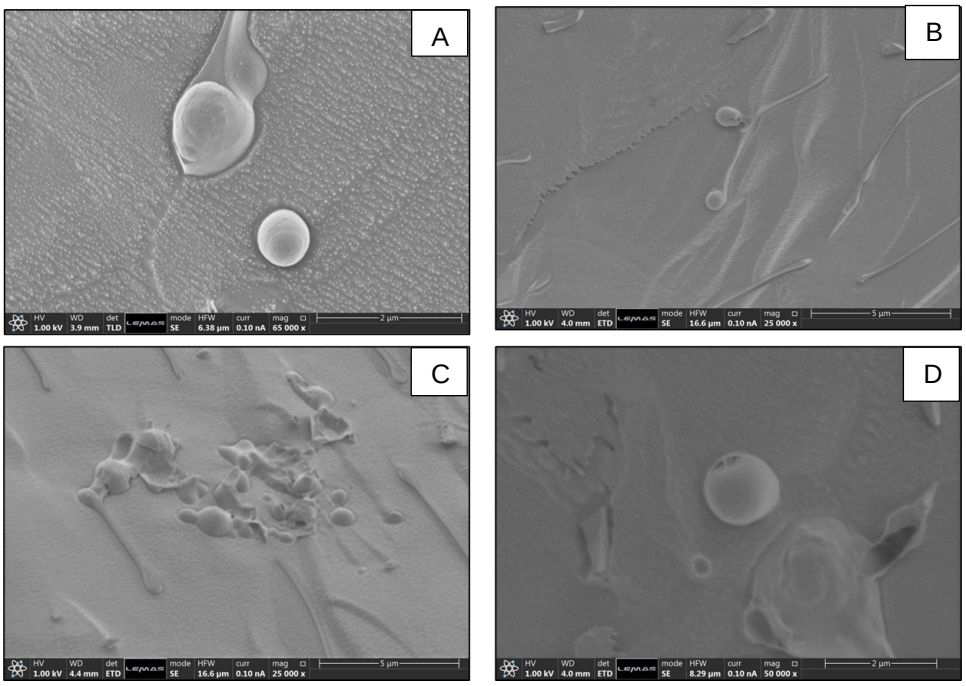

Figure 24. Cryo-SEM images for Sample 1

8.2. Optical images

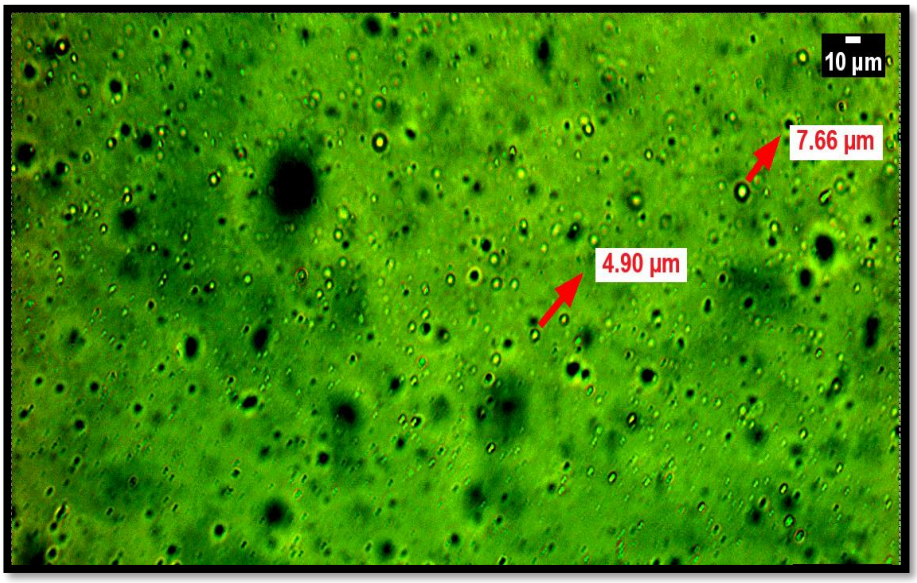

Figure 25. Microscope image from Sample 1

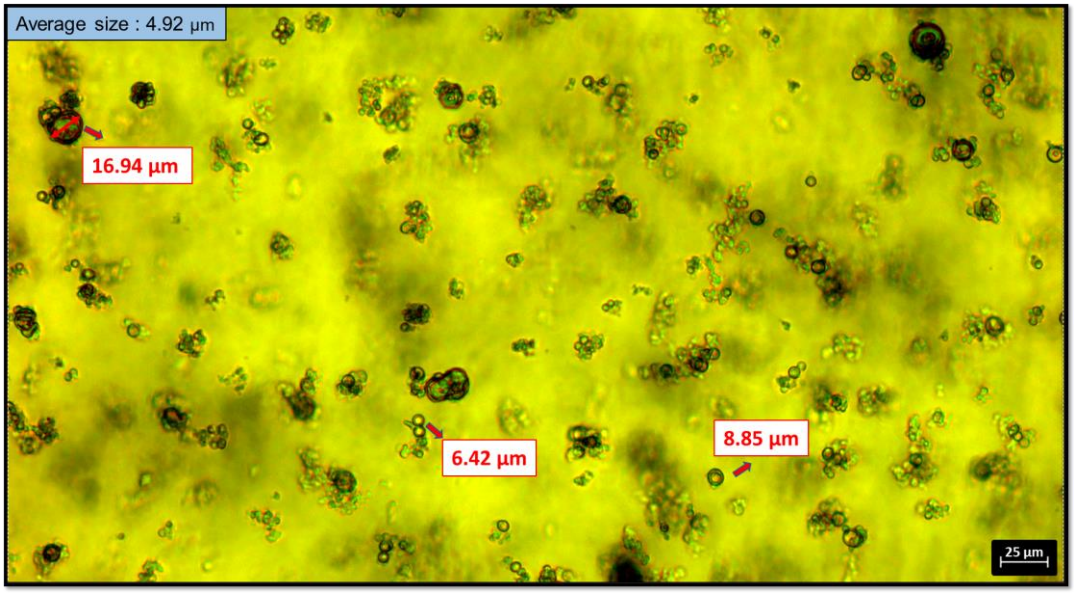

Figure 26. Microscope image from Sample 2

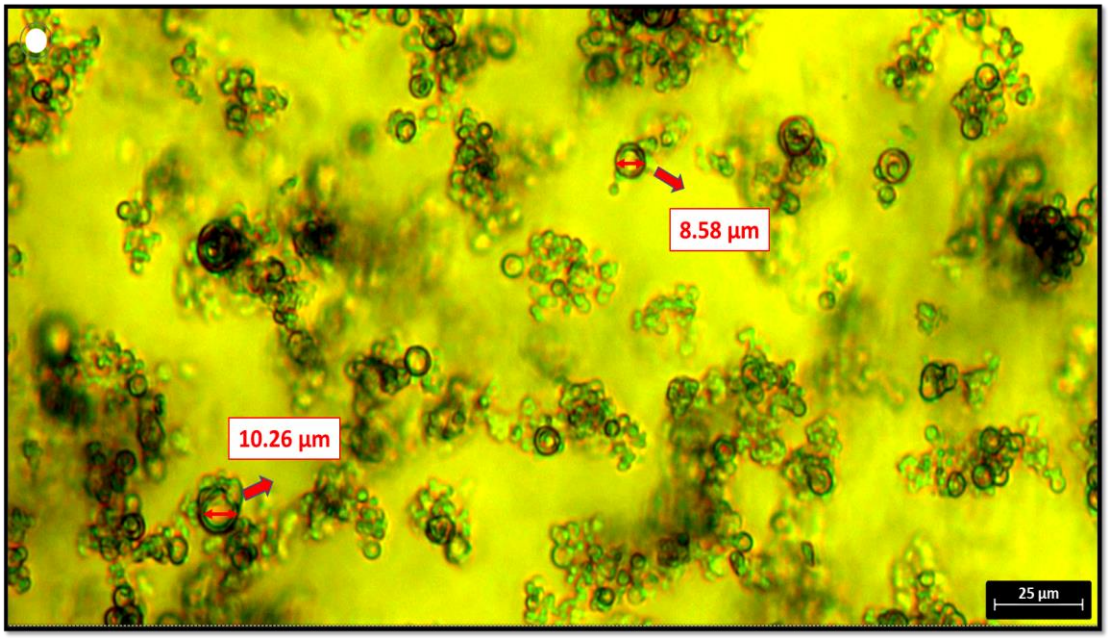

Figure 27. Microscope image from Sample 2

8.3. During process images

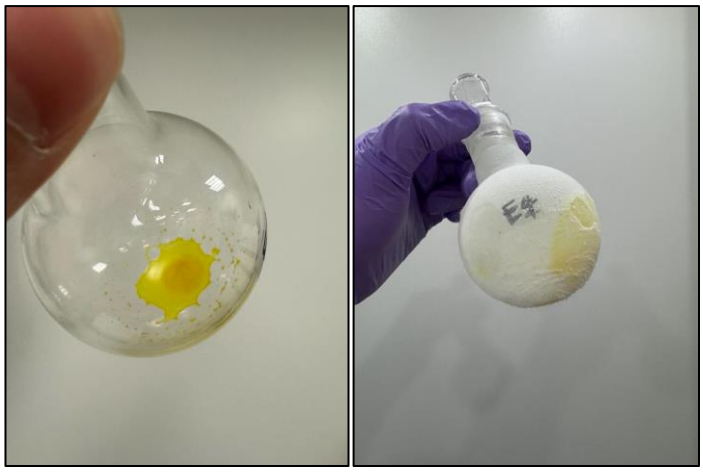

Figure 28. Picture after removing solvent and a picture after freezing by N_2.