

Elastic Liposomes Containing Cationic Surfactants

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Zásady pro vypracování

I. Teoretická část

Zpracujte literární rešerši na zadané téma. Charakterizujte elastické lipozomy, jejich složení, vlastnosti a metody přípravy. Zaměřte se na lipozomální systémy s obsahem kationických surfaktantů. Srovnajte tyto systémy se standardními kationickými lipozomy.

II. Praktická část

V praktické části práce připravte elastické lipozomy s obsahem cetyltrimethylammonium bromidu, případně dalších vybraných kationických surfaktantů. Vhodnými technikami stanovte jejich fyzikálně-chemické charakteristiky. Na základě získaných dat určete vliv složení membrány na vlastnosti elastických lipozomů a ve formulační studii vyberte složení membrány pro účinnou enkapsulaci karotenoidů, např. astaxanthinu. Získané výsledky zpracujte a formulujte závěry práce.

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Seznam doporučené literatury:

1. Kuznetsova, D. A. et al. Enhancement of the Transdermal Delivery of Nonsteroidal Anti-inflammatory Drugs Using Liposomes Containing Cationic Surfactants (2022), ACS OMEGA 7 (29), pp. 25741-25750
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ABSTRAKT

Táto diplomová práca je zameraná na prípravu a charakterizáciu kationických elastických lipozómov, ktoré majú potenciál ako nosiče aktívnych látok v kozmetike a farmácii. Pre zabezpečenie ich stability v biologickom prostredí, efektívnej enkapsulácie a netoxického správania je kľúčová optimalizácia zloženia. Práca sa preto venuje výberu vhodných kationických surfaktantov, optimalizácii postupu prípravy a analýze vlastností lipozómov s enkapsulovanými karotenoidmi (astaxantin, lutien, β -karotén). Hodnotené parametre zahŕňajú veľkosť častíc, zeta potenciál, stabilitu, antioxidačnú aktivitu a cytotoxicitu s cieľom poskytnúť relevantné poznatky pre ďalší vývoj týchto nosičov.

Kľúčové slová: kationické elastické lipozómy, kationické surfaktanty, karotenoidy

ABSTRACT

This Master's thesis focuses on the preparation and characterization of cationic elastic liposomes, which have the potential to serve as carriers of active substances in cosmetic and pharmaceutical applications. To ensure their stability in biological environments, efficient encapsulation and non-toxic behavior their composition must be optimized. The thesis addresses selecting suitable cationic surfactants, optimizing the preparation procedure, and analyzing the properties of the liposomes loaded with carotenoids (astaxanthin, lutein, and β -carotene). The criteria evaluated include vesicle size, zeta potential, stability, antioxidant activity and cytotoxicity, with the aim of providing useful insights for the further development of such delivery systems.

Keywords: cationic elastic liposomes, cationic surfactants, carotenoids

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INTRODUCTION

Elastic liposomes (also called transferosomes) are vesicular carriers with incorporated edge activators (usually surfactants) that give them unique properties including high deformability and enhanced ability to penetrate biological barriers. This allows them to deliver active substances, both hydrophilic and hydrophobic, into deeper tissue layers. They thus find application in both pharmaceutical and cosmetic formulations, offering several key advantages over conventional delivery systems. Cationic elastic liposomes are a subgroup of elastic liposomes that contain either a cationic surfactant or a cationic lipid and therefore carry a positive surface charge allowing them to interact with negatively charged biological membranes. However, cationic charge can lead to cytotoxicity and instability of vesicles, and therefore the compositions of cationic liposomes must be thoroughly investigated.

Carotenoids are naturally occurring pigments found in various fruits and vegetables. They are mostly scientifically interesting due to their antioxidant properties. Three antioxidants from the carotenoid family – astaxanthin, lutein and β -carotene – were chosen for encapsulation in this thesis.

This thesis aimed at selecting the most suitable preparation conditions and compositions of cationic liposomes followed by their analysis that included size and zeta potential measurements, as well as stability, cytotoxicity and antioxidant activity evaluation. The study began with the preparation of empty liposomes, followed by encapsulation of carotenoids into elastic liposomes with different compositions of membranes (lipid to edge activators ratio). The obtained data provided valuable information that resulted in the selection of the final formulations which underwent more thorough testing.

I. THEORETICAL PART

1 LIPOSOMES

Membranous vesicles made of phospholipid bilayers called liposomes have received a lot of attention because they possess a wide variety of properties interesting for pharmaceutical, cosmetic and food industry. (Elkholy et al., 2020) Liposomes closely resemble biological membranes with separate water and lipid-based parts, which enable them to encapsulate both hydrophilic and hydrophobic substances. Their structural properties allow these particles to be used in delivery systems. Liposomes are particularly interesting for pharmaceutical use because of their ability to improve the absorption of the encapsulated drug, their capacity to avoid enzymatic metabolism of the drug thus protecting the encapsulated substance, extending its half-life, ability to increase drug stability and drug releasing potential, control substance release and improve overall therapeutic benefits. These advantages are highly dependent on the type and composition of the specific liposome used in delivery system as well as the substance encapsulated inside the vesicle. Another advantage of liposomes is that lipid materials composing these systems are biocompatible and biodegradable. (Das et al., 2022) (Mehendiratta et al., 2024) Conventional liposomes are, however, limited by insufficient stability, low encapsulation efficiency and loading capacity, and therefore there are multiple strategies for development of a new generation of liposomal formulations with better characteristics. (Kuznetsova et al., 2022)

1.1 Classification of liposomes

Liposomes can be classified into various categories according to different criteria. They can be made up of either synthetic or natural phospholipids with the ability to assemble in liquid environment and create either a monolayer or a multilayer arrangement. (Mirtaleb et al., 2021) According to their structure and number of lamellas, liposomes can be divided into small unilamellar vesicles (SUV), large unilamellar vesicles (LUV), oligolamellar vesicles (OLV) and multilamellar vesicles (MLV). Another important classification criterion is size, which typically ranges from 30 nm to micrometer scale and therefore puts liposomes into the nanoscale size group. (Liu et al., 2022) Table 1 shows approximate size ranges of different types of liposomes. (Pattni et al., 2015)

Table 1: Sizes of different types of liposomes (Pattni et al., 2015)

LIPOSOME	SIZE [nm]
SUV (small unilamellar vesicles)	20 - 100
LUV (large unilamellar vesicles)	> 100
OLV (oligolamellar vesicles)	100 – 1000
MLV (multilamellar vesicles)	> 500

Conventional liposomes are usually prepared using for example phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine and phosphatidylglycerol. Depending on the specific ingredients included in the formulation, liposomes can be divided into three categories based on their charge – negatively charged, neutrally charged and positively charged. For instance, incorporating oleic acid into the phospholipid bilayer results in negatively charged liposomes, whereas addition of N-[1(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTAP) causes the liposomes to acquire a positive charge. (Pattni et al., 2015) The electrostatic charge of a liposome significantly influences its properties and biological interactions. Neutral liposomes generally exhibit a higher propensity for aggregation while cationic liposomes interact strongly with serum proteins and negatively charged liposomes are reasonably stable in suspensions where they show minimal aggregation. (Chauhan et al., 2020)

1.2 Elastic liposomes (transferosomes)

Elastic liposomes (EL) (also called transferosomes) are phospholipid-based nanocarriers used in active substance transport. The difference between elastic liposomes and conventional liposomes is in the membrane structure. Membranes of transferosomes contain edge activators (usually surfactants) which destabilize the phospholipid bilayer, thus increasing the deformability of the carrier. (Mirtaleb et al., 2021) Therefore we can classify liposomes into conventional and deformable/elastic liposomes. Physiochemical properties of EL as well as their efficacy and skin penetration abilities depend on their composition, specifically the type of lipid and edge activator (EA) used.

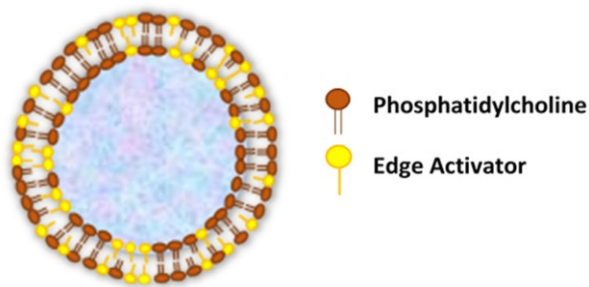


Figure 1: Structure of a transferosome

(Opatha et al., 2020)

The amount of EA should be optimal for efficient skin permeation, increased elasticity and storage stability. Due to their better chemical and pharmacokinetic qualities, ELs outperform traditional drug delivery carriers and can be used to encapsulate both hydrophilic and hydrophobic components in topical, nasal, biological and transdermal drug delivery systems. (Hussain et al., 2017)

In terms of transdermal drug delivery, conventional liposomes are less efficient because of their relatively rigid structure, which significantly limits the penetration of encapsulated substance into deeper layers of the skin. Therefore, formulations using this type of liposomal encapsulation tend to stay in *Stratum corneum*. Another limitation connected to the structure of conventional liposomes is their low stability, encapsulation efficiency and loading capacity. To address these issues, there are several strategies to design liposomes with improved characteristics. One of the possible strategies is to make deformable liposomes, such as transferosomes, ethosomes, invasomes or niosomes, involving edge activators influencing elasticity and therefore improving permeability. (Kuznetsova et al., 2022)

Most of the EL are composed of unsaturated phospholipid phosphatidylcholine with a low transition temperature. Low transition temperature of the membrane lipids causes increased permeability and leakage of the encapsulated drug, and therefore the transition temperature needs to be modified for prolonged storage stability and higher efficacy by altering the bilayer architecture. It should be set between the room temperature, which is usually 25 °C and temperature at the contact with the skin, which is approximately 32 °C. (Hussain et al., 2017)

1.3 Cationic liposomes vs neutral liposomes containing cationic surfactants

Cationic liposomes are liposomes formed of a cationic lipid in their membrane. In comparison with naturally occurring anionic and neutral phospholipids used for preparation of corresponding liposomes, cationic lipids are of synthetic origin. An example of such lipids are 1,2-dioleoyl-3-trimethylammonium propane chloride (DOTAP) and 1,2-dipalmitoyl-3-trimethylammonium-propane chloride (DPTAP).

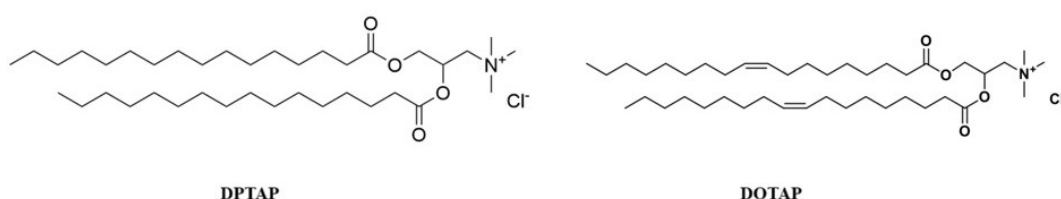


Figure 2: Structure of cationic lipids DPTAP and DOTAP

(<https://www.polysciences.com/>, *n.d.*)

According to Caritá et al. the influence of the liposome charge on drug delivery is rather controversial throughout the available scientific literature, not providing us with clear outcome. Some studies show that cationic liposomes increase permeation due to their ability to attach strongly to the skin surface and interact with the encapsulated substances, while other studies show no specific improvement. There are studies showing that cationic liposomes are more efficient because of their ability to interact with negatively charged cell membranes. (Caritá et al., 2023) (Elkholy et al., 2020) For example Caritá et al. developed cationic liposomes with incorporated DOTAP in their structure for transdermal delivery of vitamin C that showed a significantly enhanced transdermal delivery compared to conventional liposomes. Another application of cationic lipids is for instance in the delivery of genetic materials where the genetic material forms a biodegradable, biocompatible and highly stable complex with cationic liposomes. Size, charge and lipid composition all significantly influence successful delivery. Cationic liposome-DNA complex (lipoplex) is a possible alternative to viral vectors conventionally used for gene transfer. (Akhtar and Khan, 2016) (Caritá et al., 2023)

The DOTAP molecule features a quaternary ammonium head group linked to a glycerol backbone with two oleoyl chains. It was initially hypothesized that the hydrolysable ester bonds would enhance lipid biodegradability and mitigate cytotoxicity. However, it was found that formulations containing DOTAP exhibit transfection activities and cytotoxicity levels comparable to those formulations containing other types of cationic lipids. It was also reported that these monovalent lipids displayed minimal to no cytotoxic effects on near-confluent cell monolayers and maintained similar lipoplex sensitivity at 25%–35% cell confluence. The use of vesicles with 100% DOTAP in gene delivery is inefficient due to the high density of positive charges on the liposome surface. Unlike many other cationic lipids, DOTAP remains fully protonated at physiological pH (7.4), potentially increasing the energy barrier required for DNA dissociation from the lipoplex, thereby reducing transfection efficiency. Consequently, DOTAP is more effective when combined with other lipid type, a strategy commonly employed in cationic lipid-based formulations for enhanced gene delivery. Overall, cationic liposomes are particularly suitable for gene delivery, as the positively charged head groups facilitate electrostatic interactions with the negatively charged phosphate backbone of nucleic acids. (Balazs and Godbey, 2011)

Another group of positively charged liposomes includes neutral liposomes that contain cationic surfactants embedded in their membranes. As previously mentioned, conventional liposomes are rigid, unstable and have low encapsulation and loading capacity. Adding an edge activator to a membrane improves these limitations. Moreover, some studies have showed that changing the charge of liposomes increases their ability to penetrate through the skin. (Kuznetsova et al., 2022) Manosroi et al. studied the differences in transdermal penetration between neutrally, negatively and positively charged liposomes encapsulating Amphotericin B, a systemic fungal infection drug. In this study it was demonstrated that the cationic liposomal formulations exhibit high stability and enhanced transdermal penetration highlighting their potential for improved therapeutic efficacy making them a strong candidate for drug delivery. (Manosroi et al., 2004) Cationic surfactants can act as edge activators, integrate into lipid bilayer and modify its liquid crystalline behavior. Their positive charge improves drug penetration through *Stratum corneum*. Kuznetsova et al. combined phosphatidylcholine and cationic surfactants, compared characteristics and properties of different formulations and demonstrated that liposomes modified with cationic surfactants can considerably improve transdermal drug penetration. (Kuznetsova et al., 2022)

2 CATIONIC SURFACTANTS

Surfactants are amphiphilic molecules, meaning they possess both hydrophilic and hydrophobic parts which are typically well-separated within their structure. Their ability to assemble is dictated by the balance between these distinct functional regions. Surfactants are generally categorized based on their head group charge, classifying them as anionic, cationic and nonionic. For the purposes of this thesis, the main emphasis will be on the cationic surfactants. Cationic surfactants exhibit a strong tendency to adsorb onto solid surfaces since most of them carry a negative charge. Hence, they are commonly used as bactericides due to their ability to interact with negatively charged cell membranes. Additionally, cationic surfactants tend to be more cytotoxic compared to other surfactant classes. Nonetheless, they remain widely used not only in personal care products. (Gonçalves et al., 2023)

2.1 Cetyltrimethylammonium bromide

Cetyltrimethylammonium bromide (CTAB) is a cationic surfactant that contains hydrophobic tail and a positively charged quaternary ammonium head group in its structure.

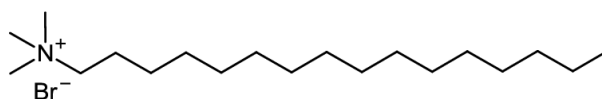


Figure 3: Structure of CTAB

(Janosevic-Lezaic et al., 2014)

Positively charged head group can react with surfaces carrying negative charge predetermining the main use of this substance. Interacting with negatively charged bacterial cell membrane, thereby effectively disrupting its structure and function, qualifies it as a good antimicrobial agent and preservative used in pharmaceutical products, personal care products and products for disinfection, sanitation and room sterilization. (Akhtar et al., 2024) Apart from being used as an antimicrobial ingredient, cationic surfactants, including CTAB, exhibit notable ability to integrate into lipid bilayers and boost lipid crystalline behavior. Positively charged head group can therefore have a significant impact on transdermal drug delivery. (Kuznetsova et al., 2022) CTAB can also be used for particle modification leading to alteration of their properties. For instance, Shilova et al. prepared magnetite nanoparticles stabilized with cetyltrimethylammonium bromide in soy lecithin liposomes and concentrated their work on the effect of the modification of these particles with CTAB. Nanoparticles modified with CTAB exhibited increase in the number of magnetite particles embedded in the lipid bilayer as a result of the interaction of the hydrocarbon chain in the CTAB molecule

and hydrophobic fatty acid residues in lecithin molecules. In conclusion, modification of nanoparticles with CTAB promoted embedding of the nanoparticles into liposomal bilayer allowing more efficient drug delivery towards targeted cells. (Shilova et al., 2022) Based on previously mentioned information it can be said that cetyltrimethylammonium bromide has an important role in both pharmaceutical and cosmetic industries. Its characteristics also highlight its potential for future advancements in drug delivery systems, antimicrobial coatings, and novel formulations.

2.2 Hexadecylamine

Hexadecylamine (HEX) is a cationic surfactant characterized by a long hydrophobic alkyl chain and a hydrophilic amino ($-NH_2$) head group. Its amphiphilic nature allows it to interact with various surfaces, making it suitable for surface modifications. For example, in a study carried out by Tabrizi et al., HEX was utilized to modify montmorillonite K10 (type of clay used as an adsorbent for cationic contaminants but also in cosmetic chemistry) to enhance its adsorption capabilities. It was concluded that modification with HEX can extensively enhance the adsorption behavior of montmorillonite. Unlike conventional surfactants, HEX exhibits low water solubility, which minimizes the risk of leaching into aqueous environments. (Tabrizi et al., 2022)



Figure 4: Structure of HEX

(NIST WebBook Chemie, SRD 69)

2.3 Benzalkonium chloride

Benzalkonium chloride (BAC) is a cationic surfactant that belongs to the quaternary ammonium compound group, widely used in pharmaceuticals and personal care products such as antiseptics, disinfectants, and cosmetics. Structurally, BAC is a combination of alkylbenzyl dimethylammonium chlorides with different lengths alkyl chains. Commercial formulations typically contain alkyl chains with 12, 14, or 16 carbon atoms.

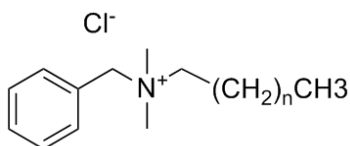


Figure 5: Structure of BAC (n =12, 14, 16, 18)

(ChemSrc, *n.d.*)

Antimicrobial properties of BAC are primarily attributed to their interactions with proteins, lipids, and guanine nucleotide triphosphate-binding proteins (G proteins) in biological membranes directly contributing to its antimicrobial properties enabling it to interact with microbial cell membranes, leading to membrane disruption and enhanced biocidal efficacy. Therefore, it is often used as a preservative in pharmaceutical and cosmetic formulations. It is commonly incorporated into ophthalmic preparations acting not only as a preservative but also as an enhancer of drug penetration and bioavailability. (Choi et al., 2018) (Mukai et al., 2024) Benzalkonium chloride can also be used as a positive membrane component for stabilization of liposomes. (NAKAMORI et al., 1993) Despite its broad utilization, BAC is also associated with potential negative effects, particularly upon repeated or prolonged exposure. Topical application of BAC-containing formulations may lead to dermatitis or skin and mucous membrane irritation. However, as Choi et al state in their paper on risk assessment of benzalkonium chloride in cosmetic products, upon compliance with current regulation guidelines, BAC is considered safe. (Choi et al., 2018)

2.4 Cetylpyridinium chloride

Cetylpyridinium chloride (CPCl) is a cationic surfactant freely soluble in water and organic solvents such as alcohol and acetone. It is a surfactant consisting of one positively charged quaternary nitrogen atom associated with hydrophobic alkyl chain with 16 carbons.

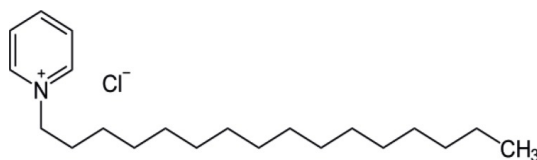


Figure 6: Structure of CPCl

(Zarei et al., 2013)

As a part of quaternary ammonium compounds group it is commonly used as an antimicrobial agent and a preservative in medical products, household products, cosmetics and hygiene products. It is able to permeate oral biofilms, reducing bacterial load in the oral

cavity and therefore is often used in oral care products such as mouth washes and toothpastes. (Kanno et al., 2020)(Yang et al., 2015)(Kaur et al., 2020)

In a study by Lavanya et al., cetylpyridinium chloride (CPCl) was incorporated into liposomes for the systemic delivery of low molecular weight heparin . These liposomes were prepared using the thin film hydration method, with CPCl acting as a penetration enhancer adding a positive charged to the nanocarriers. The positively charged liposomes showed increased ability to adhere to the negatively charged mucosal layer opening the tight junctions between epithelial cells, significantly enhancing permeation efficiency. This improved drug delivery resulted in a more effective therapeutic outcome, demonstrating the potential of CPCl-modified liposomes for enhancing bioavailability in transmucosal drug delivery. (Nallaguntla et al., 2016)

3 ENCAPSULATED SUBSTANCES

In this chapter, carotenoids that have been used in the experimental part of the thesis for encapsulation in elastic liposomes will be introduced and briefly described. Carotenoids are phytochemicals, or naturally occurring colorants found in fruits and vegetables. They belong to the group of isoprenoid pigments, which dissolve in organic solvents due to their hydrophobic nature. Their structure features an extended conjugated π -electron system specific for plant and microorganism synthesis. Humans therefore have to rely on the dietary uptake of carotenoids. Intake of carotenoids at dietary levels correlates with numerous beneficial health effects including help in prevention of cardiovascular problems and muscular degeneration. The simplest classification of carotenoids is into two main groups – provitamin A carotenoids (e.g. β -carotene) and non-provitamin A carotenoids (e.g. lutein). They exhibit several bioactivities including antioxidant, antibacterial and anti-inflammatory activity. Carotenoids are strong singlet oxygen quenchers and unpaired electron stabilizers. Within biological membranes, they can either be present in lipophilic environments or on the lipid/water interface scavenging lipid and aqueous phase radicals. (Kaulmann and Bohn, 2014)(Santos et al., 2019)(Yildiz et al., 2023)

Diverse bioactive properties of carotenoids certainly make them promising cosmetic formulation ingredients, especially with the growing demand for natural compounds. Carotenoids provide an interesting alignment between dermatological science and consumer expectations. Their cosmetic properties include antioxidant action, anti-inflammatory activity, hyperpigmentation improvement, skin regeneration and skin tone enhancement, just to name a few. Their ability to quench reactive oxygen species (ROS) makes them favorable options for skin cells protection from oxidative damage. Bioactivities assigned to carotenoids offer many benefits in cosmetic applications. Despite the limitations connected to stability of carotenoids, ongoing research and advances in technology can ensure that the unique properties of carotenoids remain preserved also in cosmetic formulations. (Adamantidi et al., 2025)

3.1 Lutein

Lutein is a plant pigment from the carotenoid family that is commonly found in fruits and vegetables. It can be present either in its free form, bound to proteins or as a monoester or diester. This so-called macular pigment is selectively accumulated in the center of the retina (macula), and its incidence is purely of dietary origin because the human body is incapable

of synthesizing it. Lutein is an antioxidant possessing numerous beneficial properties, including the ability to reduce oxidative stress. There are also inconsistent but arguable associations between high intake of lutein and lower risk of developing cardiovascular diseases. Furthermore, in some patients with different eye pathologies, the supplementation of lutein showed a certain level of improvement in the visual function. (Granado et al., 2003) The limitations of lutein include its low stability when exposed to heat or light and poor water solubility. These limitations can be overcome by using nano-encapsulation in liposomes and/or elastic liposomes, which can improve stability and bioavailability of lutein. (Zhang et al., 2023)

Lutein is a dihydroxy derivate of α -carotene, having a hydroxyl group at both terminal rings of the molecule which increases its polarity and changes its characteristics during processes of absorption, metabolism and deposition in the tissues. (Granado et al., 2003)

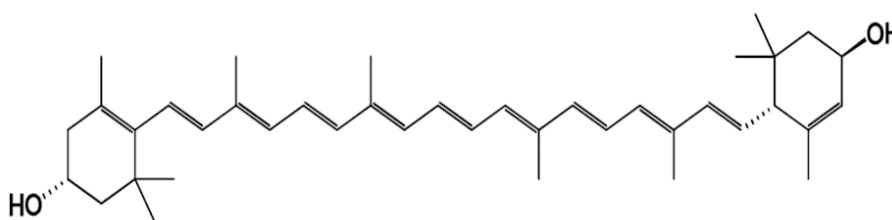


Figure 7: Structure of lutein

(Lockwood and Gross, 2005)

3.2 Asthaxanthin

Astaxanthin is a bright red-orange pigment that also belongs to the carotenoid family. As the remaining members of the group, it has powerful antioxidant activity, which is stronger than that of vitamin E or coenzyme Q10. Due to its powerful antioxidant properties, it can boost the immune system and improve muscle endurance. It has the ability to target mitochondria, regulate energy metabolism, improve muscle function and reduce oxidative stress, thereby protecting the body from inflammatory and oxidative damage. (Hasani et al., 2024) Multiple *in vitro* experiments also indicate that it is a powerful neutralizer of reactive oxygen and nitrogen molecules, including singlet oxygen, as well as single- and two-electron oxidizers. It is mainly synthesized by various algae, bacteria and fungi and its highest levels in nature have been reported in a chlorophyte alga called *Haematococcus pluvialis*. *Haematococcus pluvialis* is a freshwater green alga producing and accumulating astaxanthin under oxidative

4 PREPARATION METHODS

There are numerous approaches to how liposomes and elastic liposomes can be formulated and prepared. The process of preparation has a crucial impact on the characteristics of the vesicles. Below are listed some of the most commonly used preparation methods. (Nsairat et al., 2022) For the purposes of this thesis, only lipid film hydration method was used to prepare transferosomes.

4.1 Lipid film hydration method

Hydration of the lipid film is one of the most frequently used liposome preparation methods. Phospholipid mixture (vesicle-forming ingredients) is dissolved using an organic solvent such as chloroform or ethanol in a round-bottom flask. Afterwards, the solvent is vaporised above the lipid transition temperature and under vacuum using a rotary vacuum evaporator. A thin film is created on the walls of the glass flask and the film is then rehydrated in a buffer solution by rotation, producing liposomes. The desired size of liposomes can be reached using sonication, which effectively reduces size of the swollen vesicles and homogenizes the solution. The incorporation of encapsulated substance depends on its hydrophobicity/hydrophilicity. Lipophilic substances are added at the beginning of the process together with vesicle-forming ingredients while hydrophilic substances are added during the process of hydration. (Opatha et al., 2020) (Kuznetsova et al., 2022) (López-Cano et al., 2021)

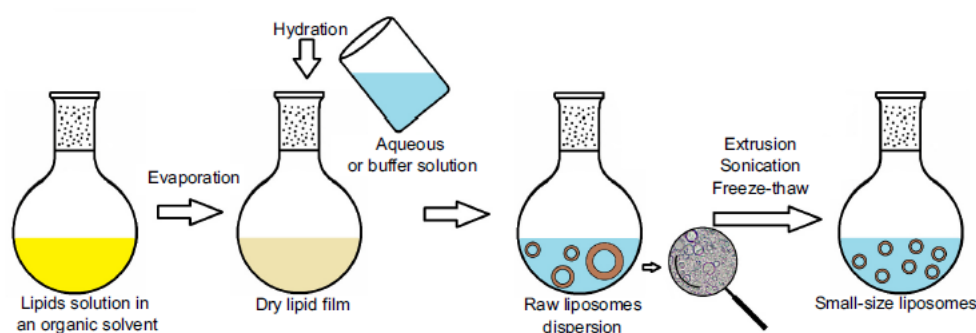


Figure 10: Lipid film hydration method

(Dymek and Sikora, 2022)

4.2 Reverse-phase evaporation method

The first step in this method is to prepare a lipid film of liposome-forming components dissolved in methanol or chloroform on the walls of a glass flask using the rotary evaporator.

The formed film is re-dissolved in organics solvent and aqueous buffer is added. Afterwards, the organic solvent is vaporised, and aqueous dispersion of liposomes is prepared. The dispersion can be further processed using sonication, extrusion or freeze-thawing. (López-Cano et al., 2021)

4.3 Dehydration-rehydration method

This method concentrates on creating new liposomes from liposomes that have already been made. The first step is dehydration of lipids followed by their controlled rehydration which can produce MLVs and SUVs. (López-Cano et al., 2021) Firstly, the lipids are dispersed at a low concentration into an aqueous solution containing the desired drug and afterwards sonicated. The water is evaporated, creating a multilayered film and the process is finalized by hydration, forming vesicles encapsulating the drug. (Nsairat et al., 2022)

4.4 Vortex-sonication method

Vesicle-forming ingredients – phospholipids, edge activator and the encapsulated substance are mixed in a buffer solution. The mixture is vortexed until a milky suspension is obtained. The cloudy appearance indicates the presence of vesicles. This process is followed by sonication in a sonication bath for a desired time and the procedure is finalized by extrusion through a polycarbonate membrane. (Opatha et al., 2020)

4.5 Ethanol injection method

Phospholipid, edge activator and lipophilic drug are dissolved in ethanol forming an organic phase which is injected in form of droplets into an aqueous phase consisting of phosphate buffer and a hydrophilic drug at a temperature of 45 – 50 °C. Ethanol is removed using vacuum evaporator and particle size reduction is done for example by sonication. (Opatha et al., 2020)

5 LIPOSOME CHARACTERIZATION

The necessity for optimization of liposomal and transferosomal formulation makes it crucial to develop optimal methods for their characterization and analysis. There are numerous methods used to determine the characterization parameters of mentioned vesicles including size, size distribution, polydispersity, zeta potential, encapsulation efficiency, degree of deformability and permeability measurements. (Opatha et al., 2020)

5.1 Size of liposomes

There are several techniques that can be used to determine the liposome size. Probably the most commonly used one is the dynamic light scattering technique (DLS). It is a non-invasive method which allows testing of the whole sample volume without changing or destroying it. It is quite popular mainly because of its simplicity and efficiency. However, the main disadvantage of this method is that the signal from large particles with much greater scattering intensity could overlay the small liposome signal. (Dymek and Sikora, 2022) Changes in the size during storage play a crucial role in determination of the formulation stability. Vesicles smaller than 40 nm tend to fuse due to high curvature of their membranes and much larger vesicles are more likely to aggregate through van der Waals interactions because of large membrane contact area. (Opatha et al., 2020)

5.2 Zeta potential

Zeta potential is one of the indicators of liposome stability. Zeta potential is normally determined by an electrophoretic mobility technique using Malvern Zetasizer. Systems with the potential value between -30 mV and $+30$ mV are considered reasonably stable and non-aggregating. However, this is only supporting indicator of stability. (Dymek and Sikora, 2022)

5.3 Antioxidant activity

In the process of transferosome preparation with the aim of developing efficient drug carriers, it is important to consider not only the amount of drug that the formulation can encapsulate, but also the stability and functionality of the encapsulated substance after incorporation into the vesicles. Therefore, in case of encapsulation of antioxidants it is crucial to assess antioxidant activity. This can be done by ABTS assay. ABTS assay is a standard colorimetric assay based on the principle of color change due to electronic transition

in atoms or molecules. The procedure is based on the assessment of the antioxidants' ability to scavenge the radical cation $\text{ABTS}^{\cdot+}$ (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)). This radical is produced when ABTS is converted to $\text{ABTS}^{\cdot+}$ by a strong oxidant. Antioxidants are then able to neutralize the radical cation and therefore this assay is used to assess the antioxidant activity. A blue-green chromophore is formed with maximum absorption at 734 nm. Both lipophilic and hydrophilic materials can be analyzed using ABTS assay because the ABTS cationic radical is soluble in organic but also aqueous solvents in a wide range of pH. (Christodoulou et al., 2022)

5.4 Cytotoxicity

Cytotoxicity testing is crucial for the determination of safety of liposomes in formulations and systems coming into contact with human cells. The viability cells after being in contact with liposomal formulations can be determined using a number of different procedures. For instance, Camera et al. tested selected liposomes using two procedures with different concentrations of liposomes. Firstly, cell death and viability of Hep G2 cells exposed to liposomes was assessed by lactate dehydrogenase release assay (LDH) and MTT assay 24 h after exposure. Additionally, potential cytotoxicity was tested on human primary blood leukocytes (PBMC) which was labeled as more relevant compared to standard Hep G2 assay. (Della Camera et al., 2021)

6 DERMAL AND OPHTHALMOLOGICAL USE OF ELASTIC LIPOSOMES

6.1 Skin delivery systems

Skin forms an efficient protective barrier between the human body and the external environment. Its functions include maintaining homeostasis, preventing excessive water loss and regulating entrance and output of different substances. The unique and complex structure of the skin is effective at blocking the entry of most topically applied drugs, especially hydrophilic ones. In general, the structure of the skin makes it easier for lipophilic substances with low molecular weight to penetrate the skin. Nanosized delivery systems provide advantages in overcoming this barrier and studies show that elastic liposomes are especially effective in transdermal drug delivery as they contain a high proportion of biocompatible excipients and possess the unique ability to deform and penetrate through the *stratum corneum* into deeper skin layers. One of the main advantages of these vesicles is their deformability since it allows them to penetrate through pores smaller than their own size. (Kotla et al., 2017) (Fernández-García et al., 2020) Thanks to higher drug loading capacity, increased stability and better permeation through skin, elastic liposomes have become noteworthy and promising delivery systems for pharmaceutical and cosmetic active ingredients. (Matharoo et al., 2024) However, it needs to be stated that their efficacy in dermal delivery is significantly influenced by many parameters including size, surface charge, number of layers and flexibility of these vesicles. (Dymek and Sikora, 2022)

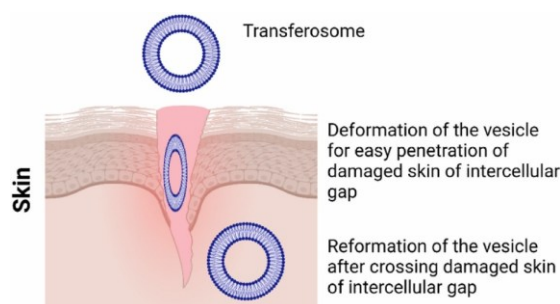


Figure 11: Transferosome permeation through the skin

(Singh and Awasthi, 2023)

In pharmaceutical practice elastic liposomes are used in the development of antipsoriatic therapeutics. For instance, Yadav et al. prepared a system of triamcinolone acetonide loaded vesicles in a carbopol gel and tested the therapeutical benefits. This system exhibited

distinguishably better penetration and distribution in rat skin compared to conventional liposomes and triamcinolone acetonide ethanolic gel. Another medicinal prepare prepared by Dhavale et al. contained fluocinolone acetonide. Again, vesicles encapsulating this drug showed high transdermal flow and improved cytotoxicity. (Yadav et al., 2021) (Dhavale et al., 2021) (Singh and Awasthi, 2023)

Apart from pharmaceutical use, transferosomes offer many advantages also in cosmetic industry, especially in case of complex formulations requiring suitable stabilization of unstable active ingredients. Transferosomes can be used in acne treatment for the encapsulation of clindamycin or vitamin C, also to encapsulate UV protectants and anti-wrinkle agents. (Rakesh et al., 2021) For example, Rodriguez et al prepared retinyl palmitate loaded transferosomes that exhibited increased skin penetration compared to free retinyl palmitate control and concluded that transferosome formulations are suitable candidates to increase the delivery of lipophilic ingredients to the epidermis. (Pena-Rodríguez et al., 2020) According to Pahwa et al, transferosomal systems have great potential in the cosmetic industry because they provide effective drug delivery while reducing dose frequency due to enhanced loading capacity and efficacy. (Rakesh et al., 2021)

6.2 Ocular applications

The ocular region is considered the most favorable site for the topical administration of drugs aimed at treating eye disorders. However, the efficacy of the drug administrated this way is frequently reduced by the eye's complex defense mechanisms, which can significantly decrease drug penetration. Ocular fungal infections are among the most prevalent eye diseases, caused by various fungal species. In the anterior part of the eye, liposomal formulations aim to improve corneal penetration, while in posterior part applications, their main aim is to prolong drug residence time, reduce toxicity, and ensure precise targeting of the drug to retinal pigment epithelium. A key obstacle in developing an effective therapeutic system is maintaining adequate drug concentration at the targeted site for a sufficient duration. The cornea and conjunctiva act as epithelial barriers, limiting the absorption of drugs into the eye. Additionally, one of the major drawbacks of ocular drug delivery systems is the rapid loss of medication following administration into the eye. (Mehendiratta et al., 2024)

Encapsulation of drugs in nanocarriers is one of the possible solutions to the challenges that traditional drug formulations bring. Vesicular drug delivery systems in ophthalmic applications offer sustained and controlled drug release on the corneal surface while

minimizing enzymatic degradation in tears and corneal tissues. Lipid-based nanocarriers are prospective drug delivery tools offering advantages such as drug protection, site-specific targeting, controlled release and enhanced bioavailability. (Das et al., 2022) Liposomes enhance drug absorption by establishing close contact with the cornea and conjunctiva, but the efficacy is substantially determined by the lipid composition in liposomes. The primary mechanisms involved in this interaction include adsorption, endocytosis, fusion, and lipid exchange. To further improve ocular bioavailability, positively charged nanocarriers can be formulated and used because their strong electrostatic attraction to the negatively charged corneal epithelium improves adhesion. (Mehendiratta et al., 2024)

Liposomes with edge activators and penetration enhancers could further boost the performance of nanocarrier drug delivery systems for ophthalmologic applications. These molecules include surfactants which can do both – promote penetration as well as render flexibility. In the case of surfactants, the main problem is their irritation potential. For instance, benzalkonium chloride can efficiently enhance drug transport through cornea when incorporated into liposomes, however it also causes severe corneal damage. (Tasharrofi et al., 2022)

7 CATIONIC ELASTIC LIPOSOMES - CURRENT STATE OF THE ADDRESSED ISSUE

Extensively studied for various applications, EL have been used for topical, transdermal, nasal, and oral drug delivery to enhance efficacy and efficiency. They have gained significant attention, especially during recent years, thanks to their advantageous properties like high drug encapsulation efficiency, improved skin permeation and penetration and exceptional deformability. These properties enable EL to optimize drug release, absorption, and therapeutic effects more effectively than traditional drug delivery systems. (Hussain et al., 2017)

Caritá et al. developed elastic cationic liposomes for dermal delivery of vitamin C combining soybean phosphatidylcholine, cationic lipid DOTAP and cholesterol. Cationic lipid was used to enhance the uptake of negatively charged vitamin C and in addition, to minimize aggregation due to the presence of electrical charge. A nonionic surfactant Polysorbate 80 was added to the formulation as an encapsulation efficiency enhancer and penetration enhancer. As a result, these liposomes not only showed good stability but also increased skin delivery, which seemed directly related to the interaction between vitamin C and cationic lipids. (Caritá et al., 2023)

Kuznetsova et al. prepared cationic liposomes containing phosphatidylcholine and cationic surfactants (cetyltrimethylammonium bromide, 3-hexadecyl-1-hydroxyethylimidazolium bromide, 1-(butylcarbamoyl)oxyethyl-3-hexadecylimidazolium bromide, and hexadecylmethylpyrrolidinium bromide for transdermal application of nonsteroidal anti-inflammatory drugs (ketoprofen, rhodamine B and diclofenac). The incorporation of cationic surfactants resulted in improvement of physiochemical properties and stability. *In vitro* testing showed that the modified nanocarriers exhibited prolonged drug release directly related to the surfactant structure. Cationic liposomes also exhibited better transdermal drug penetration. (Kuznetsova et al., 2022)

In a study by Choi et al., cationic elastic liposomes (ELs) were employed as delivery vehicles for the administration of growth factors in the treatment of chronic wounds. The findings demonstrated that topical application of these cationic ELs significantly improved the healing process, exhibiting both rapid and sustained therapeutic effects. Notably, the incorporation of highly skin-permeable, low-molecular-weight protamine (LMWP)-fused growth factors complexed with hyaluronic acid (HA) into the cationic ELs, which resulted

in a synergistic enhancement of diabetic wound healing. This formulation facilitated prolonged and efficient skin permeation of the encapsulated therapeutic agents. The elastic liposomes were fabricated *via* a microfluidization technique and were composed of hydroxypropyl cellulose (HPC), cholesterol, and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP). (Choi et al., 2017)

A review of the available literature reveals that elastic liposomes formulated with cationic lipids are more commonly studied than those incorporating cationic surfactants. In contrast, there is a notable scarcity of research focused on the preparation and application of cationic transferosomes containing cationic surfactants. This gap highlights a significant potential for future research, particularly given the promising advantages and effective applications demonstrated by the limited available studies.

8 AIM OF THE THESIS

The aim of this thesis was to first review the available literature regarding cationic liposomes (including elastic liposomes), and then to prepare and characterize positively charged elastic liposomes made of neutral phospholipids and cationic surfactants as edge activators.

The experimental part of the thesis is aimed at the optimal preparation conditions and formulation to produce stable and non-toxic elastic liposomes for encapsulation of carotenoids, namely β -carotene, astaxanthin and lutein.

II. PRACTICAL PART

9 MATERIALS

9.1 Instruments and devices

- Rotary evaporator Hei-VAP (Heidolph, Germany)
- Zetasizer Nano-ZS90 (Malvern, UK)
- Ultrasonic homogenizer (Hielscher, Germany)
- Analytical balances (Ohaus, United States)
- Magnetic stirrer (Ohaus, United States)
- Automatic pipette (Eppendorf, Germany)
- Dialysis membranes and clamps (Spectrum Laboratories, United States)
- Tensiometer (Kruss, Germany)
- See system (Advex Instruments, Czech republic)
- Multifunctional modular reader INFINITE 200 PRO (Tecan Group Ltd., Switzerland)
- MTT cell proliferation assay kit, Duchefa Biochemie B. V., Netherlands
- Infinite M200 Pro NanoQuant (Tecan, Switzerland)
- Olympus phase contrast microscope (IX 81)
- Laboratory glassware and common laboratory equipment

9.2 Chemicals and samples

- Soy L- α -phosphatidylcholine (95%) (Avanti, United States)
- 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP) (Avanti, United States)
- Cetyltrimethylammonium bromide (VWR Chemicals, United States)
- Hexadecylamine (Fluka, Switzerland)
- Benzalkonium chloride (Sigma Aldrich, United States)
- Cetylpyridinium chloride (VWR Chemicals, United States)
- α -Tocopherol (Sigma Aldrich, United States)
- β -carotene (Sigma Aldrich, United States)
- Lutein (Thermo Scientific, United States)
- Astaxanthin (Avanti, United States)
- PBS (BioSera, France)
- Sodium Chloride (Penta, Czech republic)
- Ethanol (Penta, Czech republic)

- Methanol (Chemlab NV, Belgium)
- Chloroform (VWR Chemicals, United States)
- Acetone (Penta, Czech republic)
- 2,2'-azino-bis(3-ethyl-2,3-dihydrobenzothiazole-6-sulfonate) (ABTS; >98%; HPLC), Sigma Aldrich (Merck Life Science)
- Ammonium persulfate ((NH₄)₂S₂O₈; >98%), Sigma Aldrich (Merck Life Science)
- Ultrapure water (UPW)
- Dulbecco's Modified Eagle's Medium (Biosera, France)
- Sodium hydrogen carbonate (Penta, Czech Republic)
- Calf serum (CS, mycoplasma-free; Biosera, France)
- Penicillin/Streptomycin (Biosera, France)

9.3 Solutions of starting materials used to prepare elastic liposomes

Table 2 shows all the types of components, and their concentrations prepared and used for formation of all types of samples studied in the thesis.

Table 2: Concentrations of starting materials and solvents used

Solution	Concentration [mg/ml]	Solvent
SPC	10	ethanol
CTAB	5	ethanol
CPCl	5	ethanol
HEX	5	ethanol
BAC	5	ethanol
Tocopherol	5	ethanol
Ascorbyl palmitate	5	ethanol
Tocopheryl acetate	5	ethanol
Beta-carotene	5	acetone
Astaxanthin	0,2	chloroform:methanol (7:3)
Lutein	0,2	ethanol

10 METHODS

10.1 Characterization of edge activators (surfactants)

Prior to use in liposomal formulations, basic characterization of surfactants used was performed.

Surface tension and CMC value Surface tension γ was measured with Tensiometer KRÜSS EasyDyne 20 using Wilhelmy plate method. Water solutions of cationic surfactants Cetytrimethylammonium bromide (CTAB), Cetylpyridinium chloride (CPCL) and Benzalkonium chloride (BAC) were prepared in seven different concentrations (0,5; 0,3; 0,1; 0,03; 0,01; 0,005; 0,001 %). Hexadecylamine (HEX) is not a true surfactant, and it is not soluble in water, therefore it was not included in the surface tension measurement.

As a reference substance, demineralized water was employed with tabulated $\gamma = 72 \text{ mN/m}$ at 25°C . Surfactant solution ($\sim 25 \text{ ml}$) was poured into a glass dish and placed into the instrument. During measurement, the temperature was kept at $25.0 \pm 0.2^\circ\text{C}$. At each concentration 5 readings of γ value were obtained. The measured data were evaluated by plotting average surface tension values, $\gamma \text{ [mN/m]}$, versus surfactant concentration, $c \text{ [%]}$. The CMC was then calculated through intersection of the two regression straight lines passing through the pre- and post-CMC regions.

10.2 Preparation of empty elastic liposomes

Within the framework of preformulation study, empty elastic liposomes (EL) were prepared using the lipid film hydration method in combination with sonication. Elastic liposomes consisted of soy phosphatidylcholine (SPC) and an edge activator (cationic surfactant). The following surfactants were used: Cetytrimethylammonium bromide (CTAB), Cetylpyridinium chloride (CPCL), Hexadecylamine (HEX) and Benzalkonium chloride (BAC). The EL also contained three types of antioxidants: tocopherol, tocopheryl acetate or ascorbyl palmitate. Compositions of empty EL with CTAB (different SPC:CTAB ratios) are listed in Table 3. Correspondingly to CTAB, formulations of EL with other edge activators (CPCL, BAC, HEX) were prepared and are labeled SPC:CPCL, SPC:BAC and SPC:HEX below in the text. The preparation procedure was carried out as follows:

- Stock solutions of all membrane-forming components (SPC and edge activator) were mixed in desired ratios. The total amount of membrane-forming components in each of the formulations was 100 mg.

- Using glass pipettes, the solutions were transferred into a round-bottom flask.
- Rotary vacuum evaporator conditions were set as follows: bath temperature 35 °C and the number of rotations 100 rpm and evaporation was started.
- After the evaporation was completed (approximately 15-20 minutes), the flask containing dry lipid film was left in the exhaust for at least 10 minutes for additional drying.
- Using a glass pipette, 10 ml of the hydration solution was added to the flask, and it was again placed on the evaporator, this time without evacuation. The lipid film in the flask was hydrated under rotation in the water bath (35 °C, 100 rpm) for 15-20 minutes.
- The primary dispersion formed during the process of hydration was transferred into a glass vial and sonicated on an ice bath for 4 minutes (80% amplitude, 50:50 cycling) using a sonication probe.
- The prepared EL were then stored in the fridge at 4 °C covered in aluminum foil.

Table 3: Composition of empty EL with CTAB (the corresponding formulations were prepared with CPCI, BAC and HEX as edge activators)

Formulation	SPC:CTAB ratio	SPC [mg]	Edge activator [mg]
SPC:CTAB_95:5	95 : 5	95,0	5,0
SPC:CTAB_97,5:2,5	97,5 : 2,5	97,5	2,5
SPC:CTAB_99:1	99 : 1	99,0	1,0
SPC:CTAB_99,75:0,25	99,75 : 0,25	99,75	0,25

10.2.1 Stabilization with antioxidants

In order to increase stability of EL, three different antioxidants – ascorbyl palmitate (AP), tocopherol (TOK) and tocopheryl acetate (TOAC) were incorporated into two different vesicle compositions. The same amount of each antioxidant was added, 1 ml of solution into the mixture of membrane-forming components. This volume corresponded to 5 mg of antioxidant per formulation. Size and zeta potential of vesicles were measured and compared in order to choose the most suitable stabilizing agent.

10.2.2 Sonication conditions

To evaluate the impact of sonication, three samples underwent multiple rounds of sonication with adjusted parameters, and the resulting particle sizes were compared. Based on these results, the final sonication conditions were established.

Initially, all samples were sonicated using an ultrasonic probe homogenizer for 2 minutes at 80% amplitude with a 50:50 cycle, while kept on ice. After evaluating the measurements, it was decided to proceed with modified conditions: 4 minutes of sonication at the same amplitude and cycle, also on ice. Without cooling, the samples overheated, which is undesirable; hence, all sonication was carried out in an ice bath to maintain temperature stability.

10.3 Preparation of EL with carotenoids

Three different carotenoids were chosen to be encapsulated by elastic liposomes – β -carotene (BC), lutein (LUT) and astaxanthin (AX). The preparation procedure was the same as described in chapter 10.2 concerning preparation of empty EL. The only difference was that each of the carotenoids was added to the formulation in the first step together with membrane-forming components.

Firstly, β -carotene was encapsulated into EL. The main aim was to encapsulate as much carotenoid as possible, precisely 10, 20 and 30 mg of β -carotene in EL with CTAB and CPCL. Afterwards, EL with only 10 mg of β -carotene were prepared with all edge activators and all formulations were compared. They are listed in table 4. As a hydration solution PBS was used.

Table 4: Formulations of β -carotene loaded ELs

Formulation	SPC [mg]	Edge activator	Edge activator [mg]	Carotenoid	Carotenoid [mg]
SPC:CTAB 95:5	95	CTAB	5	BC	10,20,30
SPC:CTAB 99,75:0,25	99,75		0,25		
SPC:CPCL 95:5	95	CPCL	5		10,20,30
SPC:CPCL 99,75:0,25	99,75		0,25		
SPC:HEX 95:5	95	HEX	5		10
SPC:HEX 99,75:0,25	99,75		0,25		
SPC:BAC 95:5	95	BAC	5		10
SPC:BAC 99,75:0,25	99,75		0,25		

Secondly, astaxanthin loaded EL were prepared, containing HEX or BAC as an edge activator. During the process of preparation, some samples were hydrated using water and some samples were hydrated using PBS. The influence of different hydrating solutions was compared. Compositions of samples are listed in Table 5.

Table 5: Formulations of astaxanthin loaded ELs

Sample name	SPC	Edge activator	Edge activator [mg]	Carotenoid	Carotenoid [mg]	Hydration solution
SPC:HEX 99,75:0,25	99,75	HEX	0,25	AX	2	PBS
	99,75		0,25			water
SPC:BAC 99,75:0,25	99,75	BAC	0,25		1	PBS
	99,75		0,25			water
SPC:BAC 95:5	95		5			PBS
	95		5			water

Thirdly, EL with HEX and BAC in combination acting as edge activators were prepared, loaded with β -carotene, astaxanthin or lutein as it is listed in Table 6. All these samples were hydrated with water. Two HEX:BAC ratios were prepared, one combining 0,25 mg of HEX and 0,25 mg of BAC and the other combining 0,75 mg of HEX and 0,25 mg of BAC.

Table 6: ELs combining HEX and BAC as edge activators

Sample name	SPC [mg]	Edge activator	Edge activator [mg]	Carotenoid [mg]	
SPC:HEX:BAC 99,5:0,25:0,25 BC10	99,5	HEX/BAC	0,25/0,25	BC	10
SPC:HEX:BAC 99,5:0,25:0,25 AX1	99,5	HEX/BAC	0,25/0,25	AX	1
SPC:HEX:BAC 99,5:0,25:0,25 LUT1	99,5	HEX/BAC	0,25/0,25	LUT	1
SPC:HEX:BAC 99:0,75:0,25 BC10	99	HEX/BAC	0,75/0,25	BC	10
SPC:HEX:BAC 99:0,75:0,25 AX1	99	HEX/BAC	0,75/0,25	AX	1
SPC:HEX:BAC 99:0,75:0,25 LUT1	99	HEX/BAC	0,75/0,25	LUT	1

In the next step, three samples were prepared using a cationic lipid DOTAP donating positive charge into the liposomes, instead of an edge activator. These were all loaded with 1 mg of lutein and hydrated with water.

Table 7: ELs with DOTAP

Sample name	SPC [mg]	DOTAP [mg]	Carotenoid	Carotenoid [mg]
SPC:DOTAP_50:50	50	50	LUT	1
SPC:DOTAP_90:10	90	10		
SPC:DOTAP_99:1	99	1		

Finally, based on the results obtained, six formulations were chosen as final formulations. These are described in the following chapter.

10.4 Final formulations of EL

Based on the data and information acquired during the preformulation study, six formulation compositions were chosen, prepared, characterized and compared, four of these containing surfactants as edge activators and two containing DOTAP. They are listed in Tables 8 and 9. All were hydrated using water.

Table 8: Final formulations of ELs with cationic surfactants

Formulation	SPC [mg]	Edge activator	Edge activator [mg]	Carotenoid	Carotenoid [mg]
SPC:HEX:BAC 99,5:0,25:0,25_BC	99,5	HEX/BAC	0,25/0,25	BC	2
SPC:HEX:BAC 99,5:0,25:0,25_AX	99,5	HEX/BAC	0,25/0,25	AX	2
SPC:HEX:BAC 99,5:0,25:0,25_LUT	99,5	HEX/BAC	0,25/0,25	LUT	2
SPC:HEX:BAC 99,5:0,25:0,25_AX+LUT	99,5	HEX/BAC	0,25/0,25	AX/LUT	2

Table 9: Final formulations of ELs with cationic lipid DOTAP

Formulation	SPC [mg]	DOTAP [mg]	Carotenoid	Carotenoid [mg]
SPC:DOTAP 90:10_LUT	90	10	LUT	2
SPC:DOTAP 99:1_LUT	99	1	LUT	2

The preparation procedure for these formulations is described in Chapter 10.2. After the preparation, the samples underwent the process of dialysis for approximately 12 hours followed by physical, chemical and biological characterization.

10.5 Physical and chemical characterization of EL

10.5.1 Contact angles

Contact angles of empty EL drop on hydrophobic PTFE tape were observed using a See System (Advex instruments). Prior to measurement, each sample was homogenized by sonication, after which 5 μ l of the sample was deposited onto PTFE tape in the form of a droplet using automatic pipette. The picture of the drop was immediately captured to prevent evaporation, and the contact angle of the droplet was calculated using the corresponding software.

10.5.2 Size of vesicles

The size of EL was determined using the Malvern Zetasizer Nano-ZS90, a non-invasive technique based on the dynamic light scattering principle. This method measures the fluctuations in the intensity of scattered light caused by particles undergoing Brownian motion. By analyzing these fluctuations over time, the diffusion coefficient of the particles is determined, which is then used to calculate their size expressed at z-average diameter in nm. To measure size, 10 μ l of sample was dispersed in 1 ml of either H₂O, PBS or 0,9% NaCl solution, pipetted into a plastic cuvette, and placed into the measuring cell. Measurements were conducted at 25 °C in triplicates.

10.5.3 Zeta potential

Zeta potential was measured using the Malvern Zetasizer Nano-ZS90. The Zetasizer Nano software generates a frequency spectrum from which the electrophoretic mobility is determined, and subsequently, the zeta potential is calculated.

When measuring zeta potential, 15 μ l of the sample was pipetted into 4 ml of either H₂O, PBS or 0,9% NaCl solution, mixed and dosed into the special cuvette for zeta potential analysis.

10.5.4 Antioxidant activity

Determination of antioxidant activity was carried out by ABTS assay. For the purposes of the experiment, 24-well plates (Greiner 24-well flat bottom transparent, polystyrene) were used. Each well was filled with 2000 μl of the ABTS^{•+} solution (absorbance 0,7-0,75) and 30 μl of the sample dispersion (or ultra-pure water for the blank). The analysis was done in triplicates for each sample and each well was read three times in total. Absorbance was measured after 1, 2, and 4 hours. To determine the absorbance contribution of the sample dispersions at the given dosage, the absorbance was measured for a mixture of 2000 μl ultra-pure water and 30 μl of the corresponding sample. The corrected absorbance Abs' was calculated using equation (1), where $Abs(i)$ represents the absorbance measured for the ABTS^{•+} solution mixed with EL sample and $Abs(0)$ absorbance determined for the sample mixed with water.

The antioxidant activity was then calculated using equation (2) for the percentage inhibition of radical concentration, where $Abs' (initial)$ is the corrected absorbance value of the reference ABTS^{•+} solution without a EL sample and $Abs' (final)$ is the absorbance of the sample at a given time interval.

$$Abs' = Abs_i - Abs_0 \quad (1)$$

$$\%RI = \frac{(Abs'_{initial} - Abs'_{final})}{Abs'_{initial}} \cdot 100 \quad (2)$$

Performance of the samples after storage was verified and, using above described method, antioxidant activity was also assessed on the vesicles that were stored for four weeks at 4 °C.

10.5.5 Long-term stability of vesicles

Stability of six final formulations of EL was followed over a period of one month by measuring size and zeta potential in water, PBS and 0,9 % NaCl solution. The formulations were kept in dark at 4 °C.

10.6 Biological characterization of EL

10.6.1 Cytotoxicity

Cytotoxicity was determined using the MTT assay on the mouse embryonic fibroblast cell line NIH/3T3, in accordance with ISO 10993-5 standard. The MTT Cell Proliferation Assay Kit (Duchefa Biochemie B.V., Netherlands), containing tetrazolium salt was used. Absorbance was measured at 570 nm, with a reference wavelength of 690 nm, using an Infinite M200 Pro NanoQuant spectrophotometer (Tecan, Switzerland).

Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Biosera, France), supplemented with sodium hydrogen carbonate (Penta, Czech Republic), 10% bovine calf serum (CS, mycoplasma-free; Biosera, France), and 1% Penicillin/Streptomycin (Biosera, France). Cultures were incubated at 37 °C in a humidified atmosphere with 5% CO₂.

The cytotoxicity testing was carried out over a period of three days. On the first day, cell suspensions at a concentration of 1×10^5 cells/ml were seeded into cell culture plates (TPP, Switzerland). On the second day, test samples were added to the cultured cells at concentrations of 1%, 5%, and 10% (v/v) and on the third day, cells were examined microscopically to assess morphological changes, and the MTT assay was performed to determine cell viability.

The results are expressed as the percentage reduction in cell viability relative to control cells cultivated in medium without extracts of the tested materials. Cell morphology was monitored using an inverted phase-contrast microscope (Olympus IX 81).

11 RESULTS AND DISCUSSION

The experimental work of this study has been carried out in two main parts – preformulation study, including characterization of used edge activators (surfactants), and selection and characterization of final formulations.

11.1 Preformulation study

The main aim of the preformulation study was to gather data and information about manufacturing technology and composition of cationic EL composed of neutral phospholipid SPC and cationic surfactants, and evaluate the impact of the composition on the properties of ELs. Firstly, the cationic surfactants used as edge activators were characterized. Afterwards, empty ELs were prepared combining different SPC : edge activator ratios. These were then characterized in terms of size and zeta potential. The final part of the preformulation study focused on preparation of cationic EL loaded with carotenoids, namely β -carotene, astaxanthin and lutein and their characterization.

11.1.1 Characterization of surfactants

The first part of the preformulation study included characterization of surfactants by measuring their surface tension and calculating critical micellar concentration (CMC). From the acquired data, correlations between surface tension and concentration of the surfactant were plotted (Figures 12, 13 and 14), and critical micellar concentration (CMC) of each of the surfactants was calculated (Table 10).

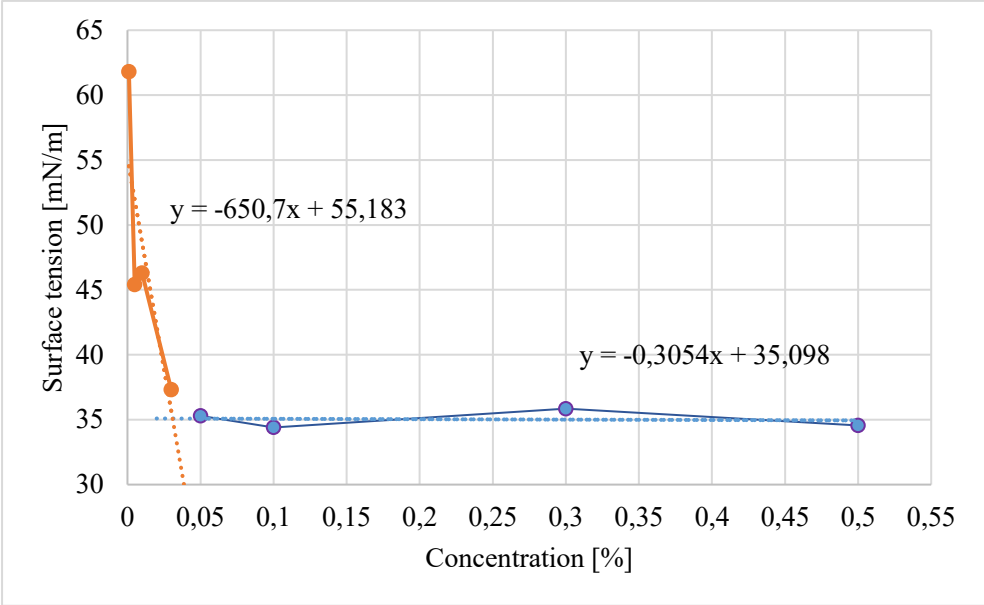


Figure 12: Surface tension curve of CTAB solutions

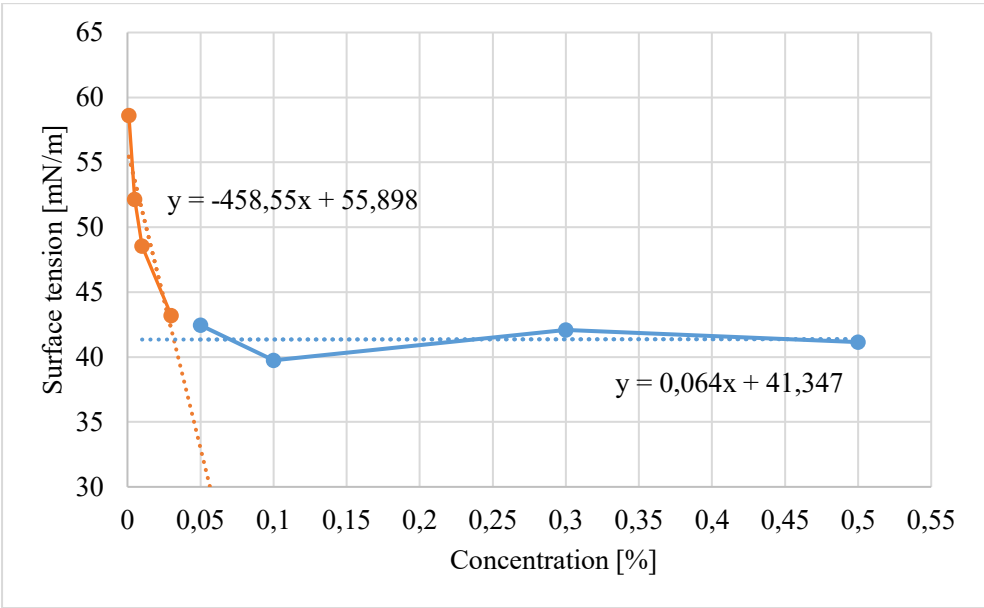


Figure 13: Surface tension curve of CPCL solutions

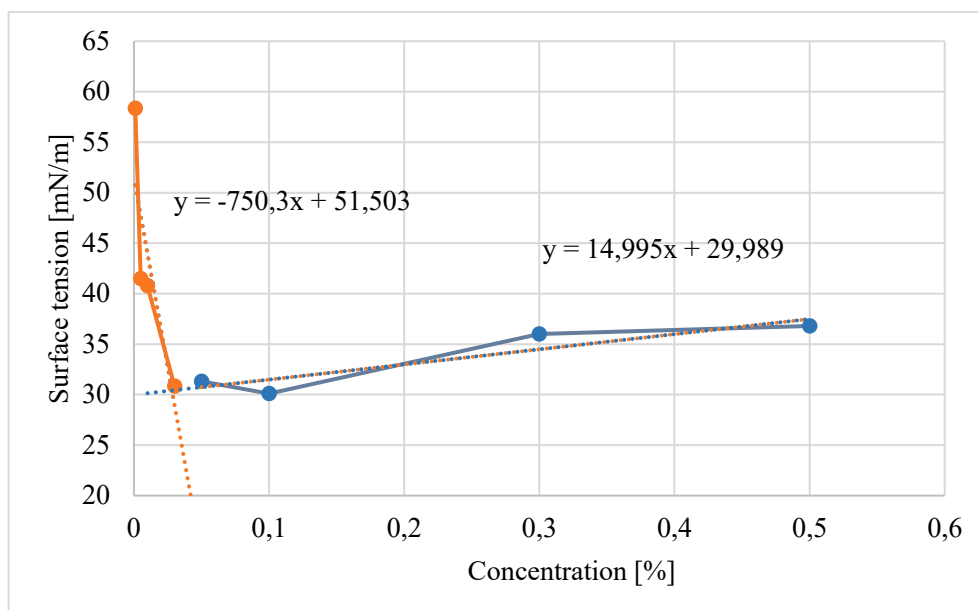


Figure 14: Surface tension curve of BAC solutions

The measured data for all three surfactant solutions showed that the critical micellar concentrations were 0,031 (CTAB), 0,032 (CPCL) and 0,028 % (BAC), which correspond to concentrations of 0,85 (CTAB), 0,94 (CPCL) and 0,82 (BAC) $\text{mmol}\cdot\text{l}^{-1}$ (Table 10). The determined values for CTAB a CPCL are consistent with the literature and exhibit only minor variations. However, the value calculated for BAC differs. This could be due to differences in the lengths of alkyl chain in our surfactant compared to published values. In summary, the results suggest that the surfactants used may perform similarly when used as edge activators.

For hexadecylamine, which is a surfactant-like long-chain primary amine, CMC value was not determined. This is mainly due to its low solubility in water and presence of amine group, which tends to form hydrogen bonds and aggregates in a manner distinct from typical surfactants. Consequently, its aggregation behavior is not as straightforward as the one of conventional surfactants.

Table 10: CMC values of surfactants used

Surfactant	Molar mass [g/mol]	Calculated CMC [$\text{mmol}\cdot\text{l}^{-1}$]	CMC in literature [$\text{mmol}\cdot\text{l}^{-1}$]
CTAB	365	0,85	0,9*
CPCL	340	0,94	0,9**
BAC	376 (C12)	0,82	0,43***

* (Li et al., 2006) ; ** (OKADA et al., 2016) ; *** (Nandni and Mahajan, 2013)

11.1.2 Empty EL

Contact angles

The influence of edge activators on the properties of empty ELs was investigated by measuring the contact angle on the hydrophobic surface of PTFE. Figure 15 displays the results, which show that when the activator content increases, the contact angle decreases. This behavior is consistent with all surfactants employed. Edge activators, even at low concentrations, improved surface wetting of vesicles made of SPC alone (contact angle $\sim 101^\circ$), indicating a change in surface characteristics of the produced EL.

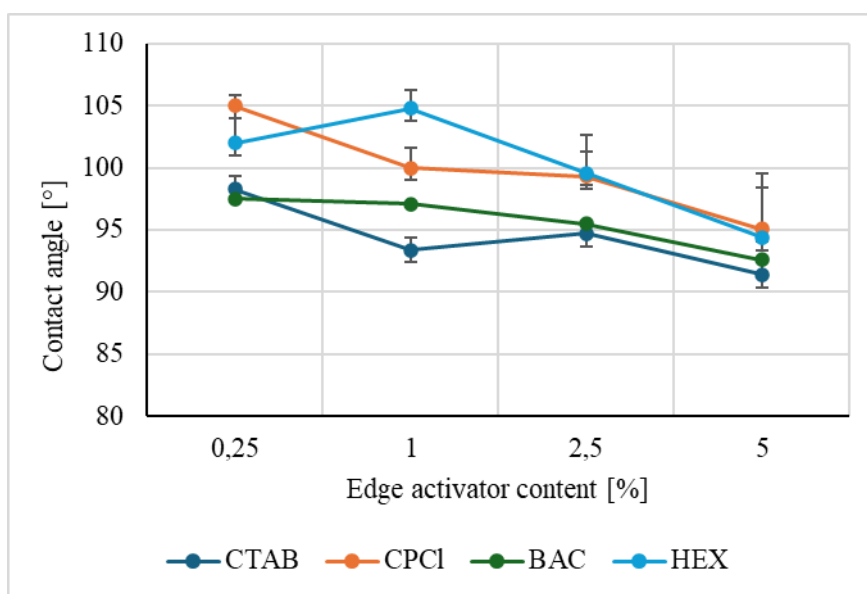


Figure 15: Dependence of contact angles on concentration of edge activators in empty EL measured on hydrophobic surface

Size and zeta potential of vesicles

The first series of EL formulations, which were prepared consisted only of a phospholipid and an edge activator in different ratios, without any encapsulated substances. The total amount of the membrane-forming components was always 100 mg.

Elastic liposomes containing SPC and CTAB (or CPCL or HEX or BAC) as an edge activator were prepared in four different ratios SPC : edge activator (95 : 5; 97,5 : 2,5; 99 : 1 and 99,75 : 0,25). Size of vesicles and their zeta potential were measured in aqueous

environment. The Figures 16 and 17 show data obtained for all the prepared formulations of empty EL.

In the Figure 16 data acquired on particle size, and their correlation with the composition of the EL are summarized. As the concentration of the edge activator decreased, vesicle size showed an increasing trend. This trend is obvious for all the formulations, irrespective of the edge activator used. However, in closer inspection of the data, behavior of EL with hexadecylamine is different in comparison with other formulations. For HEX containing EL at ratios 95 : 5; 97,5 : 2,5 and 99 : 1, the vesicle sizes were the smallest in comparison with remaining formulations. However, at the lowest activator content (99,75 : 0,25 ratio) the vesicle size increased significantly showing the biggest vesicles among the formulations (~ 350 nm). This documents that the behavior of HEX is different in comparison with other edge activators used, as it was already mentioned in part 11.1.1.

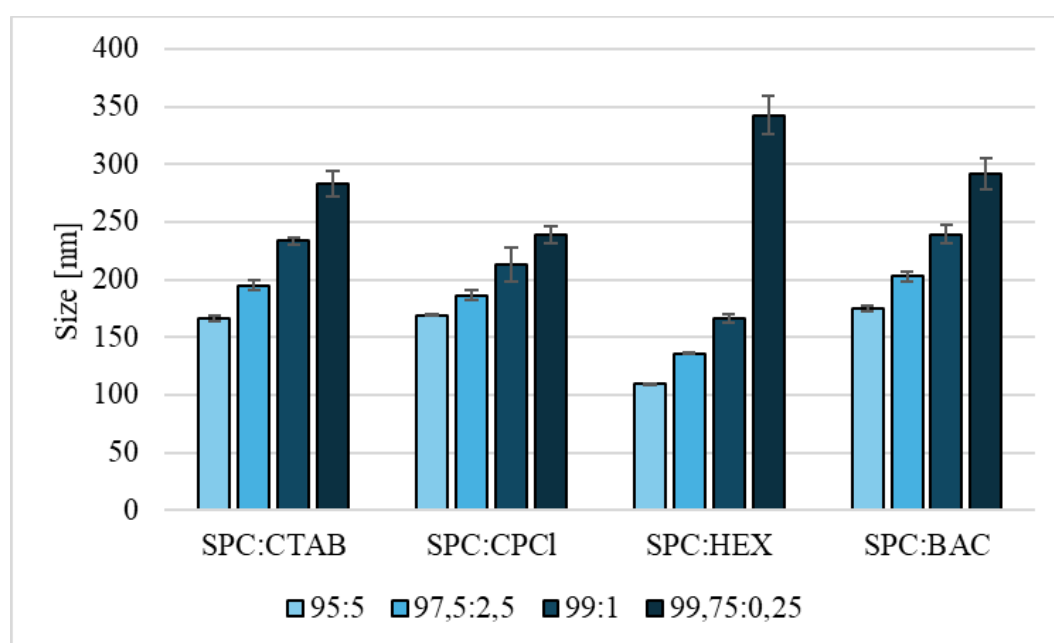


Figure 16: Size of ELs formulated with different ratios of SPC : edge activator, determined immediately after sample preparation, measured in water

The results of this part of the investigation are consistent with literature studies, which show that increased concentration of edge activator reduced EL size. This reduction is related to the ability activator to fluidize the lipid bilayer, which improves vesicle flexibility and supports smaller vesicle formation. (Opatha et al., 2020) However, when increasing activator concentration, careful optimization must be considered as too much activator can

result in vesicle instability or micelle production instead. As a result, maintaining vesicle integrity and the correct size requires an optimum activator concentration.

Data collected on zeta potential of studies empty EL are given in Figure 17. The Figure shows that in contrast to vesicle size, zeta potential decreased with lower edge activator concentrations, regardless of the type of edge activator used. Interestingly, at the lowest edge activator content the samples behaved differently. While in case of vesicles containing CTAB or CPCL, the zeta potential was negative at their lowest concentration, zeta potential of vesicles containing HEX or BAC remained positive.

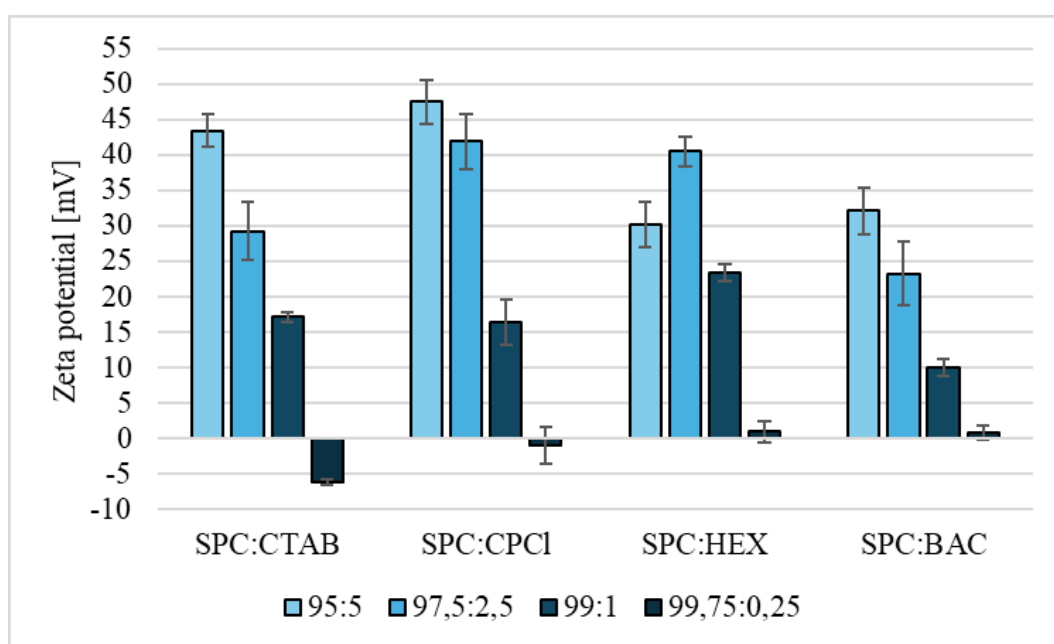


Figure 17: Zeta potential of EL formulated with different ratios of SPC : edge activator determined immediately after sample preparation measured in water

11.1.3 Influence of antioxidants on EL

Based on experience from previous work (Srnc, 2022), astaxanthin and also other carotenoids are sensitive to degradation. In order to maintain their long-term stability after encapsulation in liposomes, it is desirable to use antioxidants in the formulations. Hence, the influence of antioxidants on properties of empty EL was investigated within the preformulation study. In Table 11 there are listed and compared samples with different antioxidants used.

Table 11: Size and zeta potential of formulation SPC:CTAB with and without different antioxidants (TOK – tocopherol, TOAC – tocopherol acetate, AP – ascorbyl palmitate)

Sample name	Size [nm]	Standard deviation	Zeta potential [mV]	Standard deviation
SPC:CTAB 95:5	166,4	2,1	43	2,3
SPC:CTAB 95:5_TOK	174,0	1,6	56	2,0
SPC:CTAB 95:5_TOAC	176,9	2,1	52	1,2
SPC:CTAB 99:1	233,6	2,8	17	0,7
SPC:CTAB 99:1_AP	186,0	2,0	-28	1,1

The results on vesicle size are illustrated in Figure 18, which shows that in the formulation with 5 mg of edge activator (SPC:CTAB 95:5), type of antioxidant used had no impact on the size of the EL and in case of formulation with 1 mg of edge activator (SPC:CTAB 99:1), the size was reduced with addition of ascorbyl palmitate as an antioxidant. Zeta potential was more visibly influenced as can be seen in Figure 19. Formulation with 5 mg of edge activator showed an increase in zeta potential from ~40 mV to more than 50 mV which means more stable vesicles, while the zeta potential of formulation with 1 mg of EA changed from positive to negative.

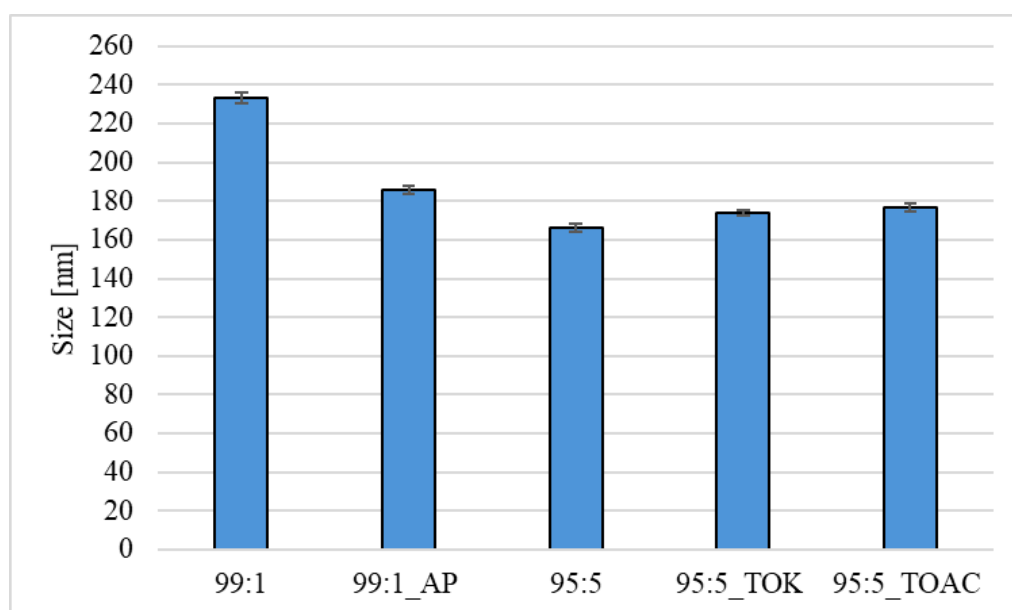


Figure 18: Sizes of SPC:CTAB vesicles without and with antioxidants

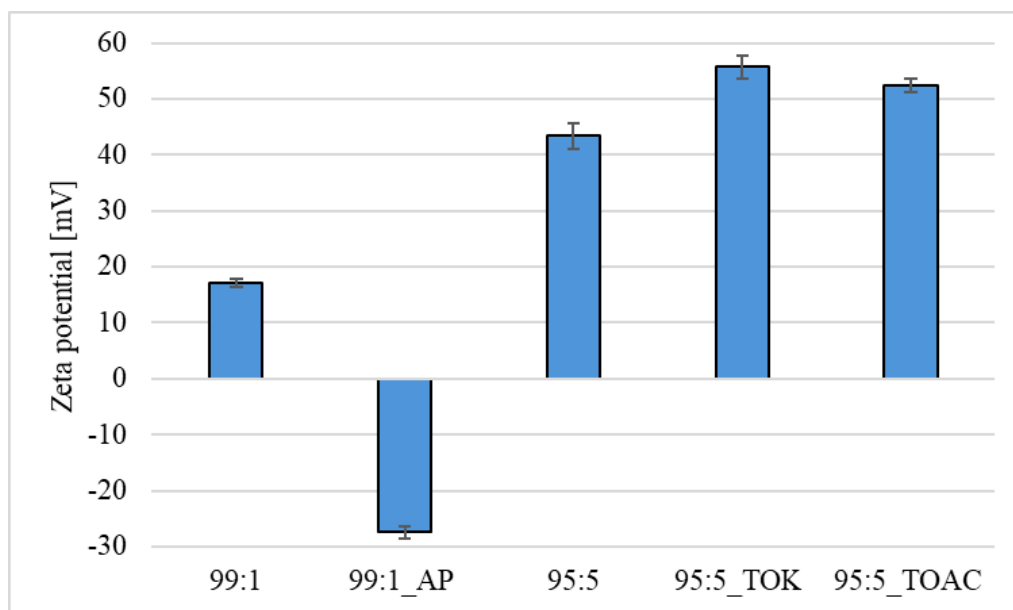


Figure 19: Zeta potentials of SPC:CTAB vesicles without and with antioxidants

Based on the observed results it was decided that all of the next formulations would contain tocopherol as a stabilizing agent. This decision was made based on the observation that tocopherol did not affect vesicle size but did increase zeta potential and therefore provided better stabilization.

11.1.4 Influence of sonication on EL

One of the key factors affecting the stability of EL is their particle size. In general, larger particles have a higher tendency to agglomerate and sediment, which reduces overall sample stability. Therefore, the goal is to produce smaller nanocarriers. This can be achieved by optimizing the sonication conditions, which was performed in this part of preformulation study. Table 12 and Figure 20 summarize sonication conditions and their influence on size and zeta potential of chosen samples.

Table 12: Influence of sonication conditions on vesicle size of EL

Sample Name	Sonication round	Sonication conditions				Size [nm]	Stand. deviation
		Time [min]	Amplitude	Cycle	Temp. [°C]		
SPC:CTAB 97,5:2,5	round 1	2	80	50:50	on ice	195,1	3,9
	round 2	2	80	50:50	on ice	181,2	1,0
SPC:CTAB 99,75:0,25	round 1	2	80	50:50	on ice	283,4	11,2
	round 2	2	80	50:50	on ice	241,1	2,12
	round 3	4	80	50:50	on ice	214,7	4,4
SPC:CPCL 99,75:0,25	round 1	2	80	50:50	35°C	239,2	7,2
	round 2	4	80	80:20	35°C	162,9	2,7
SPC:HEX 99,75:0,25	round 1	2	80	50:50	on ice	342,1	16,5
	round 2	2	80	80:20	on ice	242,3	4,0

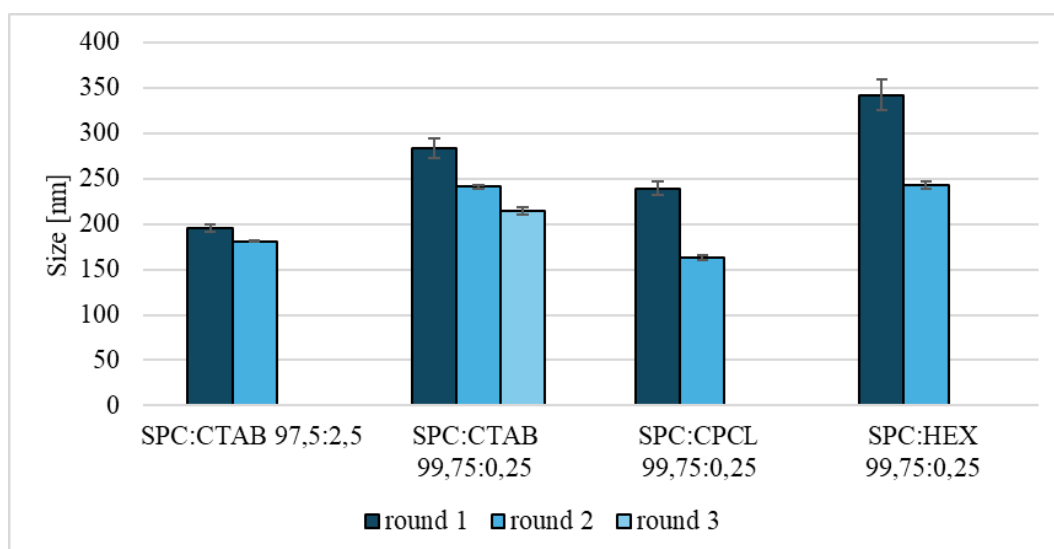


Figure 20: Relation between size and sonication rounds

Figure 20 illustrates that the longer the sonication time, the smaller the size of the vesicles. However, it was taken into consideration that the sonication time could not be more than 6 to 8 minutes due to the possibility of producing too small vesicles. Also, in the case of samples SPC:CPCL 99,75:0,25 and SPC:HEX 99,75:0,25, cycling 80:20 had an impact on vesicle size, resulting in size decrease.

Based on the observed results, the optimum sonication conditions were chosen to be 4 minutes in an ice bath with 80% amplitude and 50:50 cycling. These conditions yielded EL of the desired size.

11.2 Encapsulation of carotenoids

11.2.1 Encapsulation of β -carotene

In this part of the study, the effect of the encapsulated carotene on the vesicle size and zeta potential was tested. For this purpose, at first formulations were prepared containing 0,25 mg of edge activator (97,75 : 0,25), in which 10, 20 or 30 mg carotene were encapsulated. Due to expected application of the developed EL in biological systems and known toxicity of the cationic surfactants, the formulation with their lowest content was chosen for the test. Size and zeta potential were measured in both demineralized water and then in PBS. The purpose of the PBS measurements was to explore the behavior of vesicles under physiologically relevant environments. After vesicle preparation, all samples were hydrated with PBS and tocopherol was used as an antioxidant. Results of the size measurements are given in Figure 21 (H₂O) and 22 (PBS).

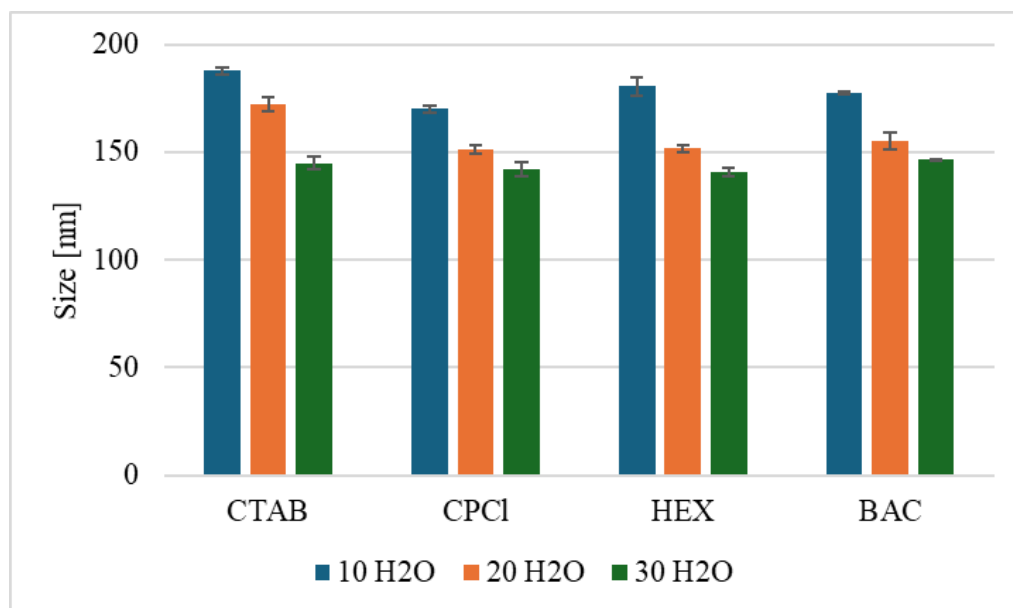


Figure 21: Influence of β -carotene (10, 20 and 30 mg) on size of EL (97,75 : 0,25) (measured in water)

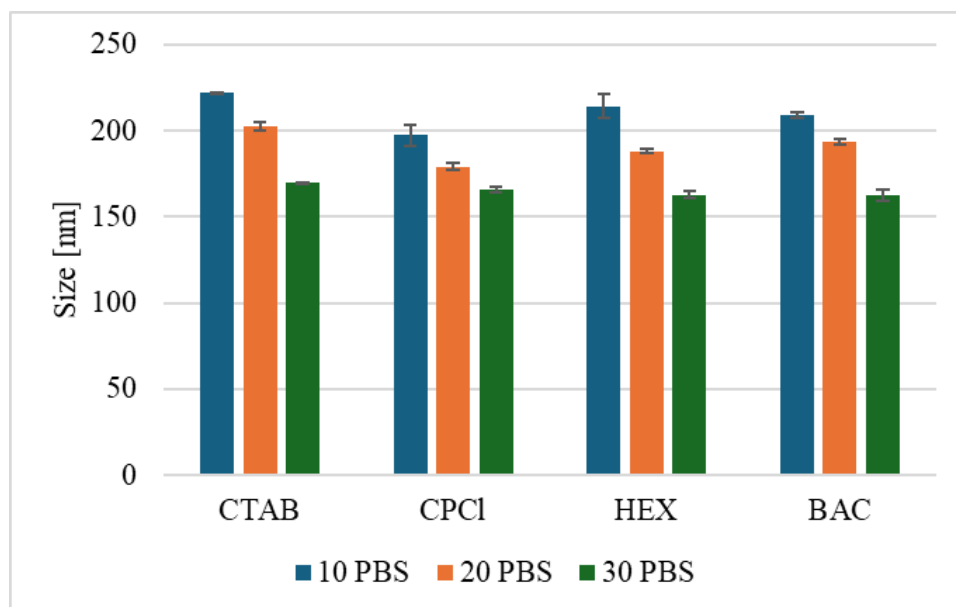


Figure 22: Influence of β -carotene (10, 20 and 30 mg) on size of EL (97,75 : 0,25) (measured in PBS)

The Figures 21 and 22 unambiguously confirm that increasing the amount of carotene influenced encapsulation ability of EL, and with increasing concentration of carotene size of EL decreased.

In general, encapsulating β -carotene in EL can lead to an increase in vesicle size due to the incorporation of this lipophilic compound into the phospholipid membrane. However, the edge activators can influence this effect by enhancing the deformability of the vesicles, potentially leading to smaller sizes. Therefore, the combined impact on vesicle size depends on the interplay between β -carotene encapsulation and the properties of the edge activators used. In the literature, it was published that the incorporation of carotenoids in liposomes can affect the membrane fluidity and permeability (Tan et al., 2014) and can affect the membrane properties. The assumption is that carotenoids with oxygen-containing groups (xanthophylls) can rigidify the membrane fluidity, whilst the influence of carotenoids with only carbon and hydrogen atoms (carotenes) are insignificant or opposite. (Kadian and Garg, 2012)

Regarding the influence of the medium used to measure vesicle size, PBS increased the size in comparison with water. This effect primarily results from the electrostatic shielding of the positive surface charge by ions in the PBS, which reduces repulsive forces between liposomes and promotes size increase.

Influence of β -carotene concentration on zeta potential for samples with SPC: edge activator ratio 99,75 : 0,25 is summarized in Tables 13 (measured in water) and 14 (measured in PBS). The tables illustrate that zeta potential measured in water was significantly higher than that determined in PBS, where the values were close to zero. This is due to the compression of electrical double layer caused by the ionic strength of the solution.

Table 13: Zeta potential of EL formulated with SPC:edge activator ratio 99,75:0,25 encapsulating 10, 20 and 30 mg of β -carotene measured in water

Edge activator	β -carotene amount [mg]	Zeta potential [mV]	Standard deviation
CTAB	10	-16,6	1,1
	20	-17,6	0,6
	30	-22,5	1,0
CPCL	10	-16,5	1,1
	20	-16,9	1,5
	30	-21,3	1,1
HEX	10	-2,0	0,1
	20	-14,5	0,4
	30	-20,1	1,7
BAC	10	-8,0	0,6
	20	-16,2	1,5
	30	-21,0	0,8

Table 14: Zeta potential of ELs formulated with SPC:edge activator ratio 99,75:0,25 encapsulating 10, 20 and 30 mg of β -carotene measured in PBS

Edge activator	β -carotene amount [mg]	Zeta potential [mV]	Standard deviation
CTAB	10	-1,8	0,9
	20	-3,7	0,6
	30	-3,6	0,6
CPCL	10	-1,1	0,2
	20	-3,2	0,8
	30	-4,0	0,1
HEX	10	-2,4	0,5
	20	-4,0	1,0
	30	-3,9	0,9
BAC	10	-2,6	1,3
	20	-4,0	0,6
	30	-4,9	0,5

The above-mentioned and discussed results demonstrated that the lowest employed concentration of cationic edge activators was unable to maintain the required positive charge of the EL. Therefore, the corresponding test was conducted with formulation with SPC : edge activator ratio of 95:5 with 10 mg of encapsulated β -carotene. Also, in this case the size and zeta potential of vesicles were measured both in water and in PBS.

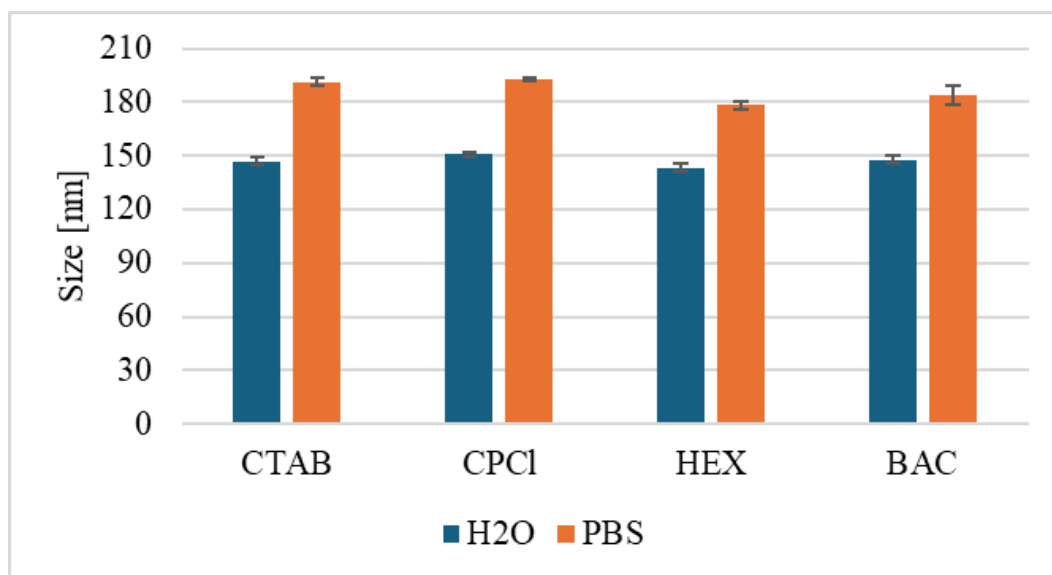


Figure 23: Comparison of size of EL formulated with SPC:edge activator ratio 95 : 5 and 10 mg β -carotene measured in water and PBS

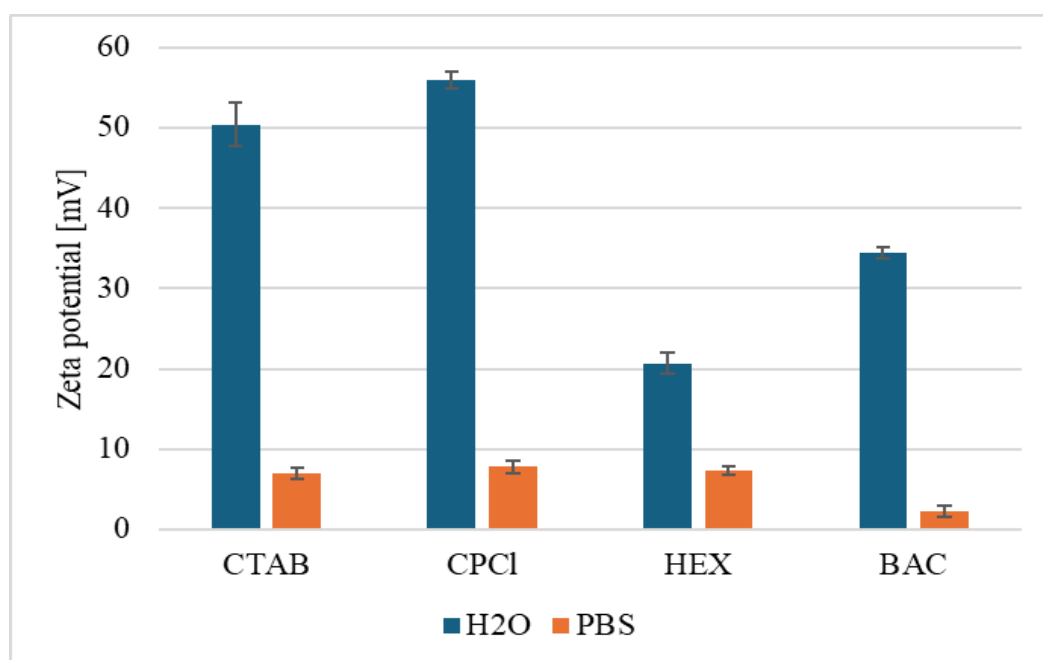


Figure 24: Comparison of zeta potential of EL formulated with SPC:edge activator ratio 95 : 5 and 10 mg β -carotene measured in water and PBS

The sizes of vesicles measured in water were similar (~ 150 nm) across all samples, regardless of edge activator type. Similarly to previously reported sample 99,75:0,25, the samples with lipid:activator ratio 95:5 measured in PBS exhibited greater size values (~ 190 nm), regardless of the activator utilized.

The zeta potential values reveal significant changes in EL properties as a function of measuring conditions (Figure 24). When measured in water, EL with CTAB, CPCL, and BAC as edge activators had all zeta potentials above 30 mV and EL with HEX above 20 mV. In contrast, when measured in PBS, all of the samples showed a considerable drop in zeta potential, with values less than 10 mV. This is induced by the environment's ionic strength, which affects the electrical double layer.

11.2.2 Encapsulation of astaxanthin

Astaxanthin (AX) was the next carotenoid to be encapsulated in EL. Two different formulations were made in accord with the testing of carotene encapsulation described above; one with 5 mg edge activator (95 : 5) and another with 0,25 mg edge activator (99,75 : 0,25). In this case HEX or BAC edge activators were applied. Compared to β -carotene, the amount of encapsulated AX was reduced; 2 mg of AX was added to EL with HEX and 1 mg of AX was added to EL with BAC. The reduced amount of AX was chosen based on experience from the previous work conducted in the ÚTTTK laboratory and literature data about regularly used AX concentrations in liposomes and ELs (Luo et al., 2024).

Furthermore, aggregates were seen to form during the EL production when the dry lipid film was hydrated with PBS. Though these aggregates were dispersed by sonication, hydration was also performed with water, and the results were compared (Figures 25 and 26). These figures illustrate that the size of vesicles was significantly reduced when hydrating with water in comparison with PBS; and therefore it was decided to continue hydrating with water, rather than with PBS, because smaller vesicles are more stable and less prone to agglomeration. As regards the medium used for measuring vesicle size (water or PBS), from Figures 25 and 26 it is visible that the sizes remained similar regardless of the measuring environment.

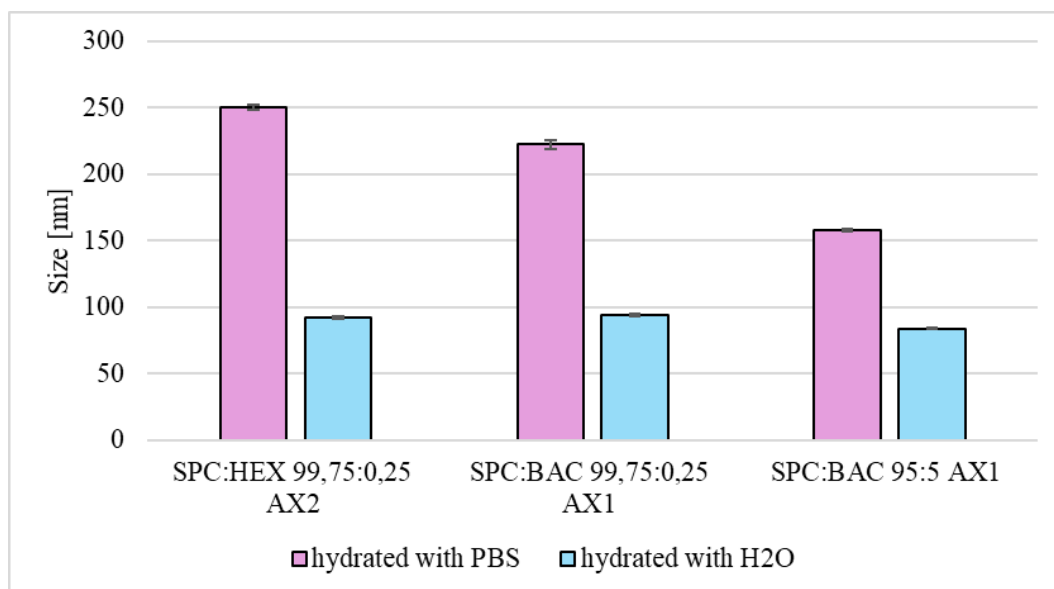


Figure 25: Comparison of EL size with encapsulated AX hydrated with PBS or water. Measurements conducted in water (AX1 = 1 mg AX and AX2 = 2 mg AX)

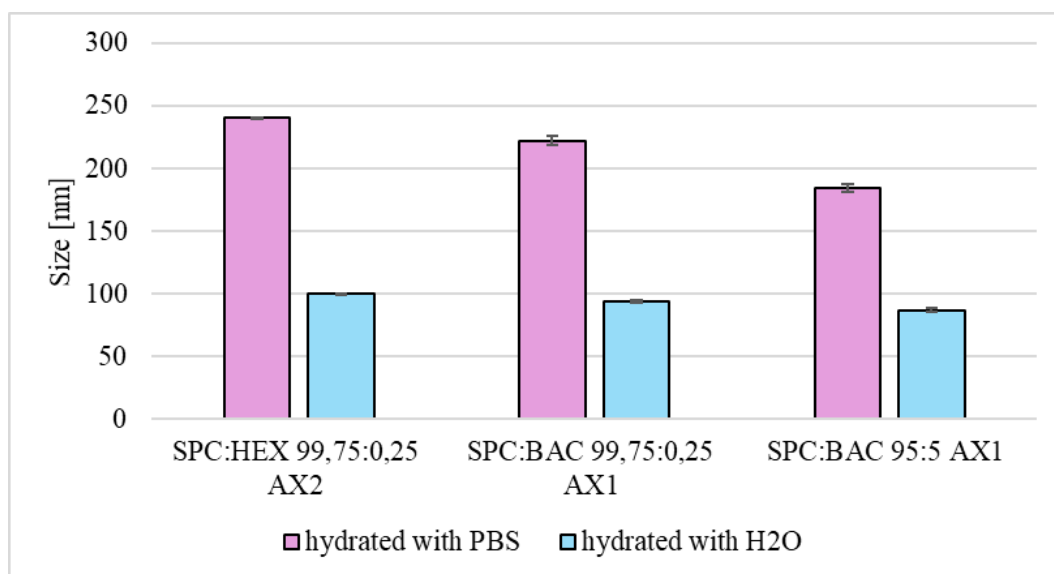


Figure 26: Comparison of EL size encapsulating AX hydrated with PBS or water. Measurements conducted in PBS (AX1 = 1 mg AX and AX2 = 2 mg AX)

Visible changes in zeta potential of samples with AX were observed (Figures 27 and 28), which were influenced both by medium used to hydrate vesicles (water or PBS) as well as by the measuring environment (water or PBS). The most significant change was seen in formulation SPC:BAC 99,75:0,25 AX1 and SPC:HEX 99,75:0,25 AX2 hydrated with H₂O or PBS when measured in water. In these two cases the zeta potential changed from positive

values when hydrated with water to negative values close to zero when in PBS (Figure 27). The situation, however changed when PBS was used to measure zeta potential. In this case negative zeta potentials were measured for these formulation irrespective of medium used for their hydration (Figure 28). As it was previously observed, measuring in PBS has an impact on zeta potential due to its ionic strength which influences the thickness of electrical double layer. The zeta potential of formulation SPC:BAC 95:5 AX1 measured in water remained positive regardless of whether the lipid film was hydrated with water or PBS (Figure 27).

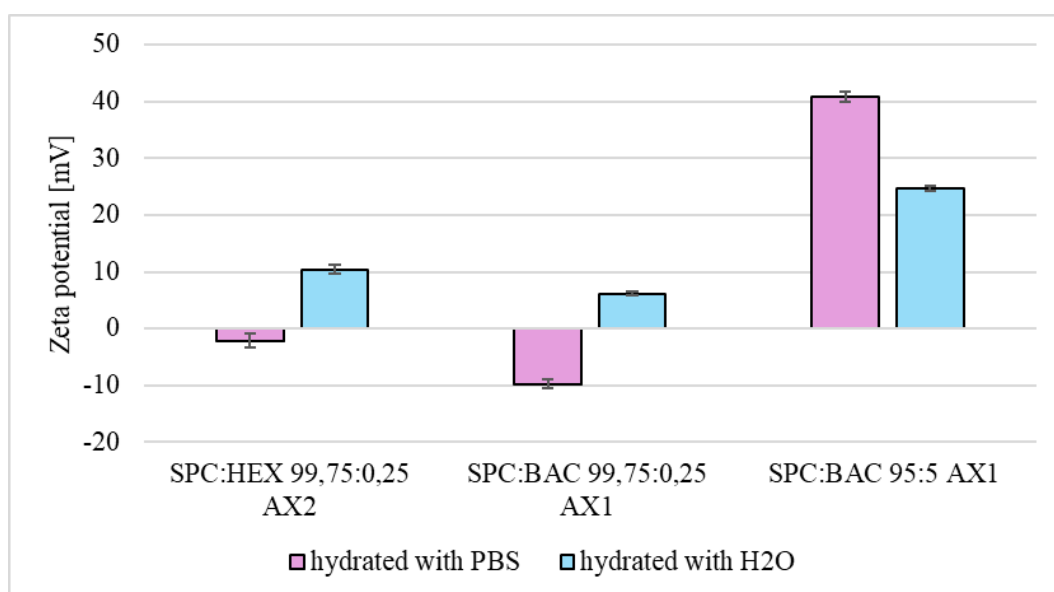


Figure 27: Comparison of zeta potential of EL with AX hydrated with PBS or water. Measurements conducted in water (AX1 = 1 mg AX1 and AX2 = 2 mg AX)

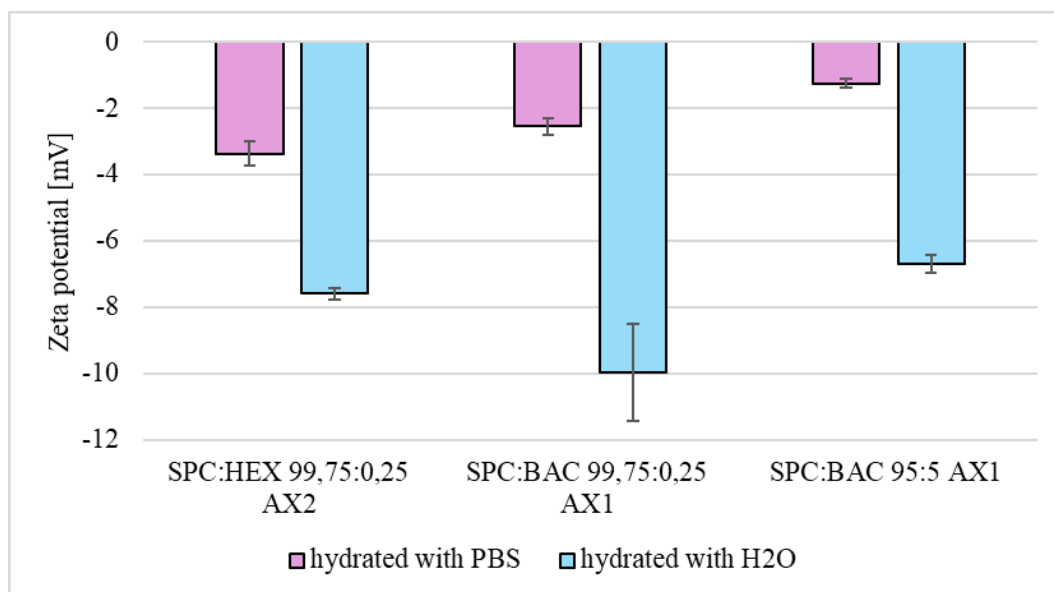


Figure 28: Comparison of zeta potential of EL with AX hydrated with PBS or water. Measurements conducted in PBS (AX1 = 1 mg AX and AX2 = 2 mg AX)

11.2.3 Combination of HEX and BAC as edge activators

In order to give EL positive charge but at the same time incorporate as little surfactant as possible, HEX and BAC were combined in the following compositions: SPC:HEX:BAC 99,5:0,25:0,25 and SPC:HEX:BAC 99:0,75:0,25. The samples were hydrated with water and size and zeta potential were measured in both water and PBS. These compositions of lipid membrane were used in combination with three different carotenoids, in addition to previously presented carotene (10 mg) and astaxanthin (1 mg), the third carotenoid lutein (1 mg) was also used. The results recorded for size of lipid vesicles are given in Figures 29 and 30 and zeta potential in Figures 31 and 32.

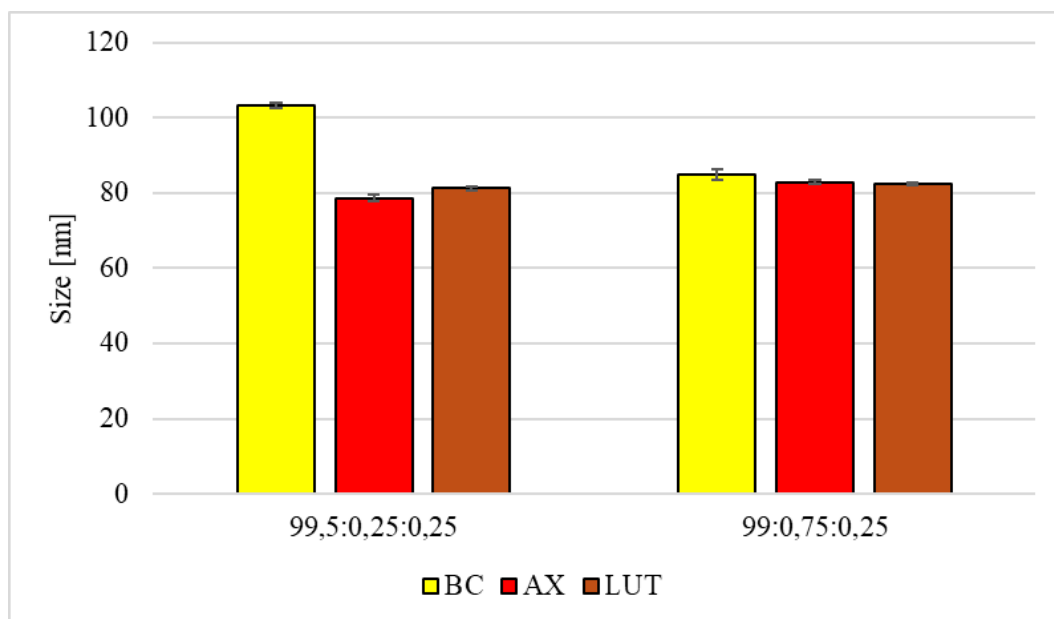


Figure 29: Size of EL combining HEX and BAC as edge activators in membrane.
Measurement conducted in water

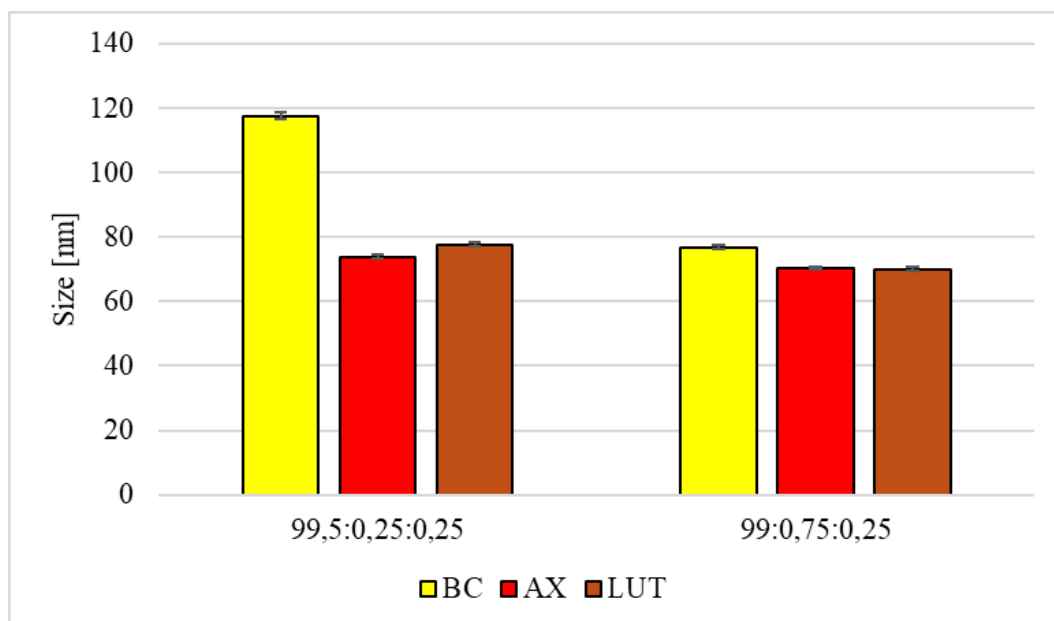


Figure 30: Size of EL combining HEX and BAC as edge activators in membrane.
Measurement conducted in PBS

Figures 29 and 30 demonstrate that the measuring environment (H_2O or PBS) didn't significantly influence the size of carriers. However, the amount and type of carotenoid encapsulated did. Formulations with 10 mg of β -carotene with HEX/BAC edge activators in ratio 0,25/0,25 displayed notably bigger size than the same formulations encapsulating 1 mg

of astaxanthin or lutein. Interestingly, the formulation containing HEX/BAC in ratio 0,75/0,25 produced approximately the same-sized carriers regardless of the amount of carotenoid used in the formulation. Compared to the measurements done in water, vesicles measured in PBS displayed only a very slight decrease in size.

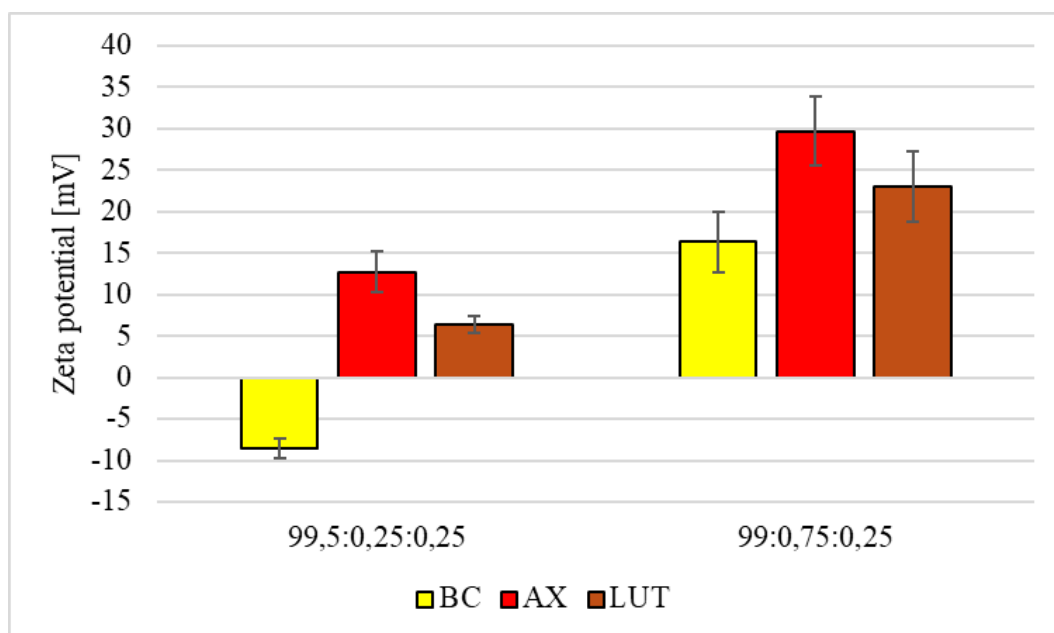


Figure 31: Zeta potential of EL combining HEX and BAC as edge activators. Measurement conducted in water

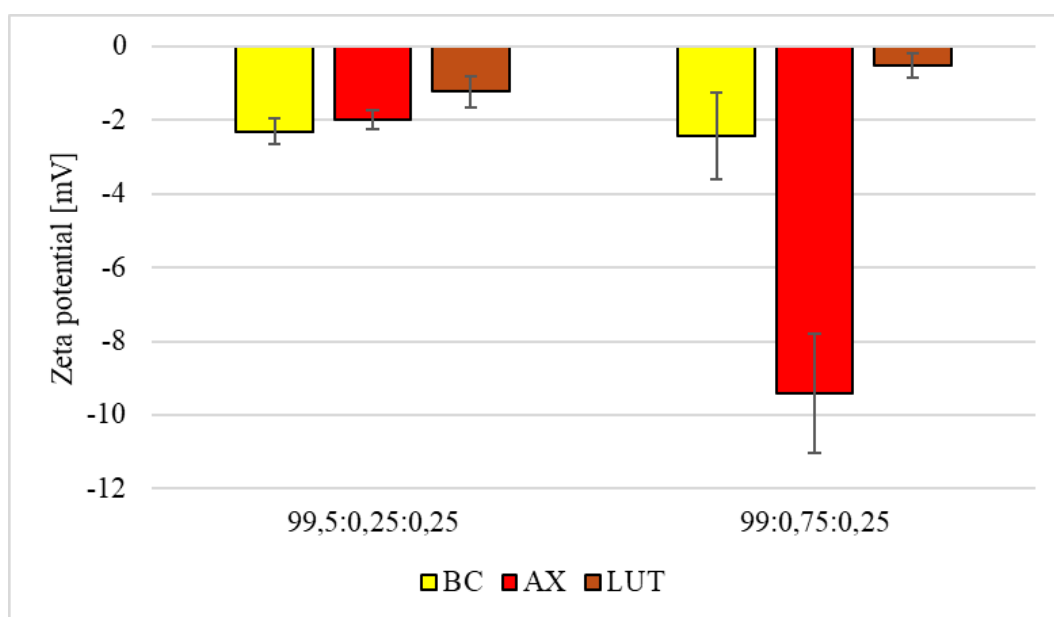


Figure 32: Zeta potential of EL combining HEX and BAC as edge activators. Measurement conducted in PBS

The only formulation that approached the zeta potential value of +30 mV and therefore can be considered stable based on zeta potential value is SPC:HEX:BAC 99:0,75:0,25 encapsulating 1 mg of astaxanthin, measured in water. The measuring environment significantly influenced values of zeta potential of all observed formulations. All samples measured in PBS (Figure 32) showed negative zeta potential values.

11.2.4 Cationic liposomes containing DOTAP

In the next part of the thesis, cationic lipid 1,2-dioleoyl-3-trimethylammoniumpropane DOTAP was used as a carrier of positive charge in phospholipid (SPC) membrane. The formulations with the following SPC : DOTAP ratios were investigated 50 : 50, 90 : 10 and 99 : 1 with encapsulated lutein. The samples were hydrated in water and their size/zeta potential determined in PBS or water.

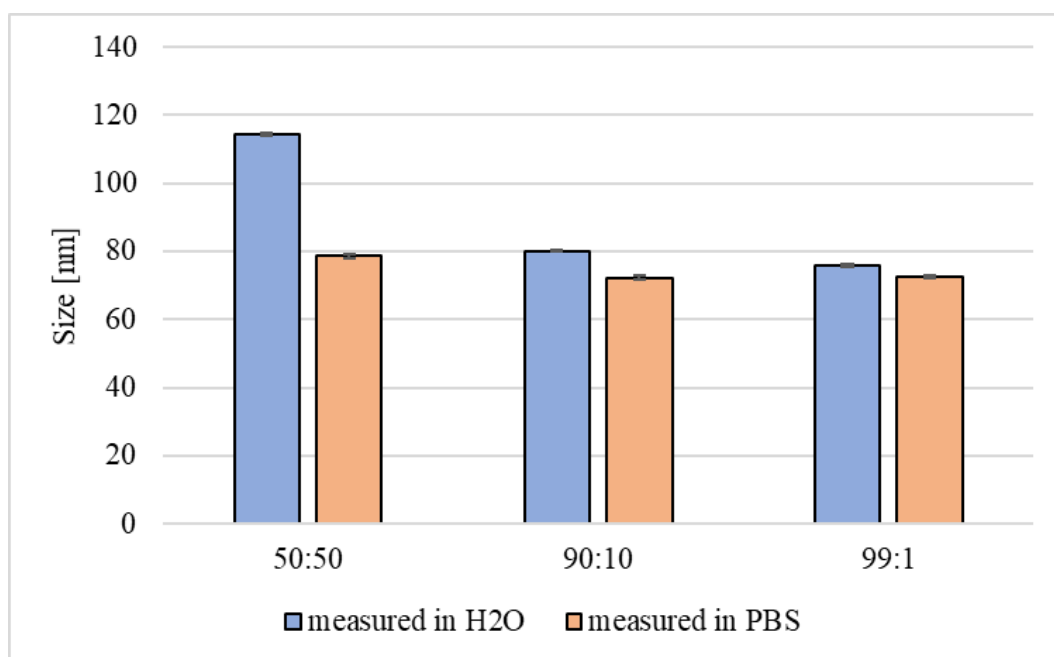


Figure 33: Size of liposomes containing different ratios of SPC : DOTAP with encapsulated LUT measured in water and PBS

The size of vesicles containing DOTAP depended on the SPC:DOTAP ratio. Vesicles containing equal amount of SPC and DOTAP were bigger in size than the other two formulations (ratios 90:10 and 99:1), which were more similar in size. The medium used for size measurement impacted on the size of the carriers, which were slightly smaller than when measured in PBS.

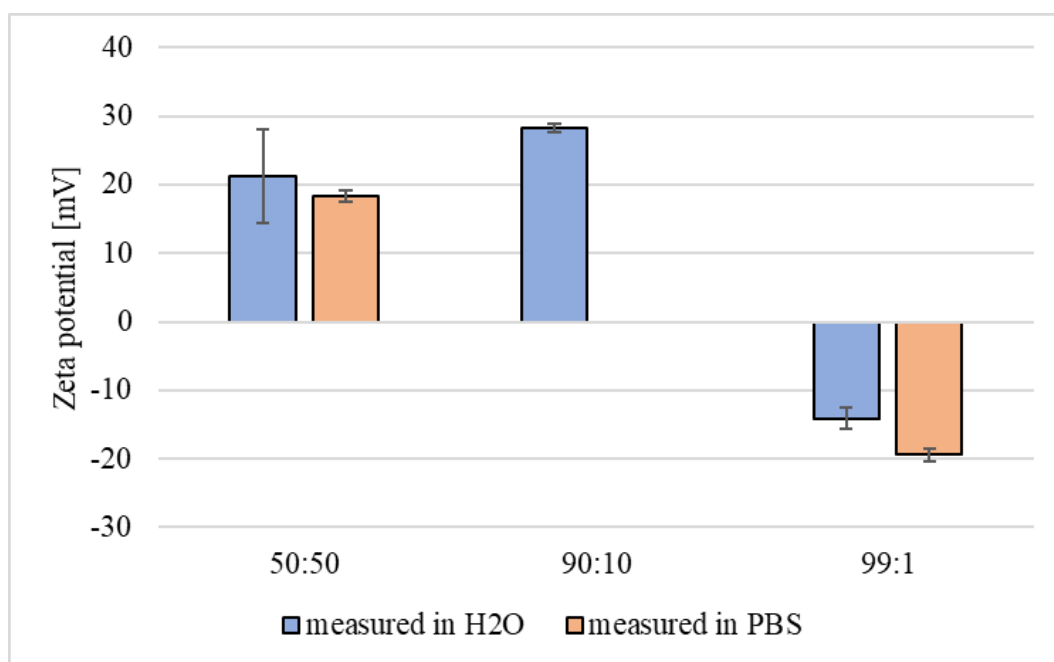


Figure 34: Zeta potential of liposomes containing different ratio of SPC : DOTAP with encapsulated LUT measured in water and PBS

Interestingly, measuring environment had a lesser impact on the zeta potential of the DOTAP containing vesicles in comparison with the vesicles gaining their positive charge from cationic surfactants (CTAB, CPCL, BAC). In the case of vesicles carrying positive charge due to the cationic lipid DOTAP, zeta potential of SPC:DOTAP 50:50 was similar when measured in water and in PBS. While in the case of SPC:DOTAP 50:50, zeta potential remained positive (~20 mV), in the case of SPC:DOTAP 99:1 (with the lowest concentration of DOTAP) zeta potential remained in both measuring negative environments under -20 mV. Zeta potential of 90:10 formulation was not measured in PBS due to a technical problem.

11.3 Final formulations

The information acquired from the comprehensive tests conducted within preformulation and carotenoid encapsulation studies was used to create the final EL formulations. The formulations were designed to preserve a membrane composition that carried a positive charge while encapsulating the regularly utilized concentrations of carotenoids. Tests with various hydration media (PBS, water), as well as media utilized to measure vesicle size and zeta potential, provided useful information for the final formulation design. Four formulations with cationic surfactants as edge activators and two formulations with cationic lipid DOTAP were prepared. The amount of each carotenoid in formulation was set at 2 mg.

Final formulations were prepared using water as the hydration medium, and their size/zeta potential were measured in water, PBS and saline (0.9 % NaCl).

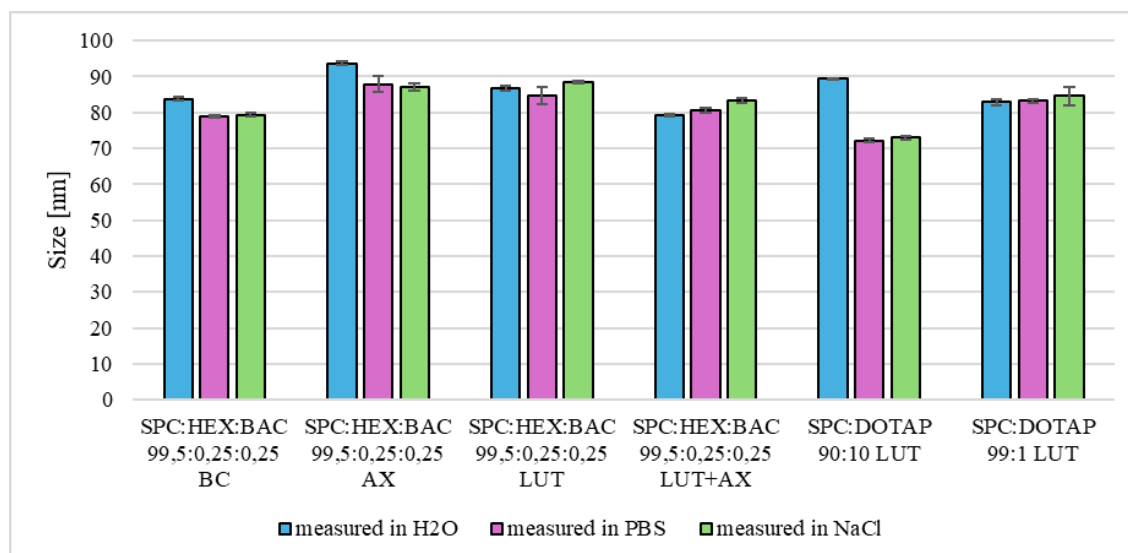


Figure 35: Size of vesicles in six final formulations immediately after sample preparation

Figure 35 shows sizes of vesicles in all six final formulations measured in each of three environments, water, PBS and aqueous NaCl. It can be said that among the six formulations, samples SPC:HEX:BAC encapsulating β -carotene and astaxanthin, and SPC:DOTAP 90:10 show a similar trend, where the vesicles measured in water are bigger in size in comparison with ones measured in PBS or NaCl. In other cases there is no consistent trend relating vesicle size and type of measuring medium. All the vesicle sizes, however, are smaller than 100 nm, which is advantageous because smaller vesicles could be an indicator of more homogenous and stable formulations. The biggest difference in terms of correlation between size and measuring environment is observed in the sample SPC:DOTAP 90:10 with encapsulated lutein, where EL measured in PBS and NaCl are significantly smaller (~ 70 nm) than those measured in water (~ 90 nm).

The behavior of samples can be described not only by size values, but also by their distribution functions. These are shown in Figures 36 (comparison of formulations SPC:HEX:BAC) and Figure 37 (SPC:DOTAP). The distribution curves in Figure 36 reveal that samples tested in PBS and aqueous NaCl contain, in addition to the major population of nanometer-sized vesicles, a fraction of micrometer-sized vesicles (peak in the 1-10 μ m range). This is most likely a fraction resulting from vesicle aggregation caused by the presence of electrolytes in both media. However, the amount of aggregates present in

samples is insufficient to alter the average size of the vesicles. Distribution curves of SPC:DOTAP in Figure 37 show bigger vesicles in samples measured in water in comparison with samples in ionic media. Moreover, in SPC:DOTAP 99:1 LUT sample, fractions with micrometer sizes are also present.

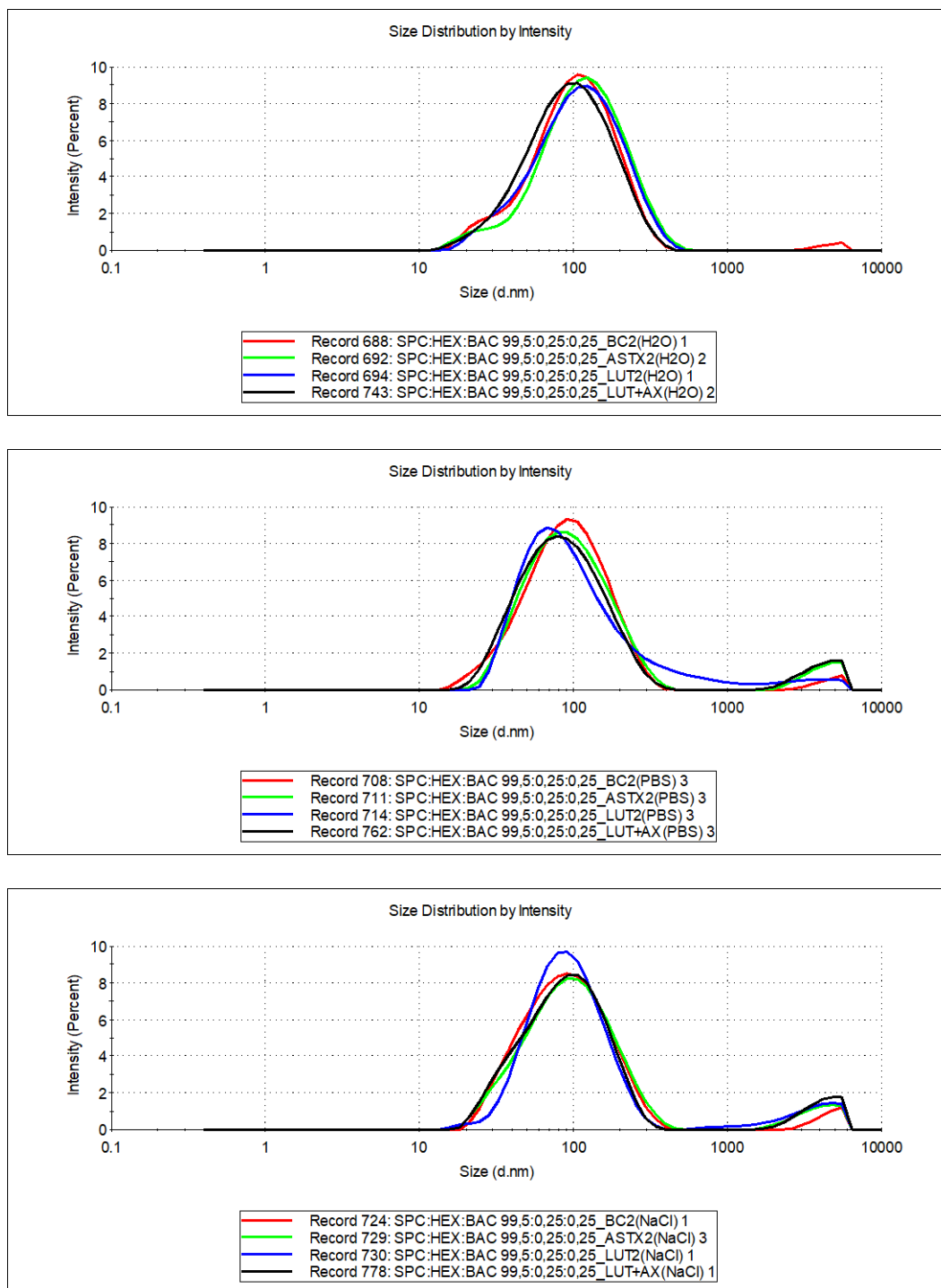


Figure 36: Size distribution of final formulations SPC:HEX:BAC measured in three different media, water (top), PBS (middle) and 0,9 % NaCl (bottom) immediately after preparation

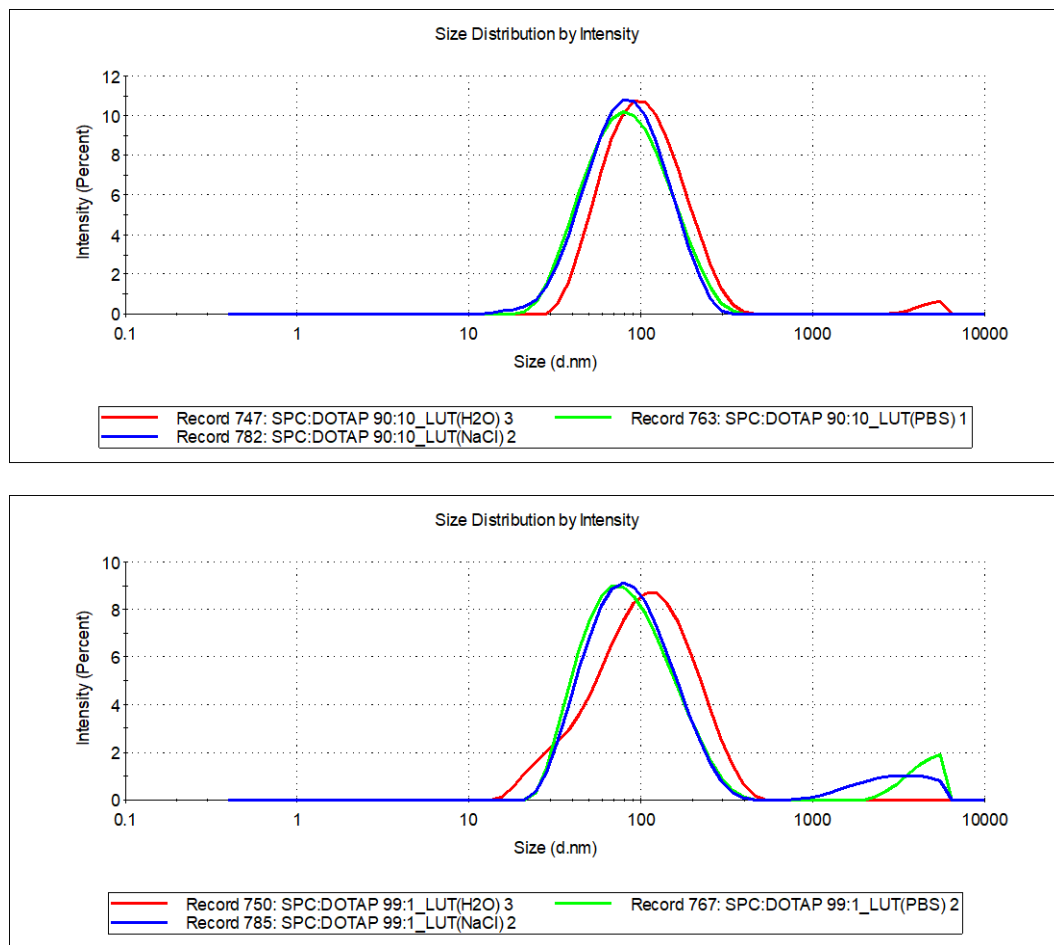


Figure 37: Size distribution of SPC:DOTAP 90:10_LUT (top) and SPC:DOTAP 99:1_LUT (bottom) measured in water (red line), PBS (green line) and NaCl (blue line) after preparation

The photos of formulations are depicted in Figure 38 (with cationic surfactants) and Figure 39 (with DOTAP). The figures show that the samples preserve the color of the encapsulated carotenoid (yellow for LUT and carotene), and orange for (AX). Sample dispersions are transparent, indicating the small size of the produced vesicles.

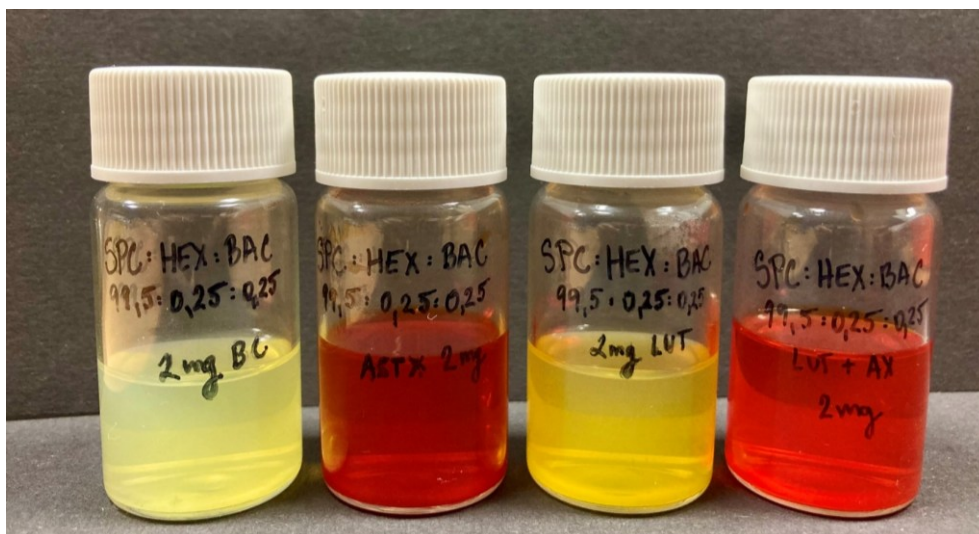


Figure 38: Final formulations with SPC/HEX:BAC with encapsulated (from the left) β -carotene astaxanthin, lutein and mixture of astaxanthin/lutein, after preparation



Figure 39: Final formulations with SPC/DOTAP 90:10 (left) and 99:1 (right) with encapsulated lutein, after preparation

Measuring zeta potential (Figure 40) in three different environments provided interesting information regarding relation between zeta potential and the measuring medium. In case of the sample encapsulating β -carotene, zeta potential in all three environments stayed negative, only PBS and NaCl caused its decrease closer to zero. Other samples measured in water exhibited positive zeta potential. When comparing the three measuring environments it can be said that both PBS and NaCl caused significant decrease in zeta potential and brought it close to zero in all of the six samples. This is caused by the ionic strength of these environments where the presence of ions causes compression of electrical double layer,

surface charge is therefore being neutralized, and zeta potential approaches zero value. However, zeta potential in formulation SPC:DOTAP 90:10 with encapsulated lutein remains positive in all media, evidencing that positive charge from higher amounts of cationic lipid is not fully screened by ionic media.

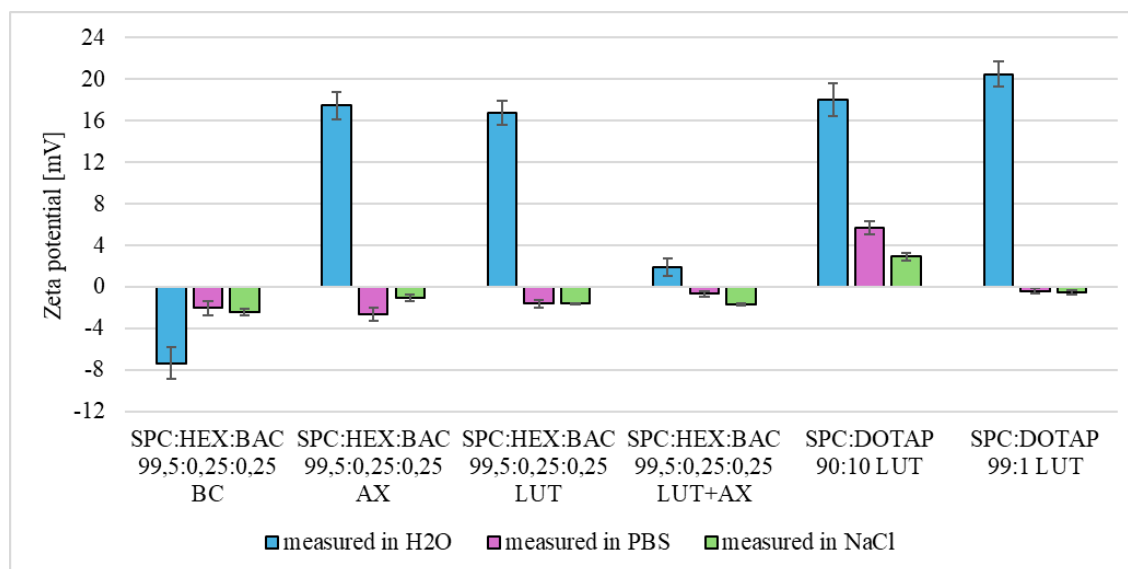


Figure 40: Zeta potential of vesicles in six final formulations determined immediately after preparation

11.3.1 Long-term stability

The size and zeta potential of six final formulations were measured in water, PBS and NaCl over period of one month – immediately after preparation, after 2 and 4 weeks. Figure 41 show changes in size and Figure 42 illustrates changes in zeta potential.

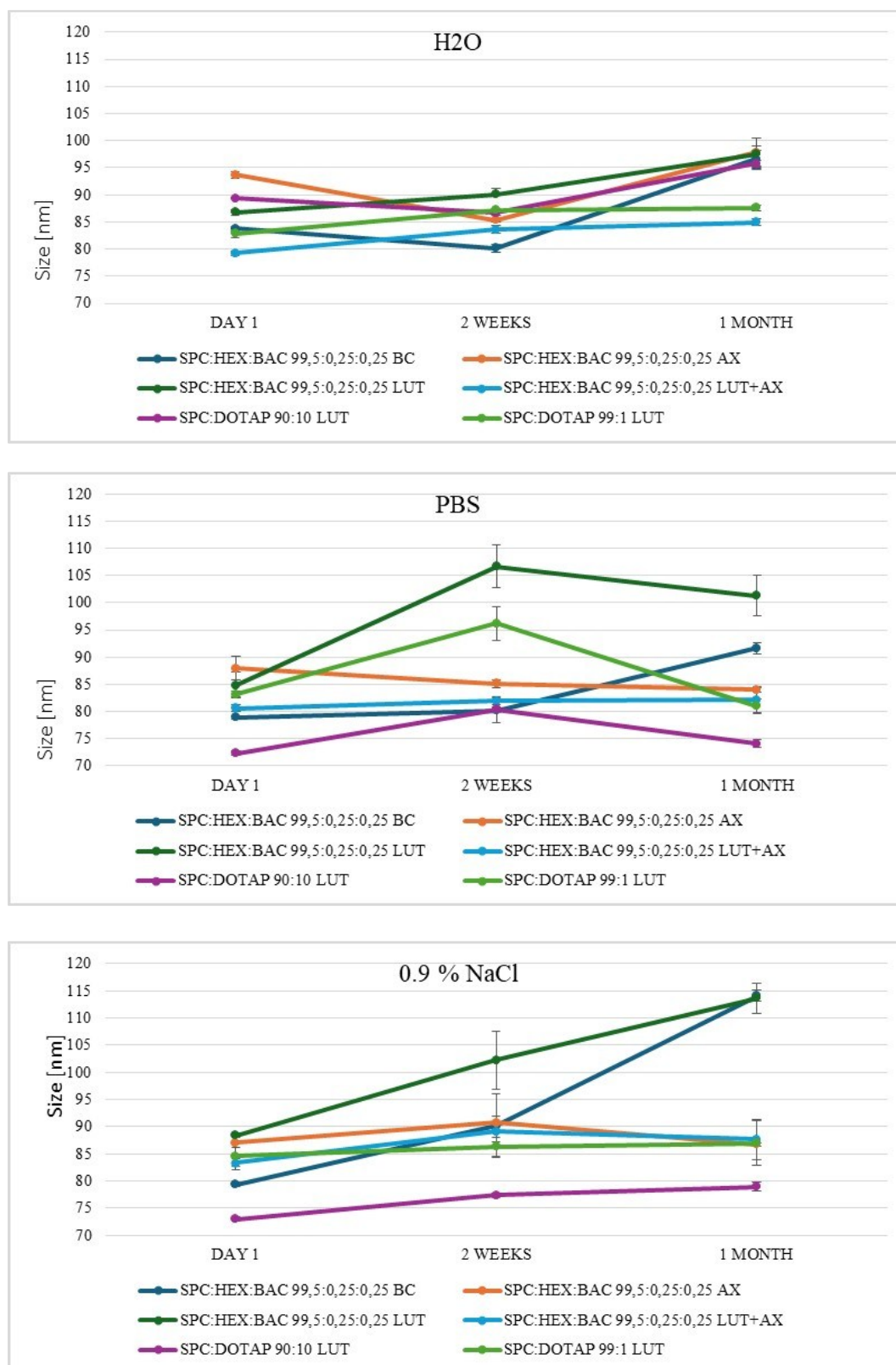


Figure 41: Changes in size over time measured in three different media (H₂O, PBS and 0,9 % NaCl)

The changes in vesicle size differed among the measuring media. Samples SPC:HEX:BAC encapsulating BC, AX and sample SPC:DOTAP 90:10 measured in water showed mild decrease in size after two weeks and increase in size after one month. The other three samples measured in water showed opposite behavior, their sizes during 4 weeks storage increased. However, the increase was still minor and the vesicle sizes lied below 100 nm.

When measured in PBS, sample SPC:HEX:BAC encapsulating BC showed decrease in size after two weeks and increase in size after one month. Sample with AX showed steady decrease in size over the monitored period of time and sample combining AX+LUT showed slight increase in size. Both samples containing DOTAP as well as sample SPC:HEX:BAC 99,5:0,25:0,25 encapsulating LUT showed increase in size after two weeks and decrease in size after one month.

When measured in 0,9% NaCl solution, the sample encapsulating AX showed a slight increase and then decreased in size again, and all other samples increased their size over the period of one month. The vesicle size increased the most in the sample SPC:HEX:BAC with encapsulated BC, from ~ 80 to 115 nm.

The variations of vesicle size can be caused by various reasons. The increase in size might have been induced by aggregation of vesicles caused by van der Waals forces or inadequate repulsive stabilization. Another reason might be fusion of adjacent lipid bilayer leading also to vesicle increase. In this respect, vesicle swelling can't be omitted as well. Decrease in size, on the other hand, could have been caused by leakage of the encapsulated content over time or instability in edge activator.

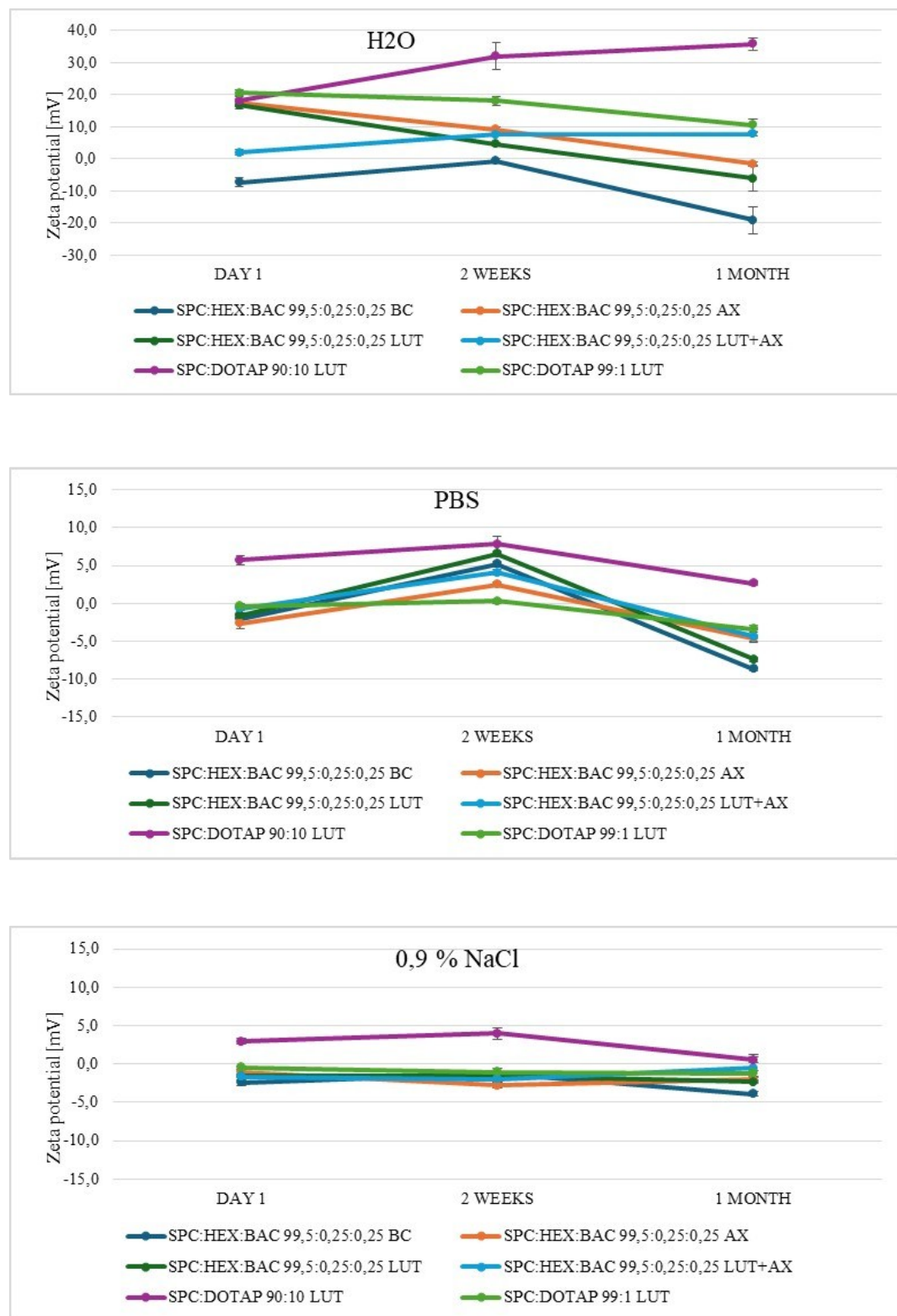


Figure 42: Changes in zeta potential over time measured three different media (H₂O, PBS and 0,9 % NaCl)

Changes in zeta potential also depended on the measuring environment. Samples with encapsulated astaxanthin, lutein and sample SPC:DOTAP 99:1 showed steady decrease in zeta potential over one month, the first two samples changed from positive to negative zeta potential when measured in water. Sample containing lutein/astaxanthin mixture and sample SPC:DOTAP 90:10 both showed an increase in zeta potential and sample encapsulating BC showed first decrease after two weeks and then rapid overall decrease after one month, when measured in water. Interestingly, when measured in PBS all samples exhibited the same trend – slight increase in zeta potential after two weeks and then decrease after one month. When measuring in NaCl solution, the zeta potentials of all samples stayed approximately the same over the period of one month keeping values close to zero. Compared to the results obtained from measurements in water, zeta potential measured in PBS and saline showed more uniform values; the only sample that differed was SPC:DOTAP 90:10, whose zeta potential remained positive in all media throughout the storage time. The changes in zeta potential over time could have possibly be caused by storage conditions, lipid degradation or edge activator degradation as well as changes in ionic strength of the surrounding medium. According to the previous text, the physical stability of the vesicles over time was systematically evaluated; however, chemical stability of the encapsulated carotenoids was not monitored. Nevertheless, the color shift of the EL dispersions over time can be used to estimate the chemical stability, albeit in an approximate and indirect manner. Visual examination of the samples (Figures 43 and 44) verified that neither sample showed any visual changes during two weeks of storage at 4 °C.



Figure 43: Final SPC:HEX:BAC formulations with encapsulated (from the left) β -carotene astaxanthin, lutein and mixture of astaxanthin/lutein, 2 weeks after preparation

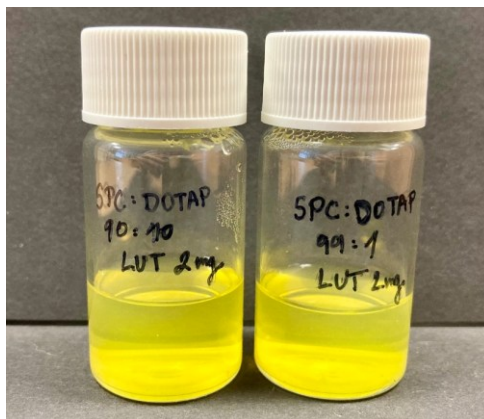


Figure 44: Final formulations with SPC/DOTAP 90:10 (left) and 99:1 (right) with encapsulated lutein, two weeks after preparation

After four weeks of storage (Figure 45), it is clearly seen, that yellow color of sample with β -carotene becomes lighter, indicating degradation of the encapsulated content. The samples with LUT and AX show only slight color change, suggesting that the encapsulated carotenoids did not degrade significantly during the storage. Of course, this visual evaluation just provides an indication of the level of degradation, and the HPLC method is required to accurately determinate the degradation process in encapsulated carotenoids. This activity is planned as a part of the ongoing research on this topic.

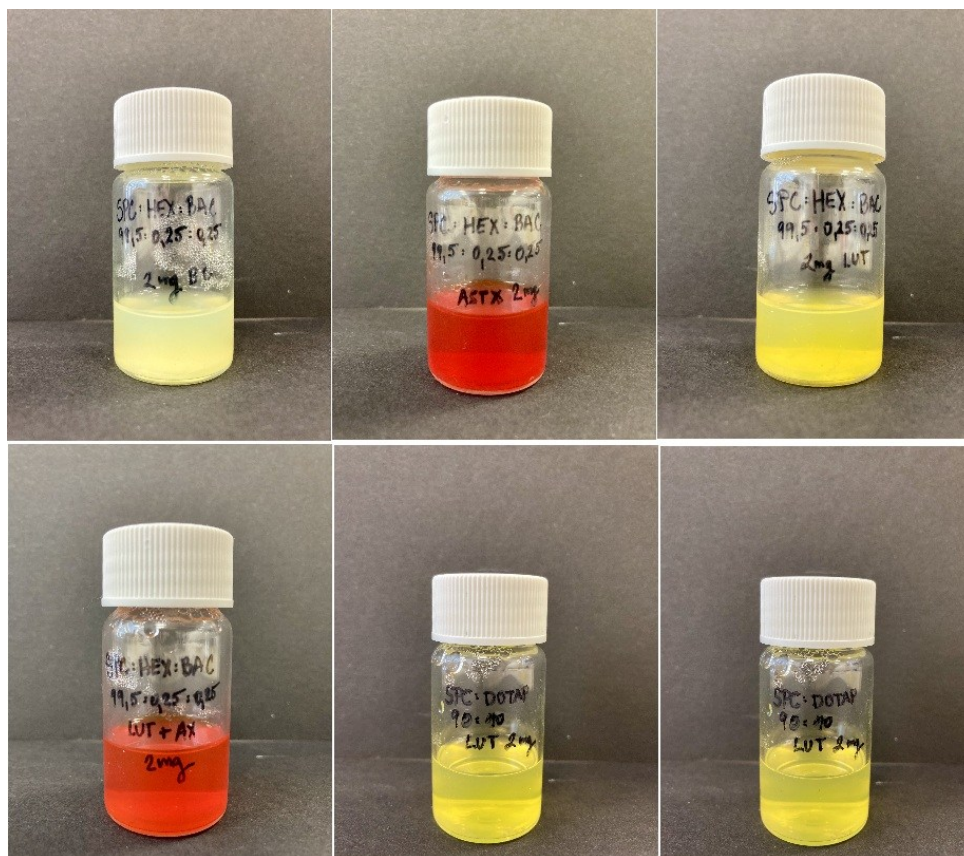


Figure 45: Final SPC:HEX:BAC formulations with encapsulated (from the top left) β -carotene astaxanthin, lutein and mixture of astaxanthin/lutein (bottom left), and with SPC/DOTAP 90:10 (bottom middle) and 99:1 (bottom right) with encapsulated lutein, four weeks after preparation

11.3.2 Antioxidant activity

Antioxidant activity of final formulations was measured after sample preparation and then after four weeks. The procedure is described in chapter 10.5.4 and the results are given in Figure 46. At first, time dependence of ABTS $\bullet$ radical scavenging activity was determined at defined time intervals of 1, 2, and 4 hours after mixing the sample with radical solution. Based on these experimental findings (data not presented), none of the tested formulations exhibited time-dependent changes in antioxidant activity in the ABTS assay. Thus, for both freshly prepared and four-week-stored samples, the relative inhibition values (% RI) determined after four hours are given.

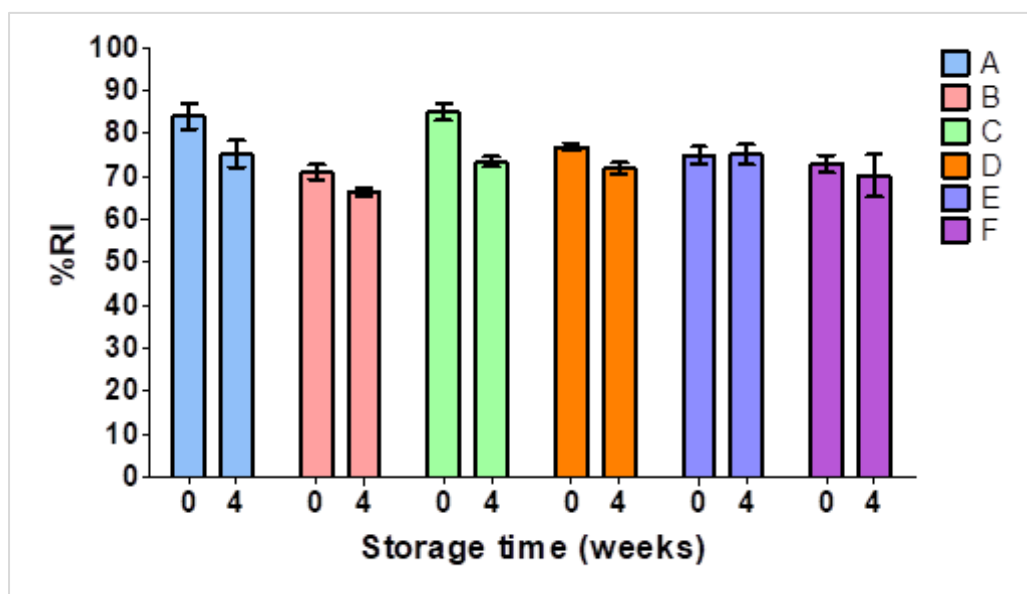


Figure 46: Comparison of relative ABTS•+ radical inhibition (% RI) after 4h in fresh (0) and 4-week stored (4) samples; A – SPC:HEX:BAC with BC, B – SPC:HEX:BAC with AX, C – SPC:HEX:BAC with LUT, D – SPC:HEX:BAC with LUT+AX, E – SPC:DOTAP (90:10) LUT and F – SPC:DOTAP (99:1) LUT

Generally, the lowest radical scavenging activity was observed for sample SPC:HEX:BAC which contained astaxanthin (AX) (sample B). In contrast, samples A and C, incorporating β -carotene (BC) or lutein (LUT) demonstrated the highest antioxidant efficiency, with inhibition values exceeding 84 %. The remaining formulations exhibited comparable radical inhibition, with values ranging between 71% and 77%.

Following four weeks of storage, the radical scavenging capacities of the formulations remained largely preserved. With the exception of sample B, all tested samples maintained relative inhibition (% RI) values above 70 %. However, a decrease in %RI was noted for all samples prepared with SPC:HEX:BAC (A, B, C and D) when compared to their freshly prepared counterparts. Interestingly, samples containing SPC:DOTAP (E, F) exhibited only negligible differences between activity of freshly prepared and stored samples. (Figure 46).

In summary, all carotenoid-loaded cationic vesicle formulations retained their ability to effectively scavenge ABTS•+ radicals, both immediately after preparation and following storage.

11.3.3 Cytotoxicity

At normal dietary or physiological concentrations, most carotenoids are non-toxic and often act as antioxidants, protecting cells from oxidative stress. However, encapsulation of carotenoids in nano-sized vesicles enhances their bioavailability and solubility in water and lipids, increases the surface-to-volume ratio by reducing vesicle size to the nanoscale range and can thus can alter their biological properties. (Elkholy et al., 2023) Therefore, cytotoxicity of our samples was also studied.

Figure 47 illustrates the relationship between relative cell viability and the tested samples diluted to 1, 5 and 10 % of the original samples containing different carotenoids . The data are presented as mean values with corresponding standard deviations, based on five independent replicates per sample. The cytotoxicity assessment was performed in accordance with ISO 10993-5 guidelines, where cell viability below 0,7 is indicative of cytotoxic effects. Cell viability higher than 0,7 correspond to non-cytotoxic samples.

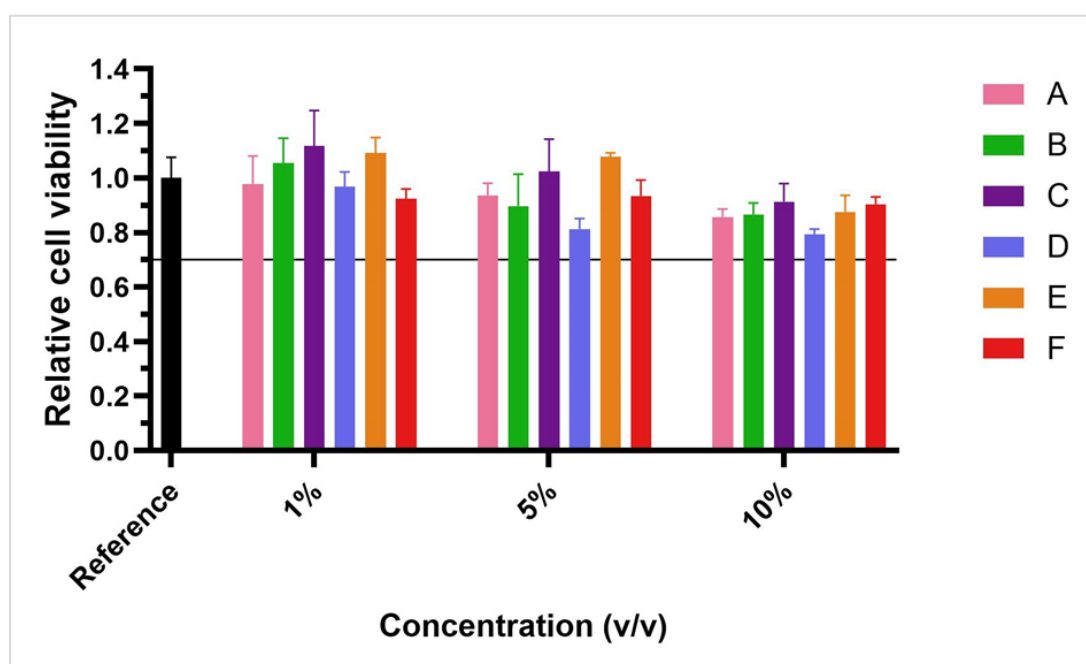


Figure 47: Relative cell viability of mouse embryonic fibroblast depending on sample volume concentration. Data are expressed as mean $\pm$ SD ($n = 5$ per group), A – SPC:HEX:BAC BC; B – SPC:HEX:BAC AX; C – SPC:HEX:BAC LUT; D – SPC:HEX:BAC LUT+AX; E – SPC:DOTAP (90:10) LUT; F – SPC:DOTAP (99:1) LUT.

The results of the cytotoxicity assay demonstrated that none of the carotenoid-loaded cationic EL exhibited cytotoxicity at the three different tested concentrations, thereby confirming their biocompatibility under the specified conditions.

The viabilities of the cells treated with 2, 10 and 20 µg/ml carotenoids (which correspond to used dilutions) for 24 h were not significantly different from the reference. These results are consistent with the findings of (Chintong et al., 2019), who investigated the cytotoxicity of astaxanthin extract from shrimp shells on human dermal fibroblasts (WS1) using the MTT assay. Treatment with astaxanthin concentrations ranging from 5-160 µg/ml for 24 hours did not significantly affect cell viability compared to the reference.

CONCLUSION

The master's thesis concentrates on the preparation and characterization of cationic elastic liposomes containing cationic surfactants or cationic lipid DOTAP as edge activators (EA), encapsulating carotenoids astaxanthin, β -carotene and lutein.

Firstly, preformulation study was conducted investigating various factors, which might influence composition of EL. Within the preformulation, following tests were run:

- Empty cationic EL were prepared each with one cationic surfactant (CTAB, CPCL, HEX and BAC) in four different SPC:EA ratios (95:5; 97,5:2,5; 99:1; 99,75:0,25). Their size and zeta potential were measured, and all four compositions exhibited similar trend: with decreasing amount of EA vesicle size increased, while zeta potential decreased.
- The carotenoids were then encapsulated. Two SPC:EA compositions (95:5 and 99,75:0,25) were used for encapsulation. The study aimed to observe the effect of the encapsulated carotenoid (10, 20, and 30 mg of β -carotene) on vesicle size and zeta potential. With increasing amounts of encapsulated carotenoid, the vesicle size decreased, a trend observed in both water and PBS used for vesicle size measurements.
- The effect of the measuring environment (H_2O vs PBS) was shown to be noticeable for both compositions. When measured in PBS, greater vesicle sizes were recorded, while zeta potential significantly decreased, indicating the influence of the solution's ionic strength on the electrical double layer.
- The EL membrane composition was modified to maintain a positive surface charge of the elastic liposomes while using the lowest possible surfactant concentration. The aim of the work was not only to prepare stable cationic liposomes but also to ensure their practical usability, meaning they should not be cytotoxic when in contact with living organisms. Therefore, another composition was chosen. The composition combined HEX and BAC as edge activators in the ratios SPC:HEX:BAC 99,5:0,25:0,25 or 99:0,75:0,25, encapsulating β -carotene, astaxanthin, and lutein.
- The EL were prepared by substituting surfactants as edge activators with cationic lipid (DOTAP).

Using the data collected during the preformulation study, six final formulations were prepared. Four included the membrane-forming composition SPC:HEX:BAC 99,5:0,25:0,25, encapsulating β -carotene, astaxanthin, lutein, or a mixture of astaxanthin/lutein, and two used compositions SPC:DOTAP 90:10 and 99:1. Size and zeta potential measurements were performed in three environments: water, PBS, and 0.9% NaCl solution. These results confirmed previous findings that the measuring environment had little impact on vesicle size but significantly influenced zeta potential. While zeta potential in water ranged from -10 mV to $+20$ mV, it was within ± 5 mV in PBS and NaCl.

Long-term stability testing (one month) revealed a gradual increase in vesicle size, likely due to vesicle aggregation, which was dependent on vesicle composition and encapsulated carotenoid. Conversely, zeta potential decreased. The antioxidant activity was determined using the ABTS assay. Even after one month, all formulations retained effective radical scavenging ability, higher than 70 %. Exception was SPC:BAC:HEX with encapsulated astaxanthin, which had less than 70 % activity after a month of storage. Cytotoxicity of final formulations was assessed using the MTT assay. The test showed that none of the formulations exhibited cytotoxicity at 1%, 5%, or 10% dilutions of original dispersion, with cell viability greater than 80 %, when compared to the non-cytotoxic reference.

The results of this thesis therefore demonstrate that cationic elastic liposomes have the potential to serve as carriers of active substances, provided that suitable preparation conditions and membrane component compositions are chosen. At the same time, the findings offer opportunities for further testing and analysis in this area.

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LIST OF ABBREVIATIONS

AP	Ascorbyl palmitate
AX	Astaxanthin
BAC	Benzalkonium chloride
BC	β -carotene
CMC	Critical micellar concentration
CPCL	Cetylpyridinium chloride
CTAB	Cetyltrimethylammonium bromide
DLS	Dynamic light scattering
DOTAP	Diioleoyltrimethylammonium propane
DPTAP	1,2-Dipalmitoyl-3-trimethylammonium-propane
EA	Edge activator
EL	Elastic liposomes
HA	Hyaluronic acid
HEX	Hexadecylamine
HPC	Hydroxypropyl cellulose
HPLC	High-Performance Liquid Chromatography
LDH	Lactate dehydrogenase release
LMWP	Low-molecular-weight protamine
LUT	Lutein
LUV	Large unilamellar vesicles
MLV	Multilamellar vesicles
NaCl	Sodium chloride
OLV	Oligolamellar vesicles
PBMC	Primary blood leukocytes
PBS	Phosphate-Buffered Saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PTFE	Polytetrafluoroethylene
RI	Relative inhibition
ROS	Reactive oxygen species
SPC	Soybean Phosphatidylcholine
SUV	Small unilamellar vesicles

TOAC	Tocopheryl acetate
TOK	Tocopherol
ÚTTTK	Department of Lipids, Surfactants and Cosmetics Technology
UV	Ultraviolet radiation

