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## „Characterization and Evaluation of a bolalipid – modified lipidnanoemulsion as drug delivery system“


verfasst von / submitted by

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# Abstract

According to the Biopharmaceutics classification system, around 40% of total drugs in the market have poor water solubility and therefore low oral bioavailability. One approach to increase this problematic factor is by introducing these compounds into drug delivery systems. Lipidnanoemulsions (LNEs) as drug delivery systems have been studied enormously due to their ability of encapsulating higher amounts of lipophilic compounds, thus increasing their oral bioavailability. A recent study has shown that synthetic bolalipids (PC – C24 – PC) are good solubilizing agents for these drugs. However, their observed $IC_{50}$ ranged between 35 – 94 µM. The aim of this study was to develop a safe bolalipid – based lipid nano emulsion to increase the solubility of a hydrophobic model compound (curcumin) due to the introduction of synthetic PC – C24- PC. The nanoemulsions produced were able to encapsulate up to 1,5 mg/ml of curcumin into the core – shell of the formulations, while maintaining the physicochemical properties of the LNEs. The produced lipid formulations showed stability at 4°C and uncontrolled room temperature for 28 days and showed no cytotoxicity on Caco – 2 cells up to 4 µM of the cosurfactant. Bolalipid LNEs are good candidates for further studies to confirm that the oral bioavailailability can be improved by increasing the solubility of hydrophobic compounds.



**Keywords: drug delivery system, bolalipid, lipid nanoemulsions, surfactant, stability, cytotoxicity**

# Zusammenfassung

Nach dem Biopharmaceutics Classification System (BCS) haben etwa 40% der auf dem Markt befindlichen Arzneimittel eine schlechte Wasserlöslichkeit und somit eine geringe orale Bioverfügbarkeit. Eine Möglichkeit dieses bestehende Problem zu verbessern ist die Einkapsulierung dieser lipophilen Substanzen in Drug Delivery Systems. Lipidnanoemulsionen (LNEs) sind hervorragende Drug Delivery Systems aufgrund ihrer Fähigkeit größere Mengen von lipophilen Verbindungen einzubringen und somit deren orale Bioverfügbarkeit zu erhöhen. Eine Studie hat kürzlich gezeigt, dass synthetisch hergestellte Bolalipide (PC – C24 – PC) gute Lösungsvermittler für diese Substanzen sind. Ihre beobachtete $IC_{50}$ lag jedoch zwischen 35 – 94 µM. Das Ziel dieser Studie war die Herstellung einer sicheren Lipidnanoemulsion mit Bolalipid als Co-tensid, um die Wasserlöslichkeit einer lipophilen Modelldroge (Curcumin) durch die Verwendung von synthetisch hergestelltem PC – C24 – PC zu erhöhen. Die hergestellten Nanoemulsionen konnten bis zu 1,5 mg/ml Curcumin in ihren Lipidkern einzubringen und haben hervorragende Stabilität von 28 Tagen gezeigt bei Lagertemperaturen von 4°C und unkontrollierter Zimmertemperatur unter Erhaltung der physikochemischen Eigenschaften der LNEs. Zusätzlich wiesen diese Formulierungen keine Zytotoxizität an Caco – 2 Zellen im Bereich der getesteten Konzentration des verwendeten Co-Tensids (bis zu 4 µM) auf. Aufgrund dieser aktuellen Beobachtungen sind Bolalipid – LNEs gute Kandidaten für weitere Studien, um zu bestätigen, dass diese Drug Delivery Systems die orale Bioverfügbarkeit von lipophilen Arzneidrogen durch die Verbesserung der Löslichkeit, erhöht werden kann.

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# Abbreviations

| | |
|---|---|
| API | active pharmaceutical ingredient |
| BCS | biopharmaceutics classification system |
| B – LNE | bolalipid lipidnanoemulsion |
| CUR – B – LNE | curcumin – loaded bolalipid lipidnanoemulsion |
| CUR – L – LNE | curcumin – loaded lecithin lipidnanoemulsion |
| cmc | critical micelle concentration |
| DDS | drug Delivery System |
| DLS | dynamic Light Scattering |
| DDPS | N-dodecyl-N,N-dimethylammonium-propanesulfonate |
| EE | encapsulation efficiency |
| IR | immediate release |
| LNE | lipidnanoemulsion |
| L – LNE | lecithin lipidnanoemulsion |
| MTT | 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide |
| NE | nanoemulsion |
| nm | nanometers |
| OSM | organic solvent mix |
| PIC | phase inversion composition |
| PIT | phase inversion temperature |
| PC | phosphatidylcholine |
| PDI | polydispersity Index |
| RR | recovery Rate |
| SMEDDS | self-microemulsifying DDS |
| SNEDDS | self-nanoemulsifying DDS |

| | |
|---|---|
| URT | uncontrolled room temperature |

# 1. Introduction

Many drug candidates with promising pharmacological activity often come with a huge challenge for their successful formulation, clinical efficacy, industrial applicability, and marketing [1]. In most cases, the limiting factor of conventional drug delivery systems (DDS), which include simple dosage forms like tablets, capsules, syrups etc. [2] is the poor water solubility of the active pharmaceutical ingredient (API), a slow dissolution rate in the gastrointestinal tract, which leads to low absorption rate and therefore low oral bioavailability [1].

Bioavailability can be defined as the portion of applied drugs that enters the blood circulation [3]. The most commonly used drug delivery is oral administration because of its high patient compliance, cost-effectiveness, and wide variety in the design of dosage forms and especially their convenient use. The restricting problem that occurs in designing these oral medications is the low bioavailability. While the bioavailability of drugs applied intravenously is 100%, the bioavailability of drugs taken orally is affected by the rate and extent of both absorption and first pass metabolism, which again determines the maximum concentration ($c_{max}$) and the time at which this is attained ($t_{max}$) [3]–[5]. Additionally, it has been studied various times, that the physicochemical properties of a drug like solubility (can be defined as the quantity of solute dissolves in a specific amount of solvent), dissolution rate (the rate at which the drug enters the solution) and gastrointestinal permeability are strongly correlated with the rate and extent of the absorption process thus improving these parameters have been the goal of many pharmaceutical studies [6], [7].

The physicochemical characters of a drug may help researchers to understand possible impairments of solubility and bioavailability. Compounds with five or more carbon atoms are expected to have a poor water solubility (<100µg/mL), a log P value of 2 or higher, a high molecular weight (>500 Da), and a high melting point (>2000°C) [8]. An estimated total of 40% of the drugs in the market have poor water solubility. The biopharmaceutics classification system (BCS), shown in Figure 1, categorizes drug substances into four classes depending on their solubility and permeability properties. Drugs with poor solubility but high membrane permeability, which are classified as BCS class II, show limited dissolution capacity, and have

therefore low absorption rate. Convenient drug delivery systems are not designed for these kinds of drugs, which often have a water solubility of 1 µg/ml. One way to improve this limiting step is designing formulations, which increase the dissolution rate or the solubility of the drugs. While BCS class III drugs have high solubility and low permeability characters, drug substances in class IV show low profile on both parameters. Optimizing their chemical structure to obtain an improved physicochemical property is a common strategy used in drug discovery [9], [10]. Only a total of 8% of new drug candidates have high solubility and permeability [11].

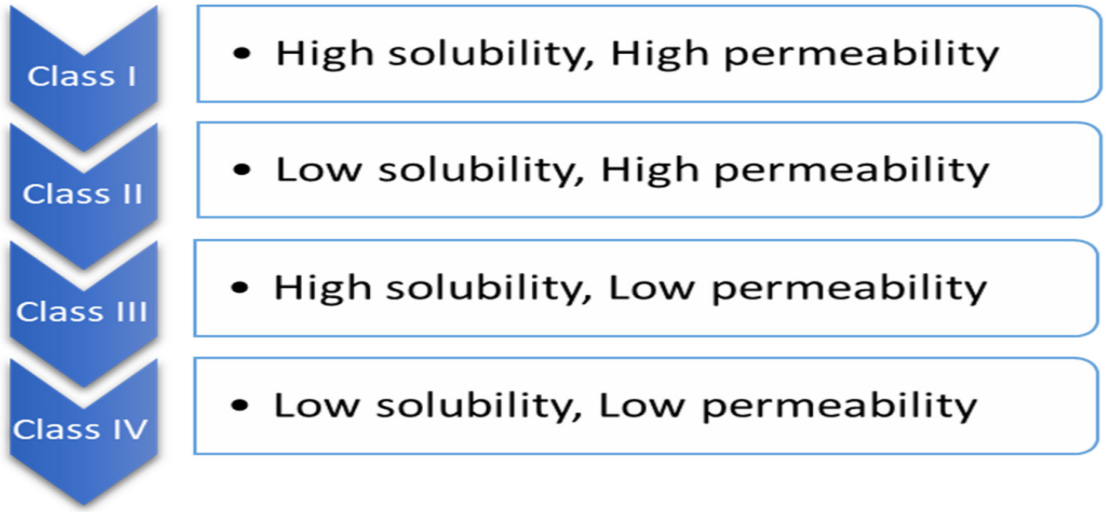


*Figure 1 Biopharmaceutics classification* system [7]

## 1.1. Drug delivery system

A drug delivery system can be described as a formulation or a device which allows the introduction of therapeutic substances into a living organism and enhances its efficacy and safety by improving the rate and time and by controlling the place of release of the API in the body. Furthermore, the administration of the pharmaceutical product, the liberation of the pharmacological active substance by the product and the transport of the drugs through biological membranes to a specific target site play a big role in the drug development [12], [13].

Conventional DDS including tablets and capsules are called immediate release (IR) formulations because of their immediate release of loaded drugs. In comparison sustained drug delivery system with especial formulations were developed for a controlled release over time. The history of DDS can be traced back to 1950 with the introduction of Spansule® 12-h release technology, which controlled the dissolution rate of the compound by a coating barrier, which limited the contact with gastrointestinal fluids. In 1970 the concept of PEGylation was developed and allowed an increased blood circulation time. Only with the introduction of nanomedicine in 2000, which was mainly developed for tumor-targeting drug delivery, many researchers have been developing various forms of nanoformulations thus producing lipidnanoemulsions, which increased the water solubility of lipophilic drugs [14].

As mentioned already, the most desirable and commonly used drug delivery is the oral ingestion because it's convenient and easy to use and have a high patient compliance. The limiting problem in drug development is the low water solubility of drugs. A low water solubility leads to low bioavailability, which limits the therapeutic potential of possible drug candidates. This leads to a higher dose requirement of drugs with very low aqueous solubility to reach an effective plasma concentration, which in combination with slow permeation may lead to gastrointestinal toxicity. While the bioavailability of drugs taken orally depends on many factors such as first – pass metabolism, presystemic metabolism and the sensitivity to efflux transport mechanism, both the water solubility and the gastrointestinal permeability have a bigger impact on the low oral bioavailability of drug compounds [5], [7].

There are numerous techniques shown in Figure 2 to improve the water solubility, the dissolution, and the bioavailability of drugs. One approach is by reducing the drugs particle size via micronization thus increasing its surface area leading to a larger interaction area with the solvent and enhanced dissolution. However, thermolabile substances will eventually degrade due to the induced thermal stress. Chemical alternations like changing the pH and using buffers, using a soluble prodrug, or by salt formation are also viable options. This approach however comes with the introduction of organic solvents such as methanol, and chloroform for the solubilization of lipophilic drugs thus increasing the toxicity and environmental pollution [8]. Other used methods are melt-extrusion, solid dispersions, the

use of nanoparticles and surfactants and formation of water-soluble complexes. The most common approach however is the lipid-based formulation [15], [16].

Based on the relation of used lipids, surfactants and cosurfactants, lipid-based formulations can be divided into four categories (Type I – IV) [16]. They include liposomes, self-microemulsifying DDS (SMEDDS) or self-nanoemulsifying DDS (SNEDDS), nanoemulsions and nanocapsules [17]. All of them have the main advantage of increasing solubility, however nanocarriers brings additional benefits such as drug degradation protection and the active transport to the target area. Additionally, lipid-based formulations stimulate the intestinal lymphatic transport pathway, altering intestinal permeability by modulating enterocytes-based transports and reduce hepatic metabolism. According to a study from 2007, lipid-based formulations inhibit P – glycoprotein efflux transporters as well, thus increasing the bioavailability [18].

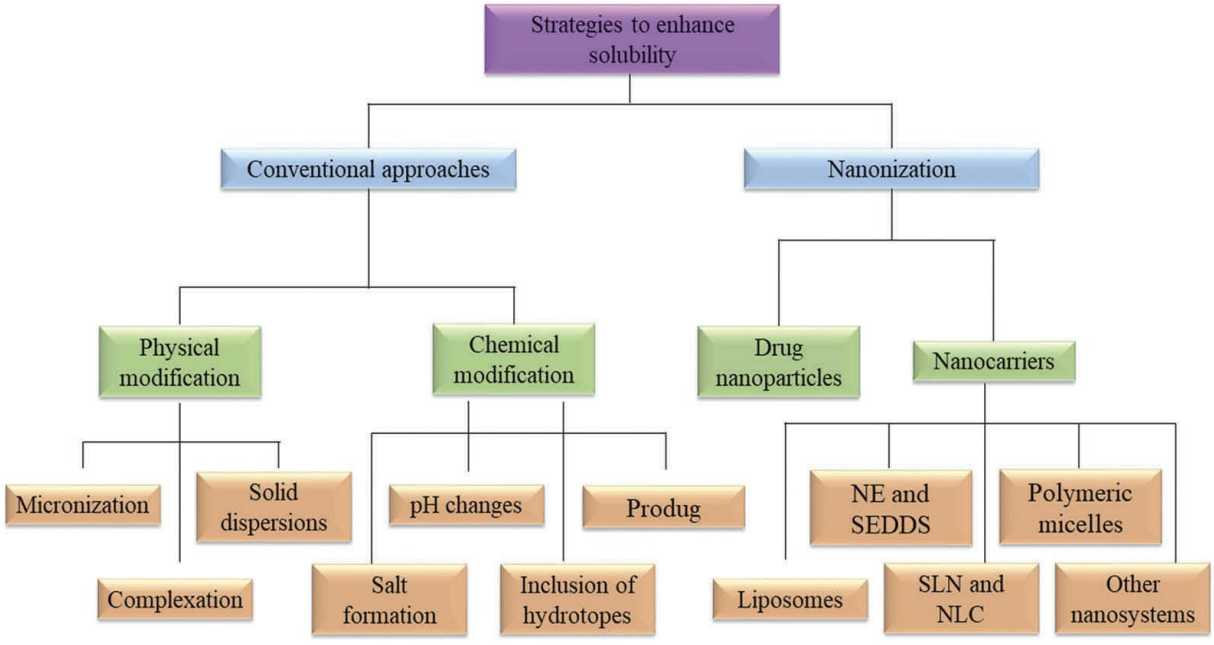


*Figure 2 Strategies to improve low aqueous- solubility of drugs* [8]

## 1.2. Lipidnanoemulsion

Nanoemulsions (NEs) are isotropic, transparent and two – phase systems with at least two immiscible liquids, mostly water and oil, with one of them being finely dispersed as small droplets in the continuous phase. NEs are also known as miniemulsions, ultrafine emulsions and submicron emulsions. The focus of this research is on oil in water (O/W) emulsions, containing small oil droplets dispersed in an aqueous phase. The produced nanoemulsions with a size range between 20- 200 nanometers (nm) are thermodynamically stable against droplet aggregation, flocculation, and coalescence with the use of surfactants and cosurfactants [1], [19], [20].

Nanoemulsions have been used in various formulations such as foams, creams, and sprays making them viable for topical applications. Their small size can penetrate rough skin surfaces and improves the penetration of pharmaceutically active ingredients. Because of their ability to better uptake lipid soluble supplements in cell cultures technology, NEs are also used to promote cell culture growth and for toxicity studies of oil soluble compounds. In drug development NEs have been utilized in drug formulations for oral drug delivery as they possess a high surface area making them effective transport systems [21].

Nanoemulsions as DDS have been studied enormously to overcome the mentioned limiting problems of conventional DDS as they can encapsulate compounds due to their size and high kinetic stability, which then again leads to an increase of lipophilic drug's solubility and therefore bioavailability [22]. Other advantages of these newly developed DDS are the direct delivery of the active pharmaceutical ingredient to its designated location and the reduction of side effects [23]. For example, studies show that when bioactive compounds like propofol, cytotoxic agents or clarithromycin are encapsulated in LNEs, the pain associated with these drugs when applied intravenously, is reduced because the tissue is exposed to a lower drug concentration. LNEs also protect their cargo, especially when they are susceptible to hydrolysis or oxidation. In an *in vitro* study with paclitaxel – loaded LNEs the $IC_{50}$, which is the concentration needed to reduce the proliferation of T47D cells by 50%, was three nM lower compared to paclitaxel in liposomes formulations. This is an indication that anti-cancer drug loaded – LNES are expected to accumulate in the tumor by passive targeting through enhanced permeability and retention effect due to their small droplet size [24]. Other study

with Doxorubicin – loaded LNEs showed 1.6x increased cytotoxic activity compared to free Doxorubicin and even higher when they were PEGylated. The higher antitumor activity was a result of the faster internalization of Doxorubicin into the cells by the lipid formulation [25].

Their safety use can be confirmed by the amount of lipidnanoemulsions (LNE) on the market as they were initially developed to supply energy to patients with nonfunctional gastrointestinal tract [26]. However, it is important to note, that potential toxicity may occur as their safety depends on the composition, structure, administration route of the NEs, and their interaction with biological systems [27]. However, an *in vivo* study (2019) on male Wistar Rats regarding the toxicity of frequently used NEs for DDS showed no changes on biochemical, hematological, oxidative stress, and genotoxicity parameters, which displays their safety use when applied orally [28].

There are several techniques that are used for the production of LNEs. They can be divided into two sections depending on the energy used for the preparation. High – energy methods use strong disrupting forces provided by mechanical devices such as ultrasonicators, microfluidizers and high kinetic homogenizers. This method allows us to produce nanodroplets with a narrow polydispersity index (PDI). According to Zhang, 2011 a homogenous system can be achieved, if the PDI value is between 0,08 - 0,3. Values higher than 0,3 mean that the particle size of the nano droplets is less uniform [29].

The method we used in our study is the solvent diffusion method (Figure 3) associated with phase inversion temperature (PIT). In this method, the compound and the oil are dissolved in an organic solvent. This mixture is then quickly injected into an aqueous phase containing the surfactant at higher temperature and under mechanical stirring. As the temperature cools, the lipid particles are formed [30]. PIT shown in Figure 4 is a low energy method. Other method described in the literature is the phase inversion composition (PIC). This method uses the physicochemical properties of the system. The solubility of non-ionic surfactants (e.g. Kolliphor HS15) depends on the temperature. If the temperature is increased the affinity of non-ionic surfactants changes from water to oil, because of the progressive dehydration of the polar head group, thus favoring the formation of a bicontinuous microemulsion. However, before achieving this change in affinity of the surfactant from water to oil, it reaches the phase inversion temperature where the solubility of the surfactant in oil and water is

approximately equal with very low interfacial tensions thus promoting the emulsification. Kinetically stable NEs can be produced when the temperate of the system is quickly removed from the PIT. While being stirred at this cooling process, surfactant molecules migrate from the oil phase into the water phase thus promoting the formation of o/w nanoemulsions [31]–[33].

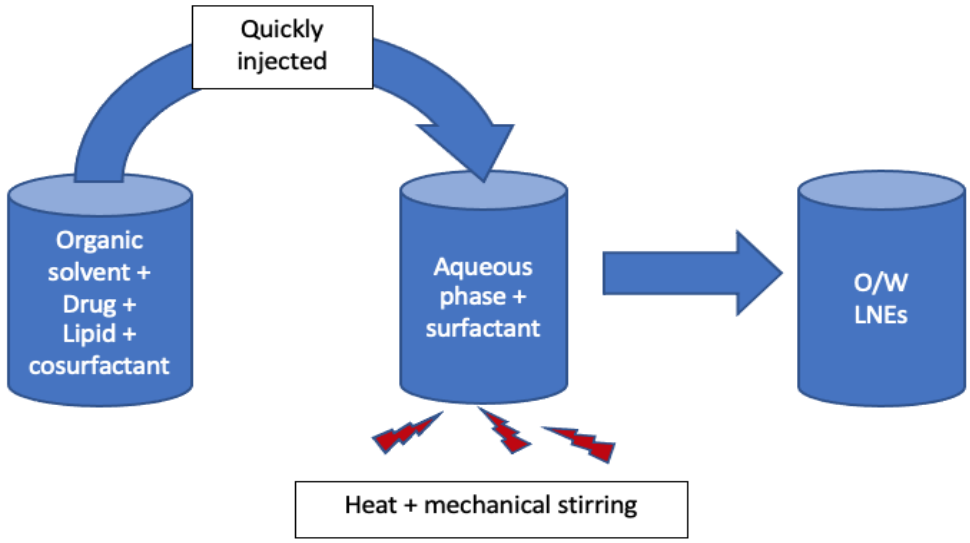


*Figure 3 hot solvent diffusion*

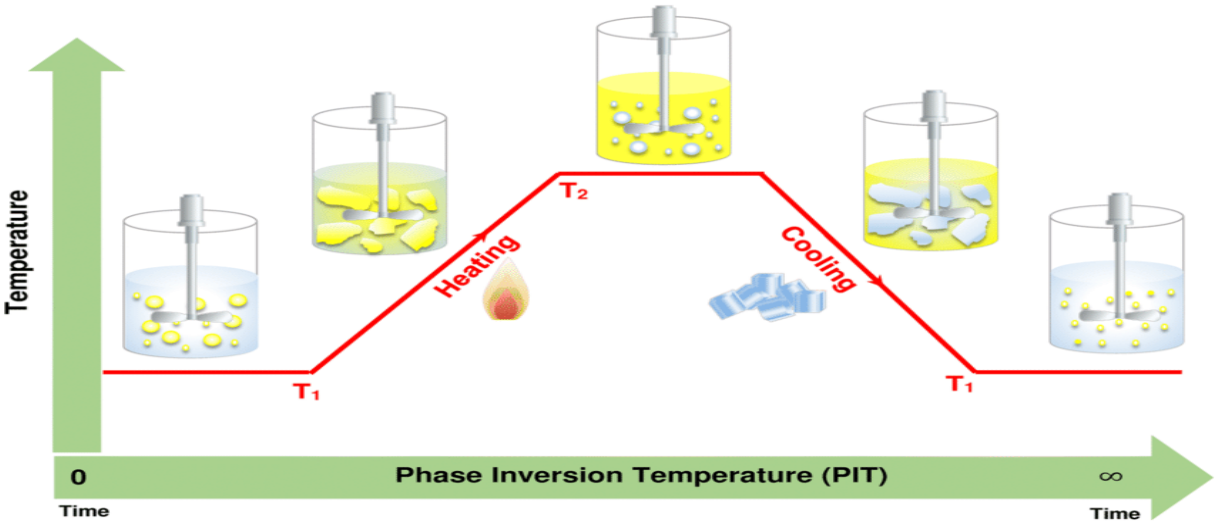


*Figure 4 Schematic depiction of Phase Inversion Temperature (PIT)* [34]

Each component of these lipid formulations plays a big role in drug loading and bioavailability. While the type of oils, whether natural or synthetic, mainly influences the loading capacity and in vivo absorption rate, surfactants and cosurfactants may have an impact on the emulsification efficiency and stability of the formulations. In O/W (oil in water) systems, surfactants are absorbed at the interface of water and oil, which leads to a formation of stabilized nanoemulsion by reducing the interface free energy and surface tension of the two immiscible liquids. Choosing the surfactant is crucial for the lipid formulation, as surfactants changes the particle size of the produced emulsions. Studies showed that using higher concentrations of surfactants can produce smaller – sized droplets due to lowered surface tension, however, they can also produce larger particles by disrupting the interface, so that water can be absorbed into the oil droplets [35].

According to studies and acute toxicity tests performed on animals, toxicity level is as follows: non – ionic < zwitterionic < anionic < cationic. Anionic and nonionic surfactants, when applied orally, are likely to cause less irritation on mucous membranes. However, this effect depends on the concentration of the used surfactants [36].

In previous studies cosurfactants like lecithin, which is naturally found in egg yolks, milk, soybeans, rapeseeds, or sunflower kernels, are mainly used to improve the water solubility of lipophilic compounds [37]. Like surfactants, these types of surface-active agents are located at the interface between the two phases, thus reducing the tension. They form a mechanical barrier and inhibit the produced nanocarriers to coalesce, which leads to a higher degree of thermodynamic stability. Additionally, their emulsifying or co-emulsifying character increases the dispersibility of hydrophilic surfactants in the lipophilic phase, which is essential for the homogeneity of the lipid formulation and encapsulation of bioactive compounds [35].

Nanoemulsions are promising vehicles for drug administration, which is supported by their emerging use in recent studies. Paclitaxel – loaded NEs when administered orally to rats showed about three times higher concentrations of paclitaxel in the systemic circulations compared to an aqueous solution. The oral bioavailability of Tamoxifen showed an eightfold increase in mice when introduced into water – soluble NEs [17]. In another study with darunavir – loaded NEs, a hydrophobic drug for AIDS – treatment the oral bioavailability was increased by 223% in comparison to the suspended form by keeping the drug in a dissolved

state thus skipping the dissolution process. The used surfactants (Tween 80) are additionally a modulator of P-glycoprotein and inhibit CYP450 enzymes activity at the intestinal region thus increasing the absorption of the drug [38]. NEs have also been used to increase the solubility of polyphenols such as curcumin and epigallocatechin gallate. Curcumin – loaded NEs for breast cancer treatment on obese women is currently clinically being tested [17]. Further a study from 2015 showed that lipid formulations could incorporate higher amounts of quercetin, proving their wide use in pharmaceutical research [39].

However, despite these promising reported advantages, relevant regulations on nanomaterials are still limited. Only a few countries within the European Union and the United States of America have a standardized guidance for toxicological evaluation based on standard toxicological assays [40]. It has been reported that excipients used in lipid-based formulations may cause unwanted side effects. As such Polysorbate 80, a solubilizer used to formulate docetaxel is associated with acute hypersensitivity reactions with respiratory distress, hypertension, and angioedema. Another study including Polysorbate 80 in Eprex® (a recombinant human erythropoietin formulation) may have induced an antibody-mediated pure red cell aplasia, a severe adverse effect that caused the product to be recalled from the market. Further, Cremophor EL®, a cosolvent used in Taxol® (Paclitaxel) showed similar acute hypersensitivity symptoms. It is very clear that further investigations are still necessary regarding drug-related toxicity [41].

## 1.3. Bolalipids and its potential to be used in LNE

One cosurfactant that has promising characteristics for a successful formulation are bolar lipids, also called bolaamphiphiles or for short bolalipids. They have a unique chemical structure with two polar headgroups, which are connected by lipophilic alkyl chains. They can be further divided into two groups: while symmetrical bolalipids have similar headgroups, asymmetrical bolalipids have different hydrophilic heads, that can differ in size, polarity, and tendency for ionization. Naturally occurring bolalipids are a component of the cell membrane of a specific species of Archaea, which are known for their outstanding stability in harsh environments like high salt concentrations, low pH values and high temperatures [42].

Lipid nanocarriers must overcome the problems that occur when applied orally. The harsh conditions in the gastrointestinal tract including gastrointestinal fluids, low pH, high salt concentrations and the presence of enzymes may affect their stability. Additionally, the microbiome that reside in the mucus layer and efflux transporters that are expressed by the intestinal epithelium may lead to a slower absorption rate [17]. The introduction of bolalipids into lipid vesicles became very promising in drug administration research, as they showed improved stability against the harsh conditions found in the human digestive system. These produced archaesomes also increased the bioavailability of encapsulated drugs, which is an important goal for every DDS. Additionally, these lipid formulations could be autoclaved without changing the size of the prepared vesicles and showed no toxicity when they were administrated orally and intravenously. However, the extraction of these naturally occurring tetraether lipids (TELS) are expensive and very time – consuming, so the synthesis of single – chained bolalipids was on demand [43].

A new general synthesis procedure of bolalipids with two phosphocholine (PC) headgroups connected by a fully saturated alkyl chain with carbon atoms (PC – $C_n$ – PC, C = 22 – 32) were developed by Drescher et al. in 2007. Upon reaching the critical micelle concentration (cmc), bolalipids tend to aggregate. PC-C32-PC, for example self assembles into different long and flexible nanofibers forming a hydrogel and when heated exhibits a reversible phase change at 48.3°C. This structural transformation leads to a formation of micelles with no gel-forming ability and is highly dependent on the length of the alkyl chain. The shorter the lipophilic chain, the more likely it is that bolalipids form micellar structures at lower temperatures [42].

However, as mentioned above, these amphiphilic surfactants may follow the safety profile of non-ionic surfactants. As Li et al (2022) described in their research that bolalipids with longer lipophilic chains are limited to oral administration, while C22 had a significant reduction in cytotoxicity in mammalian cells compared to C26.

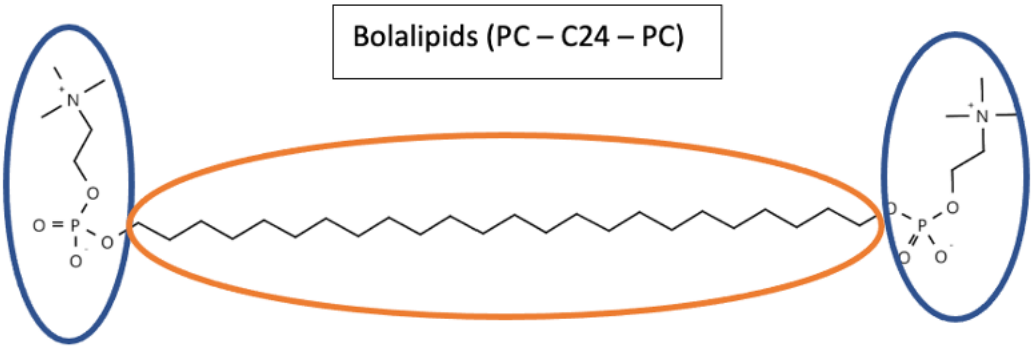


*Figure 5 Chemical structure of Bolalipid (PC - C24 - PC)*

In recent studies optically transparent aqueous nanoemulsions with high capacity to encapsulate quercetin were prepared by using the hot solvent diffusion and phase inversion temperature (PIT) by only changing the composition of the lipid formulations. According to Hädrich et al (2015), lipidnanoemulsions composed of castor oil, soybean lecithin and PEG 660-stearate were able to load up to 1,5 mg/ml Quercetin while maintaining the particle size of around 20 nm, which has been reported to be suitable for oral administration [39].

Despite numerous studies on liposomal oral drug delivery, no formulation has been approved so far for oral administration. For our study, we decided to include PC – C24 – PC as cosurfactant into our formulation, because of its outstanding ability to solubilize hydrophobic drugs. Feng et al described also in their study, that bolalipids with shorter alkyl chains have a reduced cytotoxic activity while maintaining their solubilization property. Another study with bolalipid – vesicles showed also increased stability in phosphate buffered saline (simulating gastrointestinal conditions), which nanocarriers must overcome when applied orally [43]. These results show that bolalipids have numerous advantages for their possible use in drug delivery. However, further investigations regarding bolalipids as a component of lipid formulations are necessary to provide information about their potential use.

## 1.4. Study questions and aims of the thesis

For this research, curcumin, the bioactive compound found in turmeric (Curcuma longa), has been used as a model of poor water -soluble compound to evaluate the ability of bolalipid – LNE as a drug delivery carrier. This curcuminoid has been associated with many health benefits including its' anti-inflammatory, anti-tumoral and antimicrobial effects. Curcumin has the same problem of BCS class II drugs with their low aqueous solubility (according to drug bank, curcumin has a water solubility of 0,00575 mg/ml) and therefore low bioavailability, which limits their therapeutic and pharmaceutical use [37], [39].

The main objective of this study is to provide new insights into the development of a safe curcumin – loaded LNE with improved solubility and decreased toxicity. We designed this project to characterize and evaluate a bolalipid – LNE *in vitro* to try to answer the following questions, while focusing mainly on the following aspects:

1. Does PC – C24 – PC as a co-emulsifier have any beneficial effect on the physical properties of LNE, such as particle size, zeta potential, polydispersity index and long storage stability?
2. Whether the use of bolalipid can increase the drug loading with a high encapsulation rate for hydrophobic drugs?
3. Do the produced lipid nanoemulsions exhibit *in vitro* cellular toxicity on Caco – 2 cells?

# 2. Materials and Methods

## 2.1. Materials

| Solvents | Vendors |
|---|---|
| Ethanol 96% | Brenntag Austria GmbH |
| Acetone, ACS reagent | Sigma-Aldrich |
| Distilled Water | Was provided by Uni Vienna, Department of Pharmaceutical Technology |
| Milli Q Water | Was provided by Uni Vienna, Department of Pharmaceutical Technology |
| Methanol, for HPLC ≥ 99,9% | Promochem |
| Dimethyl sulfoxide (DMSO) | Carl-Roth |
| **Consumables** | |
| 12- hydroxystearic acid – polyethylene glycol copolymer (Kolliphor HS 15) | Sigma-Aldrich |
| Castor oil (CO) | Carl-Roth |
| Hydrogenated soybean lecithin (Phospholipon 80) Lecithin | Lipoid |
| Bolalipids | Were synthesized according to ref. [44] |
| NaCl | Carl – Roth |
| 20 ml vials, ND13 Vials | VWR |
| Syringe and Syringe Filters PVDF 0,22 µm | Carl-Roth |
| Needles | Carl-Roth |
| Amicon Ultra-0.5 Centrifugal Filter Unit with Ultracel-30 membrane, 30 kDa NMWL | Sigma-Aldrich |
| FBS Superior | Sigma-Aldrich |

| | |
|---|---|
| Penicillin/Streptomycin (10,000 U/ml) | Thermo Scientific |
| Dulbecco's Phosphate Buffered Saline, Modified (DPBS), w/o calcium chloride and magnesium chloride, sterile-filtered | Sigma-Aldrich |
| Thiazolyl blue tetrazolium bromide (MTT) | Sigma-Aldrich |
| 96 Well-F, Multiwell cell culture plates, sterile VWR® | VWR |
| Cell counting Slides for TC20™ Cell Counter | Bio-rad |
| **Instruments** | |
| AREX 6 Digital PRO Heating Magnetic Stirrer and VTF Digital Thermoregulator | Velp Scientifica |
| Ultrasonic Water Bath | Elma |
| Zetasizer Nanoseries | Malvern Instruments |
| Centrifuge Rotanta 460 R | Hettich |
| TC20™ Automated Cell Counter | Bio-rad |
| Epoch 2 Microplate Spectrophotometer | Biotek |

## 2.2. Preparation of the Lipidnanoemulsions

The LNEs were prepared using the hot solvent diffusion method associated with the phase inversion temperature [39]. An Organic solvent mix (OSM) with the composition of acetone and ethanol 96% 60:40 (v/v) were used to create a lecithin (10 mg/mL) stock solution and bolalipid (2 mg/ml) stock suspension. Kolliphor HS15 (melted: 1,5 g) was dissolved in 100 ml distilled water to obtain a 1,5 % (w/v) Kolliphor HS15 stock solution as the aqueous phase. Lecithin – LNE (L – LNE) was prepared with an oil phase mixture consisting of 30 mg Castor Oil (CO), 0,2 ml Lecithin stock solution and 1,8 ml of OSM for a total volume of 2 ml, of which 1ml were quickly added to a 10 ml aqueous phase (pre-heated to 80°C) under magnetic stirring at 1500 rpm. For bolalipid – LNE (B – LNE), the oil phase was prepared similarly to L – LNE,

however, 1 ml bolalipid suspension was used instead of lecithin added OSM to a final volume of 2 ml. The oil phase of LNE 50:50 (lecithin: bolalipid, 50:50, w/w) contained 30 mg CO, 0,2 ml lecithin stock solution, 1 ml bolalipid suspension and 0,8 ml OSM. A summary of the compositions of the proposed LNEs can be seen in ***Table 1***. The resulting colloidal suspensions were then cooled to room temperature and make sure that the OSM was evaporated under magnetic stirring at 500 rpm overnight. On the following day, Milli-Q water was added to make a final volume of 10 ml. Finally, the colloidal suspensions were filtered through an 0,22 µm filter to remove impurities without influencing the particle size (data not shown). All proposed formulations were prepared in triplets on different days for better reproductivity of the experiment.

*Table 1 Composition of the proposed lipidnanoemulsions*

| LNEs | Kolliphor HS 15 | Castor Oil | Lecithin/Bolalipid |
|---|---|---|---|
| **amount (mg)** | 150 | 15 | 1 |
| **final concentration (mg/ml)** | 15 | 1,5 | 0,1 |

### 2.2.1. Characterization of the lipid nanoemulsions

The particle size and the zeta potential of the LNEs were obtained through dynamic light scattering (DLS) using a Zetasizer Nano Series from Malvern Instruments. The particle size and zeta potential measurements were performed at 25 °C after dilution of the samples in Milli – Q water and 1 mM NaCl, respectively. The hydrodynamic diameter was determined using Stokes-Einstein's equation, $D=(\kappa_B T/6\pi\eta D)$, where $\kappa_B$ is Boltzmann's constant (J/K), T is temperature in K, D is the diffusion coefficient and $\eta$ is the viscosity of the medium – water in this case ($\eta$ = 0.89 cP at 25°C).

#### 2.2.1.1. Dynamic Light Scattering

Dynamic Light scattering (DLS) also known as photon correlation spectroscopy or quasi – elastic light scattering, shown in **Fehler! Verweisquelle konnte nicht gefunden werden.**, is a common technique used to determine the size of nanoparticles dispersed in a solution. When a monochromatic light (e.g., laser) enters the solution, light is scattered in all directions, which is a phenomenon called Rayleigh Scattering. The intensity of the scattered light will be measured by a detector (usually a photomultiplier) at a certain angle, changes over time due to the Brownian motion, which is caused by the constant collision of nanoparticles with solvent molecules, which again leads to a constantly changing distance between the biomolecules. This constant collision results in a faster movement of smaller biomolecules and when measured over time, their position changes unlike larger particles, which move more slowly and adapt similar positions at different time periods. When the laser beam is directed to the dispersion, it undergoes a Doppler broadening due to the Brownian motion of the nanocarriers, which again leads to mutually destructive phases that cancel each other out, or mutually constructive phases producing a signal that can be detected. A digital autocorrelator matches the intensity fluctuations of the scattered light with time to determine the diffusion behavior of the dispersed molecules. The motion of the biomolecules is however also dependent on the temperature and viscosity of the solvent. If these parameters are known, the diffusion coefficient, which is related to the hydrodynamic diameter, can be determined by measuring the intensity fluctuations [45], [46].

$$R = \frac{kT}{3\pi\eta D}$$

The size of the particle can then be calculated using the Stokes-Einstein equation. **D ($m^2/s$)** is the diffusion velocity (Translational diffusion coefficient) of the particle, **k** ($m^2kg/Ks^2$) is the Boltzmann constant, **T** (K) is the temperature, **η** (Pa.s) is the viscosity of the solution and **r** is the hydrodynamic radius of the particle. The diffusion behavior in this formula is inversely

proportional to the size of the particle, which results in faster movement of smaller particles than larger ones [45], [46].

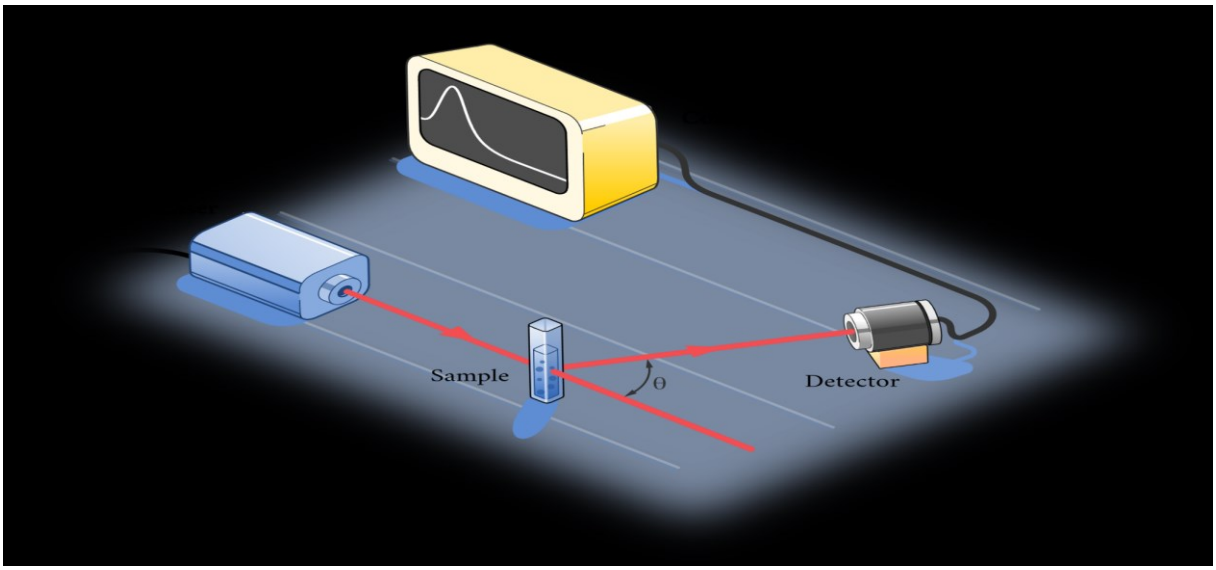


*Figure 6 Schematic representation of Dynamic Light Scattering (CC BY-NC; Ümit Kaya via LibreTexts)* [47]

## 2.3. Stability of the lipid nanoemulsions

The proposed LNEs, depicted on Figure 6, with lecithin as co-surfactant (L – LNE) and bolalipids (PC-C24-PC) (B – LNE) were prepared according to the same method described in the section 2.2. The final colloidal suspensions were divided into 3 x 3 ml in sterile glass vials, labeled them into samples A, B, C for L – LNE and D, E, F for B – LNE. All samples were stored for 28 days under sealed conditions. Samples A and D were then stored at -20°C, samples B and D were stored at 4 °C and samples C and F were stored at uncontrolled room temperature (URT). The experiment was repeated three times to ensure the reproductivity.

### 2.3.1. Characterization of the long–stored lipid nanoemulsions

Every week, both samples A and D were defrosted for at least 30 minutes. All samples were then lined up from A – F on a black background to check, if there were any visible changes in the suspensions accordingly. An additional empty vial was placed next to the lipid formulations to verify that the optical change is solely due to storage temperature conditions.

Pictures were taken with an Iphone 8 to record the visual appearance of the colloidal suspensions every week. Under the safety hood, 0,1 ml was taken out of each vial and diluted with Milli – Q water for further particle size analysis. Samples were then stored again in their different temperature conditions, accordingly.

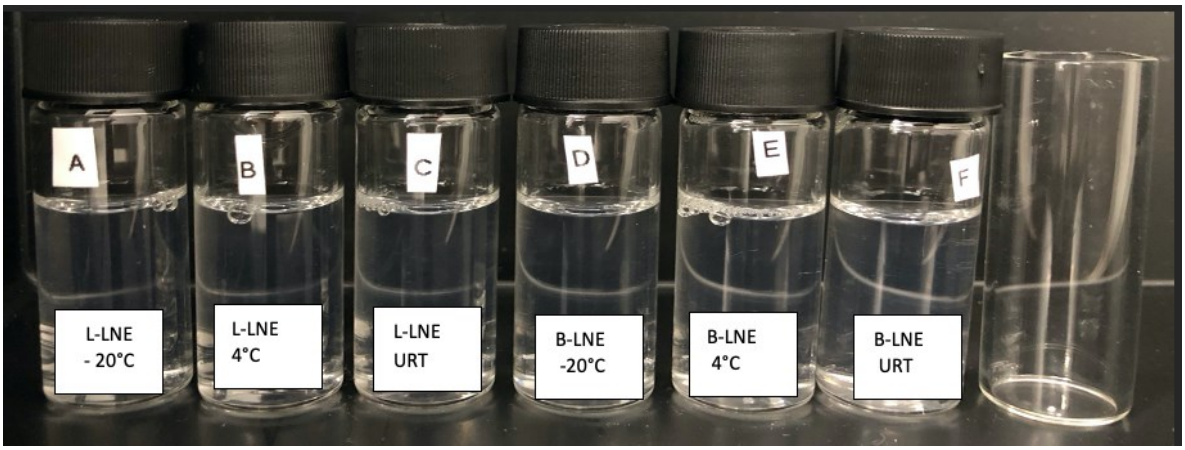


*Figure 7 Picture of the LNEs on the day of their preparation*

## 2.4. Curcumin Encapsulation

### 2.4.1. Preparation of curcumin – loaded LNE

The proposed LNEs were prepared according to the method described in 2. 2. Curcumin (30 mg) was added to the oil phase, of which 1,00 ml was quickly injected into the aquoes phase.

### 2.4.2. Preparation of standard stock solution of curcumin for calibration curve

A standard stock solution of Curcumin was freshly prepared by dissolving the compound in methanol (1 mg/ml). Different concentrations between 1 - 15 µg/ml were prepared by diluting the curcumin stock solution with methanol in 5 ml volumetric flasks.

### 2.4.3. Determination of maximum wavelength by UV/VIS Spectroscopy

To determine the maximum absorbance wavelength of curcumin solution (methanol), a curcumin 200 µg/ml solution was scanned using the UV spectrophotometer in the range of 200 to 700 nm in 1 nm steps. Methanol was used as blank. The corresponding maximum absorbance of curcumin in methanol was observed at 423 nm, which is similar described in the literature [48].

### 2.4.4. Preparation of standard calibration curve of Curcumin by UV/VIS Spectroscopy

The standard calibration curve of curcumin was obtained by measuring the absorbance of curcumin stock solution in concentration range between 1 – 15 µg/ml prepared from the stock solution in methanol at 423 nm. Calibration curve of curcumin was then plotted with absorbance on y-axis and curcumin concentration on x-axis. The experiment was repeated three times.

### 2.4.5. Determination of Curcumin Concentration in the lipid nanoemulsions by UV/Vis Spectrophotometer

After the filtration with 0,22 µm filter, 0, 5 ml of the colloidal suspension was then transferred into an Amicon Ultra-0.5 Centrifugal Filter Unit. With the parameters RPM = 3000, RAD = 299, RCF = 3008 and at a temperature of 4 °C, the formulations were centrifuged for 20 minutes. The remaining LNE without free drugs was collapse and diluted with methanol 1:1000, 1:500 and 1:200 accordingly. To determine the encapsulation efficiency (EE %) of the proposed LNEs, the mass of curcumin encapsulated into the nanoparticles was calculated using the following equation:

$$\textit{Encapsulation efficiency \% (EE \%)} = \frac{amount\ (mg)\ of\ curcumin\ in\ the\ LNE}{total\ amount\ of\ curcumin\ in\ final\ product} \textit{ x 100}$$

EE % was calculated by dividing the amount (mg) of curcumin that remained in the LNE with the total weight of curcumin recovered from the centrifugal filter. The concentration of curcumin entrapped in the LNEs was determined by monitoring the absorbance at 423 nm using a UV/Vis spectrophotometer on a 96 – well plate at 25 °C with the determined curcumin standard curve above.

$$\textit{Recovery Rate \% (RR \%)} = \frac{encapsulated\ drug\ content}{theoretical\ drug\ content} \textit{ x 100}$$

RR % was determined by dividing the amount (mg) of curcumin entrapped in the lipidnanoemulsions with the theoretical drug content.

### 2.4.6. Principle of UV – VIS Spectrophotometry

Ultraviolet – visible (UV – vis) spectrophotometry, shown in Figure 8, is a method used to identify (qualitative) or to determine the concentration (quantitative) of a solution using the absorbance of a compound. When light of UV – VIS (wavelength range between 180 – 780nm) is absorbed, chromophore molecules absorb photon, which allows an electron to change its ground state to a higher energy state [49]. By using a monochromator, a light source (usually a deuterium or hydrogen lamp), will be separated into its individual wavelength components. The filtered light is then directed into the sample's solution, in which the sample molecules absorb the light partially. The remaining light, which is the transmitted light (I), is determined by a detector. The detector in the spectrophotometer measures the intensity of light passing through the sample solution. The intensity of the transmitted light is due to absorption of light at specific wavelength, reduced, which results into a lower value than the original intensity $I_0$. According to the Beer- Lambert law, the absorbance of a solution is directly proportional to

the concentration of the absorbing substance in the solution and the path length. When the distance is a fixed parameter (1 cm) the solution's concentration can be determined. The absorbance of the compound can be determined by [50]:

$$T = I / I_0$$

$$A = \varepsilon cd = - log (T)$$

The absorbance (**A**) is then obtained by the Beer- Lambert law, where $\boldsymbol{\varepsilon}$ is the extinction coefficient, **d** is the path length, **c** is the concentration of the solution, **T** stands for transmittance, **I** for transmitted light and $\mathbf{I_0}$ for the original intensity of the light [50]

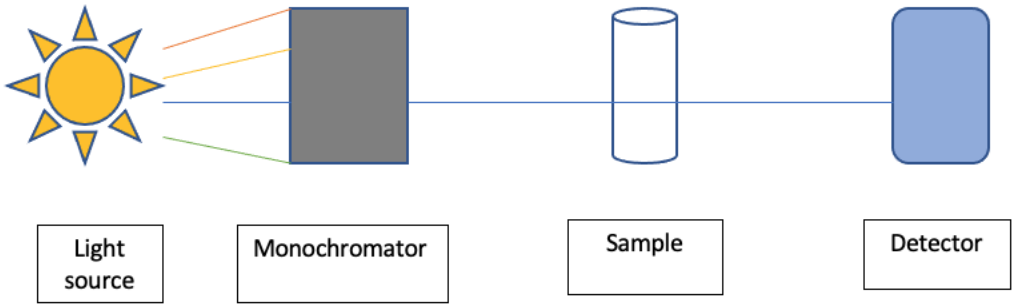


*Figure 8 Principle of UV/VIS Spectrophotometer*

## 2.5. Cytotoxicity Assay

The toxicity of the prepared lipid nanoemulsions was evaluated using Caco-2 cells through the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) cytotoxicity test.

### 2.5.1. Theory behind the MTT Cytotoxicity test

The MTT cytotoxicity test is an assay where data is recorded using a plate-reader by estimating the number of viable cells. This method is based on the conversion of the tetrazolium salt MTT into formazan crystals by mitochondrial reductase, which is an enzyme found in living cells. The MTT reagent permeates through the cell membrane and the mitochondrial inner membrane because of its lipophilic chemical structure and its positive charge. Dead cells lose their ability to convert MTT into the formazan crystals, thus making it a useful tool to measure cell viability. However, the exact mechanism of this assay has not been fully understood, as some enzymes or cell organelles may play a role in the MTT reduction. When the cells are exposed to drugs at different concentrations, which can result in the decrease of the mitochondrial activity, the cytotoxicity can then be determined by detecting the formed formazan crystals at 570 nm using an UV/Vis spectrophotometer [51].

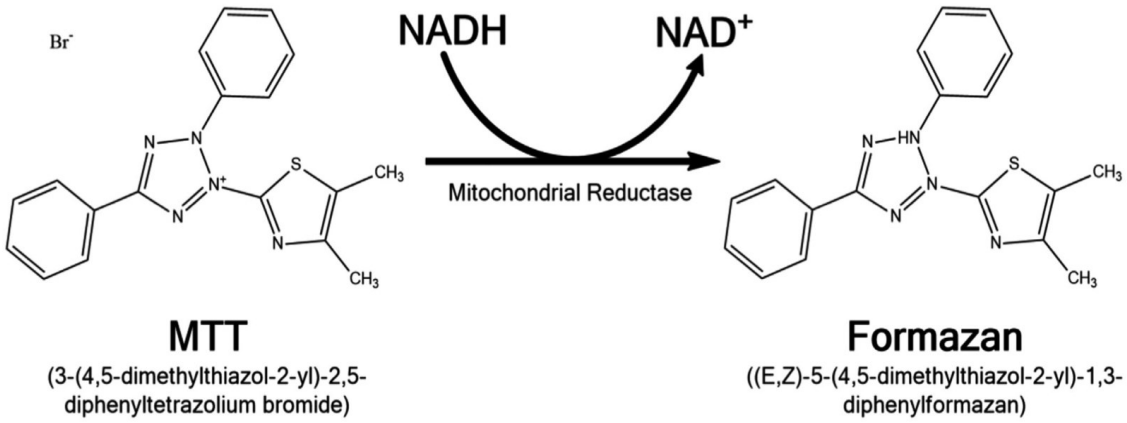


*Figure 9 Structures of MTT and formazan crystal* [52]

### 2.5.2. Caco-2 cell line

The human epithelial cell line Caco-2 (**Ca**ncer **co**lo-2) is derived from a human colorectal adenocarcinoma having the advantage of spontaneous differentiation when reaching confluence. They have been used widely especially for drug absorption studies after oral

administration. Other applications include toxicity studies of drug candidates and to determine drug transport mechanism (for example paracellular vs. transcellular) across the intestinal epithelium. They have been also used to determine the influence of formulation components on the gastrointestinal transport of APIs. Using this cell line has however several limitations. The normal intestinal epithelium has more than once cell type, the lack of mucus, the absence of bile acids and phospholipids as they have a big impact on the solubility of lipophilic compounds and the lack of Cytochrome P450 enzymes [53].

### 2.5.3. Cell Culture

Caco–2 cells were obtained from Ao. Univ. – Prof. Mag. Dr. Franz Gabor group (Department of Technology, University of Vienna). The cells were maintained in RPMI 1640 supplemented with 10 % FBS Superior and 1 % Penicillin/Streptomycin at 37°C in a humidified incubator with an atmosphere of 5 % $CO_2$. The Caco–2 cells were regularly sub-cultured using Trypsin/EDTA. The cell culture medium was changed every three days. At 80% confluence, typically after four – five days, the cells were split according to the experiment before further cultivation. The cells were first rinsed with 5 ml DPBS for a 75 $cm^2$ flask, before adding 3 ml Trypsin/EDTA to wet the whole cell layer. The cells were then trypsinize with another 4 ml Trypsin/EDTA and were then transferred into the incubator for six minutes to make sure that the cells were detached from the surface of the cell culture flask. To stop the trypsinization, 9,0 ml of the cell culture medium was added. The cell suspension was put in a falcon tube and centrifuged for five minutes at 1000 rpm. The cell pellet was resuspended in a new cell culture medium and an aliquot of 20 µl were taken out to count the cells using a TC20$^{TM}$ automated cell counter from Bio–rad [53].

### 2.5.4. MTT – Assay

Caco-2 cells were prepared in a 96- well plate containing a final volume of 200 µl and a cell density of 4 x $10^4$ cells per well. The cells were then incubated for 24 hours at 37°C in a humidified incubator with an atmosphere of 5% $CO_2$. The old medium was removed, and the

cells were then rinsed with DPBS. 200 µl of the proposed lipid formulations prepared in a mirror plate were added and incubated for another 24 hours.

### 2.5.5. Preparation of the MTT Stock Solution

Thiazolyl Blue tetrazolium bromide (500mg) was dissolved in 20 ml of sterile DPBS (Dulbecco's Phosphate Buffered Saline) to achieve a 5 mg/ml stock solution. The prepared solution was then filtered under safety hood with a 0,22 µm filter to remove undissolved particles and keep it sterile. The stock solution, which was wrapped in an aluminum foil for light - protection was stored at 4°C for frequent use.

### 2.5.6. Preparation of the Test formulations

The proposed lipid nanoemulsions were prepared according to 1. 2.. Both curcumin–loaded nanocarriers were diluted to create test formulations with an encapsulated curcumin concentration of 120 µM. To compare these curcumin – loaded lipid carriers, the vehicles, which are the formulations without drug content (L – LNE and B – LNE) were diluted with the same factor to maintain the exact concentrations of the other lipid components (e.g., cosurfactants). A 20 mM curcumin in DMSO were prepared and diluted with cell culture medium to make a 120 µM Curcumin solution. The highest concentration applied to the cells were diluted with a constant factor covering a range of 1,4 µM – 120 µM of curcumin.

### 2.5.7. Determination of the Cell Viability using UV/VIS Spectrophotometry

After the 24 hours treatment time on the cells, 20 µl of the prepared MTT stock solution, was added to each well. The plates were again incubated for another three hours. The supernatant was discarded, and 100 µl DMSO were added to dissolve the blue formazan crystals. The optic density was measured at 570 nm using a plate reader. Cells incubated in the culture medium

or with Triton X – 100 (0,5 % w/v) were used as a control of 100 % and 0 % of cell viability. The cell viability was determined by:

$$\text{Cell viability (\%)} = \frac{T-N}{P-N} x\ 100$$

The cell viability (%) can be determined with the given formula above, where **T** is the absorbance of the tested formulation, **N** is the absorbance of negative control (no viability), and **P** is the absorbance of positive control (full viability).

# 3. Results and Discussion

## 3.1. Characterization of the LNEs by DLS

The mean particle size of the nanosized emulsions prepared by the hot solvent diffusion were measured with Dynamic Light Scattering. In the present study, we developed LNEs to characterize the effect of bolalipids on the physicochemical characters of the produced formulations. To compare the bolalipid nano carrier with the traditional lecithin – LNEs, all other excipients of the emulsion system were kept at the same concentrations. Table 4 summarizes the physicochemical properties of the proposed LNEs. The data displayed showed the measurements from n = 3. The data are expressed as mean $\pm$ standard deviation. One – way ANOVA (analysis of variance) was used to determine statistically significant differences between the means of the three formulations. For follow – up experiments, we decided not to include LNE 50:50 as no statistically significant changes in the physicochemical properties of the produced nanoemulsions were observed as shown in table 4.

*Table 2 summary of the physicochemical properties of the proposed LNEs. The results are presented as a mean of 3 measurements ± standard deviation*

| | L – LNE | B – LNE | LNE 50:50 | p – value |
|---|---|---|---|---|
| **Size (d, nm)** | 16,61 ± 1,10 | 17,26 ± 0,49 | 15,98 ± 2,21 | 0, 38 |
| **PDI = Polydispersity Index** | 0,17 ± 0,07 | 0,20 ± 0,03 | 0,15 ± 0,06 | 0,06 |
| **Zeta Potential (mV)** | -4,05 ± 2,67 | -2,22 ± 0,79 | -2,25 ± 1,75 | 0,21 |

### 3.1.1. Particle size

All of prepared LNEs have similar particle size with a diameter of around 16 nm, which indicates that no significant differences of the particle size were observed. Therefore, the incorporation of bolalipid into LNEs has no effect on the particle size of the nanocarriers. The particle size is an important parameter in determining the extent and rate of drug absorption. The particle size and structure of LNEs can be influenced by various factors such as preparation procedures, type and concentration of lipids [54].

### 3.1.2. Polydispersity Index

Polydispersity index (PDI) characterizes the heterogeneity of a system based on the particle size. The determined values are under 0,20, which correlates with the measured particle size, indicating that the produced LNEs are monodisperse in their droplet size. The accepted PDI – value found in the literature is below 0.5, however, lower PDI – values (< 0.25) are preferred due to the narrow size distribution [54].

### 3.1.3. Zeta potential

The zeta potential is a parameter used to describe the stability in colloidal systems due to electrostatic repulsion or attraction between particles. As displayed in Table 4 the zeta potential of the proposed LNEs was up to -4 mV. In the literature a zeta potential value higher than 30 mV is necessary for an electrostatic stabilization. However, it has also been reported that lower zeta potential values like -4 mV are sufficient to stabilize emulsion systems if the system is provided with steric stabilizers [39].

Due to the different phospholipid components found in lecithin mixture, the surface charged of the nanosized droplets is negative, due to the presence of charged phosphatidylserine, phosphatidylinositol, and phosphatidic acid. Hädrich et al, 2015 described in their research that a decreased zeta potential was caused by the presence of polyethylenoglycol chains of PEG 660 – stearate in the surface area, which again lead to disposition of the negatively charged phospholipids [39].

The similar physicochemical properties of both nanoemulsions could be explained by the similar chemical structure of the used cosurfactants. Lecithin is a mixture of different phospholipids. Phospholipids are amphiphilic molecules and consist of a polar headgroup and a lipophilic tail. They consist of a glycerol molecule, in which sn–1 and sn–2 positions are modified with fatty acid that may differ in length and saturation degree. Sn-3 position is esterified with phosphoric acid, which again, is esterified with an alcohol. The structure of this alcohol defines the different types of phospholipids. As such, they can be divided into phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), and phosphatidylserine (PS). PC and PE are zwitterionic and have a neutral charge at pH 7 compared to PG, PI and PS that are negatively charged at this pH value [55]. The most common component found in lecithin is PC [56]. In comparison PC – C24 – PC bolalipid is composed of two hydrophilic phosphocholine (polar headgroups) connected by one alkyl chain [42].

Our results show that changing the cosurfactant has no effect on the physicochemical properties of the nanoemulsions. Microemulsification is generally dependent on many factors such as 1) nature and concentration of the oil, surfactant, cosurfactant and aqueous phase, 2)

oil/surfactant and surfactant/cosurfactant ratio, 3) temperature, and pH of the environment and lastly 4) the physicochemical properties of the compound such as pKa, polarity and hydrophilicity/lipophilicity [57]. A study from 2019 reported that parameters like oil phase composition, surfactant concentration, temperature and preparation methods had a considerable effect on the particle size and polydispersity index of the produced nanoemulsions [58]. In 2012 a group studied the effect of different PEG – 660 stearate concentration on the formation of nanosized emulsions. Dora et al, 2011 showed in their study that higher surfactant concentrations resulted into formation of smaller nano droplets. They also showed that when surfactant to oil weight ratio was greater than > 6.0, the hydrodynamic radius of the nanoparticles was even smaller [57]. It has been reported various times, that higher surfactant concentrations achieved smaller particle size due to the increased thickness of the interface and decreasing interfacial tension. However, excessive surfactant concentrations would promote flocculation and instability due to bridging or depletion [59]. Larger amounts of surfactants in the final formulation can also lead to toxic concerns [60]. *In vitro* cytotoxicity studies of unloaded nanoemulsions performed on human keratinocytes and fibroblasts showed that formulations with anionic (e.g., sodium dodecyl sulfate) and non – ionic emulsifiers (e.g., Tween 80) had comparable toxic effect while lecithin – based (amphoteric) nanoemulsions exhibited lower cytotoxicity [61]. It has been stated in the literature that removing excessive surfactants from lipid nanospheres was possible, however, this extra step may lead to loss of drugs [62].

## 3.2. Stability of the Lipidnanoemulsions

Physical stability of emulsions is maintained when the system shows resistance to physicochemical changes, which may result into sedimentation, flocculation, coalescence, and separation of phases, which reduces the quality of the product and shorten the storage life [63]. Therefore, the average droplet size of the nanoemulsions was assessed during the storage time. Longer storage studies are preferred, however as time was a limiting factor, the storage time was set at 28 days.

### 3.2.1. Repeated Freeze – Thaw Stability

Figure 10 demonstrates the change in particle size of L – LNE and B – LNE when stored at -20°C, respectively. L (lecithin) and B (bolalipids) represents the used cosurfactants in the lipid formulations respectively. The growth of z – diameter (average diameter) from around 16 nm to around 32 nm started in the first freeze – thaw cycle on Day 7. This result is also shown on Figure 11 by documenting the visual appearance of the proposed LNEs, which were taken with an iPhone 8. By the end of the experiment on Day 28, the measured size is higher than 50 nm, indicating that rapid changes in temperatures may induce thermal stress to the biomolecules, thus destabilizing the system.

Lecithin – based lipid formulations may differ in their visual appearance, which can be explained by their droplet size. Nanoemulsions with a particle size of higher than 100 nm appear milky white to semitransparent, while visible separation of the emulsion system into two phases (an upper concentrated emulsion and a clear, transparent solution) upon long – term storage was also described in the literature [56]. More Freeze – Thaw cycles may lead to even greater z – diameter than 50 nm and may even exhibit a milky optical appearance as mentioned above.

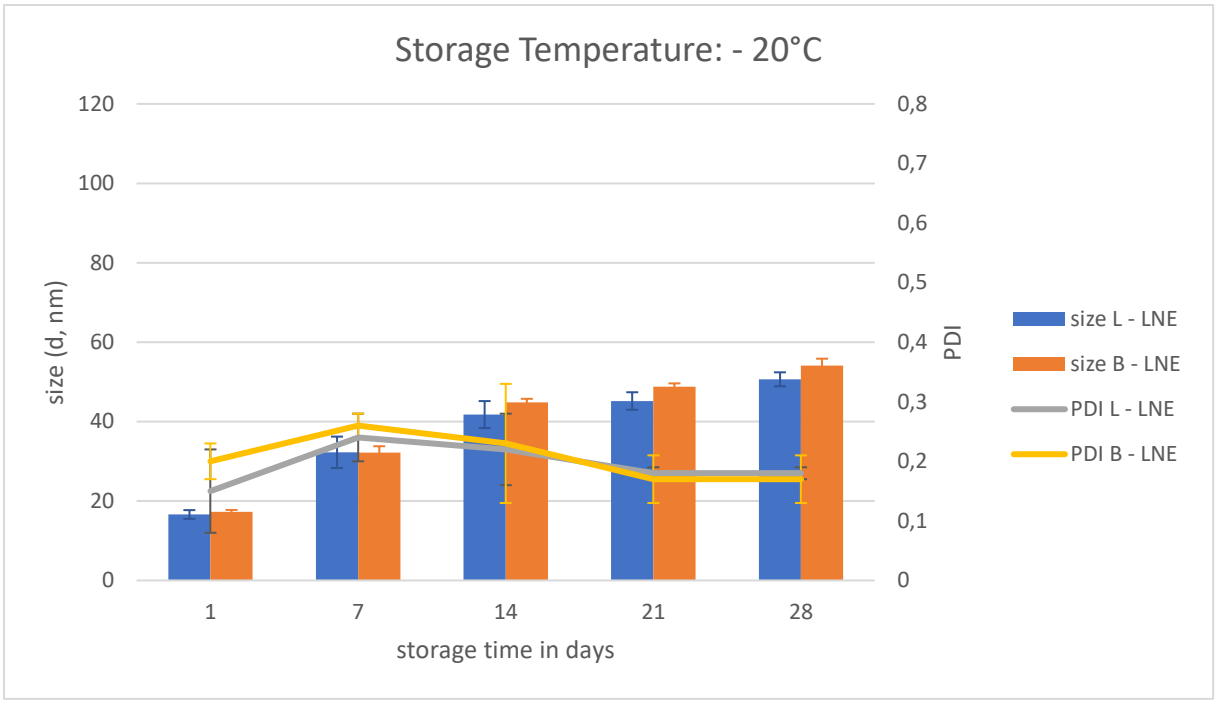


*Figure 10 repeated Freeze - Thaw stability of the proposed LNEs. The data are expressed as mean ± standard deviation (n=3). L - LNE contains lecithin as cosurfactant, B - LNE as PC - C24 - PC.*

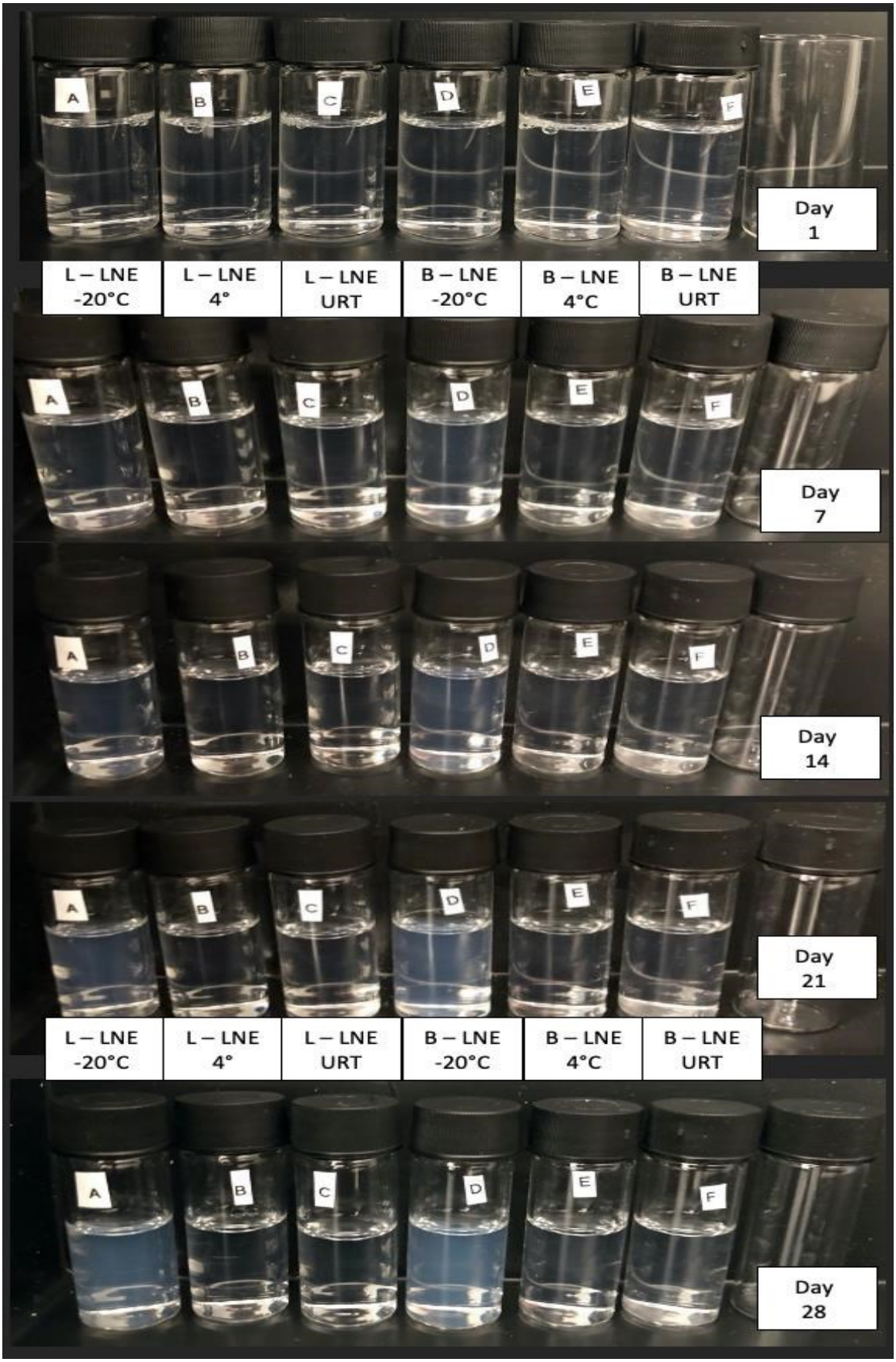


*Figure 11 visual appearance of the proposed LNEs over the storage time of 28 days*

It has been reported that lecithin – based nanoemulsions showed instability to Freeze - Thaw cycles. One cycle was sufficient to observe increased z – diameter and a phase separation, indicating the occurrence of some destabilization [64], which is similar to the observed data shown in Figure 10 and Figure 11. Figure 11 shows the change in the optical appearance of the prepared LNEs over the period of 28 days. At the beginning of the experiment, all samples were clear, transparent and monodisperse. We observed, that both samples A (L – LNE) and D (B – LNE), which were stored at a temperature of -20°C respectively, exhibited a cloudier appearance (compared to the other samples), upon defrosting at uncontrolled room temperature for 30 minutes. This thermal stress caused by freezing and thawing changed the optic appearance of samples A and D, which is already visible in the first cycle in the first week. This result correlates with the measured data from the particle size analysis using DLS shown in Figure 10. The measured mean particle size in the first Freeze – Thaw cycle was around 32 nm compared to their original size at around 16 nm. As time progressed, the opaque- look of the suspensions were more defined and visible, indicating the growth of the particle size of the nanocarriers. By the end of the experiment on Day 28 with 4 cycles, the mean size was higher than 50 nm.

Freeze – Thaw stability is necessary in pharmaceutical research, as lipidnanoemulsions may undergo rapid temperature changes during storage and transportation. According to recent studies lipid crystallization in oil – in water (O/W) emulsions, which depends on the chemical structure of the surfactants, may induce system destabilization [64]. The formed lipid crystals interact with the interfacial membrane negatively, thus promoting aggregation of the nano particles. Further, the Brownian motion of the lipid particles, caused by the constant collision between nano – droplets with solvent molecules, enhances the formation of lipid bridges of residual oil (partially coalescence). These lipid bridges lead to complete merging of the droplets (true coalescence) when the lipid crystals are then introduced to higher temperatures [65]. One study from 2020 showed that protein – chlorogenic acid stabilized nano emulsions were more resistant to aggregation, as this negatively charged agent changed the surface charge of the microemulsions, thus promoting electrostatic repulsion between the particles [66]. One other way to achieve Freeze – Thaw stability is by incorporating Purity Gum (a modified maize starch) into the formulation, which promotes the formation of a thick emulsifier layer, which inhibits the coalescence of the droplets [64].

### 3.2.2. Long – term stability

Samples B and C with lecithin as cosurfactant, E and F with bolalipids were stored at 4°C (B and E) and URT (C and F) accordingly, as shown in Figure 11. The visual appearance of these samples looks unchanged (transparent and clear) over the storage time of 28 days, which represents their stability at these given temperatures, which is similar to the observed data shown in Figure 12 (URT) and Figure 13 (4°C). The calculated p – value between Day 1 and Day 28 for L – LNE at 4°C was 0,45, and 0,43 for URT. Similar p – values were calculated for B – LNE.

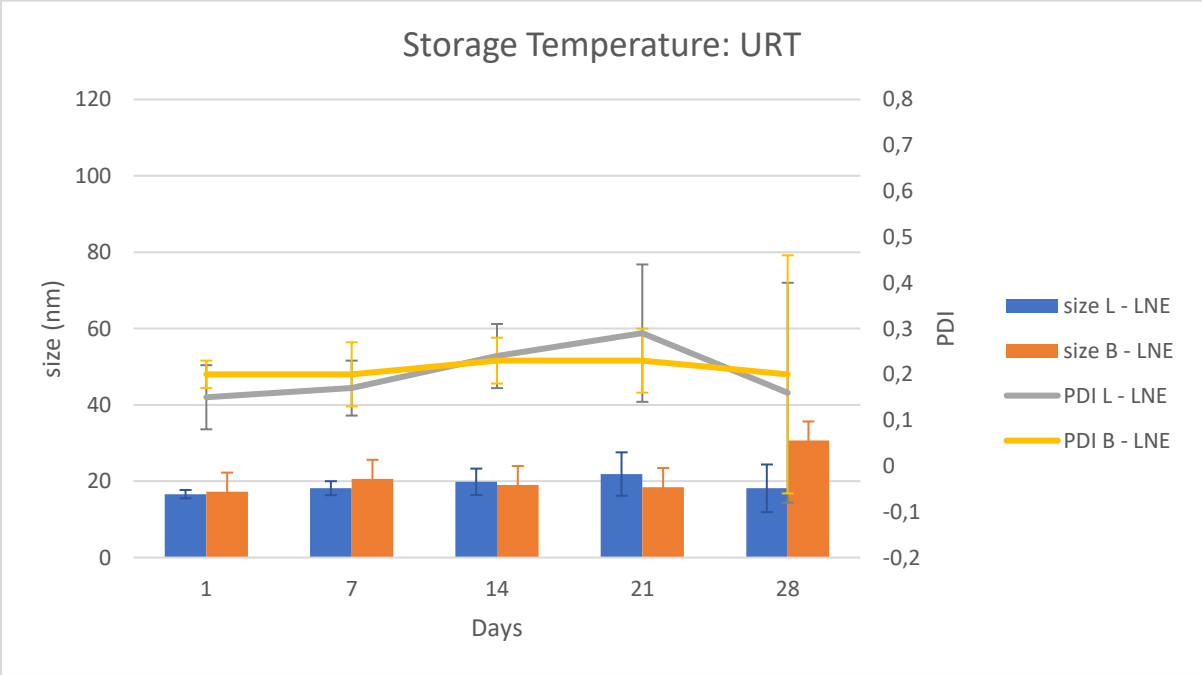


*Figure 12 Evolution of the particle size at storage temperature of URT. The data are expressed as mean ± standard deviation (n=3). L – LNE contains lecithin as cosurfactant, B – LNE contains PC – C24 – PC.*

Figure 12 demonstrates the evolution of the particle size of the LNEs stored at uncontrolled room temperature (URT). L (lecithin) and B (bolalipids) represents the used cosurfactant in the lipid formulations respectively. L – LNE maintained its size at around 20 nm over the course of 28 days with a calculated p – value of 0,43. B – LNE showed an increased particle size on day 28 (compared to its original particle size on day 1) with a calculated p value of 0,39. The large standard deviation of the particle size of B – LNE on day 28 indicates that some aggregation

or contamination of the samples may have occurred. However, aggregation would contradict the results of Dora et al [57]. Dora et al (2012) described in their study, that the particle size of lecithin based nanoemulsions, showed no significant changes in the physicochemical properties over a time of three months at room temperature and 4°C, which can be explained by the presence of non – ionic surfactant (PEG 660 – stearate) due to steric stabilization [57]. For our study, we used Kolliphor HS15, which is a high – molecular – weight PEGylated macrogol surfactant, which should stabilize the system due to surface repulsion caused by their long fatty acid chains, which has also been reported in the literature [67]. Our proposed LNEs should exhibit sufficient stability at this given temperature due to presence of Kolliphor HS15, which contamination is more likely to explain the large standard deviation of the particle size.

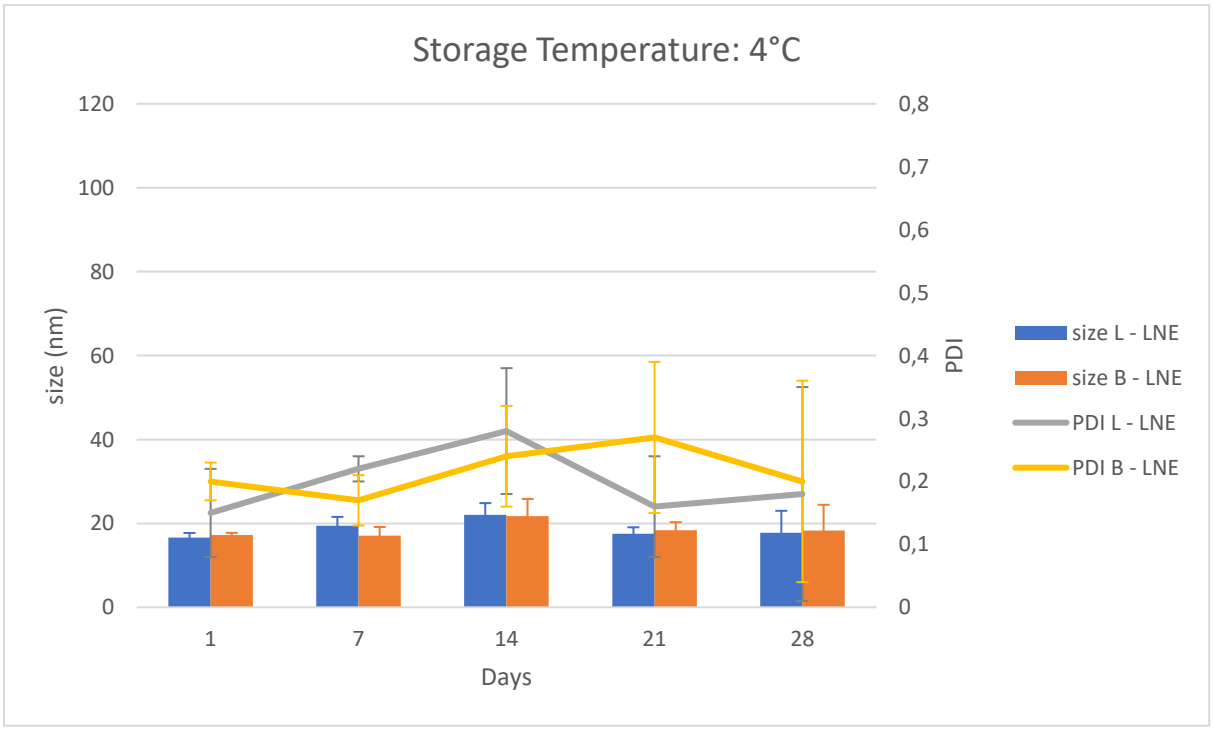


*Figure 13 Evolution of the particle size of the proposed LNEs at storage temperature of 4°C. The data are expressed as mean ± standard deviation ( n=3). L – LNE contains lecithin as cosurfactant, B – LNE contains PC – C24 – PC.*

Figure 13 depicts the evolution of the nanoparticles at storage temperature of 4°C. As mentioned above already, lecithin based nanoemulsions at this temperature should be stable due to steric stabilization. Our results indicates that bolalipids nanoemulsions exhibit sufficient stability over the given time. The PDI value remained under 0,3 over the course of the experiment.

In the literature, one mechanism of emulsion destabilization is described when droplets sizes are small and a certain solubility of the dispersed phase in the continuous phase is given, which is a phenomenon called Ostwald ripening or molecular diffusion. However, it has been reported that lecithin – based nanoemulsions are resistant to Ostwald ripening (diffusion of smaller particles to larger one due to differences in solubility) as lecithin is insoluble in water. Therefore, the diffusion of the dispersed nano droplets should be limited [56]. This effect may also apply to PC – C24 – PC. The hydrophobicity of PC – C24 – PC bolalipids may follow the hydrophobicity of lecithin as amphiphilic molecules show higher degree of hydrophobicity with more carbon atoms [42]. Another possible explanation for the sufficient stability is the presence of Kolliphor HS15 due to steric stabilization as already mentioned.

## 3.3. Evaluation of the calibration curve

The standard calibration curve of curcumin obtained by measuring the absorbance of curcumin solution in concentration range (1 – 15 µg/ml) prepared from stock solutions in triplicate. The determined equation of line of regression is y = 0,0771x + 0,0037. The calibration curve was highly linear with $R^2$ = 0,9992.

### 3.3.1. Limit of detection (LOD) and Limit of quantification (LOQ)

LOD and LOQ are terms used to describe the lowest concentration of an analyte that can be reliably measured by an analytical method. While Limit of detection (LOD) is the smallest

amount of analyte in the sample that can be detected, Limit of quantification (LOQ) is the smallest amount of analyte in the sample that can be quantitatively determined. LOQ and LOD were determine using the following equation [48]:

$$LOQ = 10 * S.D. / Slope$$

$$LOD = 3.3 * S.D. / Slope$$

*Table 3 LOQ and LOD by UV Visible spectroscopy*

| | **UV Visible spectroscopy** |
|---|---|
| **LOD** | 1,07 µg/ml |
| **LOQ** | 3,24 µg/ml |

## 3.4. Encapsulation Efficiency

One of the main objectives of this study, was the encapsulation of curcumin into the LNEs while maintaining the concentration of 1,5 mg/ml as low solubility of hydrophobic compounds remains a challenging problem in pharmaceutical research. Improving the aqueous solubility of such drugs enhances their oral bioavailability. T – test was used to determine statistically differences between the two proposed formulations.

*Table 4 summary of the physicochemical properties of the curcumin - loaded LNEs. The data are expressed as mean ± standard deviation ( n=3).*

| | CUR – L – LNE | CUR – B – LNE |
|---|---|---|
| **Size (nm)** | 21,81 ± 2,59 | 21,59 ± 1,48 |
| **PDI (Polydispersity Index)** | 0,37 ± 0,08 | 0,36 ± 0,03 |
| **Zeta potential (mV)** | -2,37 ± 2,29 | -3,29 ± 1,29 |
| **Encapsulation Efficiency (%)** | 100 ± 0 | 100 ± 0 |
| **Recovery Rate (%)** | 78 ± 10 | 85 ± 7 |

A summary of the physicochemical properties of the curcumin – loaded LNEs is presented in Table 5. The presented data displays the mean of n =3 ± standard deviation. The measured mean particle size and polydispersity index were around 22 nm and 0,37 respectively. The zeta potential was measured up to -3 mV. The encapsulation efficiency (%) was determined by dividing the curcumin concentration in the supernatant by total concentration of curcumin. The formulations studied demonstrated high encapsulation efficiency with both having the ability to incorporate 100% of the curcumin. The recovery rate (%), which was calculated by drug content in the LNEs divided by theoretical amount multiplied by 100. In both formulations, around 80% could be recovered. All of the measured data were compared to each other (p – value > 0,05). Therefore, changing the surfactant had no effect on the physicochemical properties of the produced LNEs. These results are similar for unloaded nanocarriers shown in Table 4. As PDI is an important parameter to determine the quality of the nanodroplets, this value should be as small as possible. In the present study, as shown above, the measured PDI is 0,37 for L – LNE and 0,36 for B – LNE, respectively. In the literature, PDI values < 0,5 are acceptable with a suitable particle size, however values lower than 0,25 are preferred due to the narrow size distribution [54].

In a recent study, the solubilization capacity of three synthetic bolalipids (PC – $C_n$ – PC, n = 22,24 and 26) were evaluated. All three bolalipids showed increased solubilizing capacity at

concentrations above 0,1 mM surfactant. This observation may indicate the pharmaceutical application of synthetic bolalipids [42]. The pharmaceutical significance and therapeutic efficacy of curcumin is limited due to its poor bioavailability. In the present study, an attempt has been done to overcome the problematic bioavailability of curcumin by introducing the compound into bolalipid based nanoemulsions. Formulations were prepared by using hot solvent diffusion method associated with PIT, which are both two low – energy methods. Our nanoemulsions have shown the ability to incorporate a large amount of curcumin (1,5 mg/ml) with a z – diameter of 20 nm. No previous experiments have been done to formulate bolalipids in nanoemulsions. As such no comparison to the literature are available. However, the encapsulation of quercetin in lecithin NEs have been studied various times. Hädrich et al and their group obtained a quercetin (1,5 mg/ml) – loaded nanoemulsions composed of castor oil, lecithin, and PEG 660 – stearate [39]. Vaz et al (2022) and their group were able to encapsulate both curcumin (0,632 mg/ml) and quercetin (0,702 mg/ml) in NEs with components similar to the presented formulations [68]. In microemulsification systems, curcumin molecules are typically located within the lipophilic part of the oil droplets, but some of them may also be located close to the oil – water interface due to the polar groups of curcumin [69].

## 3.4. Cytotoxicity Assay

The cytotoxicity mechanism of surfactant is generally explained by their interaction of with phospholipid bilayer leads to an expansion of the outer layer of the membrane, thus changing its structure. This decreased in function and its resistance to withstand maximum stress, promotes the formation of small transient holes. When the cells are exposed to even higher surfactant concentrations, these small holes turn into permanent holes, thus beginning the disintegration of the cell membrane [42].

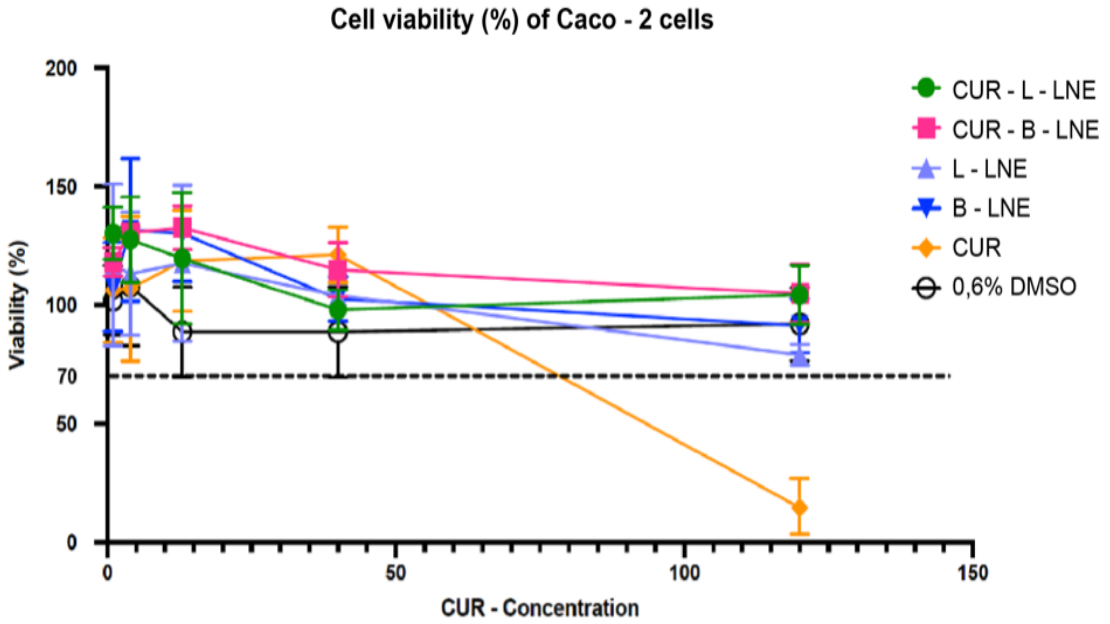


*Figure 14 concentration - dependent MTT reduction of the proposed LNEs; CUR – L – LNE and CUR – B – LNE represent the curcumin – loaded nanoemulsions, L – LNE and B – LNE represent the unloaded nanoemulsions, CUR stands for free curcumin*

Figure 14 displays the cell viability (%) on the y – axis and curcumin concentrations (mM) on the x -axis. The data are expressed as mean $\pm$ standard deviation from three independent experiments using Caco – 2 cell line on three different passage numbers (p55, p57, p59). The cytotoxicity of the proposed LNEs was evaluated based on their cell viability. The accepted cell viability described in the literature is above 70% [70]. The cell viability was calculated with the given formula in 2.5.6. Triton X 0,5% were used as negative control, while untreated cells as positive control. DMSO was used to dissolve curcumin, and as such applied to the cells as a negative control to determine that the cytotoxic effect is solely due to curcumin. According to the literature, treatments with up to 1% DMSO has no lethal effect on Caco – 2 cells [71].

As shown in Figure 14 the highest applied free curcumin concentration (120 µM) exhibited cytotoxic effect on Caco – 2 cells with a calculated cell viability of only 15%. The described $IC_{50}$ (inhibiting concentration, at which 50% of the cells die) of free curcumin in the literature ranges between 50 µM – 250 µM. The inhibiting concentration differs between the tested cell types, as such $IC_{50}$ of free curcumin on HeLa cells is above 250 µM [72], while 50 µM is

sufficient to inhibit cell proliferation of Hep – 2 cells [73]. Based on this finding, we can assume that $IC_{50}$ of curcumin on Caco -2 cells is way lower than 120 µM, as the calculated cell viability is only at 15%.

For our study, we based the concentration of the applied lipid formulations on curcumin to determine that the cytotoxic effect of the prepared nanoemulsions was solely due to the surfactant concentrations. The curcumin – loaded lipidnanoemulsions were diluted to have the same curcumin concentration in the NEs as the free curcumin. For comparison the unloaded LNEs (L – LNE and B – LNE) were diluted with the same factor to keep the same concentrations of other excipients applied to the cells. As displayed in Figure 14 the unloaded LNEs showed no toxic effect on Caco – 2 cells (calculated cell viability was above 70%) in the concentration range tested, which is similar to their loaded formulations (CUR – L – LNE and CUR – B – LNE). Our calculated concentration of bolalipids was lower than the mention $IC_{50}$, which however showed no cytotoxic effect on Caco – 2 cells, indicating the safety profile and excelled biocompatibility of our proposed lipid formulations. The highest applied concentration of PC – C24 – PC was 4 µM, which did not affect cell cytotoxicity as shown in Figure 14. Comparing the cytotoxicity of curcumin – loaded LNEs with free curcumin, it is noticeable, that drug – loaded nanoemulsions exhibit less cytotoxic effect (70 % cell viability compared to 15%). A possible explanation of the non – cytotoxicity of the curcumin – loaded LNEs is due to curcumin release kinetics. To evaluate this hypothesis, follow – up release studies should be considered.

One study from 2011 reported that the *in vitro* toxicity of DDPS (N-dodecyl-N,N-dimethylammonium-propanesulfonate, a zwitterionic surfactant), on mammalian cells (including Caco – 2 cells) depends on the concentration and exposure – time of surfactants. They reported that the cytotoxicity of DDPS was not observed up to concentration close to critical micelle concentration (cmc) and as such DDPS may cause structural changes in the cell membrane unlike cationic surfactants, which toxicity can be explained by membrane partitioning, translocation across the membranes on the intracellular level [74]. In fact, Li et al (2022) reported that bolalipids seem to follow the cytotoxicity pattern of non – ionic surfactants, which $IC_{50}$ – values were way higher than their cmc (cmc value of PC – C 24 – PC is around 0,003 – 0, 005 mM for fibrous structures and micelles) [42]. Based on Li and Inacio's

study [74], we can assume that since lecithin and bolalipids are both zwitterionic like DDPS, they may follow these observations.

Li et al described in their study, that the mean $IC_{50}$ of the tested bolalipids (C22 – C26) is between 0,035 – 0,094 mM [42]. It has been reported that potential toxicity of LNEs depends on many factors, such as composition, structure, administration route and their interaction with biological systems [27], while the cytotoxicity of free zwitterionic surfactant may depend on the concentration and exposure time [74]. In the present study, our bolalipid – LNEs exhibited excellent excapsulation efficiency and showed no cytotoxic effect in the concentration range tested on Caco – 2 cells.

# 4. Conclusion

Many drug candidates with promising pharmacological activity often come with challenge regarding their successful formulation. In most cases, the limiting factor is the poor water solubility of these compounds. [1]. According to the biopharmaceutics classification system, an estimated total of 40 % of the drugs in the market have a poor aqueous solubility [9].

In the literature lipidnanoemulsions as DDS have been studied enormously because of their ability of encapsulating higher amounts of lipophilic compounds, the direct transport of the compound to its designated location, the reduction of unwanted side effects [23] and can protect their cargo, especially when this is susceptible to hydrolysis or oxidation [24].

Evidence from this study shows that the introduction of these synthetic cosurfactants into lipid formulations were able to encapsulate curcumin up to 1,5 mg/ml, while maintaining the physicochemical properties of the prepared nanoemulsions. This indicates that synthetic bolalipids like PC – C24 – PC as cosurfactant were able to replace lecithin successfully by keeping the ability of encapsulating curcumin, a model of poor – water soluble drug by improving its solubility. This work demonstrated a promising strategy to formulate drugs with poor water solubility by introducing these compounds into the core – shell of the bolalipids. As such BTZ - =43, a compound with promising pharmacological activity against tuberculosis showed low efficacy in mouse model because of its poor aqueous solubility and low bioavailability [75]. While our prepared biomolecules may show insufficient Freeze – Thaw stability, due to lipid crystallization [64], our proposed drug – free lipid nanocarriers showed sufficient stability at 4°C and uncontrolled room temperature.

*In vitro* cytotoxicity study performed on Caco – 2 cells showed that both loaded and unloaded lipid formulations showed no toxic effect with a calculated cell viability of higher than 70%, indicating their safety profile and biocompatibility in the tested concentration range (up to 4µM). However, *in vitro* cytotoxicity studies are only the first step in evaluating their safety use. Additional *in vitro* cytotoxicity assays should be performed and compared with the $IC_{50}$ of bolalipids observed from a previous study [42]. Follow – up experiments regarding their

permeability property, drug release kinetics, *in vivo* bioavailability studies and *in vivo* toxicity are an aspect worth clarifying to fully evaluate their potential pharmaceutical applications.

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