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APPLICATION OF THE QUALITY BY DESIGN METHODOLOGY IN THE DESIGN AND DEVELOPMENT PROCESS OF LIPOSOMAL DELIVERY SYSTEMS

Ph.D. Thesis

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I. **Zsófia Németh**: A Quality by Design-based approach to developing an intranasal liposomal formulation; *Medical Conference for PhD Students and Experts of Clinical Sciences 2018, 27. October 2018. Pécs, Hungary*

II. **Zsófia Németh**, Dorina Gabriella Dobó, Edina Pallagi, Ildikó Csóka: Basic research methods in the development process of the liposomal formulations; *I. Symposium of Young Researchers on Pharmaceutical Technology, Biotechnology and Regulatory Science, 31. January 2019. Szeged; Hungary*

III. **Zsófia Németh**, Dorina Gabriella Dobó, Edina Pallagi, Ildikó Csóka: Preparation and characterization of liposomes and the importance of their compositions; *XVI. János*

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V. **Zsófia Németh**, Dorina Gabriella Dobó, Edina Pallagi, Ildikó Csóka: Quality by Design-based approach for liposomal development; *II. Symposium of Young Researchers on Pharmaceutical Technology, Biotechnology and Regulatory Science, 23-24, January 2020. Szeged, Hungary*

VI. **Zsófia Németh**, Dorina Gabriella Dobó, Edina Pallagi, Ildikó Csóka: How can the application of Risk Assessment support the development process of liposomes?; *Medical Conference for PhD Students and Experts of Clinical Science 2020, 17. October 2020. Pécs, Hungary*

VII. **Zsófia Németh**, Dorina Gabriella Dobó, Edina Pallagi, Ildikó Csóka: Quality-focused formulation - QbD-based liposome design and development; *III. Symposium of Young Researchers on Pharmaceutical Technology, Biotechnology and Regulatory Science, 20-22. January 2021. Szeged, Hungary*

VIII. **Zsófia Németh**: How can the application of the Quality by Design approach assist the design and development process of liposomes*?; Scholars Webinar on: Drug Delivery and Nanomedicine, 24-25. March 2021. online*

IX. **Németh Zsófia**, Sipos Bence, Reza Semnani Jazani, Dobó Dorina Gabriella: Liposzóma alapú nano-hordozórendszerek kockázatbecslésre épülő optimalizálása; *XIV. Clauder Ottó Emlékverseny, 2021. november 11-12. Budapest, Magyarország*

X. **Zsófia Németh**, Reza Semnani Jazani, Bence Sipos, Dorina Gabriella Dobó, Edina Pallagi, Ildikó Csóka: Risk-based optimization of liposome-based nano-carrier systems; *IV. Symposium of Young Researchers on Pharmaceutical Technology, Biotechnology and Regulatory Science, 19-21. January 2022. Szeged, Hungary*

Poster presentations

I. Edina Pallagi, Orsolya Jójárt-Laczkovich, **Zsófia Németh**, Piroska Szabó-Révész, Ildikó Csóka: Risk Assessment based nano-sized liposome formulation development; *12th Central European Symposium on Pharmaceutical Technology and Regulatory Affairs and Satellite Symposium on Pharmaceutical Biotechnology, 20-22. September 2018. Szeged, Hungary*

II. **Zsófia Németh**, Dorina Gabriella Dobó, Edina Pallagi, Ildikó Csóka: Analytical investigation techniques in the service of liposome development; *26th International Symposium on Analytical and Environmental Problems, 23-24. November 2020. Szeged, Hungary*

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IV. **Zsófia Németh**, Reza Semnani Jazani, Dorina Gabriella Dobó, Ildikó Csóka: Factorial Design-based liposome optimization applying vesicle-modifying membrane additives; *13th Central European Symposium on Pharmaceutical Technology, 16-18. September 2021. Gdansk, Poland*

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III. Reza Semnani Jazani, **Zsófia Németh**, Dorina Gabriella Dobó, Ildikó Csóka: Essential oils and liposomes – Potentials in their pharmaceutical use*; 4th Young Technologists' Forum 2021, 26. May 2021. Budapest, Hungary*

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IV. Reza Semnani Jazani, **Zsófia Németh**, Dorina Gabriella Dobó, Ildikó Csóka: Pharmaceutical study of essential oil-loaded liposomal formulations; *IV. Symposium of Young Researchers on Pharmaceutical Technology, Biotechnology and Regulatory Science, 19-21. January 2022. Szeged, Hungary*

V. Bence Sipos, **Zsófia Németh**, Edit Kisvári, Ildikó Csóka: Knowledge Space development and Risk Assessment-based evaluation on hyperlipidaemia-related patient adherence; *XXV. Spring Wind Conference, 6-8. May 2022. Pécs, Hungary*

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ABBREVIATIONS

ΔH_m	Enthalpy changes
AFM	Atomic Force Microscopy
ANOVA	Analysis of Variance
API	Active Pharmaceutical Ingredient
BBB	Blood-Brain Barrier
CARPA	Complement Activation-Related Pseudoallergy
CH	Cholesterol
CMAs	Critical Material Attributes
CPPs	Critical Process Parameters
CQAs	Critical Quality Attributes
DCP	Dicetyl phosphate
DLS	Dynamic Light Scattering
DoE	Design of Experiments
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
DOPE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
DPPE	1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine
DPPE-PEG$_{2000}$	1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]
DS	Design Space
DSC	Differential Scanning Calorimetry
DSPC	1,2-distearoyl-sn-glycero-3-phosphocholine
DSPE-PEG$_{2000}$	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]
DSPE-PEG$_{3000}$	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-3000]
EE%	Encapsulation Efficiency
FITC	Fluorescein isothiocyanate
FT-IR	Fourier Transform Infrared Spectroscopy
GDNF	Glial cell-line derived neurotrophic factor
GUV	Giant Unilamellar Vesicle
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use

LUV	Large Unilamellar Vesicle
MAs	Material Attributes
MUV	Medium-size Unilamellar Vesicle
MVL	Multivesicular Liposome
NBCDs	Non-Biologically Complex Drugs
PBS pH 4.5	Phosphate-Buffered Saline pH 4.5
PBS pH 5.6	Phosphate-Buffered Saline pH 5.6
PBS pH 7.4	Phosphate-Buffered Saline pH 7.4
PC	L-α-phosphatidylcholine
PdI	Polydispersity Index
PE	Phosphatidylethanolamine
PEG	Polyethene glycol
PPL	Phospholipid
PPs	Process Parameters
QbD	Quality by Design
QbT	Quality by Testing
QTPP	Quality Target Product Profile
RA	Risk Assessment
RES	Reticuloendothelial System
SA	Stearylamine
saline solution	Sodium Chloride Physiological Solution
Span 60	Sorbitan monostearate
SSA	Specific Surface Area
SUV	Small Unilamellar Vesicle
T_c	Gel to Liquid-Crystalline Phase Transition Temperature
T_g	Glass Transition Temperature
TGA	Thermogravimetric Analysis
T_m	Phase Transition Temperature
Tween 20	Polyethylene glycol sorbitan monolaurate/Polysorbate 20
Tween 80	Polyethylene glycol sorbitan monooleate/Polysorbate 80
ZP	Zeta Potential

1. INTRODUCTION

The pharmaceutical industry has complex areas evolving dynamically due to research and development trends, market dynamics, and regulatory requirements leading to new scientific and technical issues to be addressed[1]. Whilst in basic research, phenomena are examined thoroughly, and their possible further utilisation is evaluated; applied research deals with technological and production developments.

Besides the comprehensive collection of the available biopharmaceutical knowledge, the 'unmet therapeutic needs' should also be revised to find possible therapeutic goals at the beginning of product development. The formulations must meet the requirements of the trio of the active pharmaceutical ingredient (API), the administration route, and the dosage form. The stakeholders' expectations should be kept in mind: a solution should be provided for the patient, and a profitable plan for the industry. Regulatory requirements should also be considered even at the early stage of development. In terms of production, besides quality, affordable costs, and challenges of upscaling processes, the novel pharmaceutical developments should consider following social responsibility trends, e.g. applying green technologies. The product properties should be designed into the product and integrated into the development process when the target product profile is defined for a product with expected quality and functionalities, helping to maintain patient adherence. Conscious and strategic analysis and planning are needed to evaluate and incorporate these input requirements into the development plan[2]. This is ensured by the Quality by Design (QbD)-based approach that can be used even in the early stages of pharmaceutical research and development and results in time- and cost-effective implementation from research to product marketing and industrial-scale manufacturing.

Liposomal formulation development is a challenging process. Liposomes have been proven to be successful nanocarriers for targeted gene and drug delivery since their first fabrication in the early 1960s; however, the level of the challenges rises in parallel to the number of information and developments in this field. Certain factors have critical influences on the characteristics of liposomes. The various production techniques require different material attributes (MAs, characteristics of the components) and process parameters (PPs, settings of the preparation process). To get a high-quality product, knowledge of medical, pharmaceutical, chemical, biological and physical sciences should be combined with technological experiences[3]. All this information needs to be considered, organized, and evaluated to develop a successful liposome-based formulation. Adopting the QbD concept and the Risk Assessment (RA) method to the initial development phase of the liposomal formulations assists in optimizing the vesicles and rationalising their design and study[4].

2. THEORETICAL BACKGROUND

2.1. Liposomes

Liposomes are described as artificially prepared vesicles composed of one or more concentric lipid bilayers enclosing one or more aqueous compartments by the European Medicine Agency[5]. Liposomes as drug carrier systems have several advantages[6] and thus are at the centre of up-to-date pharmaceutical investigations. The main potentials originate from their wide variety and various application possibilities[7]. These vesicles can be illustrated as microscopic phospholipid (PPL) 'bubbles' surrounded by a bilayered membrane structure and an aqueous core in the centre[8]. Liposomes provide a suitable delivery system for hydrophobic drugs (in the nonpolar membrane) and hydrophilic compounds (in the polar central phase). Liposomal administration of the API can reduce the potential side effects and toxicity, improve the efficacy and provide more favourable pharmacokinetic profiles and targeted therapy.[9] PPLs (natural or artificial) and cholesterol (CH) are the main components of liposomes and cell membranes. The bilayer structure is spontaneously formed from the PPLs in an aqueous milieu due to their amphiphilic property: a hydrophilic 'head' (including a phosphate group) and a hydrophobic 'tail' (two fatty acid chains) connected via a glycol molecule. Phosphatidylcholine (PC), the most common neutral PPL of the biological membranes, has a choline molecule in its structure as the 'head' group. The most often negatively or neutrally charged apolar carbon chains of the PPLs are oriented toward each other while the polar heads condense into a layer and the outer and inner aqueous phases. The rigidity and the permeability of this bilayer depend on the length and the saturation of the carbon chains. The more saturated chains build up the bilayer, the more stable the liposome is. The integrity of the membrane originates from its CH content. The fluidity of the bilayer can be changed by the interaction between the PPL fatty acid chains and CH.[10] Charged liposomes can be formed from cationic and anionic PPLs completing the neutral lipids and causing electrostatic repulsion between the layers[11]. The release of the active agents of the liposomes is modified by the phase transition temperature of the lipid complex. The CH builds into the membrane and fills the gaps between the PPLs, which decreases the fluidity of the membrane[12]. To increase their circulation time, liposomes are also commonly PEGylated, i.e., polyethene glycol (PEG) chains are attached to the PPL surface during the production process, which results in steric inhibition of immune response phagocytosis[13].

2.1.1. Differentiation of liposomes

From the discovery of liposomes made by Alec D. Bangham in 1965[14] until today, four different generations of liposomes have been distinguished. The first-generation liposomes (conventional liposomes) are made up of neutral and/or negatively charged PPLs and CH[15]. In cases of intravenous administration, these vesicles are taken up by the reticuloendothelial system (RES) (phagocytes); thus, their circulation time is short[16]. The second generation consists of long-circulating liposomes, while the third generation is made from surface-modified liposomes that can avoid the defence mechanism of the immune system. The fourth generation is built from the polyethene glycol-attached PEGylated or so-called 'stealth' liposomes[7,15]. The surface of these vesicles is coated with a hydrophilic polymer, such as PEG, that increases the repulsive forces between the liposomes and thus avoids the protein adsorption and opsonisation of the liposomes by the RES[16,17]. In this way, a longer residence time is provided for the liposomes to remain in the tumour tissues[17]. Beyond the generational grouping of the liposomes, liposomes can be classified regarding their compositions and drug delivery mechanisms such as conventional liposomes, long-circulating liposomes, polymorphic or bioresponsive liposomes[18–20] (pH-sensitive, thermosensitive, cationic liposomes), and decorated liposomes (surface-modified vesicles and immunoliposomes)[9,21]. Recent studies focus on immunoliposomes that bind to antibodies or antibody fragments on their surface, cationic liposomes composed of positively charged PPLs, and stimuli-responsive vesicles sensitive to local environmental conditions[22,23]. Liposomes are used for the application of highly potent medications. Their pharmaceutical application is essential in cancer therapy, and several studies are in progress in newly targeted medical areas focusing on alternative delivery routes[24–26].

2.1.2. Challenges of liposome development

Liposomal formulations have a highly focused role in the therapy development of unmet clinical needs and diagnostic imaging techniques; however, the regulatory authorities need to meet several challenges regarding the quality, safety, and efficacy of liposome-based products that make liposome development a more challenging task[27,28]. Although more and more liposomal products are entering clinical trials and registration, the regulation of this area is not complete yet[29]. The development of liposome-based products, the equivalence studies for the follow-on versions and their official authorisation procedure are hampered by the fact that these systems belong to the group of non-biologically complex drugs (NBCDs). Due to the complexity and diverse clinical use of NBCDs, it is not possible to establish a general regulatory procedure

for these products; only product-specific guidelines are available.[30] The guideline of the European Medicines Agency[5] provides information on relevant clinical and non-clinical data required for the authorisation of intravenous liposomal products; however, it does not specify concrete analytical and testing strategies or criteria systems, only general principles for the evaluation of liposomal products made only for the traditional intravenous application. The guideline is noncompliant with the research on the development process of liposomes targeting alternative drug delivery route administration. As even small changes in liposomes have a significant effect on the result parameters, delivery route-specific criteria, a well-defined manufacturing process and optimal process control are required to ensure that the quality of the product meets the quality requirements. Answerable questions appear from the development side as well. Production of long-term stable formulations, thus reaching proper zeta potential, and recovering the original quality of the freeze-dried samples during reconstitution prior to application, are still challenging for the researchers. Although liposome research has a nearly 60-year-old history, the composition, proportions, and amounts in the literature are still based on tradition. The liposome recipes work but differ from research group to research group and have not been optimized in comprehensive studies. The applied compositions, the chosen production methods, and the opted parameters greatly influence the experimental results. The reason why those formulations were previously studied and how the circumstances were selected are important for further utilisation of the results. Finding the most appropriate compositions for the purposes and achieve the best results are one of the challenges of these times. Thus, as it was done in the present work, the critical parameters influencing the liposome characteristics need to be identified and set to maintain all the necessary parameters for an applicable formulation, e.g. the vesicle size, the polydispersity and the zeta potential.

2.2. Quality by Design approach

There was a paradigm shift in the early 2000s in the pharmaceutical industry. The classical Quality by Testing (QbT) method, when the quality was assessed only after the product was designed and manufactured, has been replaced by the QbD approach, a process control and quality management system built up from knowledge, design and risk analysis.[31] This concept focuses on the prior definition and design of the target product, considering the needs and requirements emerging from the clinical side (patient), the industrial processes, and the regulatory aspects. The QbD is a holistic, systemic, structured, knowledge-and RA-based quality management approach, followed mainly in the pharmaceutical industrial production process.[32,33] However, it also can be extended and applied in the early pharmaceutical research and

development phase[2,4,34,35]. In general, following the QbD concept results in a better understanding of the product, the process, and the relationship between the material- and production technique-related factors[36]. The scientific data-originated information network provides proper background and applicability for the results from studies following the QbD principles. Organized, reasoned and well-designed experimental plan and work are needed to combine the knowledge and the requirements for development studies on nanoscale drug delivery systems if the API, the administration route, or the carrier has limitations. Nowadays, including the QbD elements during the submissions of the marketing authorisation documents is a regulatory requirement.

2.2.1. Elements of the QbD process

The whole QbD method is described in the guidelines of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH)[37–39]. Briefly, the complete QbD method includes the following steps[37]: (1) Defining the Quality Target Product Profile (QTPP) based on the knowledge space developed from the relevant scientific literature according to the appropriate in vivo relevance and deciding on the manufacturing process. (2) Identifying the Critical Quality Attributes (CQAs), the potentially critical MAs and PPs. (3) Performing the RA[38], finding the Critical Material Attributes (CMAs) and the Critical Process Parameters (CPPs), and setting up the Design of Experiments (DoE) based on the RA results. (4) Determining the Design Space (DS). (5) Setting up a process Control Strategy to ensure consistent product quality. (6) Accomplishing Product Lifecycle Management complemented with Continuous Improvement in the industry.

2.2.2. Advantages of the QbD approach

The primary focus of the method is on the profound preliminary target product design. Prior knowledge (from literature and former research experiences) and risk estimation is added to the theoretical design phase of a development process. This accurate design, especially the implementation of the RA, helps set up the practical experiments correctly in practice. It is advantageous in the early nano-pharmaceutical developments of complex or sensitive drugs and systems with special considerations[2,40–45]. The development process of liposomes is challenging due to their complex manufacturing processes. The tools of the QbD approach can guide the formulation process to obtain better quality for the liposomal products[46]. A well-built Knowledge Space and the RA provide a stable base to classify the formulation requirements, e.g. stability.

2.3. Stability aspects: the crucial role of zeta potential

Solid surfaces can possess a non-zero surface charge due to dissociative surface groups, specifically adsorbed ions, even without an external potential bias. At the investigation, exponentially decreasing potentials are measurable around the nanoparticles. The highest value is located at the particle surface (surface potential). The so-called Stern layer borders the particle, and the surface potential is neutralised by its closely associated ions of opposite charge. The rigid inner Stern layer proceeds in a looser diffuse layer of ions. The boundary layer, which separates the species attached to the surface and the mobile medium, is the slip(ping) or shear plane. The ion layers are referred to as the electrical double layer.[47–50] The electrokinetic potential for colloidal systems is defined as the zeta potential (ZP). That is the electric potential measured between the double layer interfacial at the slipping plane around the particle related to a point in the medium; and refers to the magnitude of the surface charge. As it is thus the average electrostatic potential at the hydrodynamic plane of shear, ZP is between the Stern plate and the diffuse layer, generally 0.2 nm from the surface.[51,52] ZP characterises not only the electrical double layer and the nanoparticle but the colloidal formulation itself in surface adhesion and stability studies giving information about the stability, circulation time, protein interactions, permeability, and biocompatibility of the nanoparticle[47,49]. However, the ZP analysis technique is sensitive, and therefore the measurement conditions need to be defined and repeated for comparable results. The ZP value is influenced by the temperature, solvent viscosity, pH, ionic strength, and surface characteristics of the nanoparticles. Even minor differences in these parameters can result in significant changes in the ZP values.[47]

2.3.1. The optimal zeta potential value

The magnitude of the ZP can predict the stability of a nanoformulation. High values show highly charged particles that prevent aggregation and ensure redispersion due to repulsive electric forces, while at low ZP, coagulation may form[53–55]. As a general rule, ZP above 30 mV in absolute value is considered a sign of good stability. Above 60 mV shows excellent stability properties for the formulation[53,54,56]. ZP greater than ±30 mV indicates monodisperse formulations without aggregation [50]. Nanodispersions with absolute ZPs around 20 mV are prone to have only short-term stability, and under 5 mV tend to aggregate rapidly[54].

Nevertheless, the ZP value is not the absolute sign of nanoparticle stability. The observations above were made for electric stabilisation and low molecular weight surfactants. In the case of higher molecular weight surfactants that apply steric stabilisation or combined

electrostatic and steric stabilisation techniques, 20 mV or lower absolute ZPs are enough for good stability because the large molecules or polymers adsorbed onto the particle shift the slipping plane further from the surface reducing the potential value.[54] The aggregation of the particles may be prevented by steric stabilisation via the addition of non-ionic media or changing the conditions of the dispersion medium[56].

2.3.2. In vivo behaviour of nanoparticles

The shape, size and charge of the nanoparticles influence their cellular uptake. The attraction between the nanoparticles and the cellular membrane is based on electrostatic interactions. The ZP of the particles influences the cell and tissue binding processes. Particles with a positive charge can attach to the cell membrane as it has negatively charged sites. Due to the repulsive forces, the negatively charged particles bind to the cationic domains in clusters. Higher ZPs lead to stronger membrane binding and result in higher cellular uptake.[52,57] Electrostatic interactions also influence the protein adsorption of the nanoparticles. Particles with positive ZP were found to adsorb well to proteins, while negatively charged ones did not show a significant level of bondage. Protein-binding can be influenced by changing the surface charge[54]. The aggregation of the particles with near-neutral ZP determines their elimination by the RES[50]. Surface modification can mask the particles and provide a more extended stay in the systemic circulation.[52,54] Particles with positive ZP are suitable for mucoadhesive formulations as these systems can firmly adhere to the negatively charged mucosa[58]. Nanoparticles with cationic charge are reported to have enhanced cytotoxicity compared to the negatively charged ones; however, liposomes constituted from negatively charged lipids were found to be less stable at intravenous administration than the neutral and positive ones[22,54]. Besides the fact that conventional anionic liposomes are eliminated rapidly by the RES, their application can cause pseudoallergy manifesting in vasoconstriction, pulmonary hypertension, dyspnoea, platelet and leukocyte number decrease; thus, liposomes have not been widely applied without modifications for intravenous drug delivery.[22] Particles with both positive and negative ZP have their benefits. It is the task of researchers to optimize formulations with effective intracellular delivery and a minimal level of toxicity.[54]

The properties of the nano-delivery systems, i.e. circulation, release, and absorption, are influenced by the characteristics of the nanoparticles, like the particle size and the ZP[52]. At the liposome-cell interaction, the vesicle wall can adsorb into the cell membrane, degrade, and then the released content can diffuse to the cytoplasm. The molecules are delivered directly into the cytoplasm when the liposome fuses with the cell membrane. From a maximum diameter of 150

nm vesicles, the drug content can be transported into the cell by receptor-mediated endocytosis. The mechanism of the liposome-cell interaction depends on the features and charge of the liposome surface.[22] For a proper drug release, the affinity between the drug and the medium must be higher than between the pharmaceutical ingredient and the drug delivery system. Loading a drug into the nanoparticle can alter its ZP.[50]

2.3.3. Importance of zeta potential in therapy

Reaching the suitable ZP value is essential for effective nanomedicines. The ZP of the nanomaterials can influence the targeted therapy, the stability and the drug release profile of the dosage form.[52,54] Due to enhanced penetration properties, charged liposomes have been developed for transdermal delivery. The negatively charged vesicles diffuse to the dermis faster than the positive liposomes.[59,60] Gene delivery is implemented via the application of cationic liposomes since Felgner's research group described in 1987 that the positively charged liposomes tend to form complexes with the negatively charged nucleic acids and trigger the cellular uptake and the expression of the carried DNA/RNA content[22,61]. Due to the negatively charged endothelial cell surface, tumour cells favourably take up positively charged nanoparticles which retain longer than negative or neutral ones. Cationic drug delivery systems also take part in the passive targeting of tumours.[52] Other studies showed that particles with a slightly negative ZP and a vesicle size of 150 nm are prone to accumulate in the tumour. The electrostatic interactions between the nanocarrier systems and the membrane of cells can be utilised in the transportation through the blood-brain barrier (BBB). The negatively charged BBB cell membrane attracts particles with positive ZP at the adsorptive mediated endocytosis.[52]

2.4. Modification of zeta potential

ZP can be modified by many factors, such as the liposome composition, charged lipids, the pH and ionic strength of the hydration media, and the production parameters. The addition of charged molecules to the composition of the nanocarriers is one of the best practices for stabilising the formulations. By incorporating various charge-inducing agents into the PPL bilayer of the liposome (stearylamine (SA) or dicetyl phosphate (DCP)), the ZP of the vesicles can be modified, its absolute value, and thus the stability of the samples can be increased[62,63]. SA gives a positive/cationic charge to the vesicles, while DCP a negative/anionic charge, thus preventing their aggregation by imparting stability due to electrostatic mechanisms[64]. Experimental results demonstrated the oxidative stability-enhancing effect of these substances[65]. The inclusion of the charge inducers into the membranes can suppress the aggregation process

by electrostatic stabilisation[66,67]. DCP as cetyl alcohol comprises 16 carbons without double bonds and is a negatively charged PPL that maintains a net negative charge and possible long-term stability to the liposomes[10,68–71]. Cationic, synthetic lipids can incorporate a positive charge into the liposome membranes and are thus commonly used in nucleic acid delivery[10]. The polar head groups of the cationic lipids form electrostatic interactions with the negatively charged phosphate groups of the genes[72]. SA contains an ionisable nitrogen atom with a positive charge on physiological pH[67]. It has been observed that SA distributes asymmetrically in the lipid bilayer, located mainly on the outer surface of the liposomes[73]. Studies on SA-nanoparticles showed increased stability, minimised drug leakage, and a controlled release profile[74].

2.4.1. Limitations of charge imparting agents

Cytotoxicity limits the clinical use of SA as the hydrophilic nitrogen 'head' group of the molecule interacts with specific enzymes[63,74]. Studies reported apoptosis induced by SA generating reactive oxygen species, activating protein kinase C, or enhancing the release of apoptosis-dependent proteins[75,76], and haemolysis arising from the interaction between the SA and the negatively charged erythrocyte membrane. Human red blood cells are less sensitive to SA; thus, the addition of small amounts can be safe[77]. Adams and his colleagues showed that intracerebrally administered SA liposomes led to respiratory failure and brain damage, while DCP caused epileptic seizures and rapid death in mice[78,79]. In contrast, DCP is a safe cosmetic ingredient, even if it has lower skin permeability than SA due to the negatively charged mammalian skin[63]. Inglut et al. made a review study on the immunological and toxicological effects of liposomes. Their conclusion was that relatively low mol% of CH and polyethene glycol is recommended for the intravenous application of chemotherapeutic agents. Liposomes with ZP less than 30 mV should be considered for gene delivery to minimize toxicities.[79] The toxicity of the formulations can differ based on the compositions, the delivery routes, and the applied models; thus, they should be evaluated individually in relevant circumstances.

2.4.2. Overview of the SA- and DCP-liposomes

The literature on SA- and DCP-containing liposomal formulations was accurately checked. The relevant findings were collected in ***Tables 1 and 2*** after performing calculations if that were necessary to express the compositions in molar ratios. The applied ratios and the results varied mainly from study to study, justifying the importance of a time- and material-saving experimental design-based research and later development of liposomal formulations containing SA and DCP.

Table 1. *Summary of data on SA-containing formulations compared with the optimized composition of this present study*

Composition	Molar ratio	Drug	Size (nm)	PdI	ZP (mv)	Source
PC:CH:SA	1:1:3.85	ketorolac tromethamine	7060 ± -	0.43 ± -	-	Mehanna et al.[80]
PC:CH:SA:Span 60	1:1:0.15:1	flucytosine	135 ± 12	0.27 ± -	+42.5 ±2.1	Salem et al.[81]
PC:SA:Tween 20	20:6.3:2.4	curcumin	252 ± 52	0.17 ± 0.01	+34.0 ± 0.6	Ternullo et al.[82]
PC:SA:Tween 20	20:6.3:2.4	curcumin	232 ± 68	0.22 ± 0.04	+33.7 ± 1.1	Ternullo et al.[83]
PC:CH:SA	5.5:1.0: 1.5	butamben	240 ± 65	0.22 ± -	+30.2 ± 3.9	Mura et al.[84]
PC:CH:SA	6.6:10.3:11.13	sumatriptan	349 ± 100	0.28 ± 0.24	+37.9 ± 3.7	Villasmil-S. et al.[62]
PC:CH:SA	7:3:1.5	amphotericin B	940 ± 40	-	+28.4 ± 0.3	Soni et al.[85]
PC:SA	2:0.5	amphotericin B	140 ± 4	0.24 ± 0.04	+32.0 ± 0.2	Mishra et al.[72]
PC:SA	1:0.5	amphotericin B	202 ± 6	0.39 ± 0.03	+63.0 ± 0.4	Mishra et al.[72]
PC:CH; SA	9.13:1; 5.18 mg	paclitaxel	193 ± 2	0.17 ± 0.03	+38.2 ± 3.5	Ingle et al.[64]
PC:CH; SA	7:2; 5.00 mg	resveratrol	146 ± 10	-	+38.0 ±9.1	Jagwani et al.[73]
PC:SA	7:2	doxorubicin	148 ± -	-	+43.1 ± -	De et al.[86]
DSPC:CH:SA	7.5:2.5:0.5	prednisolone, methotrexate	159 ± 2	0.09 ± -	+6.3 ± 0.4	Verma et al.[87]
PC:CH:SA	7.8:2.6:2.9	pemetrexed disodium	220 ± 5	0.23 ± 0.02	+22.2 ± 0.5	He et al.[77]
PC:CH:SA	8:1:2	risperidone	209 ± 16	-	+22.4 ± 1.5	Narayan et al.[88]
PC:CH:SA	8:1:0.25	risperidone	99 ± 7	-	+15.6 ± 1.4	Narayan et al.[88]
PC:CH:SA	7:3:1.1	monensin	121 ± 20	0.25 ± 0.01	+43.9 ± 0.9	Rajendran et al.[89]
DOPC:CH:SA	10:6:1	GDNF	149 ±11	-	+30.0 ± 3.0	Migliore et al.[90]
DOPC:CH:SA	10:6:1	ovalbumin	299 ± 26	-	+19.0 ± 1.5	Migliore et al.[91]
DOPE:PC:CH:SA	10:45:29:16	-	95 ± 9	0.24 ± 0.03	+52.8 ± 3.7	Vhora et al.[76]
PC:CH:SA: DSPE-PEG_{2000}	11:7:0.6:1.4	-	209 ± 2	-	+48.7 ± 4.3	Tran et al.[92]
PC:CH:SA	7:3:1.1	-	77 ± 2	0.21 ± -	+32.9 ± 2.1	Sharma et al.[93]
PC:SA	7.3:1	-	81 ± 6	0.24 ± 0.02	+17.5 ± 1.8	Caddeo et al.[94]
PC:SA	7:2	-	146 ± -	0.20 ± -	+52.0 ± -	De et al.[95]
PC:SA	3:1	-	140 ± 49	-	+11.4 ± 0.4	Lotosh et al.[96,97]
PC:CH:SA	12:5:5	-	108 ± 15	0.20 ± 0.04	+30.1 ± 1.2	OPT-SA

Table 2. *Summary of data on DCP-containing formulations compared with the optimized composition of this present study*

Composition	Molar ratio	Drug	Size (nm)	PdI	ZP (mv)	Source
PC:CH:DCP	1:1:3.85	ketorolac tromethamine	8350 ± -	0.45 ± -	-	Mehanna et al.[80]
PC:CH:DCP	1:1:0.7	tretionin	318 ± 3	0.43 ± -	-41.2 ± 1.2	Rahman et al.[98]
PC:CH:DCP	6:1:1.5	silymarin	756 ± -	0.61 ± -	-77.3 ± -	Kumar et al.[99]
PC:CH:DCP	6.6:10.3:5.49	sumatriptan	549 ± 10	0.37 ± 0.09	-68.1 ± 0.4	Villasmil-S. et al. [62]
PC:CH:DCP:Span 60	1:1:0.1:1	flucytosine	159 ± 5	0.26 ± -	-59.1 ± 1.7	Salem et al.[81]
PC:CH:DCP:Tween 80	9:3:1:1	5-fluorouracil	108 ± 11	0.31 ± 0.05	-16.3 ± 1.5	Alomrani et al.[100]
PC:CH: DCP:DSPE-PEG_{2000}:DPPE	7:2:1:1:0.025	FITC-dextran	116 ± -	0.12 ± -	-29.0 ± -	Togami et al.[101]
PC:CH: DCP: DSPE-PEG_{2000}:DPPE	7:2:1:1:0.025	rhodamine B	125 ± -	0.09 ± -	-32.0 ± -	Togami et al.[101]
PC:CH:DCP:DSPE-PEG_{2000}	11:7:1.4:0.6	-	191 ± 4		-45.1 ± 2.5	Tran et al.[92]
PC:CH:DCP	15:8:1	-	195 ± 5	0.28 ± -	-47.0 ± 1.0	Calvo et al.[102]
PC:CH:DCP	10:4:1	-	146 ± 6		-18.6 ± 0.5	Ethemoglu et al.[103]
PC:CH:DCP	7:2:1	-	134 ± 4	0.12 ± 0.03	-49.4 ± 3.5	Togami et al.[104]
PC:CH:DCP	8.5:4.5:6.5	-	88 ± 14	0.21 ± 0.02	-36.7 ± 3.3	OPT-DCP

3. EXPERIMENTAL AIMS

The objective of my Ph.D. work was to develop and investigate liposomal formulations applying Risk Assessment and the Quality by Design principles. Two main research approaches were carried out during my work. The first was adapting the QbD concept and the RA methodology to the early development phase of a liposomal formulation. Then the second part was adjusting the zeta potential of liposomes to an adequate value maintaining the stability of the vesicles.

The first part of the research focused on the first four stages of the QbD implementation:

1. Developing the Knowledge Space and defining the Quality Target Product Profile.
2. Identifying the Critical Quality Attributes, the potentially critical Material Attributes and Process Parameters.
3. Performing the RA, finding the Critical Material Attributes and Critical Process Parameters, and setting up the Design of Experiments based on results.
4. Determining the Design Space.

In the studies, the requirements of liposome formulation prepared via the thin-film hydration preparation technique were investigated. After the universal initial RA[4], the highlighted critical parameters were investigated from new perspectives in an updated RA targeting 'intermediate' API-free liposomal formulations[35]. Comparative characterization studies were carried out, and the general effects of the selected CMAs and CPPs were determined on the properties of the liposomes: the PC-CH weight ratio, the PEGylated PPL content, the quality of the PEGylated PPL, the quality of the hydration media and the cryoprotectants, and the working temperature. The formulations were characterised based on their size, surface charge, thermodynamic behaviour, formed structure and bonds.

In the second part of the research work, as it was necessary for a stable formulation, the ZP value of the liposomes was improved:

The optimal molar ratios of the PC, CH and the charge imparting membrane additive SA or DCP were determined in a 3^2 fractional factorial design. The following primary outcomes were required for the develop liposome formulations to be accepted:

- vesicle size under 150 nm
- polydispersity index less than or equal to 0.30
- zeta potential higher than |30| mV

4. MATERIALS AND METHODS

4.1. Materials

Liposomes for the RA studies and the ZP optimization were made from the alcoholic solutions of the following materials in different combinations and ratios according to the DoE in ***Figure 1***: cholesterol (CH) (Molar Chemicals Kft., Budapest, Hungary), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DPPE-PEG_{2000}) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-3000] (ammonium salt) (DSPE-PEG_{3000}) (Avanti® Polar Lipids Inc., Alabaster, AL, USA), L-α-phosphatidylcholine (PC), and octadecylamine (=stearylamine, SA), or dihexadecyl phosphate (=dicetyl phosphate, DCP) (all purchased from Sigma-Aldrich Chemie GmbH, Munich, Germany). The lipids were dissolved in ethanol 96%(V/V) (Molar Chemicals Kft., Budapest, Hungary).

Phosphate-buffered saline pH 7.4 (PBS pH 7.4), pH 5.6 (PBS pH 5.6) and pH 4.5 (PBS pH 4.5); furthermore sodium chloride physiological solution (saline solution) pH 5.5[105] were used as hydration media. The composition of these solutions are the followings: PBS pH 7.4: 8.0 g/L NaCl, 0.20 g/L KCl, 1.44 g/L Na_2HPO_4 x 2 H_2O, 0.12 g/L KH_2PO_4; PBS pH 5.6: 0.65 g/L K_2HPO_4, 8.57 g/L KH_2PO_4; PBS pH 4.5: 6.80 g/L KH_2PO_4; physiological saline solution: 9.0 g/L NaCl dissolved in purified water. The materials used to make these hydration media are the following: sodium chloride (NaCl), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4) (Molar Chemicals Kft., Budapest, Hungary), disodium hydrogen phosphate dihydrate (Na_2HPO_4 x 2 H_2O), and dipotassium phosphate (K_2HPO_4) (Spektrum-3D Kft., Debrecen, Hungary). Four carbohydrates were used as cryoprotectants in 5% of the PPL mass for lyophilisation: D-glucose, D-sorbitol (Hungaropharma Zrt., Budapest, Hungary), D-trehalose and inulin (Sigma-Aldrich Chemie GmbH, Munich, Germany).

4.2. Methods

4.2.1. Application of the Quality by Design method

4.2.1.1. Knowledge Space development and definition of the QTPP

The initial step of a QbD-guided development process is to define the target product profile of the aimed formula and its quality criteria. For this purpose, a primary knowledge space development[43] must be carried out, which means collecting and systematising all the relevant information regarding the aimed product and the production. In order to see all the influential

parameters of the desired liposome product together, an Ishikawa (cause-effect or fishbone) diagram was constructed to collect and visualize these factors for further steps.

The QTPP is a prospective summary of the quality characteristics of the product that ideally will be achieved, ensuring the aimed quality, taking into account the safety and efficacy criteria. The selection of the QTPP factors is based on the requirements of the interested parties. It contains the essential parameters of the formulation from the patient's point of view, the requirements from the clinical field and the regulatory aspects. It usually covers the administration route, the dosage form, the dose, the appearance, the bioavailability, the strength, and the stability[38].

4.2.1.2. Determination of the CQAs

The second step is the selection of those factors, the CQAs, which have critical effects on the targeted product quality (QTPP). It forms a definitive list of the formulation characteristics derived from the QTPP and related to the safety and efficacy of the product. The selection of these parameters is based on prior knowledge and previous experiences. According to the definition of the ICH guideline, the CQAs are physical, chemical, biological or microbiological properties that should reach an appropriate range or limit to ensure constant end-product quality. The CQAs may include information about the particle or the vesicle size, the size distribution, the API, and the drug release[38].

4.2.1.3. Determination of the CMAs and the CPPs

In the next step, the CMAs and/or the CPPs should be determined, which are the factors relating to the chosen materials and the selected production method, thus may influence the CQAs and, therefore, should be monitored or controlled to ensure that the process leads to the targeted quality. As the nomination of a factor as a CQA, choosing the CMAs and CPPs depends on the predefined goals, the expected quality of the product, the therapeutic needs, and the selected production process[37,38].

4.2.1.4. Risk Assessment

The key step of the QbD-driven development process is the RA (initial, recurrent/updated or finalised) that assists in identifying and ranking the CMAs and CPPs based on their impact on the CQAs of the product[32]. The RA is typically performed as the first step during an early phase of the pharmaceutical development process and is re-evaluated when more information becomes available, and higher knowledge is obtained[37,38]. The LeanQbD software (QbD Works LLC,

Fremont, CA, USA) was used for the RA process. The procedure started with the interdependence rating between the QTPP and the CQAs, and the CQAs and the CMAs/CPPs. A three-level (1-3-9) scale was used to describe whether the relationship between the parameters is 'high' (H), 'medium' (M) or 'low' (L) and the results were presented in Risk Estimation Matrices. Risk is defined as the combination of the probability of the occurrence of harm and the severity of that possible damage. The scoring was done for each parameter pair individually. The qualitative three-level scale is linked to a numeric scale in the software to provide the severity scores of the evaluated risk factors based on mathematical calculations. After categorising the interdependence, a risk occurrence rating was made for the CMAs/CPPs using the same three-grade scale (H/M/L) for the analysis. During the risk evaluation, the software transformed the established risk levels into numerical scores that combined the provided information[106]. Pareto diagrams[107] were generated, presenting the ranking of the CQAs and the CMAs/CPPs according to their potential impact on the final product (QTPP). Due to that generated origin of the severity scores (influenced by the scale levels used for the analysis), the relative position of the factors should be considered in the evaluation of the charts instead of the values. The Pareto charts show the differences between the effects of the CMAs and the CPPs and help select the potential experimental design factors. Although software supports the RA process, identifying the risks and estimating their severity and occurrence is the task and responsibility of the research group. The software only makes the calculations and visualises the final results. These results form the basis of the DoE. The initial RA should be refreshed in an updated RA when new knowledge is found, or the research goal is changing.[38]

4.2.1.5. Design of Experiments

Based on the results of the initial RA, the DoE was built up[37]. The DoE is a practical development plan designed and carried out according to the most relevant influencing factors (CQAs, CMAs, CPPs) selected by the priority ranking of the RA in order to define the DS. The characterisation results of the liposomes made for the study were used as a base for updating the existing RA.

Six factors were chosen to investigate the significance of their effect on 'intermediate' API-free liposomal formulations and thus to validate their role as CMAs/CPPs: the PC-CH weight ratio, the PEGylated PPL content, the quality of the PEGylated PPL, quality of the hydration media and the cryoprotectants, and the working temperature.

The effects of the working temperature and the PC-CH weight ratio were investigated on conventional, only PC- and CH-containing compositions. Using the information obtained from

these early studies, the effects of the PEGylated PPL content, the type of PEGylated PPL, the quality of the hydration media and the type of the cryoprotectant were investigated under improved conditions (pre-set temperature (60°C) and PPL-CH weight ratio (60:40 or 80:20).

Two membrane additives, SA and DCP, were studied according to the 3^2 fractional factorial design to optimize the ZP of the liposomal formulations. The liposomes were formed at 60°C and hydrated with PBS pH 5.6. The selected independent variables were the molar quantities of the liposome components: PC, CH, and SA/DCP. These experimental factors were systematically varied at 3 levels and 9 runs. The molar value of PC ranged from 7.5 to 12.5 mmol, of CH from 3.5 to 5.5 mmol, while the amounts of the membrane additives (SA/DCP) were adjusted between 3 and 9 mmol. The effects of these independent factors on the vesicle size (Z-average), the polydispersity index (PdI) and the ZP values were investigated as a primary outcome. In the case of the ZP values, one-one quadratic response surface was plotted, and the second-order polynomial models were constructed. The relationship between the variables on the response could be analysed following this second-order equation:

$$Y = \beta_0 + \beta_1 x_1 + \beta_{11} x_1^2 + \beta_2 x_2 + \beta_{22} x_2^2 + \beta_3 x_3 + \beta_{33} x_3^2, \quad (1)$$

Where Y is the response variable; β_0 is a constant; β_1, β_2, and β_3 are linear coefficients; and β_{11}, β_{22}, and β_{33} are quadratic coefficients. Response surface plots for ZP were plotted according to the regression model for SA/DCP.

4.2.2. Preparation and lyophilisation processes of liposomes

The liposomes were prepared via the thin-film hydration method[14], the most common liposome production process described by Bangham's research group for the first time. This technique ensures a stable and straightforward way for liposome preparation[108] and, according to previous experiences[43], can be easily adapted for liposome studies. Several modified versions of the original technique exist[108]; however, the basic steps of the process are mutual: (1) preparation of the lipid film from PPLs and CH, (2) hydration of the thin lipid film with a hydration medium, and (3) modification of the numbers of lamellas and the size of vesicles. During the process, the ethanol was evaporated from the alcoholic compositions applied in the different ratios (***Figure 1***) at 150 mbar, at the investigated temperature or in general at 60°C in a Rotavapor® R-210/215 (BÜCHI Labortechnik AG, Flawil, Switzerland) rotary evaporator at 25 rpm rotation speed. The lipid film was hydrated with the selected hydration media. The formulations were subjected to a 30-minute ultrasonication (Elmasonic S 30 H ultrasonic bath, Elma Schmidbauer GmbH, Singen, Germany).

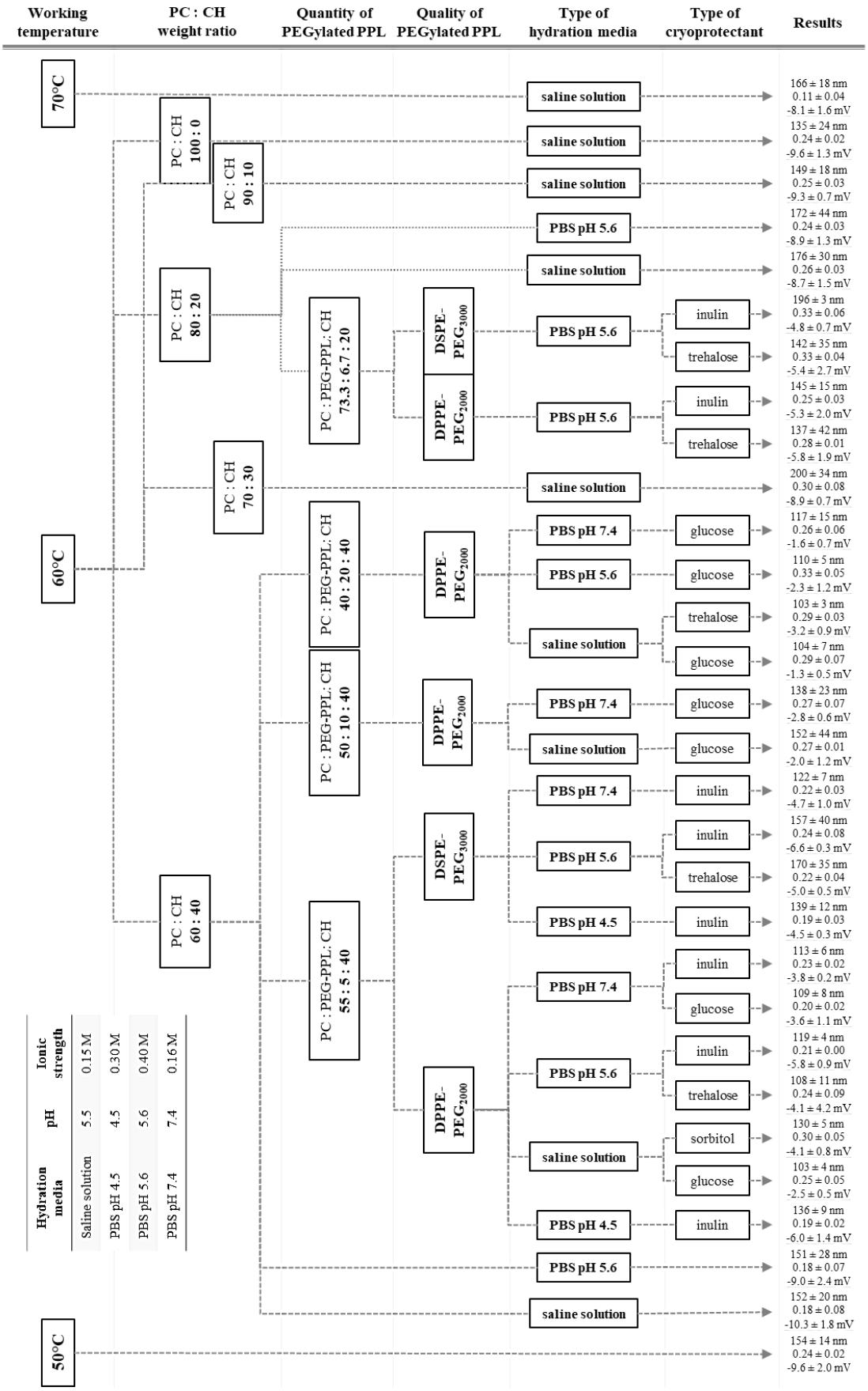

Figure 1. *A summary flowchart that combines the DOE presenting the investigated factors and applied combinations with the results of the liposome characterization studies.*

The liposomes were shaped in two steps via vacuum membrane filtration (Rocker 400 oil-free vacuum pump, Rocker Scientific Co., Ltd. New Taipei City, Taiwan) using a 0.45 μm (nylon membrane disk filter 47 mm, Labsystem Kft., Budapest, Hungary), then a 0.22 μm membrane-filter (Ultipor® N66 nylon 6.6 membrane disk filter 47 mm, Pall Corporation, New York, NY, USA). The obtained samples were immediately investigated for vesicle size, polydispersity and ZP in the liquid state and then lyophilised for further investigations (SanVac CoolSafe freeze dryer, LaboGene™, Lillerød, Denmark). During lyophilisation, the temperature was gradually decreased from +25°C to −40°C at atmospheric pressure, and then the pressure was reduced to 0.01 atm. The samples were dried for 8-10 hours before the temperature, and the pressure was increased step by step to +25°C and 1 atm, respectively. The lyophilised samples were stored at 2-8°C.

4.2.3. Characterization of the liposomes

4.2.3.1. Vesicle size and zeta potential analysis

The vesicle size (expressed in Z-average) and the PdI of the liquid liposome formulations were measured using the dynamic light scattering (DLS) technique. The International Organization for Standardisation defined the nanoscale size as the range extending between 1 and 100 nm. Based on their definitions, nanoparticles are those nano-objects that have all of their external diameters in the nanoscale, and there is no significant difference between the lengths of the longest and shortest axes of the particle[109]. Therefore, the size and size distribution of the liposomes are fundamental features of the systems. Our acceptance criterion for liposomes was size under 200 nm in general and under 150 nm for the optimized samples. The PdI is a dimensionless value theoretically between 0.00 and 1.00, providing information about the uniformity of the particles. In the case of lipid-based nanocarriers, formulations with a PdI of 0.30 and below are acceptable, indicating a homogenous population of the vesicles[110]. The ZP, the potential difference between the investigation media and the adsorbed stationary fluid layer on the particle surface, is one of the indicators of formulation stability. Absolute ZP values above 30 mV indicate good formulation stability[54]. The measurements were carried out using a Malvern Zetasizer Nano ZS system (Malvern Panalytical Ltd., Malvern, Worcestershire, UK) equipped with a 633 nm wavelength laser from 1 mL of samples in folded capillary zeta cells (Malvern Panalytical Ltd., Malvern, Worcestershire, UK) at 25°C.

4.2.3.2. A three-dimensional surface profile investigation

Atomic force microscopy (AFM) images of liposomes were obtained under normal ambient conditions using the tapping mode of an NT-MDT SolverPro Scanning Probe Microscope (NT-MDT, Spectrum Instruments, Moscow, Russia) from one drop of the formulations applied on a freshly cleaved mica surface (Muscovite mica, V-1 quality, Electron Microscopy Sciences, Washington, DC, USA). AFM tips (type PPP-NCHAuD-10, thickness: 4.0 μm, length: 125 μm, width: 30 μm, nominal radius of curvature: 2 nm; NanoWorld AG, Neuchâtel, Switzerland) were applied for the measurements. The non-contact silicon cantilevers had a typical force constant of 42 N/m and a resonance frequency of 330 kHz.

4.2.3.3. Thermal analysis

The DSC studies can give information about the phase transition temperature (T_m) of the liposomes[111]. The glass transition temperature (T_g) is an essential parameter regarding the stability of the lyophilised samples[112]. The thermodynamic state of the liposomes made for the RA studies was checked in the temperature range of 25-300°C via differential scanning calorimetry (DSC) technique (TA Instruments DSC Q20, TA Instruments, New Castle, DE, USA). The possible T_m and T_g temperatures were determined from 6-10 mg freeze-dried samples using a 10°C/minute heating rate in dry nitrogen gas flow. During the thermogravimetric analysis (TGA), 8-10 mg of the lyophilised samples were heated up at a temperature range of 25-300°C at a 10°C/minute heating rate and investigated for mass changes in the dry nitrogen gas atmosphere using the Setaram Labsys TG-DTG-DTA analyser (SETARAM Instrumentation, Caluire, France).

In the case of the samples made for the ZP optimization study, the DSC measurements were done with a Mettler-Toledo DSC 3^+ Star^e System DSC analyser (Mettler-Toledo International, Inc., Columbus, OH, USA) in the temperature range of 10-65°C at 2°C/min heating rate. 6-10 mg of the lyophilised samples were investigated in hermetically sealed aluminium sample pans under a 150 mL/min constant argon flow. The settings of the TGA studies were identical to the previously described one, but the measurements were carried out via a Mettler-Toledo TGA/DSC 1 thermogravimetric analyser (Mettler-Toledo International, Inc., Columbus, OH, USA). Empty aluminium pans were used as control. Every measurement was normalized to the sample size and was evaluated with the STAR^e 9.30 software (Mettler-Toledo International, Inc., Columbus, OH, USA).

4.2.3.4. Investigation of chemical bonding

Mid-infrared spectroscopy was used to get information about the chemical bonds forming between the liposome materials. The interactions between the compounds of the liposome products were measured via an Avatar 330 Fourier transformed infrared (FT-IR) Thermo Nicolet spectrometer (Thermo Electron Corporation, Waltham, Massachusetts, USA) equipped with an infrared light source and optics. Spectra were recorded on the freeze-dried powder samples in 4000-400 cm^{-1} wavenumber range with 4 cm^{-1} spectral resolution in absorbance mode. Samples were prepared using a hydraulic tablet press by compressing the lyophilised powders into pellets with potassium bromide (KBr) powder at 10 kN for 2 min (Specac Ltd., Orpington, UK). Pure KBr pellets were used as references.

4.2.3.5. Residual ethanol measurement

The liposome components were dissolved in ethanol 96% (V/V). The residual concentration of ethanol as a Class 3 solvent (solvent with low toxic potential) in pharmaceuticals must be less than 50 mg, i.e. concentration of 5000 ppm (0.5%) in the daily dose of the final product according to the requirements of the International Council of Harmonization guideline[113]. The amount of residual ethanol in the samples was determined using a Shimadzu GCMS-QP2010 SE gas chromatograph-mass spectrometer (Shimadzu Corporation, Kyoto, Japan) equipped with a Zebron ZB-5MSi column (Phenomenex, Torrance, CA, USA). The initial oven temperature was 80°C for 2 min, which was then increased to 180°C at 20°C/min and held at 180°C for 2 more minutes. The mass spectra were recorded in continuous scans from 0.5 to 1.6 min in the 25-46 m/z region. For the measurements, 1 mg/L sample solutions were made in toluene, and 5 μL aliquots were injected in each run. The system was calibrated using a 0.01 mmol L^{-1} ethanol solution in toluene.

4.2.4. Statistical analysis

Data analysis and graphs were made in Microsoft® Excel (Microsoft Office Professional Plus 2013, Microsoft Corporation, Redmond, WA, USA), OriginPro® 8.6 (OriginLab Corporation, Northampton, MA, USA) and JMP® 13 software (SAS Institute, Cary, NC, USA). One-way analysis of variance (ANOVA) statistical analysis was performed using the TIBCO Statistica® 13.4 software (Statsoft Hungary, Budapest, Hungary). For the significance study, the results were evaluated according to their p-value. Variables with p less than 0.05 at a 95% confidence level were considered significant. All experiments were performed in triplicates, and the corresponding mean and standard deviations were indicated.

5. RESULTS AND DISCUSSION

5.1. Quality by Design-based liposome development

5.1.1. Knowledge Space development and definition of the QTPP

In the first step of the QbD-based liposome development, the factors that may influence the quality of the liposome product were collected in the Knowledge Space development process. An Ishikawa diagram was assembled to illustrate the relationship between the information on liposomes and the elements of the liposome preparation process (***Figure 2***). The data were sorted into 4 groups regarding:

- the carrier system: monodisperse and stable liposomal formulation
- the liposomal formulation: physical characteristics of the vesicles
- material properties: liposome components and excipients
- preparation process: essential points of the thin-film hydration technique

These categories formed the base of the later definitions of QTPP, CQAs, CMAs and CPPs, respectively.

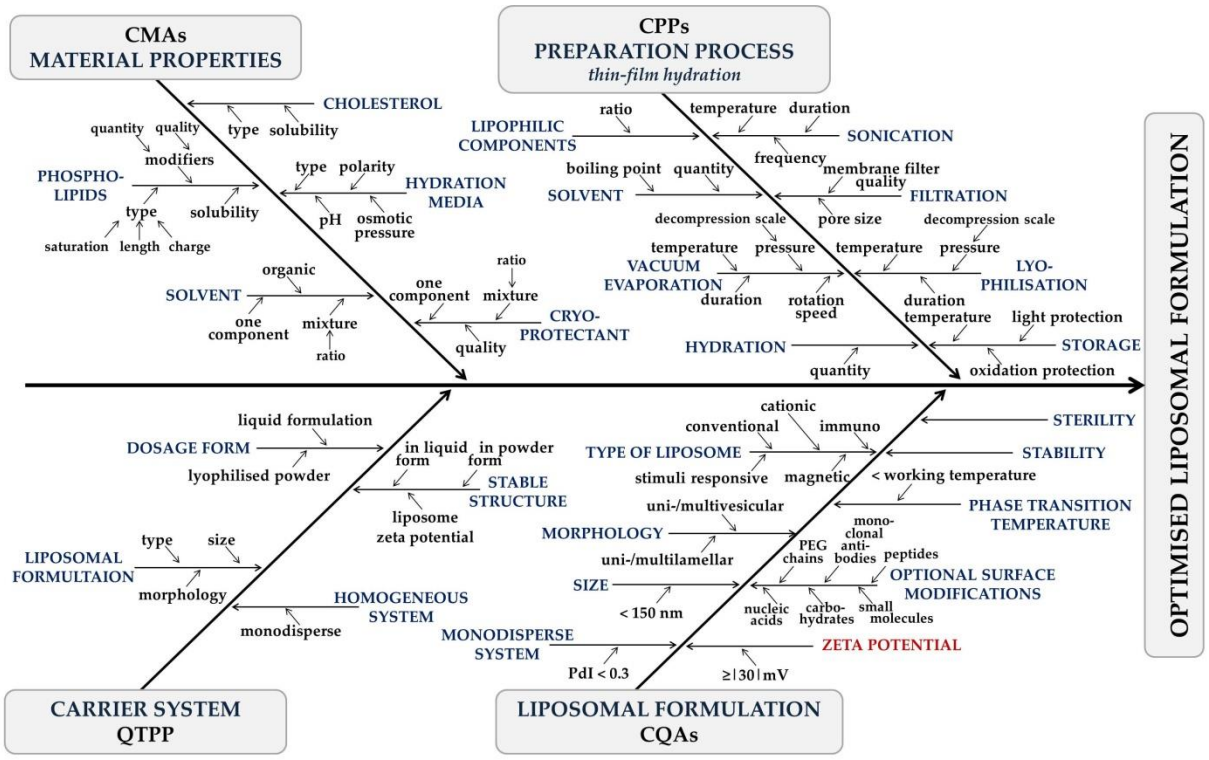

Figure 2. *Ishikawa diagram of the factors that impact the quality of the liposomes made in the thin-film hydration method.*

The factors that can describe a liposome formulation were collected, and a general QTPP was established (see ***Table 3***).

This universal selection was concretised for 'intermediate' liposomal products that can later provide an applicable base for carrier systems. Liquide formulation and stable lyophilised powder was accepted as the form of preparation. The structure and the size of the vesicles are related to the potential final dosage forms, and administration routes can be modified by the excipients and can affect the incorporation of an API. Vesicles in the 100-200 nm size range can be suitable for several application methods. The stability of a formulation influences the safety, efficacy, and quality profile of the product. Thus a stable, large unilamellar vesicle (LUV)-containing, monodisperse and homogeneous, API-free formulation was determined as the QTPP of 'empty' liposomes free from active substances. The changes in the liposome attributes due to the factors found to be critical in the RA were investigated on these vesicles in the characterization studies.

5.1.2. Determination of the CQAs

The CQAs made up the list of those quality attributes that are critically related to the QTPP. That is why the CQAs are also unique and depend on the QTPP. The potential CQAs were collected and presented in ***Table 4***.

The elements that were identified as the CQAs of the 'empty', 'intermediate' liposomes are the followings:

- type of the liposome: determines the quality of the lipids – conventional liposomes
- compatibility with targeted drug delivery: formulations must be suitable for the requirements of enclosing an API later
- size of the vesicles: mean particle size between 100-200 nm
- lamellarity: unilamellar liposomes
- morphology: spherical vesicles
- polydispersity index: monodisperse system – PdI≤0.30
- zeta potential: around ±30 mV
- surface modifications: maintain targeted delivery – traditional and PEGylated liposomes
- phase transition temperature: ideal if the working temperature is higher than T_m
- sterility: to fulfil the microbiological requirements – not necessary to be pyrogen-free
- stability: vesicles with stable physical and chemical attributes, no aggregation in the aqueous phase

Table 3. *Collection of the possible elements of the quality target product profile (QTPP) for a liposome-based formulation.*

QTPP	Details	Comments
Indication/therapeutic effect	based on the API	its characteristics may necessitate the use of liposomes
	not important for empty liposomes	empty liposomes are used, e.g., in cosmetology
Target patient population	based on the indication	suitable dosage form for each age group
Route of administration	compositions may differ based on the target	can be determined by the API and the target patient population
Site of activity/target	based on the indication	targeted delivery
	based on the API	
Dosage strength	based on the API	differs even in the same pharmaceutical subgroup
	based on the target patient population	needed dose changes with age and health condition
	based on the indication	appear in the case of preparation with a wide range of indications
	based on the administration route	depends on the adsorption circumstances, e.g. in the case of nasal application, the needed dose is less than per os
Dosage form/appearance	liposomes in aqueous solution	transparent, light scattering liquid (vesicles in colloidal size)
	lyophilised powder	solid powder; colour based on the API and the excipients
Viscosity	applicable ranges based on the administration route	sign of stability; maintains efficient drug release; higher viscosity indicates smaller size, a narrow PdI, slower drug release, and lower clearance rate
Osmolality	applicable ranges based on the administration route	be tolerable, ideally 300 ± 30 mOsm/kg
Physical attributes of the liposomes	morphology, particle size, and ZP	change with the adjustment of the composition
Pharmacokinetics	liberation, adsorption, distribution, metabolism, elimination	necessary for API-loaded liposomes
Safety	complement activation-related pseudoallergy (CARPA)	all types of intravenous liposomes can cause CARPA; enhanced by increasing size in the 70–300 nm range; more than 71 mol% CH; PEG-PE insertion
	chemical/biological decomposition	needs to be investigated
	degradation products	concentration must be under the legal limit
Sterility	based on the administration route	sterile and pyrogen-free formulations are needed for intravenous application
Stability	in aqueous solution	needs to be stable; duration of stability is decisive
	in lyophilised powder form	
Solubility/dissolution	in aqueous solution	media: non-toxic, non-irritable
	in lyophilised powder form	immediate release
Homogeneity	homogenous formulation	sign of stability
Drug release	based on the treatment type	site and timing can be modified

Table 4. *Collection of the possible factors of critical quality attributes (CQAs) of liposomes.*

CQAs	Details	Comments
Type of liposomes	conventional liposomes	neutral or negative PPLs
	immune liposomes	antibodies, antibody fragments
	cationic liposomes	positive PPLs
	magnetic liposomes	metal particles
	bioresponsive liposomes	thermosensitive (37°C < T_m)
		pH-sensitive (acidic milieu)
		LiPlasome (secretory phospholipase A2)
Number of lamellas	more layers	multilamellar (>0.5 μm)
		oligolamellar (0.1–1.0 μm)
	one layer	unilamellar
Size of vesicle	small unilamellar vesicle (SUV)	20–100 nm
	medium-size unilamellar vesicle (MUV)	between SUV and LUV, >100 nm
	large unilamellar vesicle (LUV)	>100 nm
	giant unilamellar vesicle (GUV)	>1 μm
Surface modifications	no modification	rapid elimination
	PEG chains (stealth liposomes) (quality and quantity of the chains)	steric exclusion (decreased opsonisation and phagocytosis); prolonged circulation
	monoclonal antibodies, antibody fragments, peptides, nucleic acids, carbohydrates, small molecules	provide targeted delivery by biding to the targeted receptors
Morphology of liposomes	spherical vesicles	self-organized structure
	concentric layers	multi-layered vesicles
	spherical with multiple non-concentric lipid vesicles inside	multivesicular liposome (MVL)
Particle size and size distribution	d(0.1), d(0.5), d(0.9), span, surface and volume weighted means (D[3,2]; D[4,2])	mean particle size <200 nm
Polydispersity index (PdI)	indicating polydispersity of the system	below 0.5 is acceptable
Specific surface area (SSA)	influences drug release	smaller vesicles maintain a higher surface area-to-volume ratio than larger particles
Zeta potential	indicating stability	stable formulation from around ±30 mV
Phase transition temperature (T_m)	influences drug release	determined by the composition of the liposome; CH0 reduces this value
Empty liposomes/ API content	modifies the physical attributes of the liposomes	the characteristics of the API determine its position
Position of the API	hydrophilic API	in the hydrophilic aqueous centre
	lipophilic API	in the lipophilic double membrane
	surface-bounded	monoclonal antibodies, antibody fragments, peptides, nucleic acids, carbohydrates, small molecules
Encapsulation efficiency (EE%)	higher EE% is the goal to increase the drug concentration in the final formulation	manufacturing costs can be reduced, and more flexible dosing can be provided by higher EE%
Permeability, targeted drug delivery	semi-permeable membrane target specificity	the highest permeability is at T_m; increases effectiveness
Drug release profile	maintains therapeutic activity	site and timing can be modified
Sterility	if necessary	even for the materials; in the case of aseptic preparation
Stability	chemical, biological, microbiological	characteristic values must remain in the recommended ranges until use

5.1.3. Determination of the CQAs and the CPPs

The preparation method defines the CPPs of the liposome formulation process; thus, the production technique that enables the target CQAs needs to be selected prior to investigating the possible CMAs and CPPs. The thin-film hydration method[108] was chosen for these studies. The application of a quality management visualisation tool (a fishbone diagram, process mapping, or a flow chart) is helpful for the identification of the potential CMAs and CPPs. A flow chart was built to show the systemic collection and presentation of the potential CMAs and CPPs (***Figure 3***). The thin-film hydration liposome technique steps are presented in the middle of the figure. The left side of the flow chart shows the MAs, while the right side the PPs. These MAs and PPs can affect the result of the liposome manufacturing process. The critical ones must be selected and labelled as CMAs and CPPs in the RA.

- MAs with an impact on the liposome features: the quality of the PPLs and the CH derivatives, the ratio between the wall forming agents, the surface modifiers, the phase transition temperature of the lipids, the quality of the solvent, the hydration media, the cryoprotectants and the further additives
- PPs that can affect the vesicle properties: the working temperature, the quantity of the solvent and the hydration media, and the settings of the thin-film hydration method (dissolution, vacuum evaporation, sonication, filtration, lyophilisation, and storage)

Knowledge, practical experiences and a thorough literature background survey of the field[4] were necessary to establish the information presenting tools (the Ishikawa diagram, the flow chart and the tables of the QTPP and the CQAs). The knowledge was used further for the decisions of the RA. Although the main points of the tables and figures are composed of evidence from the literature mixed with practical experiences, the systemic collection of all the relevant factors and data is the novelty of this work.

5.1.4. Initial and updated Risk Assessment

The criticality of the factors was identified in the RA. The severity of the risks that the elements meant to each other was determined in a three-grade scaled (H/M/L) interdependence rating between the items of the QTPP and the CQAs plus the CQAs and the CMAs/CPPs. As the decisions are based on complex knowledge, suggestions from more researchers were synthesized in a research group-level brainstorming.

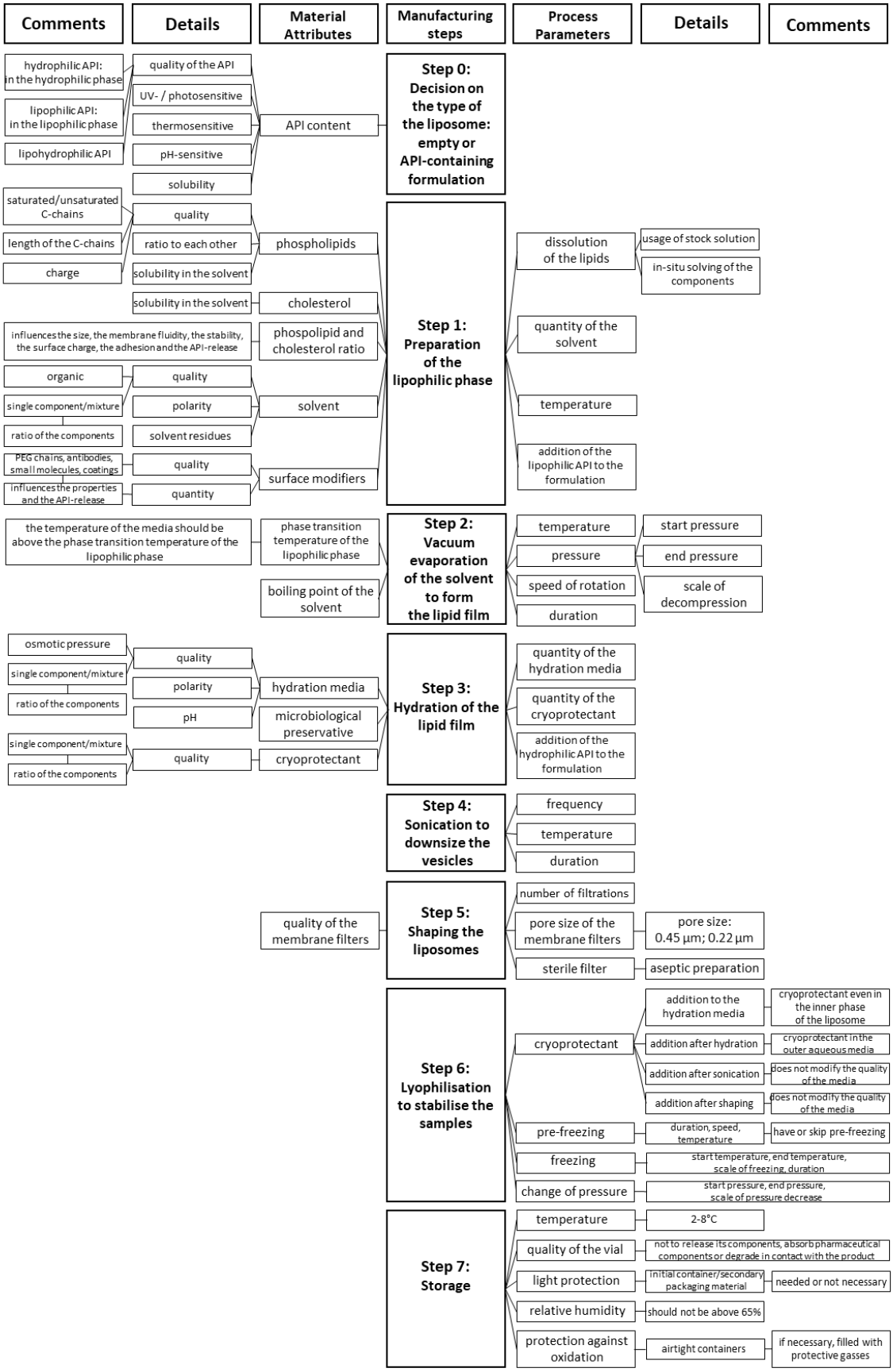

Figure 3. *Collection of the liposome components (MAs) and the preparation method (PPs) that may affect the result of the thin-film hydration liposome manufacturing technique.*

Based on the combination of the relation intensity* provided in the Risk Estimation Matrices (***Figure 4***) and the results of the occurrence rating, the software transformed the given data into numerical information and calculated the overall severity of the risks.

A

CQAs \ QTPP	dosage form/ appearance	physical attributes	stable structure	homogen-ous system
type of liposomes	high	high	medium	medium
targeted delivery compatibility	high	high	high	medium
size of the vesicles	high	high	medium	medium
number of lamellas	low	high	low	low
morphology	medium	high	medium	medium
monodispersity	high	medium	low	high
zeta potential	high	medium	high	medium
surface modifications	high	high	medium	medium
specific surface area	medium	high	low	low
phase transition temperature	high	low	high	low
sterility	high	low	medium	low
stability	high	medium	high	high

B

Process	Preparation of the lipophilic phase						Vacuum evaporation of the solvent		
CQAs \ CMAs/CPPs	phospho-lipids	surface modifiers	cholesterol	PPL-CHOL ratio	solvent	dissolution of lipids	phase transition temperature	working temperature	settings of vacuum evaporation
type of liposomes	high	high	high	medium	low	low	high	high	low
targeted delivery compatibility	high	high	medium	medium	medium	low	high	low	low
size of the vesicles	high	high	high	medium	low	low	medium	medium	low
number of lamellas	high	low	low	low	low	low	medium	medium	low
morphology	high	medium	medium	medium	low	low	medium	high	low
monodispersity	high	medium	medium	medium	medium	low	medium	medium	low
zeta potential	high	medium	high	high	medium	low	medium	medium	low
surface modifications	high	high	low	medium	low	low	medium	medium	low
specific surface area	high	medium	medium	medium	medium	low	medium	high	low
phase transition temperature	high	high	medium	high	medium	high	high	high	low
sterility	high	low	high	low	high	medium	low	low	low
stability	high	high	high	medium	low	medium	medium	low	low

Process	Hydration of the lipid film		Sonication	Shaping of vesicles		Lyophilisation		Storage
CQAs \ CMAs/CPPs	hydration media	addition of additives	settings of sonication	properties of filtration	sterility	cryo-protectant	settings of lyophili-sation	storage
type of liposomes	low	low	low	low	low	low	low	low
targeted delivery compatibility	medium	low	low	low	high	medium	low	low
size of the vesicles	high	medium	high	high	low	medium	low	low
number of lamellas	low	low	high	medium	low	low	low	low
morphology	medium	medium	high	medium	low	medium	low	low
monodispersity	medium	low	medium	high	low	medium	low	low
zeta potential	high	medium	medium	low	low	medium	low	low
surface modifications	low	low	medium	low	low	low	low	low
specific surface area	medium	medium	high	high	low	medium	low	low
phase transition temperature	medium	medium	low	low	low	medium	low	low
sterility	high	medium	low	medium	high	high	low	medium
stability	medium	high	medium	low	high	high	medium	medium

Figure 4. *Results of the interdependence investigations between the elements of the QTPP and the CQAs(A), and between the CQAs and the CMAs/CPPs (B).*

Notes:

*: A three-grade scaled interdependence rating was done between the elements of the QTPP and the CQAs plus the CQAs and the CMAs/CPPs to determine the severity of the risks that the factors mean to each other. The evaluation was done for each parameter pair individually. E.g., sonication has a strong impact on the lamellarity and the size of the liposomes (interdependence evaluated as “high”), while it does not influence the phase transition temperature of the lipid formulation (effect estimated as “low”).

The generated Pareto charts presented in ***Figure 5*** illustrate the ranking of the critical factors. The estimated criticality of the CMAs and CPPs obtained from the initial RA were verified in experiments. The results of the experimental studies were utilised in the updated RA.

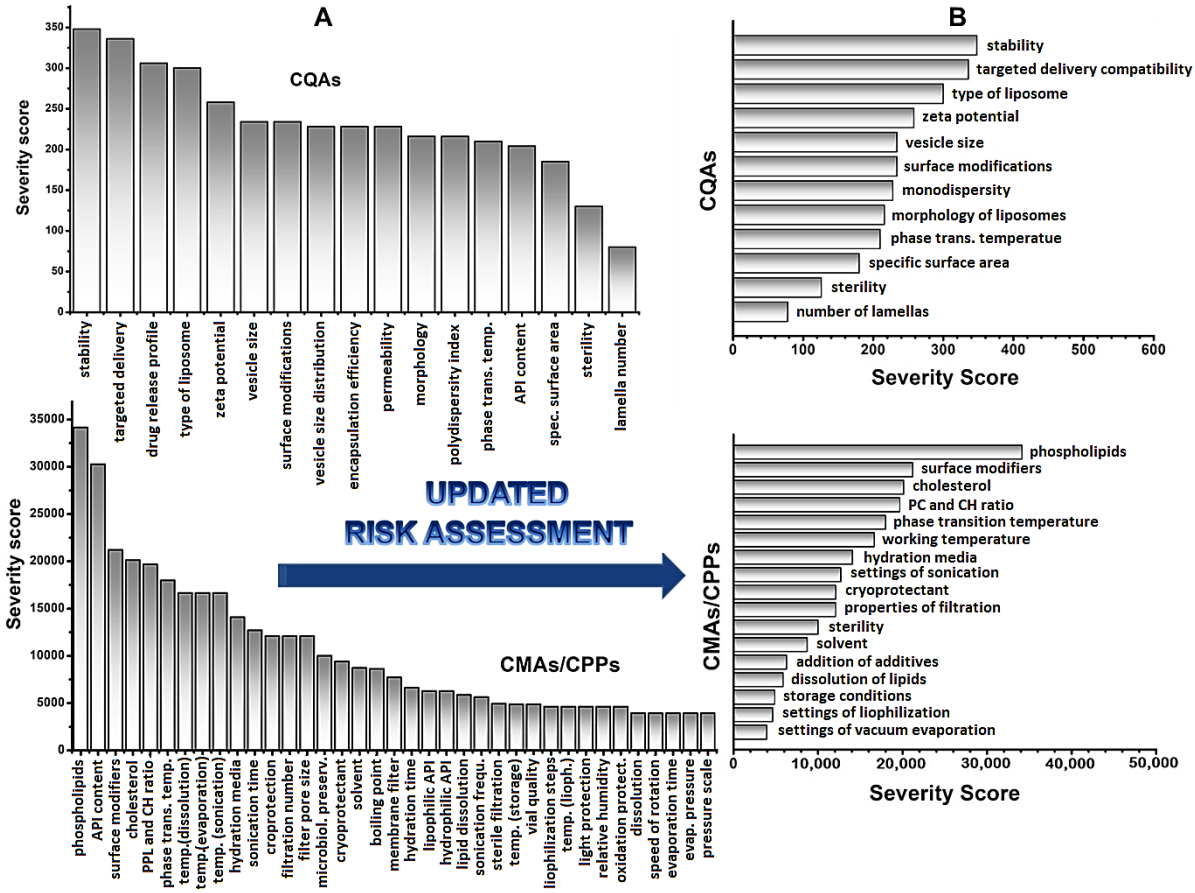

Figure 5*. List of the CMAs and the CPPs of the thin-film hydration liposome preparation method ranked by their calculated severity scores in the universal case (A) and the case of the 'intermediate' API-free liposomal carrier system (B).*

The Pareto diagrams set up the factors in a relative order based on the severity scores. The Pareto principle states that 80% of the results generally come from 20% of the inputs. The distribution could be closer to the 90/10 or the 70/30 ratio in individual cases. In this study, approximately 30% of the investigated factors reached at least 70% of the highest risk severity score; thus, those factors were considered critical for the liposome characteristics.

The API content impacts the vesicle properties, as our previous study showed[43]. If in the case of an 'empty' delivery system, the API-related factors are neglected, the stability, the targeted delivery compatibility, the type of the liposome, the zeta potential, the vesicle size, the surface modifications (in this study: application of PEGylated phospholipids), and the monodisperse size distribution were obtained as the relevant CQAs of the 'intermediate' liposomal formulation in the updated RA. Among the CMAs/CPPs, the PPL and the API content

have the highest impact on the quality of the liposomes. Besides the PPLs, the surface modifications, the CH type, the PC:CH ratio, the T_m value of the composition, the working temperature, and the hydration media can critically influence the 'empty' liposomes generated via the thin-film hydration method.

The effect of the CMAs/CPPs can be accurately investigated if some of the values are set on the same level while the ones under the scope of the study are changed according to the DoE.

The findings of this study (the influencing role of the lipid concentration, the CH ratio, and the type of the PPLs) are consistent with the conclusions of other research groups. Porfire et al. provided a general overview of the QbD approach for liposomes without a defined production process and methodologies for characterization as a control strategy. Xu et al. performed RA on liposomes prepared via the thin-film hydration technique enclosing API. The factors that affect the size, encapsulation efficiency, and stability of the liposomes were analysed in their work. They checked the properties of the formulation, the process, the analytical method, and the instrumentation reliability and found that the latter two categories can be well-controlled; therefore, the factors of these categories are not critical; however, they are non-negligible when the characterization methods are selected[114]. The findings of these studies were built into the theoretical part of this research.

5.1.5. Characterization of the liposomal products

The settings of the thin-film hydration process were kept in formerly set stable values, except the working temperature, which was chosen for further investigation based on its high severity score. The PPL-CH ratio, the effect of a PEGylated PPL, and the quality of the hydration media and the cryoprotectant were investigated from the relevant CMAs. The factors of the CMAs/CPPs were studied according to the levels and parameters presented in ***Table 5***.

Table 5. *Critical factors and their levels investigated in the liposome formulation processes.*

	critical factors	**investigated levels or parameters**				
CPP	working temperature	50°C		60°C		70°C
CMA	PC : CH weight ratio	60 : 40	70 : 30	80 : 20	90 : 10	100 : 0
	PEGylated PPL content (w/w%)	5%		10%		20%
	PC : PEGylated PPL : CH weight ratio	55 : 5 : 40		50 : 10 : 40		40 : 20 : 40
	quality of PEGylated PPL	$DPPE\text{-}PEG_{2000}$			$DSPE\text{-}PEG_{3000}$	
	quality of hydration media	saline solution	PBS pH 4.5	PBS pH 5.6		PBS pH 7.4
	quality of cryoprotectants	glucose	sorbitol	trehalose		inulin

5.1.5.1. Vesicle size and zeta potential analysis

The results of the investigations are shown on the DoE map in ***Figure 1*** and illustrated in ***Figures 6 and 7***.

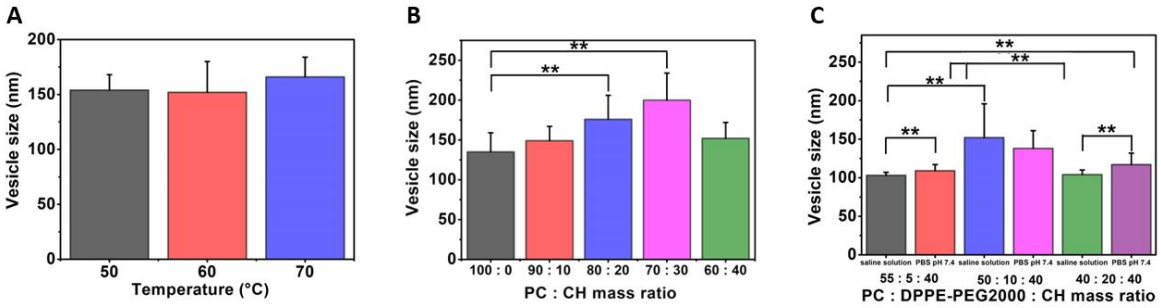

Figure 6. *Results of the DLS measurements in the case of the PC:CH = 60:40 samples hydrated with saline solution prepared at different temperatures (50-70°C) (A), the different amount (0-40%) CH-containing samples made at 60°C and hydrated with saline solution (B), the 5%, 10% or 20% DPPE-PEG2000-containing formulations made at 60°C and hydrated with saline solution or PBS pH 7.4.*

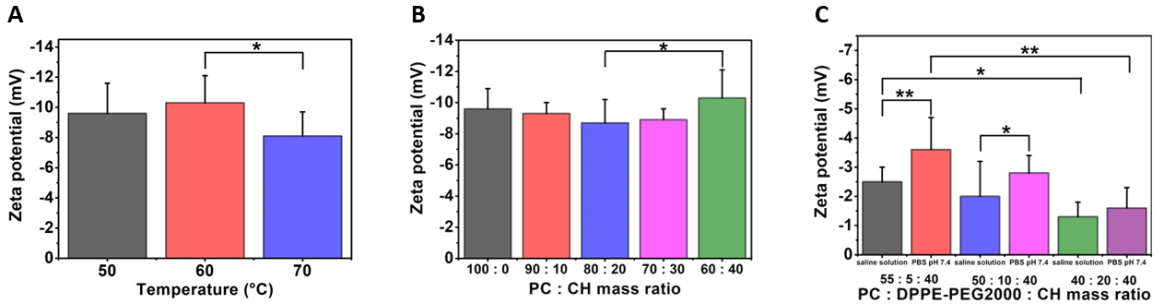

Figure 7. *Results of the zeta potential measurements in the case of the PC:CH = 60:40 samples hydrated with saline solution prepared at different temperatures (50-70°C) (A), the different amount (0-40%) CH-containing samples made at 60°C and hydrated with saline solution (B), the 5%, 10% or 20% DPPE-PEG2000-containing formulations made at 60°C and hydrated with saline solution or PBS pH 7.4.*

5.1.5.1.1. Effects of different working temperature

The effect of the working temperature is a slightly investigated parameter in the liposome preparation field. In this research, PC-CH (weight ratio: 60:40) vesicles were prepared at 50, 60, and 70°C. The mean vesicle size was not affected by the temperature change; the values were between 154-166 nm with no significant difference (***Figure 6-A***). The PdI values were under the acceptance limit of 0.30 in all cases showing homogenous formulations. However, the formulation prepared at 60°C had significantly more negative ZP (-10.3±1.8 mV) than the one at 70°C (-8.1±1.6 mV) ($p<0.05$), while it did not differ from the sample made at 50°C (-9.0±2.0 mV) (***Figure 7-A***). Based on these data, 50-60°C can be the DS for the liposomes with PC origin. According to this observation, 60°C was chosen as the working temperature for further studies.

The general practice suggests preparing liposomes above their transition temperature to receive efficient dispersion. The phase transition temperature of PC ranges between 50-55°C[115,116]. Pandey et al. studied the formulation process of liposomes prepared via the ethanol injection technique in the temperature range of 40-70°C. Their optimal working temperature range was 55-70°C. In their case, the increase in the temperature resulted in insignificantly smaller particle size, which suggests that the formulation settings should be determined case-by-case for the different preparation methods.

5.1.5.1.2. Effects of different ratios of wall-forming agents

Applying different PPL-CH weight ratios was investigated on samples prepared at 60°C, hydrated with saline solution, decreasing the CH content from 40% to zero. The size of the liposomes decreased as the PC:CH ratio was changed from 70:30 to 100:0 (***Figure 6-B***). The only PC-containing sample had significantly ($p<0.01$) smaller vesicles (135±24 nm) than the 20% (176±30 nm) or 30% (200±34 nm) CH-containing ones. Our results correlate with the work of López-Pinto et al., who found that increasing CH concentration increases the size of the vesicles investigating DPPC-CH liposomes prepared via the hydration technique[117]. This study indicated a decreased particle size (152±20 nm) for the PC:CH 60:40 vesicles. The lowest PdI value (0.18±0.08) was also measured in the same case.

Our observations from 30% CH strengthen the statement that the ZP value decreases with reduced CH% content (***Figure 7-B***). This phenomenon may appear due to the presence of a higher number of PC on the vesicle surface[118]. Significantly higher ($p<0.05$) absolute ZP values (-10.3±1.8 mV) were reached in the 40% CH-containing samples than in the 20% case (-8.7±1.5 mV), which strengthened the findings on the ZP increasing effect of the CH made by Sahu et al. screening the 20-50% of CH ratio[119]. As the CH maintains the mechanical strength of the membrane, it is suggested to complete the liposome compositions with CH. CH can maintain the rigidity of the liposomal membrane and improve its mechanical strength and packing density, thereby decreasing the permeability of water and small molecules through the membrane[120]. Thus, the application of CH is recommended to stabilise the PPL bilayer[121]. Therefore further investigations were carried out on CH-containing compositions.

5.1.5.1.3. Effect of different concentrations of PEGylated phospholipids

5-10-20% of the formulation weight was changed from PC to DPPE-PEG_{2000} to investigate the effect of the PEGylated PPL on the characteristics of the liposomes. Samples made at 60°C and hydrated with saline solution and PBS pH 7.4 were used to study the effect of

different concentrations of PEGylated phosphatidylethanolamine (PE). A non-linear relationship was detected between the PPL ratios and the vesicle size. The increase in the concentration of the PEGylated PPL from the 55:5:40 weight ratio meant first larger vesicles (50:10:40 weight ratio), then a decrease in the size (40:20:40 weight ratio) (***Figure 6-C***). The significantly largest ($p<0.01$) vesicle size was measured in the case of the formulations made with PC:DPPE-PEG_{2000}:CH = 55:10:40 weight ratio for both hydration media (saline solution: 152±44 nm; PBS pH 7.4: 138±23 nm). Increasing the amount of the PEGylated PPLs to this certain ratio enlarges the size of the vesicles; however, further addition causes a sharp decrease in the mean size value due to the formation of PEGylated PPL-based micelles as Garbuzenko et al. described for DSPE–PEG_{2000}-containing vesicles[122]. Our results show the same phenomenon for DPPE-PEG_{2000}. Sriwongsitanont et al. described that at higher than 10 mol% concentration of DSPE-$PEG_{2000,}$ liposomes formed with micelles[123]. In our research, the highest concentration of PEG-PPL was 4.50 mol% in the case of PC:DPPE-PEG_{2000}:CH=40:20:40 formulation.

The PdI was the lowest in the 55:5:40 case (0.25±0.05; 0.20±0.02); thus, this formulation is the best regarding the particle size and uniformity, providing vesicles around 100 nm with uniform size. Even the ZP values were significantly more negative in this case than at the 40:20:40 ratio (saline solution: -2.5±0.5 mV, $p<0.05$; PBS pH 7.4: -3.6±1.1 mV, $p<0.01$). The larger proportion of DPPE-PEG_{2000} was applied; the less negative was the measured ZP value (**Figure 7-C**).

The addition of the PEGylated PE to the PC-CH formulation significantly decreased ($p<0.01$) the vesicle size (152±28 nm; 103±5 nm), slightly increased the polydispersity and resulted in a significantly less negative ($p<0.01$) ZP value (-10.3±2.0; -2.5±0.5). Changing a part of the PC content to PE decreases the size of the liposomes, as Akizuki and Kaneko wrote down in their work[124]. Our results show that the usage of PEGylated PE could decrease the vesicle size as well. The reason behind this size-decreasing ability, according to Li et al., is that the bilayer structure can be stabilised by the application of non-bilayer lipids like the unsaturated PE[125]. Our finding that the addition of PEGylated PPLs can decrease the size of the formulated vesicles agrees with the report by Tsermentseli et al. for DSPE-PEG_{2000}[17].

5.1.5.1.4. Effect of different PEGylated phospholipids

Changing the PEGylated PPL from DPPE-PEG_{2000} to DSPE-PEG_{3000} in the compositions did not affect the ZP of the formulations. Significant differences ($p<0.05$ in both cases) were only measurable regarding the size of the PC:PEG-PPL-CH = 55:5:40 sample made with PBS

pH 5.6 and trehalose (107±13 nm; 170±38 nm) and the 73.3:6.7:20 formulation made with PBS 5.6 and inulin (145±43 nm; 195±4 nm).

The obtained results were in accordance with the recommendation from Kowalska et al., pointing out that besides the size of the liposomes, their stability, permeability, and surface charge can change by the addition of different lengths of polymer chains on the vesicles. In their study, the ZP values increase as the length of the PEGylated chains grows [126]. As the PEGylated PPLs carry a negative charge, some reduction of the cationic charge and some increase in the anionic charge was observed by Lei et al.; however, at low mol percentages, it had only a minor effect on the vesicles[127].

5.1.5.1.5. Effect of different hydration media

The effect of the quality of the hydration media was studied on the PC:DPPE-PEG_{2000}:CH = 55:5:40 and 40:20:40 weight ratio formulations made at 60°C. The size of the particles hydrated with saline solution (pH 5.5) (104±7 nm) and PBS pH 5.6 (110±5 nm) did not differ. In contrast, the application of the PBS pH 7.4 (117±15 nm) resulted in a significantly larger size ($p<0.01$) than that was reached with saline solution. The largest vesicle size was detected in the case of PBS pH 4.5 (136±9 nm); significantly greater ($p<0.05$) liposomes were obtained than with PBS pH 7.4.

Vesicles made with PBS pH 5.6 (-2.3±1.2 mV) according to the 40:20:40 ratio had significantly higher ($p<0.05$) ZP than those hydrated with saline solution (-1.3±0.5 mV). Significantly higher ZP values were reached with PBS pH 4.5 (-6.0±1.4 mV, $p<0.01$) and PBS pH 5.6 (-5.8±0.9 mV, $p<0.05$) applying the 55:5:40 ratio. The ionic strength of the hydration media influences the ZP; the higher the ionic strength, the more compact the ion layer formed around the vesicles, and thus, the higher the ZP, as Tefas et al. described[118]. In the presented case, the ionic strengths of the hydration media increased like: saline solution (0.15 M) < PBS pH 7.4 (0.16 M) < PBS 4.5 (0.30 M) < PBS pH 5.6 (0.40 M), which proves that the increasing ionic strength of the media increases the value of the ZP of the hydrated lipid vesicles. Therefore the ionic strength of the hydration media should be considered when it is chosen for the formulation besides the required pH.

5.1.5.1.6. Effect of different cryoprotectants

5% of the PPL mass was given to the hydration media from glucose, sorbitol, trehalose or inulin to investigate the difference between the effects of different cryoprotectants. Both the PC:DPPE-PEG_{2000}:CH = 55:5:40 and 40:20:40 weight ratios were used for the study. Trehalose

and glucose resulted in the same size of the vesicles (103±3 nm, 104±7 nm, respectively). In this study, sorbitol (130±5 nm) caused a significant increase ($p<0.01$) in the mean vesicle size compared to glucose (103±4 nm). The higher was the ratio of the DPPE-PEG_{2000} in the formulations, the less negative were the ZPs; however, it could have been determined that by using these cryoprotectants, the ZP values became more negative in the following order: glucose (-1.3±0.5; -2.5±0.5) > trehalose (-3.2±0.9) > sorbitol (-4.1±0.8). The sorbitol and the trehalose increased the ZP significantly compared to the glucose-containing formulations ($p<0.01$ in both cases). There was no significant difference between the ZP values of the samples made with trehalose and inulin; however, in this latter case, the vesicle size was significantly larger ($p<0.05$) in the PC:PEG-PPL:CH = 73.3:6.7:20 ratio for both the DPPE-PEG_{2000} (137±42 nm; 145±15, nm respectively) and the DSPE-PEG_{3000} (142±35 nm; 196±3 nm, respectively).

Hau et al. made quantitative observations about cryoprotectants on PC-CH liposomes[128]. The protective effect of various cryoprotectants is different. They suggested using 5% glucose, 10% sucrose, 15% mannitol, or 10% trehalose to achieve the best protective effect, as the smallest diameters for the vesicles were measured in these cases. They found that the mean vesicle diameter of liposomes prepared with glucose was the biggest, with trehalose the smallest of these four carbohydrates. Sylvester et al. applied the QbD concept to understand the freeze-drying process more and found that combining the cryoprotectants (trehalose + mannitol) leads to better results[129]. Thus, consideration of the quality is suggested for the cryoprotectants.

5.1.5.2. Thermal analysis

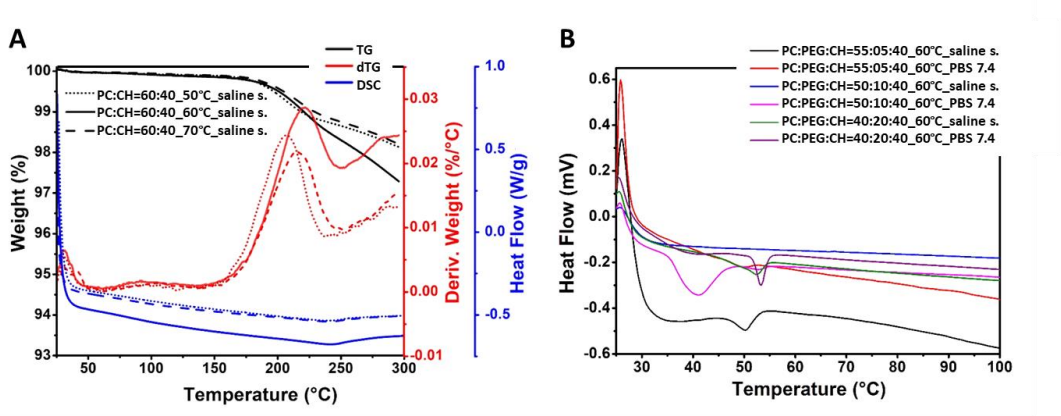

Figure 8. *TG, dTG, and DSC curves presenting the thermal behaviour of the PC:CH = 60:40 samples hydrated with saline solution prepared at different temperatures (50-70°C) (A), and DSC results of the 5%, 10% or 20% DPPE-PEG_{2000}-containing formulations made at 60°C and hydrated with saline solution or PBS pH 7.4.*

Thermal analysis has an important role in characterising liposomes due to the phase transitions of the PPLs. The gel to liquid-crystalline phase transition temperature (T_c) is typical

for the wall-forming lipids. Below the T_c value, the CH mobilises the hydrocarbon chains hindering them from crystallisation that modifies the T_m and indicates a separation before the phase transition. In contrast, above the T_c value, CH maintains the rigidity of the bilayer membrane[130]. Increasing the CH content broadens the endotherm curve; thus, the observed phase transition decreased[131,132].

The DSC and TGA measurement results (***Figure 8-A,-B***) were similar in the investigated cases and harmonised with the previous findings described in the literature. The T_c value of the samples made from the same compositions at different temperatures was ~30-33°C. Below the T_c value, the presence of the CH makes the chains more mobile in the liposomes preventing the hydrocarbon chains from crystallisation, which modifies the T_m and causes a separation before the phase transition – that is the reason why the curve was smooth and not stepping – while above the T_c value it maintains the rigidity of the membrane[130]. A remarkable phase transition was observed in the case of the PEGylated PPL-containing vesicles. This phase transition was detected at 52°C in the PC:DPPE-PEG_{2000}:CH = 55:5:40 case; at 40°C for the 50:10:40, and at 50°C for the 40:20:40 samples hydrated with PBS pH 7.4 and made with glucose. The observed increase in the phase transition temperature originated from the decreasing lateral pressure as the hydrocarbon chains of PC and DPPE-PEG_{2000} became growingly mismatched while the membrane enriched in the PEGylated PPL[133].

Based on the TG and dTG curves, the desorption of the physisorbed water content has been completed at around 100°C for all the samples. Another change was detected in the shape of the curves at 200-225° representing the molecular alterations[134]. 2-5% of the weight of the samples was lost during the heat treatment up to 300°C[135,136].

5.1.5.3. Investigation of chemical bonding

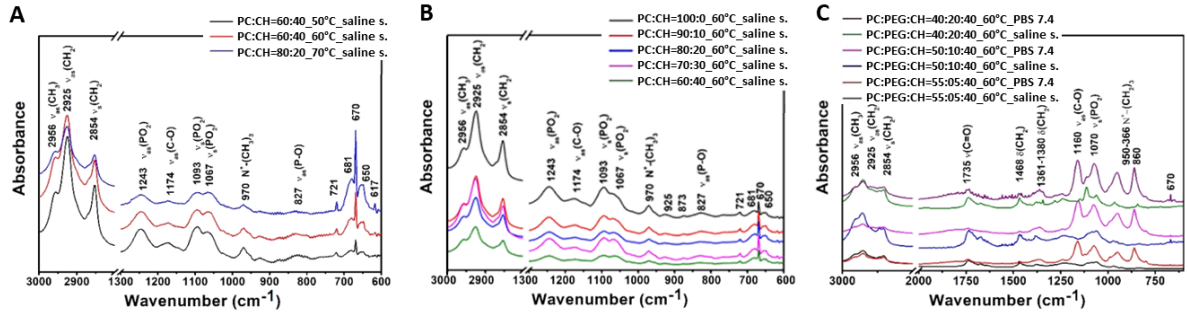

Figure 9. *FT-IR spectra of the PC:CH = 60:40 samples hydrated with saline solution prepared at different temperatures (50-70°C) (A), the different amount (0-40%) CH-containing samples made at 60°C and hydrated with saline solution (B), the 5%, 10% or 20% DPPE-PEG2000-containing formulations made at 60°C and hydrated with saline solution or PBS pH 7.4.*

The FT-IR spectra differ from type to type of wall-forming lipids. In the presented formulations, PC was the main component; thus, the FT-IR curves (***Figure 9-A,-B,-C***) demonstrated two distinct, separate regions in the spectra[137]: the fingerprint region observed at ~900–600 cm^{-1} and the C-H stretching vibrations originated mainly from the hydrocarbon chains in the 3000-2800 cm^{-1} wavenumber domain. The lower wavenumber region of the spectra (below 1800 cm^{-1}) represented the polar head groups of the PPLs. The shape of the measured spectra was the same as those gained from the PC:CH = 60:40 formulations produced at different temperatures. At 827 cm^{-1} ν_{as}(P-O), at 970 cm^{-1} ν_s(N+-$(CH_3)_3$), at 1067 cm^{-1} and 1093 cm^{-1} ν_s($(PO)_2$), at 1174 cm^{-1} ν_{as}(C-O) and at 1243 cm^{-} ν_{as}(PO_2) stretchings were detected, that is typical for the polar head groups[138]. The symmetric ν_s(CH_2) at 2854 cm^{-1} and the asymmetric ν_{as}(CH_2) stretchings at 2925 and 2956 cm^{-1} were reported as characteristics of the apolar hydrocarbon chains[137]. The traces of the FT-IR curves were consistent despite the different sample production temperatures; all the investigated samples contained similar bonds.

Differences among the FT-IR spectra of the samples hydrated with different hydration media were well-detectable. The typical ν_{as}(PO_2) and ν_s(PO_2) stretchings appeared in the case of the liposomes hydrated with the PBS solutions. The differing ionic strengths can cause the differences between the spectra of the samples hydrated with PBS of different pH. Some overlaps with other vibrations were detected between the 3050 and 2800 cm^{-1} part of the spectra. Usually, these vibrational modes are uncoupled from the other modes; thus, they are not influenced by the lipid head groups but are sensitive to the structure of the chains[137].

5.1.5.4. Residual ethanol measurement

The ethanol residue was quantified via GC-MS, and the investigated samples found ethanol concentrations under the detection limit, proving possible safe application.

5.1.6. Determination of the Design Space

The evaluation of the experimental findings leads to the determination of the DS[139], which has remarkable regulatory benefits. If the alterations in the production parameters are still in the DS, the deviation does not require modifications in the marketing authorisation documentation of the product.

In this case, we could make observations and recommendations for the parameters of a thin-film hydration-based liposome preparation process:

- A working temperature between 50-60°C and higher than the T_m of the formulation is suitable for liposomes of PC origin.

- Decreasing CH content results in smaller vesicles. The only PC-containing sample has a significantly smaller ($p<0.01$) vesicle size than those with 20-30 w/w% CH content.
- Vesicle size ~150 nm and low PdI is reachable by applying PC and CH in a 60:40 mass ratio.
- The ZP decreases as the amount of CH is reduced. Significantly higher ($p<0.05$) absolute ZP values are reachable with 40% CH content than 20%.
- Changing a part of the PC content to PE or PE-PEG_{2000} decreases the size of the liposomes.
- Increasing the PEGylated PPL content to a specific ratio enlarges the size of the vesicles, which decreases at further addition due to micelle formation. Changing 5% of the PC content to DPPE-PEG_{2000} results in the significantly smallest ($p<0.01$) vesicle size (~100 nm) than applying 10% or 20% PEG-PPL with low PdI.
- The absolute ZP value decreases by applying larger DPPE-PEG_{2000} proportions. The ZP is significantly more negative ($p<0.05$) in the PC:DPPE-PEG_{2000}:CH = 55:5:40 case than in the 40:20:40 mass ratio.
- Changing the PEGylated PPL from DPPE-PEG_{2000} to DSPE-PEG_{3000} in the compositions does not affect the ZP of the formulations. Significant differences ($p<0.05$ in both cases) were only measurable regarding the size of the vesicles increased by the larger PEG number.
- The size of the particles hydrated with saline solution and PBS pH 5.6 does not differ. The application of PBS pH 7.4 results in significantly larger ($p<0.01$) size values than the previous two, but from the four hydration media, the significantly largest ($p<0.05$) vesicles are from the usage of PBS pH 4.5.
- The ZP of the liposomes parallelly increases with the ionic strength of the hydration media due to the ion layer forming around the vesicles. In the case of the following four hydration media: saline solution (0.15 M) < PBS pH 7.4 (0.16 M) < PBS 4.5 (0.30 M) < PBS pH 5.6 (0.40 M); the application of PBS pH 5.6 ($p<0.05$) and pH 4.5 ($p<0.01$) leads to significantly higher ZP than saline solution.
- There is no difference in size when adding 5 w/w% of PPL from trehalose and glucose as cryoprotectants; however, sorbitol causes a significant increase ($p<0.01$) in the mean vesicle size compared to glucose. In the PC:PEG-PPL:CH = 73.3:6.7:20 ratio (for both the DPPE-PEG_{2000} and the DSPE-PEG_{3000}), the addition of inulin leads to significantly larger ($p<0.05$) vesicle size than trehalose.

- The addition of sorbitol or trehalose in 5 w/w% of PPL increases the ZP significantly ($p<0.01$) compared to the effect of glucose, while there is no significant difference between the ZP values of the samples made with trehalose or inulin.

5.2. Adjusting the zeta potential of liposomes

As the ZP of the prepared formulations were under the required ±30 mV for a stable formulation, the second part of the study shows the applied way to improve it.

5.2.1. Factorial experiment design for zeta potential optimization

The 3^2 fractional factorial design was chosen to optimize the ZP of the liposomes. The molar ratio between the wall-forming lipids (PC, CH) and the special additives (SA/DCP) was investigated in the experimental design. The samples were prepared via the thin-film hydration method with three independent parallels and investigated for the primary outcomes: vesicle size (Z-average), polydispersity index, and zeta potential. The investigated levels of the independent variables and the experimental results are shown in ***Table 6***.

Table 6. *Investigation levels and responses of the 3^2 fractional factorial design (Results are expressed in mean ± standard deviation from three independent parallels).*

Run	Composition (molar ratio)			Responses		
	PC	CH	SA	Vesicle size (nm)	Polydispersity index	Zeta potential (mV)
1	7.5	3.5	3.0	121 ± 28	0.22 ± 0.02	+22.0 ± 7.8
2	7.5	4.5	9.0	106 ± 21	0.23 ± 0.03	+17.6 ± 3.4
3	7.5	5.5	6.0	116 ± 14	0.23 ± 0.02	+24.6 ± 1.4
4	10.0	3.5	9.0	93 ± 6	0.22 ± 0.03	+25.0 ± 3.5
5	10.0	4.5	6.0	113 ± 16	0.23 ± 0.06	+25.8 ± 3.7
6	10.0	5.5	3.0	112 ± 7	0.16 ± 0.01	+26.6. ± 2.7
7	12.5	3.5	6.0	111 ± 6	0.19 ± 0.03	+26.3 ± 1.2
8	12.5	4.5	3.0	109 ± 7	0.17 ± 0.03	+26.6 ± 0.8
9	12.5	5.5	9.0	100 ± 17	0.17 ± 0.01	+27.1 ± 2.8
Run	**Composition (molar ratio)**			**Responses**		
	PC	CH	DCP	Vesicle size (nm)	Polydispersity index	Zeta potential (mV)
1	7.5	3.5	3.0	98 ± 11	0.20 ± 0.03	-29.9 ± 1.6
2	7.5	4.5	9.0	82 ± 16	0.24 ± 0.02	-29.6 ± 3.4
3	7.5	5.5	6.0	108 ± 9	0.21 ± 0.04	-32.5 ± 6,5
4	10.0	3.5	9.0	87 ± 16	0.24 ± 0.03	-32.6 ± 2.7
5	10.0	4.5	6.0	93 ± 23	0.23 ± 0.03	-29.7 ± 6.2
6	10.0	5.5	3.0	119 ± 25	0.21 ± 0.07	-29.7 ± 3.3
7	12.5	3.5	6.0	95 ± 8	0.18 ± 0.03	-29.2 ± 3.2
8	12.5	4.5	3.0	104 ± 25	0.18 ± 0.02	-27.6 ± 1.3
9	12.5	5.5	9.0	105 ± 2	0.18 ± 0.02	-17.7 ± 3.1

Polynomial equations were generated from the results to describe the individual main and interaction effects of the independent variables on the dependent factors. The relationships between the variables were investigated and described on the zeta potential (Y) according to the one-way ANOVA and regression analysis of the data. As all the compositions fulfilled the size and PdI acceptance criteria 150 nm and 0.30 PdI value, respectively, the impact of the experimental factors was analysed only on the ZP of the liposomes.

The relationship of the variables on the ZP (Y) in the case of the SA-containing formulations could be described with the following equation:

$$Y(SA) = 24.622 + 2.633x_1 + 0.883x12 + 0.833x_2 - 0.967x_2^2 - 0.917x_3 + 0.708x_3^2 \quad (2)$$

The regression coefficient, R^2=0.920, showed a good correlation for the surface plot. The molar ratio between the PC (x_1), CH (x_2) and SA (x_3) have no significant effect on the ZP (0.05<p). The ZP increases with the positive coefficients (x_1, x_1^2, x_2, x_3^2) of the independent variables in Equitation 2, while in contrast, the negative coefficients (x_2^2, x_3) have the opposite effect. Liposomes with SA get a positive charge.

The following equation describes how the independent factors affect the ZP (Y) of the DCP-containing formulations:

$$Y(DCP) = -29.833 + 1.250x_1 - 0.625x_1^2 + 0.300x_2 + 0.650x_2^2 - 0.450x_3 - 0.475x_3^2 \quad (3)$$

A high regression coefficient (R^2=0.984), indicating a good correlation, was found in this case, too. Similarly to the SA formulations, no significant relationship between the molar ratio values PC (x_1), CH (x_2) and DCP (x_3) and the measured ZPs (0.05<p) was found. As the DCP liposomes possess a negative charge, the negative coefficients (x_1^2, x_3, x_3^2) of the independent variables have a favourable effect on the outcome values, and the positive coefficients (x_1, x_2, x_2^2) decrease the absolute ZP values.

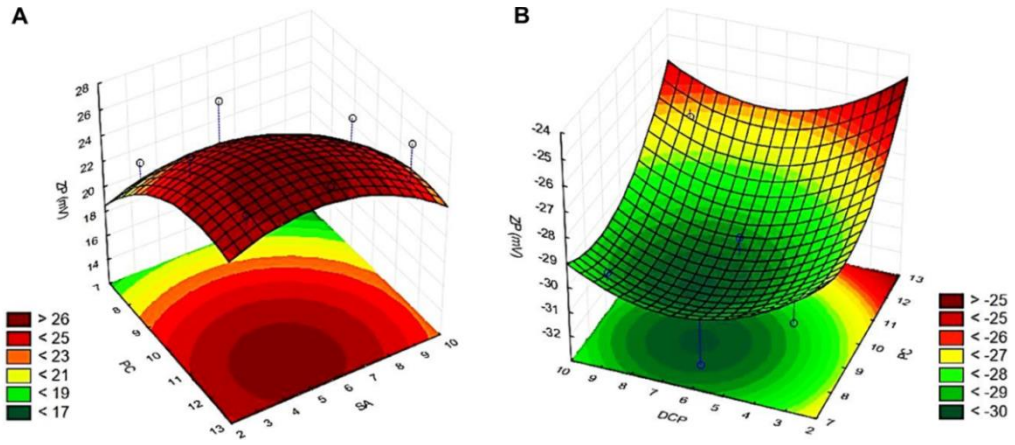

Figure 10. *Three-dimensional surface plots of the effect of independent variables on the ZP value in the 3^2 fractional factorial designs for the compositions made with the membrane additives: stearylamine (A) and dicetyl phosphate (B).*

The contour plots in ***Figure 10*** indicate the proper molar ratios for the independent factors. These 3D response surface plots visualise the main and the interaction effects of two factors, maintaining the other factor at a fixed level. The 3D response surface plots for the ZP values (Y(SA) – ***Figure 10-A*** and Y(DCP) – ***Figure 10-B***) were plotted according to the regression model by keeping one variable at a certain level. As the regression models show no factor with a linearly or a quadratically significant effect on the ZP values, the optimized compositions were read from the design spaces drawn out on the contour plots: the dark red area for the SA- and the green one for the DCP-containing formulations on ***Figure 10***. The optimal formulation for the SA-containing liposomes (hereinafter OPT-SA) was made from a 12.0:5.0:5.0 molar ratio of PC, CH, and SA, respectively. The optimized DCP-liposomes (hereinafter OPT-DCP) contained PC, CH, and DCP in an 8.5:4.5:6.5 molar ratio. The proper concentrations of the liposome-forming agents resulted in favourable formulations that meet the size, low PdI, and high ZP criteria. Detailed characterisation was done on these optimized formulations.

5.2.2. Characterization of the zeta potential optimized formulations

5.2.2.1. Vesicle size and zeta potential analysis

The ZP, the vesicle size, and the PdI as CQAs have a high impact on the quality of a liposomal formulation. All of the critical product parameters of the liposomes, such as the vesicle size (108 ± 15 nm; 88 ± 14 nm for the OPT-SA and the OPT-DCP samples, respectively), the low PdI (0.20 ± 0.04; 0.21 ± 0.02) that indicated monodisperse size distribution and the ZP (+30.1 ± 1.2 mV; -36.7 ± 3.3 mV) met the requirements of the nanosized drug delivery systems (***Table 6***). The positively charged vesicles were larger than the negatively charged counterparts, which can be explained by the spacing difference between the bilayers and the bulkiness of the charge imparting membrane additives[62,80,85]. The vesicles with ZP between 20 and 40 mV ensured stable systems by decreasing the chance of aggregation due to the high charge–charge repulsion among the liposomes[53]. The OPT-SA formulation had a significantly larger ($p<0.05$) vesicle size than the OPT-DCP, while the DCP-containing formulation reached significantly higher ($p<0.05$) ZP in absolute value.

Only some of the literature-derived results of compositions applied by other research groups (***Tables 1 and 2***) met the requirements we set. Mostly one of the three basic characteristic values (size, PdI and ZP) was not investigated or out of our acceptance range. The following conclusions were made by comparing the formulations with the acceptance criteria range

characteristics. Salem et al. and Vhora et al. reached higher ZPs but complemented their formulations with other surfactants and PPL. Their formulations had +42.5 ±2.1 mV and +52.8 ± 3.7 mV ZP at PC:CH:Span 60:SA = 1:1:1:0.15 (+flucytosine) and DOPE:PC:CH:SA = 10:45:29:16 molar ratios respectively.[76,81] Mishra et al. formulated only PC-and SA-based (2:0.5 molar ratio) amphotericin B-liposomes without adding CH to the compositions and reached +32.0 ± 0.2 mV with vesicle size 140 ± 4 nm[72]. Sharma et al. formulated liposomes with +32.9 ± 2.1 mV of ZP from PC:CH:SA applied in a 7:3:1.1 molar ratio with a smaller vesicle size (77 ± 2 nm) than OPT-SA (108 ± 15 nm)[93]. Inserting monensin as an API into the same liposomes by Rajendran et al. resulted in higher ZP (+43.9 ± 0.9 mV) and vesicle size (121 ± 20 nm)[89]. Salem et al. reached high ZP values (-59.1 ± 1.7) by adding Span60 to the DCP-containing flucytosine liposomes following the molar ratios of PC:CH:DCP:Span 60 = 1:1:0.1:1[81]. Calvo et al. formulated almost 200 nm large liposomes (195 ± 5 nm) with -47.0 ± 1.0 mV of ZP from PC:CH:DCP = 15:8:1[102]. Togami et al. reach approximately the same charge -49.4 ± 3.5 mV with a smaller vesicle size (134 ± 4 nm) applying PC:CH:DCP in 7:2:1 molar ratio[104]. To evaluate the results, the modifying effect of the API needs to be considered where it was applied. The OPT-SA and the OPT-DCP formulations reached the minimal requirements of the ZP based on the stability requirements with small vesicle sizes.

Further conclusions could be made upon better examination of the formulation compositions. Although the optimization was done in molar ratio, changing the values into weight ratio allows direct comparison with previous findings (***Table 7***). The QbD-based liposome development study concluded that the optimal PPL-CH ratio for liposome formations is the PC:CH = 60:40 and 80:20 weight ratios[35]. The weight ratio of PC in the optimized formulations was essentially the same, i.e. 60 weight units (59.9 and 60.3 in the OPT-SA and the OPT-DCP samples, respectively), while 80 weight units were found for the PC:CH ratio alone (82.7 and 78.9 in OPT-SA and the OPT-DCP, respectively) in good agreement with the previous results. Investigating the PC ratio only for the PC-CH content of the formulations and not for the whole formulas, the weight unit of PC was given close to 80 (82.7 and 78.9). As the results of an independent study, these findings can prove that the development program led to the correct answers. The DLS measurements showed the effect of the membrane additives on the SA-and DCP-free formulations. The addition of SA and DCP to the PC-CH compositions resulted in significant changes. The size of the vesicles decreased significantly: the Tukey inference was $p<0.05$ for the OPT-SA PC-CH-60-40 relationship and $p<0.01$ for the other relations between the OPT-SA, the OPT-DCP, the PC-CH-60-40 and the PC-CH-80-20 samples. The absolute ZP values increased significantly with $p<0.01$ inference for all pairs.

Table 7. *Results of the DLS measurements of the SA-and DCP-containing and SA-and DCP-free formulations indicate their compositions. (Results are expressed in mean ± standard deviation from three independent parallels.)*

	OPT-SA	**OPT-DCP**	**PC-CH-60-40**	**PC-CH-80-20**
	PC : CH: SA	PC : CH : DCP	PC : CH	PC : CH
Molar ratio	12.0 : 5.0 : 5.0	8.5 : 4.5 : 6.5	1 : 1.32	2.01 : 1
Molar % (n/n%)	54.5 : 22.75 : 22.75	43.6 : 23.1 : 33.3	43.1 : 56.9	66.8 : 33.2
Weight ratio	**59.9** : 12.5 : 8.8	**60.3** : 16.1 : 32.8	**60.0** : 40.0	80.0 : 20.0
Weight % (w/w%)	73.8 : 15.4 : 10.8	55.2 : 14.7 : 30.1	60.0 : 40.0	80.0 : 20.0
PC:CH weight ratio	**82.7** : 17.3	**78.9** : 21.1	60.0 : 40.0	**80.0** : 20.0
Vesicle size (nm)	108 ± 15	88 ± 14	151 ± 28	172 ± 44
Polydispersity index	0.20 ± 0.04	0.21 ± 0.02	0.18 ± 0.07	0.24 ± 0.03
ZP (mV)	+30.1 ± 1.2	-36.7 ± 3.3	-9.0 ± 2.4	-8.9 ± 1.3

Clear formulations were obtained with a visible opalescence indicating colloidal size systems. DLS measurements and ZP analysis were first done on the fresh samples and repeated weekly for a month. The liquid preparations could be considered stable for two weeks (results are shown in ***Table 8***). The formulations kept their good characteristics in a liquid state within this period. By the end of the fourth week, the surface charge of the vesicles decreased significantly ($p<0.05$ for OPT-SA, $p<0.01$ for OPT-DCP).

Table 8. *2-week stability changes in the optimized formulations followed by the DLS technique. Alterations were detected in vesicle size, polydispersity and ZP. (Results are from three independent parallels).*

2-week stability	**OPT-SA**	**OPT-DCP**
Vesicle size (nm)	118 ± 17	113 ± 9
Polydispersity index	0.23 ± 0.07	0.30 ± 0.09
ZP (mV)	+26.7 ± 3.1	-39.0 ± 3.2

The developed liquid liposomal formulations were lyophilised.

The AFM measurements provided a three-dimensional surface profile of the optimized samples. The AFM records of the OPT-SA (***Figure 11-A***) and the OPT-DCP (***Figure 11-B***) samples showed homogeneous size distribution with a mean vesicle size of around 100 nm. These images support the information obtained from the DLS measurements.

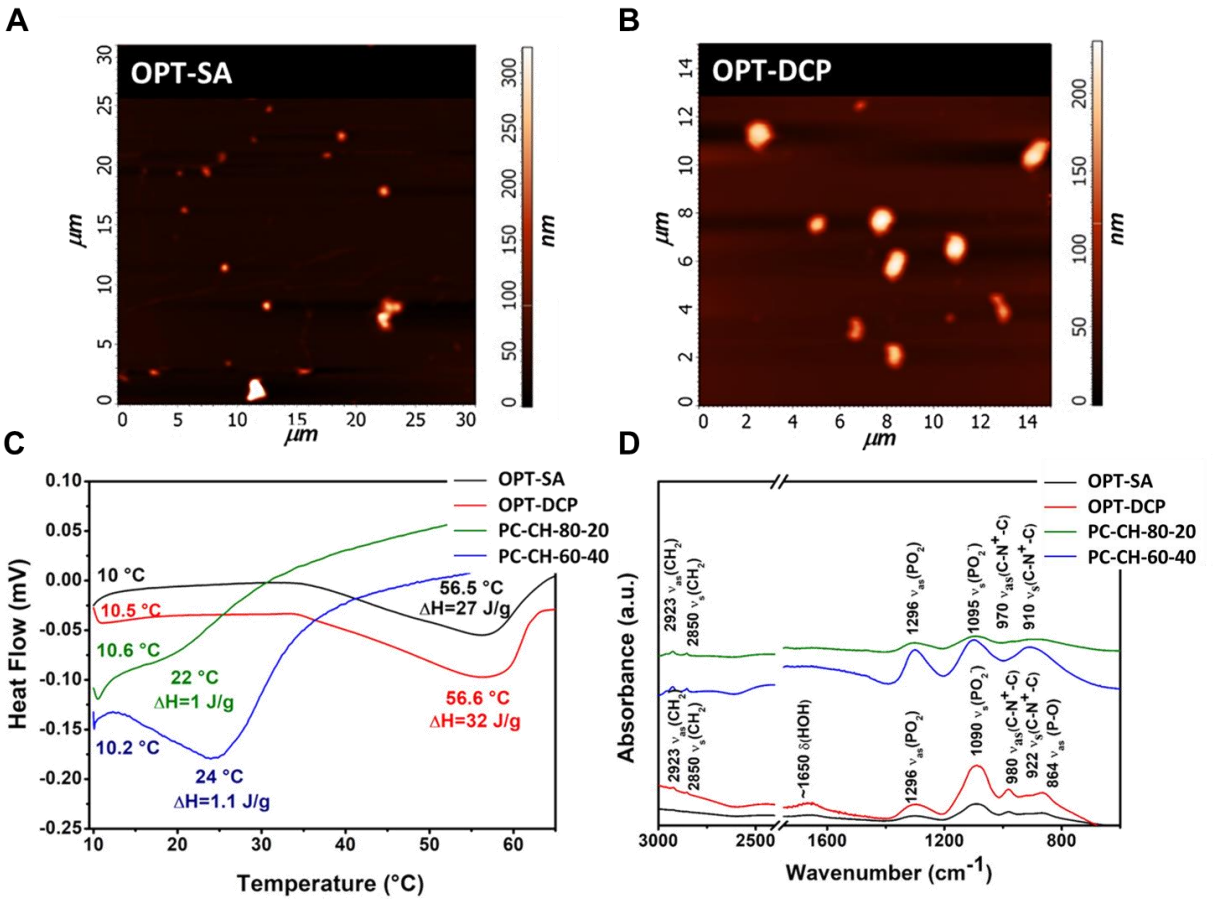

Figure 11. *AMF images were taken of the optimized liposome samples: OPT-SA (A) and OPT-DCP (B), and DSC (C), and FT-IR (D)curves show the optimized liposome samples and the membrane additive-free compositions: PC-CH-80-20 and PC-CH-60-40*

5.2.2.2. Thermal analysis

DSC measurements were done in the 10-65°C range. These studies provide information about, among others, the phase transition of the liposomes. The so-called glass transition temperature (T_g) is an important parameter in characterising the stability of the lyophilised samples. T_m and the corresponding enthalpy change (ΔH_m) influence the pharmacokinetics of the pharmaceuticals and the stability of the liposomes as well[112]. A high ΔH_m value implies a more rigid phospholipid bilayer, while similar transient enthalpies predict a bilayer phase with a similar structure[130]. The calorimetric results (***Figure 11-C***) show that the T_g value for the OPT-SA sample (black line) was 10°C, and for the OPT-DCP sample (red line) was 10.5°C. For the membrane additive-free compositions (PC-CH) 10.6 °C (PC-CH-80-20 (green line)) and 10.2 °C (PC-CH-60-40 (blue line)) were found. The T_m values were 56.5°C, 56.6°C, 22.0°C and 24.0°C, respectively. Similar ΔH_m values were given; the enthalpy change was 21 J/g for the SA-containing and 32 J/g in the case of the DCP-based optimized liposomes. Compared to the PC-CH formulations (1.0 J/g and 1.1 J/g), these compositions formed more rigid bilayers.

The thermal stability of the formulations was further investigated via TGA in the 0-300°C temperature region. The shape of the TG curves was similar to each other. The weight of the OPT-SA liposomes started to decrease at 75°C, and 4% of the original weight was lost until 300°C. The OPT-DCP formulations lost 6% of weight starting from 80°C. The weight loss happened in two steps. The first step at 75-80°C indicated the physisorbed water content desorption. The other change in the shape of the curves was detected at 200-225°C representing molecular changes and chemical degradation in the structures. The degradations occurred at high-temperature ranges only, well above the limit of any practical applications, so the optimized formulations are considered stable against temperature during production and storage.

5.2.2.3. Investigation of chemical bonding

Similar FT-IR spectra were recorded for both optimized formulations (***Figure 11-D***), indicating two separate regions according to the PC as its vibrational bonds dominate due to its highest concentration in the composition. C-H bond stretching vibrations were found from 3000 to 2800 cm^{-1}, whereas the ~900–600 cm^{-1} regime is the fingerprint region. The former mainly originates from the hydrocarbon chains, while bonds corresponding to the vibrations of the polar phospholipid head groups appear at lower wavenumbers (<1800 cm^{-1}). At 864 cm^{-1}, the asymmetric ν_{as}(P-O), and at 922 $cm^{-1,}$ the $\nu_{as}(N^{+}\text{-}(CH_3)_3)$, at 980 $cm^{-1,}$ the symmetric $\nu_s(N^{+}\text{-}(CH_3)_3)$, and at 1090 cm^{-1} the $\nu_s((PO)_2)$, while at 1296 cm^{-1} the asymmetric $\nu_{as}(PO_2)$ stretching can be seen. The symmetric $\nu_s(CH_2)$ stretchings of the apolar hydrocarbon chains appear at 2850 cm^{-1}, while its asymmetric counterpart, $\nu_{as}(CH_2)$, is seen at 2923 cm^{-1}[1138]. Bonds referring to the SA and the DCP were not distinguishable as the spectral peaks at 2900-2960, and 2850 cm^{-1} represented the DCP, while vibrations from SA appeared at 2920 and 2850 cm^{-1}. Thus FT-IR spectra were not conclusive in detecting these charge imparting agents in the formulations.

5.2.2.4. Residual ethanol measurement

The ethanol residue was quantified via GC-MS, and ethanol concentrations of 11.1 and 23.9 μmol L^{-1} (0.51 and 1.10 ppb) in the acceptance range were found in the OPT-SA and OPT-DCP samples, respectively. The amount of remaining ethanol in the samples of PC-CH-60-40 and PC-CH-80-20 was below the limit of detection in the current setup.

6. SUMMARY

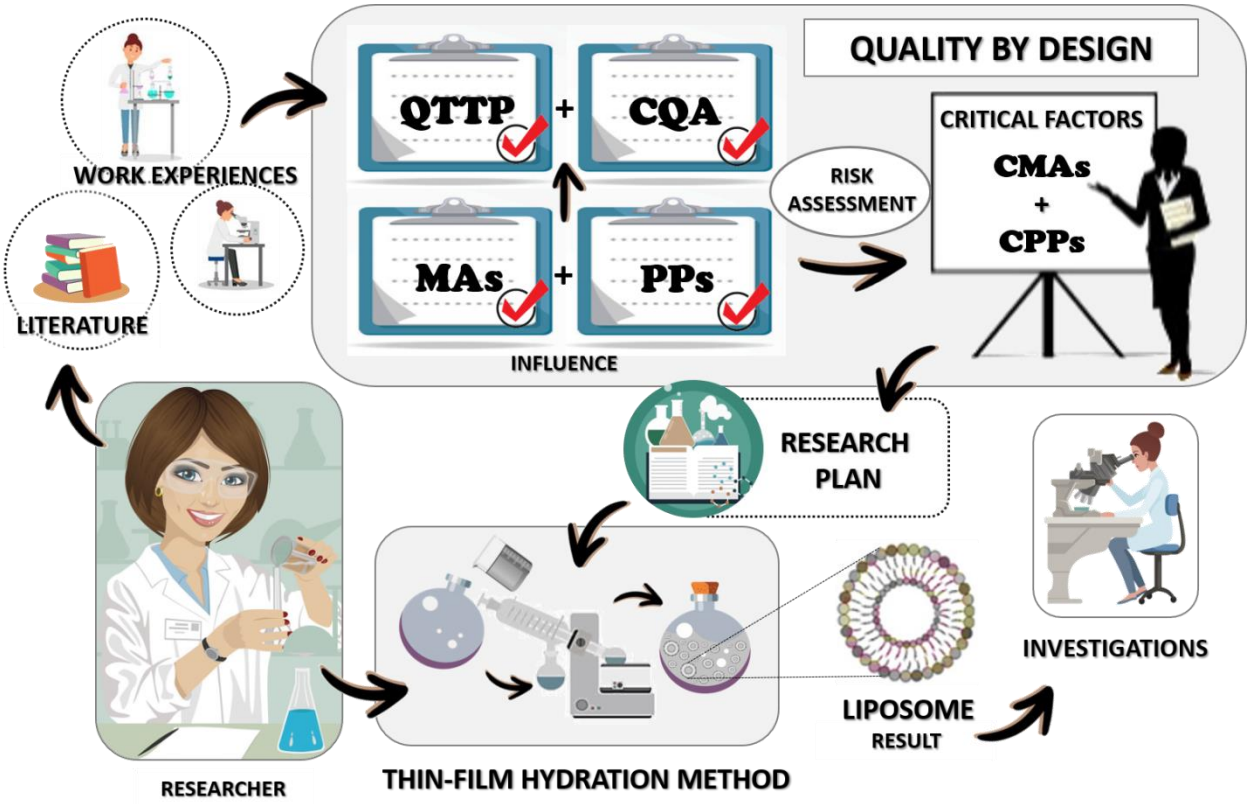

Figure 12. *Flow chart of the QbD methodology-driven liposomal formulation development.*

The requirements for the liposomal formulations vary depending on the chosen medical need and the selected route of administration. The proper liposome formulation design is assigned to the therapeutic needs. Identifying those factors that impact the final product is essential.

The aim of my Ph.D. work was to develop liposomal formulations based on the Quality by Design methodology. The requirements of the thin-film hydration liposome preparation technique were investigated in the studies. The QbD concept and the RA methodology were implemented in the early phase of liposomal formulation development (***Figure 12***), and the zeta potential of the liposomes was improved following the principles of a 3^2 fractional factorial design and utilizing the obtained knowledge on the critical product quality influencing factors.

Following a universal initial RA for the thin-film hydration method, an updated RA was carried out concerning the quality requirements of the ‘empty’ liposomal carriers. Experimental characterization studies were done to evaluate the severity of the impact that the selected CMAs and CPPs have on the properties of the liposomes. Changes in the working temperature settings, the PC-CH ratio, the PEGylated PPL content, the quality of the hydration media and the cryoprotectants resulted in a significant difference in the product quality.

The observations were built into the updated RA and the ZP optimization study. The necessary PC, CH and SA or DCP molar ratios were determined to get liposomal formulations with the predefined CQAs: vesicle size under 150 nm, PdI less than 0.30 and ZP higher than |30| mV.

The results fit into this scientific research area, extend the knowledge and give a detailed overview of the QbD methodology application in liposome development. The presented concept helps to establish and perform studies on liposomes with less effort and more success, and the observations can provide a valuable base for further developments.

New findings and practical relevance of the work:

- This is the first QbD model-based study which successfully applied an updated RA to determine the critical parameters of an 'intermediate', API-free liposomal formulation and identified the CMAs and CPPs of the thin-film hydration liposome preparation process.
- The study presented liposomal compositions described first in this work.
- The effect of some factors and liposome components was investigated for the first time or the first time in the presented combinations.
- Observations and recommendations on the parameters of a thin-film hydration-based liposome preparation process are presented regarding the working temperature, the PC-CH ratio, the PEGylated PPL content, the quality of the hydration media and the cryoprotectants of the liposomal formulations.
- Novel liposomal carriers were developed with characteristics complying with the predefined QTPP and CQAs providing vesicles with ZP in the optimal ratio and vesicle size under 150 nm in the liquid form for up to two weeks.
- The described PC:CH:SA =12.0:5.0:5.0 and PC:CH:DCP = 8.5:4.5:6.5 molar ratios provide a base that can be successfully adapted to other lipid components and formulation requirements.
- The results excellently illustrated the relevance and the potential of applying the Quality by Design methodology in liposomal research and development.

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9. DECLARATION OF OWN WORK

Name: **Dr. Zsófia Németh**

Title of the doctoral thesis: **Application of the Quality by Design methodology in the design and development process of liposomal delivery systems**

I, **Dr. Zsófia Németh**, fully aware of my responsibility, declare that my doctoral (Ph.D.) dissertation prepared at the Doctoral School of Pharmaceutical Sciences of the University of Szeged is based on my own research results. During my research work, the publication of my results, and the writing of my dissertation, I acted according to the basic principles and recommendations laid down in the Code of Scientific Ethics of the Hungarian Academy of Sciences.

Szeged, 05.09.2022.

……

<Dr. Zsófia Németh>

Ph.D. candidate

ANNEX

RELATED PUBLICATIONS

I.

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Application of the QbD-based approach in the early development of liposomes for nasal administration

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ABSTRACT

In this study, authors adapt the Quality by Design (QbD) concept as well as the Risk Assessment (RA) method to the early development phase of a new nano-sized liposomal formulation for nasal administration with brain target. As a model active agent, a BCS II class drug was chosen to investigate the behaviour of the drugs with lipophilic character. This research presents how to apply this risk-focused approach and concentrates on the first four stages of the QbD implementation. In this way the quality target product profile was defined, the critical factors were identified and an RA was performed. The RA results helped in the factorial design-based liposome preparation by the lipid film hydration method. The prepared liposomes were evaluated (vesicle size, size distribution, and specific surface area). The surface characteristics were also investigated to verify the exactness of the RA and critical factors based theoretical prediction.

The results confirm that the QbD approach in liposome development can improve the formulation process. The RA focused predictive approach resulted in a decreased number of studies in practice but in an effective product preparation. Using such innovative design and development models can help to optimise and rationalise the development of liposomes.

1. Introduction

The Quality by Design (QbD) is a risk- and knowledge-based quality management approach of the pharmaceutical formulation development described in ICH guidelines (Ich. Pharmaceutical, 2009; Türker et al., 2004), which starts with the expected quality and therapeutic goals. In this case, the product and the manufacturing process are designed and developed according to the previously defined expectations, which means a change in paradigm (Yu, 2008; Yu et al., 2014). A complete QbD study involves the following stages (Ich. Pharmaceutical, 2009): (1) Define the Quality Target Product Profile (QTPP) based on the knowledge space developed from the relevant scientific literature and with appropriate in vivo relevance. (2) Design the product and the manufacturing process in accordance with the predefined profile. (3) Identify the potential Critical Quality Attributes (CQAs), the potential Critical Material Attributes (CMAs) as well as the potential Critical Process Parameters (CPPs), and perform the Risk Assessment (RA) (Quality Risk, 2005), which is the key element of the QbD approach.

(4) Set up the Design of Experiments (DoE) based on the RA results. DoE is a practical development planned and executed according to the most relevant influencing factors (CQAs, CPPs) selected by the priority ranking of the RA in order to define the Design Space. (5) Set up a process control strategy to ensure consistent product quality. (6) In the industry, the last step is the product lifecycle management. Overall, the application of the QbD concept results in a better product, process, and interrelation understanding (Patil and Pethe, 2013). The QbD-indicated theoretical prediction and the risk-focused approach help to conduct a more effective practical research activity, which is adaptable even in the early phases of pharmaceutical developments (Pallagi et al., 2015; Gieszinger et al., 2017; Pallagi and Karimi, 2016; Karimi and Pallagi, 2016; Csóka et al., 2018).

Liposomes- as nano-carriers have many (Akbarzadeh et al., 2013). They are artificial spherical particles with an aqueous core surrounded by a hydrophobic membrane, which forms a special lipid bilayer

Abbreviations: API, active pharmaceutical ingredient; BCS, Biopharmaceutics Classification System; CMAs, Critical Material Attributes; CNS, central nervous system; CPPs, Critical Process Parameters; CQAs, Critical Quality Attributes; D[3,2], surface weighted mean; D[4,2], volume weighted mean; DoE, Design of Experiments; PdI, polydispersity index; Ph Eur, European Pharmacopoeia; QTPP, Quality Target Product Profile; RA, Risk Assessment; RPN, risk priority number; rpm, rotation per minute; SEM, scanning electron microscope; SSA, specific surface area; WF1, wall-forming agent 1 (in this study: natural oil/cholesterol); WF2, wall-forming agent 2 (in this study: Phospholipon 90G®)

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structure and has an amphiphilic character (Deamer, 2010), thus they are able to enclose both lipophilic (located in the nonpolar wall) and hydrophilic compounds (placed in the aqueous polar inner phase) into their structure (Laouini et al., 2012). The encapsulation of the molecules protects the active agents and allows better targeting (Torchilin, 2007; Maherani et al., 2011). Lecithin or other phospholipids (natural or artificial) and cholesterol are the main components of liposomes (Briuglia et al., 2015). The bilayer structure is formed from phospholipids in an aqueous milieu. Its rigidity and the permeability depend on the length and the saturation of the carbon chains. The release of the active agents of liposomes is modified by the phase transition temperature of the lipid compound. Cholesterol builds into the membrane and decreases the fluidity of it (Briuglia et al., 2015). By surface modification (eg Polyethylene glycol chains) the in vivo circulation time can be increased (Torchilin, 2007), so the therapeutic index can be raised and the toxicity can be reduced. Usually, liposomes as carriers are used in the case of drugs with low permeability (according to the Biopharmaceutics Classification System (BCS) these belong to BCS classes III and IV Rodriguez-aller et al., 2015; Zylberberg et al., 2016; Akbarzadeh et al., 2013). The drugs which exhibit high permeability (BCS Class II and Class IIb weak bases) may precipitate (in a very complex and poorly understood method) in the small intestine (Tubic-grozdanis et al., 2008). The intestinal precipitation of a Class IIb drug can depend on numerous factors (e.g. formulation, physiological factors, time of administration, etc.) and complicates in vivo prediction (Tsume et al., 2014). The incorporation (entrapment) of lipophilic BCS II drugs into liposomes and the choice of an alternative administration route like the nasal administration can offer several advantages in the therapy. Compared to the conventional administration routes (Allen et al., 1993) (e.g. intravenous administration, dermal or pulmonary intake), *nasal administration* is a non-invasive, simple and painless manner of drug delivery, able to reach an immediate and local effect, but also to get a long-term and systemic therapeutic effect (Türker et al., 2004). The active agent administered into the nasal cavity can be absorbed into the systemic blood circulation, reach the central nervous system via the blood-brain barrier (“nose-to-blood”), or reach the brain tissue directly by axonal transport. This is the so-called “nose-to-brain” route, which avoids the blood-brain barrier and results in the targeted delivery of the active pharmaceutical ingredient (API) into the central nervous system (CNS) from the nasal cavity (Bartos et al., 2018) which may help in the treatment of Alzheimer’s and Parkinson’s disease or epilepsy in the future (Agarwal et al., 2013). However, the “nose-to-brain” way has several limitations. Water-soluble materials and molecules with molecular weights higher than 40-500 Da have no, or only very limited potential to pass the blood-brain barrier, while hydrophilic and polar molecules have several limitations in the paracellular transport between the epithelial cells (Bartos et al., 2018). Lipophilic molecules can pass easily via the transcellular pathway by passive diffusion. Further general characteristics of nasal delivery are the following: Successful formulation and the therapeutic effect depend on molecular weight and size, solubility, lipophilic characteristic, the pKa value and the distribution coefficient of the API because these features influence absorption. The nasal absorption of the API depends on pH. In general, better absorption can be achieved if the pH of the API is lower than its pKa value. The normal physiologic pH is 5.5-6.5 in the nasal region, thus the product should have a pH value of 4.5-6.5. The volume of the nasal cavity is limited, so only a small amount but a highly concentrated product can be administered there (200–300 µl pro dosi) (Bartos et al., 2015). The mucus on the nasal mucosa is renewed in every 15–20 min due to the continuous mucus secretion and mucociliary activity. So, if the bioadhesivity of the product is poor, the half-life of the API is 10-15 minutes. That can be solved by using mucoadhesive excipients. Better solubility and permeability values can be expected from nanosized particles (or nanosized drops) The the optimal particle size range for nasal administration is 200–500 nm and 100–400 nm if the target is specifically the nose-to-brain route (Sonvico et al., 2018, 2018).

This work was aimed to adapt the QbD as well as Risk Assessment to the early development phase of a new liposomal formulation for brain target with nasal administration. A lipophilic drug (lamotrigine) was chosen as a model active agent in order to study the incorporation of drugs with high permeability into the liposomes, and investigate the potential advantages of this process. This research focuses on the first four stages of QbD implementation, so the aimed tasks were to define the target profile, select the critical factors, perform a Risk Assessment and a factorial design-based liposome preparation, and evaluate the results in order to verify whether the QbD approach in a liposome development can improve the formulation process and can help to make it more effective and efficient.

2. Materials and methods

2.1. Materials

The applied model API was a lipophilic (BCS Class II) drug with low solubility and high permeability properties (lamotrigine, $C_9H_7Cl_2N_2$, Meditop Pharmaceutical Ltd., Pilisborosjenő, Hungary). The molar weight of the API is 256.09 g/mol, the melting range is 216–218 °C, the solubility is 170 mg/L in water and 12 mg/L in ethanol at 25 °C, the pKa is 5.7, and the value of the logP is 2.5 measured in distilled water.

The following materials were used as excipients: Phospholipon 90G® lipid mixture (Phospholipid GmbH, Köln, Germany), cholesterol (in an alcoholic solution) (Molar Chemicals Ltd., Budapest, Hungary), natural organic apricot kernel oil (Primavera Life GmbH, Oy-Mittelberg, Germany) as components of the liposomal wall, ethanol 96% (Molar Chemicals Ltd., Budapest, Hungary, official in the Ph. Eur.) was chosen from “green chemistry” considerations (Green, 2018; Bacher, 2014), as a relatively nontoxic solvent and sodium chloride physiological solution (NaCl, Molar Chemicals Ltd., Budapest, Hungary). The excipients were used for different purposes and in different ratios according to the sample preparation process (Table 1).

2.2. Methods

2.2.1. Definition of the QTPPs and knowledge space development

The initial step is to define the target product profile and its quality criteria. The selection of the QTPPs is based on the requirements of the

Table 1
Compositions of the prepared API-containing liposome samples

Preparation process number	Concentration of phospholipid (Phospholipon 90G®)	Temperature of the ultrasound treatment	Number of the (second) filtration	Samples prepared E = Empty A = API containing
1	95	65	1	E-95-1-65 A-95-1-65
2	95	55	1	E-95-1-55 A-95-1-55
3	85	65	1	E-85-1-65 A-85-1-65
4	85	55	1	A-85-1-55 E-85-1-55
5	95	65	3	E-95-3-65 A-95-3-65
6	95	55	3	E-95-3-55 A-95-3-55
7	85	65	3	E-85-3-65 A-85-3-65
8	85	55	3	E-85-3-55 A-85-3-55

The amount of cholesterol, oil component and API (in the API containing samples) were constant in each preparation.

interested parties (clinical expectations, patients' and industrial needs, regulatory aspects). It usually includes the route of the administration, the dosage form, the dose, the appearance, and the dissolution or the pharmacokinetic data of the drug, furthermore, some special safety or quality requirements, etc. In order to see all the influential parameters of the desired liposome product, a cause and effect (Ishikawa) diagram Tague (2005) was constructed to collect and visualize these factors for the further steps. This diagram is especially useful for selecting the QTPPs and the critical factors during the next QbD-guided development steps.

2.2.2. Determination of the CQAs

The second step is the selection of the factors which have critical effects on the targeted product quality. These are the Critical Quality Attributes. The selection of these parameters is based on prior knowledge and previous experience. Usually, CQAs are physical, chemical, biological or microbiological properties that should reach an appropriate range or limit to ensure constant end product quality. CQAs may include information about particle or vesicle size, size distribution, drug release, etc. The selection of a factor as a CQA always depends on the predefined goals, the expected quality of the product and the therapeutic needs.

2.2.3. Determination of the CMAs and the CPPs

In the next step, the critical material and/or the Critical Process Parameters should be determined, which are the factors relating to the materials and the selected production methods and processes, thus may influence the CQAs.

2.2.4. Initial Risk Assessment

After the identification of the QTPPs, the CQAs, and the CMAs/CPPs, an initial RA was carried out via the following steps: interdependence estimation between the QTPPs and the CQAs and between the CQAs and the CMAs/CPPs using a three-grade scale, whether the estimated interdependence is high, medium or low. The next task is the estimation of the occurrence of the CPPs/CMAs, using the same scale. RA was performed through the Lean QbD Software® (QbD Works LLC, Fremont, USA). According to the calculation of the software, all of the critical factors were quantified and ranked by their influence. The rankings of the CQAs and CPPs were visualized on Pareto charts Powell et al. (2014) generated by the software. The Pareto charts not only show the relationships between the CMAs or the CPPs and the CQAs but also help to select the factorial design parameters of the experiments.

2.2.5. Design of experiments

For the design of the practical experiments, the JMP® 13 Software (SAS Institute, Cary, USA) was used and the Main Effect Screening Design 2 programme was chosen. For this screening design, the variable factors were selected based on the RA results. These were the concentration of phospholipids (X1, lower limit: 85 w/w%, upper limit: 95 w/w%), the temperature of evaporation during the lipid film formation (X2, lower limit: 55 °C, upper limit: 65 °C) and the number of the second filtration with the 0.22-μm membrane filter (X3, lower limit: 1 filtration, upper limit: 3 filtrations). For a response, the size and the size distribution of the vesicles were investigated. The pattern of the factorial design was the following (X1, X2, X3, – is the lower limit and + is the upper limit), sample 1: + + -, sample 2: +–, sample 3: - + -, sample 4: —, sample 5: + + +, sample 6: + - +, sample 7: - + +, sample 8: – +. The same pattern was used during the preparation of API-containing and API-free liposomes as well.

2.2.6. Preparation of liposomes

The liposomes were prepared by using the lipid film hydration method (also called as thin film method) (Dua and Bhandari, 2012). The first step was the preparation of the lipid suspension, which was a mixture of the established amount of Phospholipon 90G®, cholesterol, and natural oil. API-free (empty) and API-containing liposomes were prepared (Table 1). The preparation was based on the pattern of the factorial design. In the case of the API-containing product, the defined amount of API (250 mg) was added to the lipid phase. 50 ml of ethanol 96% was used as a solvent agent. Ethanol was evaporated under vacuum, in a water bath, with Rotavapor® R-210/215 (BÜCHI Labortechnik AG, Flawil, Switzerland), at 55°C or 65°C. The rotation speed was 25 rpm. The vacuum creation was gradual (from 1100 mbar to 300 mbar, with steps of 100 mbar, from 300 mbar to 150 mbar with steps of 50 mbar). Then, the dry lipid film was hydrated with 3x50 ml of physiological saline solution. Hydration was supported by ultrasonication (Elma Transsonic Digital S D-78224 ultrasonic bath, Elma Schmidbauer GmbH, Singen, Germany) at 55°C or 65°C, 120% power, 48 kHz, for 15 minutes. The shaping of the liposomes was performed in two steps. First, a 0.45-μm membrane-filter was used for one filtration (Millipore® SLHV033NS Millex® HV Syringe Filter with Durapore® PVDF Membrane, Sigma-Aldrich) with a 10-ml syringe, then a 0.22-μm membrane-filter for 1- or 3-time filtration (FilterBio PES Syringe, FilterBio Membrane Co. Ltd, Lab-Ex Labortrading Ltd.). This step was followed by freeze-drying (lyophilization) to stabilise the prepared samples and fit for the SEM investigation.

The preparation conditions of the liposome samples are presented in Table 1. Besides the API-free, empty samples API-containing formulations were also prepared, thus 16 liposome sample were prepared all together (Table 1).

2.2.7. Characterization of the liposomes

2.2.7.1. Particle size and zeta potential analysis. The vesicle size of the prepared liposomes was determined by using two instrumental methods. The first was the laser diffraction (Mastersizer 2000, Malvern Instruments Ltd, Worcestershire, UK) method. This technique was applied to determine the most commonly used units, the d-values, namely d(0.1), d(0.5) and d(0.9) values. These data describe particle size distribution and mean 10% undersize, 50% undersize (median diameter) and 90% undersize, respectively of the total values. The equipment calculates the span value as well. The span value provides the width of distributions (Rawle), namely span = (90% undersize − 10% undersize)/50% undersize. The higher span value may indicate greater polydispersity of the system. The uniformity of the samples can also be measured from size distribution, as well as specific surface area (SSA), surface weighted mean (D[3,2]) and volume weighted mean (D[4,2]). These two values, D[3,2] and D[4,2], both mean an average of the particle size and have an important role in flow dynamics. The second instrument, which was used for the determination of particle size and zeta potential of some of the samples, was based on the dynamic light scattering technique (Malvern Zeta Nano ZS, Malvern Instruments Ltd, Worcestershire, UK, Refraction Index: 1.33, 25°C). This investigation resulted in the mean vesicle size, polydispersity index (PdI) values and the surface charge of the prepared samples.

2.2.7.2. Scanning electron microscopy (SEM). We used a Hitachi S-4700 FE-SEM scanning electron microscope (Hitachi High-Technologies Europe GmbH, Krefeld, Germany) for this process. The freeze-dried samples were coated with gold-palladium by a sputter coater (Bio-Rad SC502; VG Microtech, Uckfield, UK). The used air pressure was 1.3–13.0 mPa. Freeze-drying (Van Winden et al., 1997) is necessary before the SEM investigation, it was made by Coolsafe 100-9 Pro ScanVac (LaboGene ApS, Lynge, Denmark, from 25 °C/normal pressure to −40 °C/0.01 atm under vacuum, after 8 hour the reheating steps were: −40 °C, −20 °C, 0 °C, +10 °C, +20 °C +30 °C until reaching the normal pressure).

Drug entrapment efficiency

The selected sample prepared by the lipid film hydration method was centrifuged with a Hermle Z 323 K centrifuge (Hermle LaborTechnik GmbH, Wehingen, Germany) for 10 minutes at 2240 rcf.

1 ml of 10-time dissolved samples was investigated and washed 2 times with sodium chloride physiological solution. The spectrophotometric investigation of the samples was conducted with a Unicam UV/VIS spectrophotometer (Unicam, Cambridge, UK) at a wavelength of 296 nm (r (Ich, 2009) = 0.998963, absorbance value = 0.0190 × concentration).

2.3. Statistical analysis

The statistical analysis of the results of the investigational data was performed with Microsoft® Excel® (Microsoft Office Professional Plus 2013, Microsoft Excel 15.0.5023.100, Microsoft Corporation, Washington, USA) and the JMP® 13 Software (SAS Institute, Cary USA).

3. Results and discussion

3.1. Definition of the QTPP, knowledge space development, identification of the CQAs, CMAs and CPPs

In the first step of this QbD-based liposome development, all the factors which could influence the aimed product were collected. This data collection process is knowledge space development. As a result, an Ishikawa diagram, namely a cause and effect diagram, was constructed from the collected factors, which is presented in Fig. 1. The influencing factors were classified into 4 groups, such as therapeutic aim, material characteristics, formulation technology, and product characteristics. All the mentioned items could have an impact on the quality of the lipophilic model drug-containing liposomes to be produced.

After knowledge space development, the QTPP was defined. The predefined goal of this study was to develop a lipophilic drug-containing liposomal liquid formulation for nasal administration with brain target. Another aim was to prepare this nasally administrable target product in a proper volume and API dose, with adequate pH values, with an optimal dissolution and API release profile, and to reach the required stability of the product. These elements and the whole profile of this target liposomal product are presented in Table 2.

In the following step, according to the QbD-based formulation development, the Critical Quality Attributes (CQAs) were identified and selected. These factors have potential critical effects on the desired quality of the final product (Table 3).

Parallel to the identification of the CQAs, the type of the liposome production was selected. For the defined aim, the lipid film hydration method seemed to be suitable. The steps of this method are presented by the authors on a process map in Fig. 2. The map shows the steps and the procedures of this liposome preparation route, involving the methods, the materials, and the process parameters. In this study, the construction of the process map formed part of knowledge space development and helped in the selection of the CMAs and in the identification of the CPPs. The material and process parameters which were found as critical are listed later in Fig. 3. Moreover, this process map

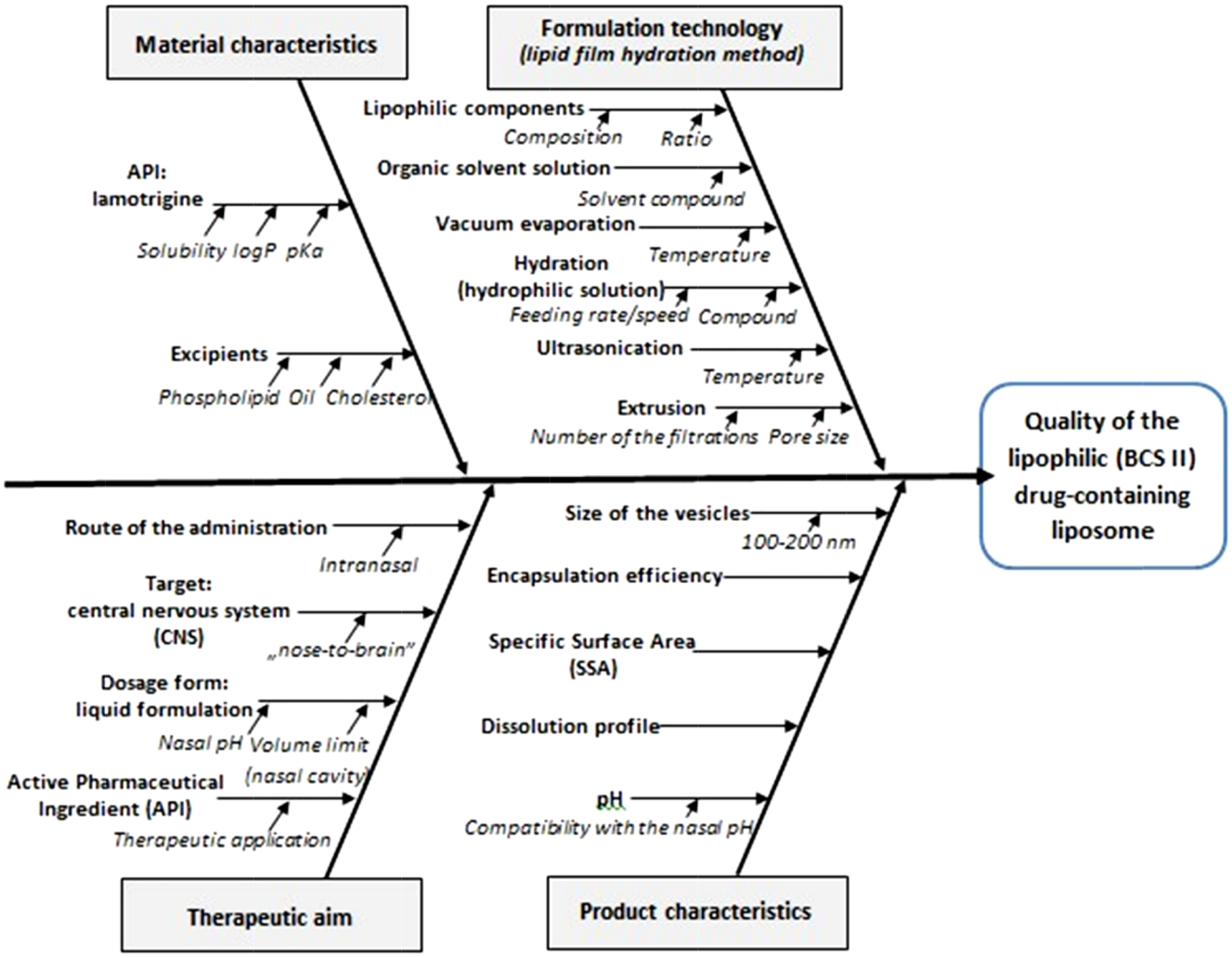

Fig. 1. Ishikawa diagram: it shows a cause and effect relationship between the material and process variables to specify the quality properties of the aimed liposomal product

Table 2
Quality Target Product Profile (QTPP) of the aimed lipophilic drug-containing liposome product.

QTPP parameter	Target	Justification
Therapeutic effect	Reach the brain tissue (in the case of this model lipophilic API, the potential target is the antiepileptic effect)	Direct or axonal transport of the API from the nose to the brain instead of the route through the blood-brain barrier Nunes et al. (2012)
Patient group	Adults or even children	The chosen model API is safely administrable in both age groups Schlumberger et al. (1994)
Route of administration	Nasal route	The nasal route offers direct access to the CNS Serralheiro et al. (2015)The nasally applied single dose of the API can be decreased compared to the orally administered dose.
Dosage form	Liquid formulation	The usage of the liquid form of the liposomal formulation offers a comfortable method for the administration of the drug into the nasal cavity Illum (2003)
Volume of one dose	200–300 µl	The nasally administrable volume is limited by the capacity of the nasal cavity Pires and Santos, (2017). The volume of the preparation is linked to the efficacy of the formulation. It is necessary that the total volume of the formulation be enough to contain the optimal dose of the API
Dissolution profile/absorption time	10–15 min	The dissolution profile of the product is related to the efficacy of the formulation. The proper amount of the active agent has to be dissolved in 10-15 minutes, which is the periodic renewing time of the nasal mucosa Hussain (1998), Arora et al. (2002).The characteristics of the optimal formulation fit to the conditions of the nasal cavity, which results in a controlled dissolution of the API
Stability (size of the vesicles)	100–400 nm	The size stability of the prepared liposomes is linked to the efficacy and quality of the preparation.The targeted size range is optimal according to the literature Sonvico et al. (2018)
pH	4.5–6.5	The optimal pH of the preparation fits to the normal pH of the nasal environment. The proper pH value ensures comfort during the application, and determines the quality and the in vivo efficacy of the product Parvathi (2012)

Table 3
CQAs of the aimed liposome product, their target and justification.

CQA	Target	Justification
Wall-forming agent 1 (Natural oil/ Cholesterol)	Optimal vesicle size	Natural oil and cholesterol influence membrane fluidity and the size of the vesicles Jójárt-Laczkovich et al. (2018) These components take part in the formation of the phospholipid bilayer Briuglia et al. (2015)
Wall-forming agent 2 (Phospholipon 90G®)	Optimal vesicle size	Phospholipon 90G® is a phospholipid complex and takes part in the formation of the phospholipid bilayer, furthermore, it influences the size of the vesicles Khale et al. (2011)
Hydration media	Optimal stability (no aggregation) of the liposomes	The hydration of the lipid film is one step of the formation of the phospholipid bilayer.
API	The successful incorporation of the lipophilic API into the liposomal wall	The incorporation of the API into the liposomal product could influence the size of the vesicles Jouyban and Soltanpour (2010)
Surface charge	Optimal for the liposomes to pass through the nasal membrane	The charge of the surface influences the stability of the vesicles, thus affects membrane transport Bozzuto and Molinari (2015)
Vesicle/particle size (and size distribution)	Around 200 nm	The size of the vesicles was chosen with regard to the aimed CNS target of the API Zawada (2004)
Stability of vesicle size	No aggregation, constant vesicle size	The stability of the vesicle size is important regarding the safety, the efficacy and the quality of the product Ich. Pharmaceutical, (2009), Winterhalter and Lasic (1993)

also indicates the step of stabilization after the production of the liposomal product. Liposomes are usually stable for a short period of time. To eliminate this problem, the final step was lyophilisation in order to obtain a stable, solid product.

3.2. Initial Risk Assessment

In the RA-based formulation development, the critical factors were analysed, the interrelations between them were estimated, and finally, the factors were ranked by their critical effect on the final product. The process of RA and the stages are presented in Fig. 3. After the determination of the QTPPs and the selection of the CQAs, CMAs, and CPPs, the impact (High, Medium or Low) of the QTPPs was estimated. That was the risk identification phase. As the following step of the risk analysis, a Risk Assessment matrix was created according to the estimated interdependence between the QTPPs and CQAs, and between the CQAs and CMAs/CPPs, and their impact. Each value had to be examined based on the interdependences between them and was rated as to whether the impact of their interdependence is “high”, “medium” or “low”. The estimation of this impact was based on the knowledge of the research group members, their experience gained from experimental practice, and scientific knowledge in the literature. The uncertainty of the critical factors was also estimated (Fig. 3).

As a result of the above-presented procedure, the QbD software calculated the risk priority number (RPN) of each factor and ranked them according to their critical effect on the liposomal product. The RPNs and their rankings are presented on the Pareto charts in Figures 4 and 5. Fig. 4 presents the ranking of the CQAs, while Fig. 5 shows the ranking of the critical factors related to the liposome production process (CMAs/CPPs).

Based on the result of this initial RA, the factors for the factorial design of the experiments could be selected.

3.3. Design of Experiments, factorial design

The RA results helped to set up the experimental design. The Main Effect Screening Design 2 programme was chosen for screening the effects of the formulation parameters on the quality of the final

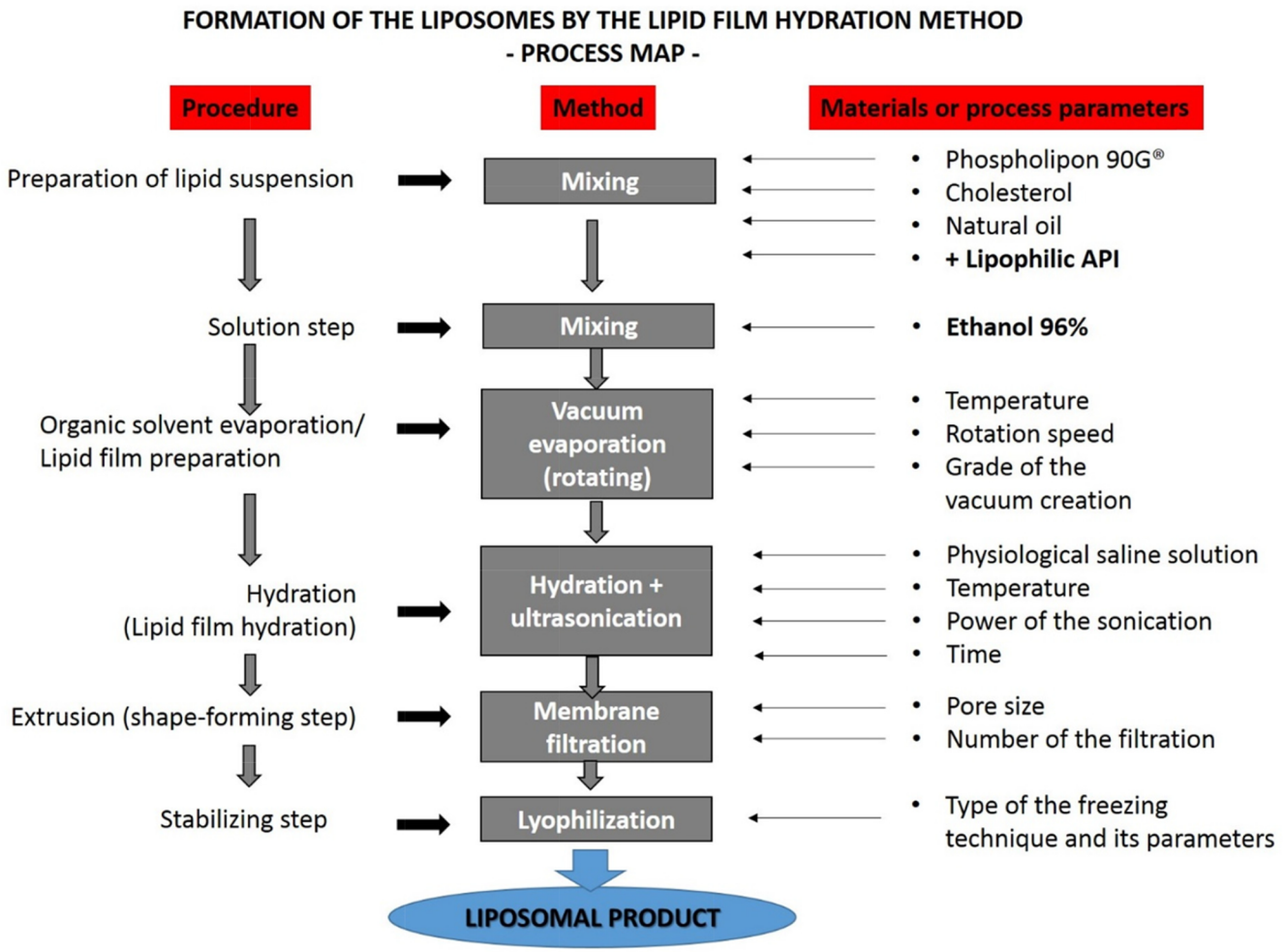

Fig. 2. Process Map of the lipid film hydration method for the preparation of liposomes

liposomal product. The variables were the following: the concentration of the phospholipids (Phospholipon 90G®), which was the third data on the list of the CQAs and was chosen as variable X1. According to our selection methodology, the size of the liposome vesicles and size distribution, which factor was in the first position on the critical list of the CQAs, were selected as the response variable and as the second factor, while the amount of the API was kept constant. From the CPPs, the temperature of ultrasonication was chosen as variable X2, and because the type of the organic solvent was also constant, the following critical factor, the number of the second filtration was designated as variable X3. The pattern of sample preparation and the variables with the selected lower (-) and upper (+) limits are shown in Fig. 6.

3.4. Characterization results of the liposomal products

The samples, made by the above-mentioned method, were investigated with the two particle size determination analytical method. The results are shown in Table 4.

The integration of the lipophilic API into the liposomal wall decreases the size of the vesicles (presented by d-values in Table 4 and Fig. 7B). Lower diameter values were measured in the case of the API-containing liposomes than in the empty ones. Based on the variable vesicle size values, it can be concluded that the modification of the preparation temperature has no notable effect on particle size distribution. During the investigations, it was observed that the reduction of the phospholipid or the elevation of the cholesterol and the natural oil component ratio decreases the heterodispersity of the systems. The span value describes the width of the particle size ranges. It indicates how far the 10 percent and the 90 percent points are apart and normalised with the midpoint, so the smaller the span value the narrower the particle size distribution range. Using the 85% phospholipid-containing formulations instead of the 95% ones resulted in systems with better monodispersity values (lower span and PdI values). Uniformity gives information about the absolute deviation from the median size. The monodispersity value of the system increases by the elevation of the number of the filtrations. In these experiments, this connection was stronger among the empty liposomes. D[3,2] and D[4,2] values indicates the central point around which the (surface area or volume/mass) distribution would rotate. The SSA values have been measured also for the particles. Nanospheres have high SSA. SSA data also show that the incorporation of the lipophilic API into the liposomes decreased the size of the vesicles due to the higher SSA values (averages for the empty vesicles are respectively 38.5 and 36.5 m (Ich, 2009)/g, and for the API-containing ones are 45.15 and 42.36 m^2/g for samples produced at 55 °C and 65 °C). Data from dynamic light scattering measurements show the typical size of the vesicles in an intensity-based size distribution (d (Peak 1) nm) and the polydispersity index (PdI) which indicates slightly heterodispers (systems) (Table 4). Zeta potentials show that the originally negative charged empty liposomes turn into positive charged vesicles by the c (Table 5).

E = 'empty liposomes', A = 'API-containing liposomes', 85 = '85 w/w% phospholipid concentration', 95 = '95 w/w% phospholipid concentration', 1 = 'one-time filtration', 3 = 'three-time filtration' were made with a 0.22-μm membrane filter, 55 = '55 °C as the preparation

QTPP parameter	Impact	CQA	CMAs and CPPs	Occurrence
Therapeutic effect	**High**	Wall-forming agent 1 (Natural oil/ Cholesterol)	Organic solvent type	**Low**
Patient group	**Low**	Wall-forming agent 2 (Phospholipon 90G®)	Temperature of vacuum evaporation	**High**
Route of administration	**High**	Hydration media	Grade of vacuum creation	**High**
Dosage form	**Medium**	API	Hydration step: feeding rate	**High**
Volume of one dose	**Medium**	Surface charge	Temperature rising during the ultrasound	**High**
Dissolution profile/absorption time	**High**	Vesicle/particle size (and size distribution)	Filtration number in shape-forming phase	**Medium**
Stability (product stability)	**High**	Stability of the vesicle size		
pH of the product	**High**			

1. Risk Identification

2. Risk Analysis
- using the Risk Estimation Matrix (REM) and the Uncertainty Estimation

CQA	QTPP							
	Therapeutic effect (H)	Patient group (L)	Route of administration (nasal) (H)	Dosage form (M)	Volume of one dose (M)	Dissolution profile/ absorption time (H)	Stability (H)	pH (H)
Wall-forming agent 1 (Natural oil/ Cholesterol)	Low	Low	Medium	Low	Low	High	High	Low
Wall-forming agent 2 (Phospholipon 90G®)	Medium	Low	Medium	Low	Low	High	High	Low
Hydration media	Low	Low	Medium	Low	Low	Medium	Low	High
API	Low	Low	High	Low	Medium	Medium	Low	Medium
Surface charge	Low	Low	Medium	Low	Low	High	Medium	Low
Vesicle/Particle size (and size distribution)	Medium	Low	High	Low	Medium	High	High	Low
Stability of the vesicle size	Low	Low	Medium	Low	Low	High	High	Low

CQA	CMAs/CPPs					
	Organic solvent type (CMA) (23%)	Temperature of the vacuum evaporation (16%)	Grade of vacuum creation (7%)	Hydration step: feeding rate (12%)	Temperature rising during the ultrasound treatment (26%)	Filtration number in the shape-forming phase (16%)
Wall-forming agent 1 (Natural oil/ Cholesterol)	High	High	Low	Low	High	Medium
Wall-forming agent 2 (Phospholipon 90G®)	High	High	Low	Low	High	Medium
Hydration media	Low	Low	Low	Low	Medium	Low
API	High	Low	Low	Low	Low	Low
Surface charge	Low	Low	Low	Low	Low	Low
Vesicle/Particle size (and size distribution)	Low	Low	Medium	High	High	High
Stability of the vesicle size	Low	Low	Low	Low	Medium	Medium
UNCERTAINTY of CMAs/CPPs → →	Low	High	High	High	Medium	Medium

Fig. 3. The selected QTPPs, CQAs, CMAs, and CPPs, and the interdependence rating results of these critical parameters as part of the Risk Assessment (High = the interdependence and the impact of the interaction is high between the examined factors, Medium = the interdependence has a medium effect, Low = the interdependence is low between the factors)

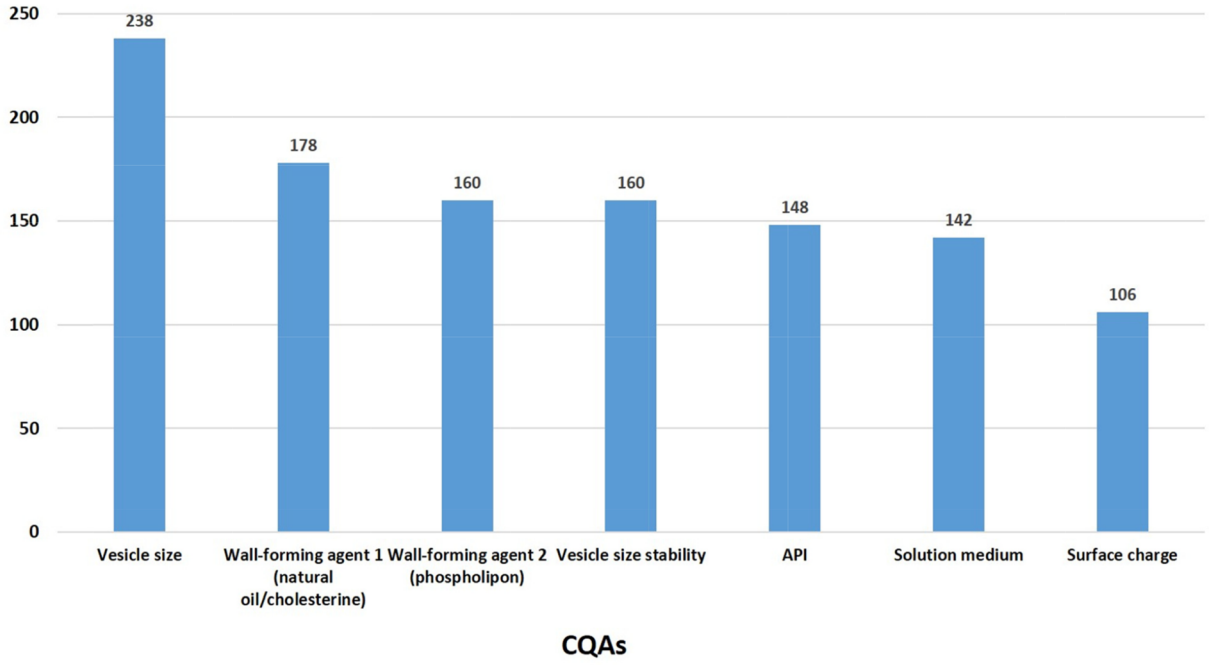

Fig. 4. Pareto chart about the ranking of the CQAs based on their potential critical effect on the aimed liposomal product

temperature', 65 = '65 °C as the preparation temperature', d (Peak 1) and PdI were measured by the dynamic light scattering technique, while the other parameters were measured by the laser diffraction method.

The produced liposomes and the measurement results were further evaluated to understand better the interrelations between the production process parameters and the product performance. A software-supported data evaluation was carried out, which resulted in a scatterplot matrix (Fig. 7A). Fig. 7-A specifically presents the alteration of the liposomal vesicle size values related to the phospholipid concentration, the number of the filtrations and the temperature of the evaporation.

The increase of the phospholipid concentration (which was named as wall-forming agent 2 –WF2 – during the design phase) resulted in

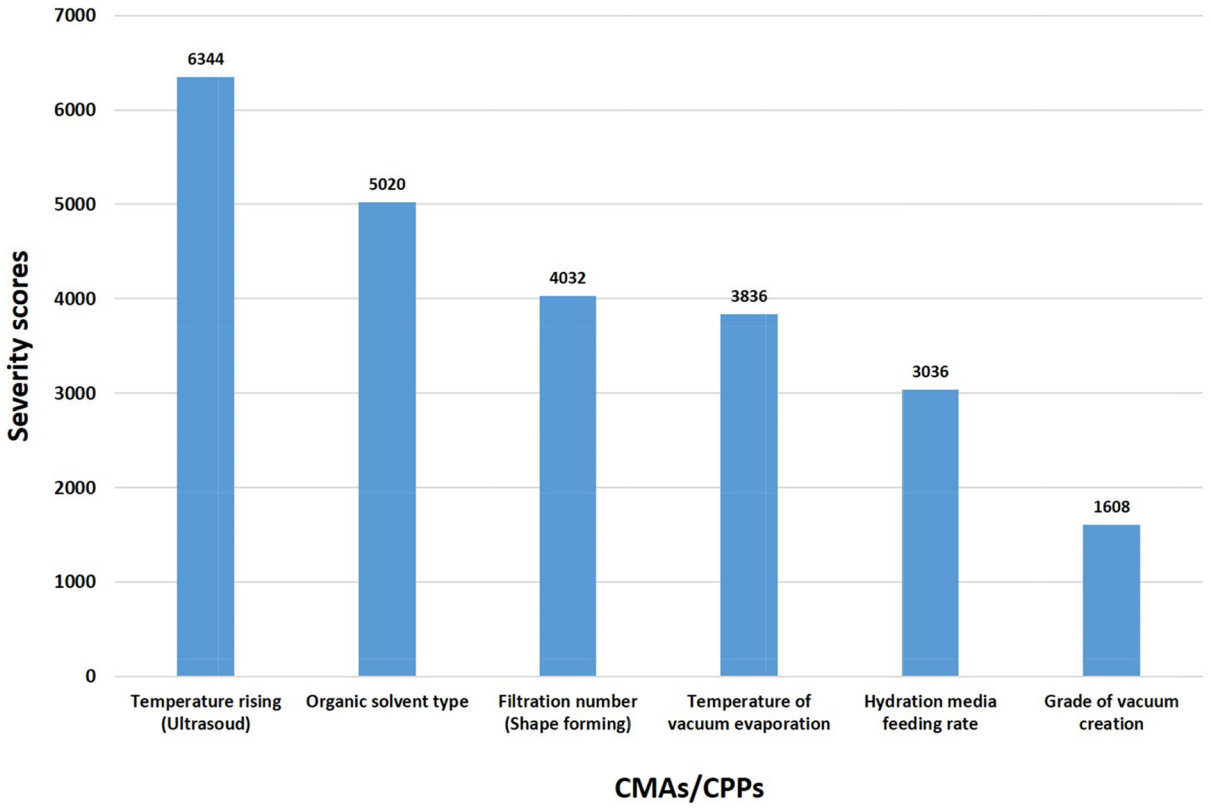

Fig. 5. Pareto chart about the ranking of the CPPs based on their potential critical effect on the aimed liposomal product

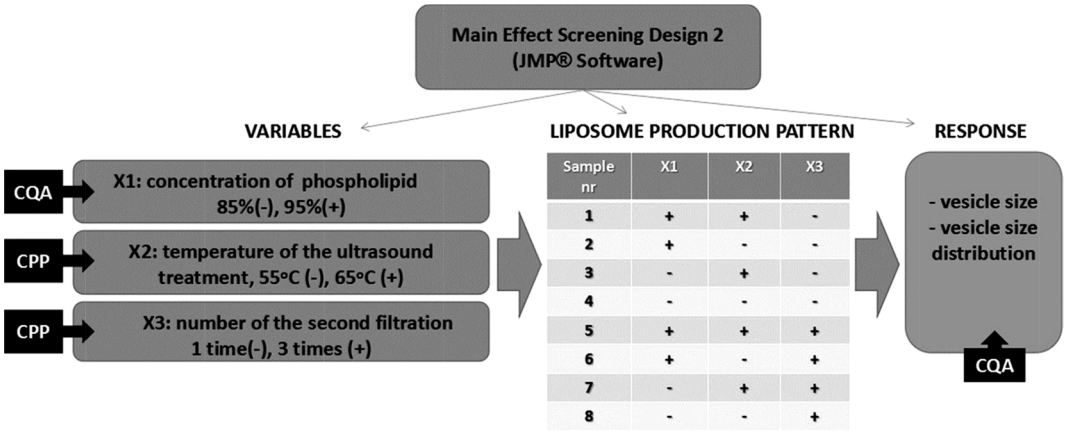

Fig. 6. Design of the experiments: factorial design of liposome preparation

Table 4
Data of the size determination of the vesicles by the laser diffraction and the dynamic light scattering methods.

Samples	d(0.1) (nm)	d(0.5) (nm)	d(0.9) (nm)	Span	Uniformity	Specific surface area (m^2/g)	Surface weighted mean D [3,2] (nm)	Volume weighted mean D [4,2] (nm)	d (Peak 1) (nm)	PdI
E-95-1-55	77	***184***	587	2.776	0.828	40.3	149	266	314.0	0.435
E-95-3-55	76	***143***	242	2.516	0.760	41.9	143	242	203.4	0.416
A-95-1-55	72	***157***	455	2.437	0.747	45.5	132	219	172.1	0.441
A-95-3-55	74	***139***	517	2.665	2.580	43.2	139	535	165.9	0.698
E-85-1-55	84	***217***	648	2.602	0.793	35.7	168	301	188.4	0.815
E-85-3-55	84	***214***	607	2.455	0.746	36.1	166	287	176.1	0.614
A-85-1-55	72	***156***	214	2.322	0.725	45.9	131	214	190.3	0.389
A-85-3-55	72	***156***	422	2.255	0.704	46.0	130	210	176.3	0.429
E-95-1-65	80	***200***	625	2.728	0.824	37.9	158	285	183.6	0.702
E-95-3-65	79	***193***	563	2.510	0.761	38.9	154	265	221.1	0.438
A-95-1-65	79	***154***	279	2.801	0.840	38.9	154	279	174.7	0.491
A-95-3-65	78	***188***	563	2.584	0.779	39.7	151	262	175.4	0.673
E-85-1-65	88	***180***	326	2.570	0.791	33.4	180	326	242.0	0.879
E-85-3-65	85	***215***	600	2.399	0.735	35.8	168	287	188.3	0.471
A-85-1-65	72	***131***	212	2.291	0.712	45.9	131	212	157.2	0.371
A-85-3-65	73	***160***	441	2.300	0.707	45.0	133	216	210.6	0.525

decreased size and increased SSA values. On the other hand, the higher number of the filtrations led to liposomes with a lower size but caused a moderate increase in the SSA (the slope of the line is lower). Lastly, part A of Fig. 7 (Fig. 7A) shows that the increase of the evaporation temperature during the production process has only a little impact on vesicle size and the SSA. Section B in the same figure (Fig. 7B) presents the effect of temperature and the presence of the API on the vesicle size (d (0.5) nm) of the liposomes. The graphics demonstrate that the presence of the API, built in the structure of the liposomes, resulted in smaller vesicles, so the lipophilic API-containing vesicles are smaller than the API-free ones. The same result was observable with both production temperature values (55 and 65°C).

Part C of Fig. 7 (Fig. 7C) presents the same results as Table 4, namely, the usage of a higher amount of WF2 slightly decreased the vesicle size of the liposomes and increased the heterodispersity of the system. The monodispersity values of the produced liposomal systems increased by the elevation of the number of the filtrations and with the usage of less WF2 in the compositions.

3.5. Results of the SEM investigation

After the lyophilisation step of liposome preparation, a SEM investigation was performed. The images taken about the freeze-dried samples proved the results obtained from the particle size characterization. The average size of the liposomes was within the range of 200 nm. Furthermore, the spherical shape of the separated liposomes is visible in Fig. 8.

3.6. Results of the drug entrapment efficiency

The absorbance value based on the spectrophotometric investigation of the selected liposomal product (A-85-3-65, Table 4) was 0.34 ± 0.01. According to this, the calculated drug entrapment of the sample was 98.27 ± 0.26%.

4. Conclusion

In this study, the QbD-based and mostly RA-focused approach was successfully adapted in the early development phase of a lipophilic API-containing liposomal formulation. An RA-based theoretical design and development were created before the practical phase of the liposomal formulation started in practice. The liposomes were designed for nasal application, and the expectation related to the model API, which was a lipophilic BCS Class II drug, was to be able to reach theoretically the brain tissue (in the further investigations) via the nasal administration route. To achieve these long-range goals this first step formulation design was performed, the elements of the QTPP were defined, and the lipid film hydration method was selected to prepare the aimed liposomes. The CPPs/CMAs related to the process, as well as the CQAs, were determined and the RA was fulfilled. Based on the initial RA results, in practice, the preparation of the liposomes was carried out following a factorial design plan. The factors of the design plan were

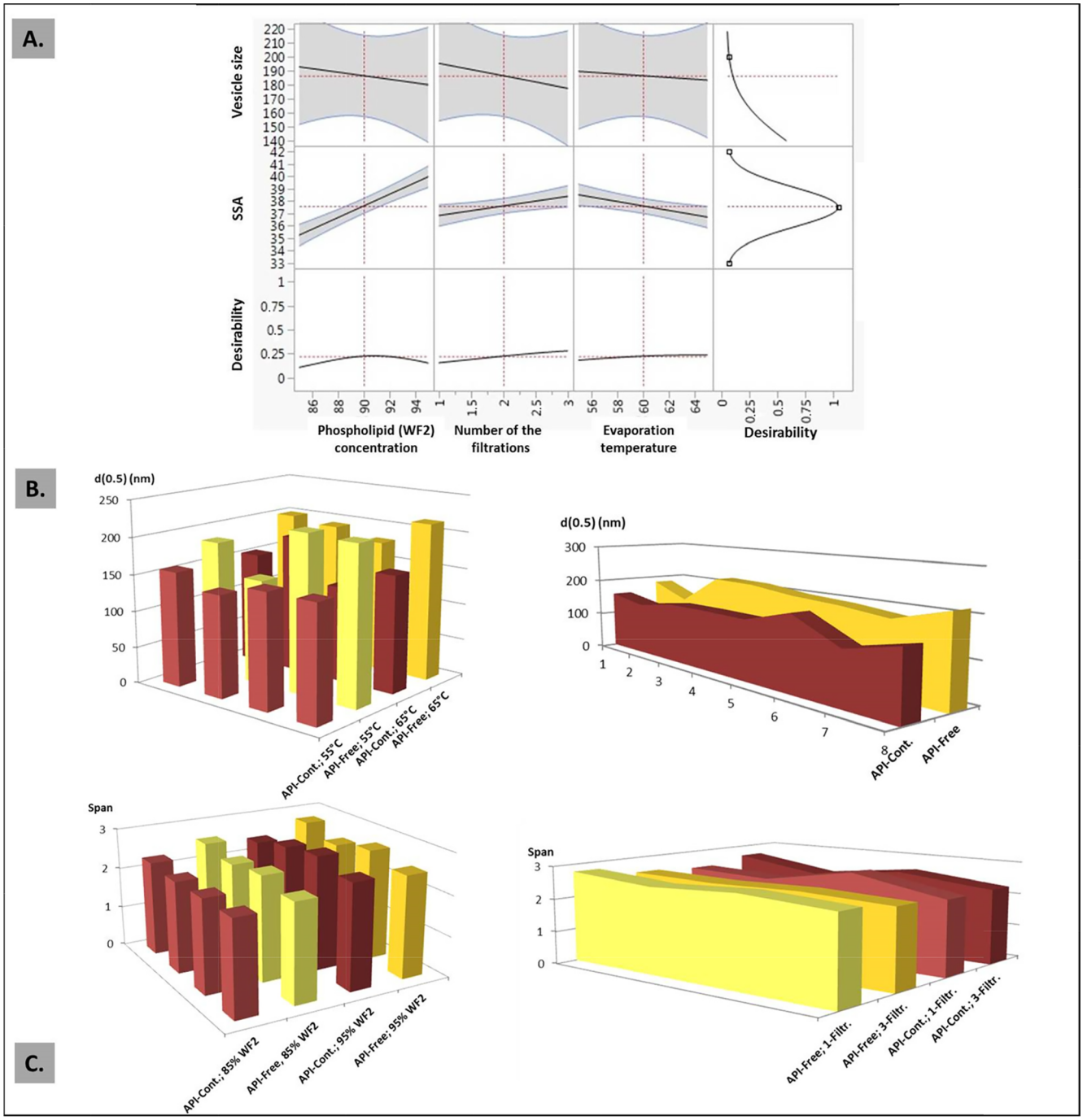

Fig. 7. Scatterplot matrix of the investigation results of the prepared liposomes (vesicle size related to the phospholipid concentration, number of the filtrations and the evaporation temperature) (A), the effect of the temperature and the API on the vesicle size of the liposomes (B), and the impact of the Phospholipon 90G® (WF2) concentration and the number of the filtrations on the heterodispersity of the liposomes(C)

Table 5
Size detection and zeta potential data of the selected vesicles.

Samples	Span	d(0.5) (nm)	Zeta potential
E-95-1-65	2.728	**200 ± 2**	−2.14 ± 0.39
E-95-3-65	2.510	**193 ± 2**	−2.52 ± 0.27
A-95-1-65	2.801	**154 ± 3**	2.81 ± 0.31
A-95-3-65	2.584	**188 ± 3**	2.63 ± 0.11
E-85-1-65	2.570	**180 ± 12**	−3.82 ± 0.35
E-85-3-65	2.399	**215 ± 16**	−3.90 ± 0.39
A-85-1-65	2.291	**131 ± 13**	2.13 ± 0.7
A-85-3-65	2.300	**160 ± 18**	1.44 ± 0.11

derived from the most critical elements of the CQAs and CPPs and formed the pattern of the experimental design, thus liposome preparation was focused only on the most highly critical parameters. The following of the theoretical screening and selection method of the critical factors led to a lower number of investigations, but an even higher rate of successful sample preparation was achieved. The investigational results of the prepared liposomes (API-free and API-containing samples were prepared following the same factorial design pattern), namely vesicle size, size distribution, specific surface area, and surface characteristics, verified the exactness of the RA and the critical factor-based theoretical prediction, and showed clear relations between the product-design (composition of the liposomes, temperature, and process

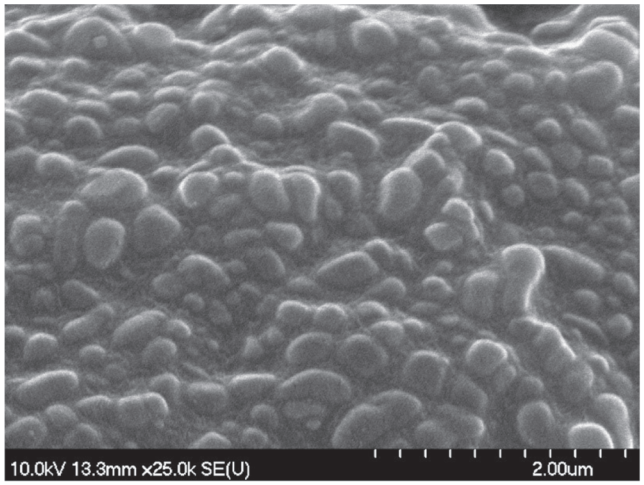

Fig. 8. SEM picture about the produced and freeze dried (lyophilised) liposomal product sample

parameters, such as temperature, or the number of filtrations, etc.) and the product characteristics of the prepared liposomes. The results proved that the QbD approach can improve the formulation process in the development of liposomes, lead to an effective product preparation process, and help in the optimization and the rationalization of liposomal developments even in those special cases when a lipophilic active ingredient is incorporated into the liposomes.

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A Proposed Methodology for a Risk Assessment-Based Liposome Development Process

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Abstract: The requirements of a liposomal formulation vary depending on the pharmaceutical indication, the target patient population, and the corresponding route of administration. Different preparation methods require various material attributes (MAs) (properties and characteristics of the components) and process parameters (PPs) (settings of the preparation method). The identification of the quality target product profile for a liposome-based formulation, the critical quality attributes of the liposomes, and the possible MAs and PPs that may influence the key characteristics of the vesicles facilitates pharmaceutical research. Researchers can systematise their knowledge by using the quality by design (QbD) approach. The potential factors that influence the quality of the product can be collected and studied through a risk assessment process. In this paper, the requirements of a liposome formulation prepared via the thin-film hydration preparation technique are presented; furthermore, the possible factors that have an impact on the quality of the final product and have to be considered and specified during the development of a liposomal formulation are herein identified and collected. The understanding and the application of these elements of QbD in the pharmaceutical developments help to influence the quality, the achievements, and the success of the formulated product.

Keywords: quality by design; quality planning; risk assessment; critical factors; liposome formulation; thin-film hydration method

1. Introduction

Liposomes are described as artificially prepared vesicles composed of one or more concentric lipid bilayers that are enclosing one or more aqueous compartments by the European Medicine Agency [1]. Liposomes as drug carrier systems have several advantages [2]. These formulations can be used, among others, to protect active pharmaceutical agents (API), incorporate both lipophilic and hydrophilic drug molecules, and maintain targeted drug delivery [3]. From the beginning until the present day, four different generations of liposomes have been distinguished. The first-generation liposomes (conventional liposomes) are made up of neutral and/or negatively charged phospholipids and cholesterol [4]. These vesicles are taken up by the reticuloendothelial system (RES) (phagocytes) in cases of intravenous administration; thus, their circulation time is short [5]. The second generation consists of long-circulating liposomes, while the third generation is made from surface-modified liposomes that can avoid the defence mechanism of the immune system. The fourth generation is built up from polyethylene glycol (PEG)ylated or the so-called "stealth" liposomes [3,4]. The surface of these vesicles is coated with a hydrophilic polymer, such as polyethylene glycol (PEG), that increases the repulsive forces between the liposomes and thus avoids the protein adsorption and opsonisation

of the liposomes by the RES [5,6]. In this way, longer residence time is provided for the liposomes to remain in the tumour tissues [6]. Beyond the generational grouping of the liposomes, they can be classified regarding their compositions and drug delivery mechanisms such as conventional liposomes, long-circulating liposomes, polymorphic or bioresponsive liposomes [7–9] (pH-sensitive, thermos-sensitive, cationic liposomes), and decorated liposomes (surface-modified vesicles and immunoliposomes) [10,11]. Liposomes are used for the application of highly potent medications. Their pharmaceutical application is essential in the field of cancer therapy, besides that of the already marketed liposomal drugs in this field, and several new studies are in progress in the above-mentioned and newly targeted medical areas as well [12–14]. Nano-system development, including nanoscale liposome research, is receiving increasing attention nowadays. Nano-sized liposomal formulations can play a highly focused role in the therapy development of unmet clinical needs and diagnostic imaging techniques in the future. However, the regulatory authorities need to meet several challenges in terms the quality, safety, and efficacy aspects of the liposome-based products [15,16]. There is still no well-defined regulatory authorisation process for liposomes; however, several international groups are working on this. The International Organisation for Standardisation (ISO) defined the nanoscale size as the range extending between 1 and 100 nm [17]. On the basis of their definitions, nanoparticles are those nano-objects that have all of their external diameters in the nanoscale, and there is no significant difference between the lengths of the longest and shortest axes of the particle [17]. Therefore, the size of the liposomes and their homogeneity (size distribution) are fundamental features of the systems. The polydispersity index (PdI), a dimensionless value theoretically between 0.0 and 1.0, provides information about the uniformity of the particles. PdI values less or equal to 0.3 are supposed to be the indicator of distribution with acceptably low polydispersity. In the case of lipid-based nanocarriers, formulations with a PdI of 0.3 and below are acceptable and are an indicator of a homogenous population of the vesicles [18]. The zeta potential value is used to define the repulsion or the attraction between the vesicles, and in this way, to predict the stability of the liposome system [19]. Liposomes with an average surface charge higher or equal to 10 mV in absolute value are considered as negative or positive vesicles, while between these values are considered neutral liposomes [20]. Nanoparticles with zeta potentials higher than +30 mV or lower than -30 mV are considered as a stable system [21]. The lamellar structure of the liposomes can also have an impact on their therapeutic application (e.g., incorporated API selection, dosage form selection, administration route definition).

The quality by design (QbD) approach is a quality management concept in the pharmaceutical industry that focuses on the prior definition and design of the target product considering all of the needs and requirements emerging from the clinical side (patient), the industrial processes, and the regulatory aspects [22,23]. QbD is a systemised, structured, knowledge- and risk assessment-focused approach, and the potentials of its extension have previously been shown by Csóka et al. [24]. The QbD approach is efficiently applicable during nano-pharmaceutical research as well [25–30]. The development process of the liposomes is challenging due to their complex manufacturing processes. The tools of the QbD approach can guide the formulation process to obtain higher quality liposomal products [31].

The whole QbD method is specified in the guidelines of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) [32–34] Briefly, the QbD method includes the following general steps:

(1) Quality target product profile (QTPP) definition: the QTPP is a prospective summary of the quality characteristics of the drug product that ideally will be achieved to ensure the desired quality, taking into account the safety and the efficacy of the drug product, considering, e.g., the route of administration, the dosage form, bioavailability, strength, and stability [33].

(2) Identification of the critical elements, such as the critical quality attributes (CQAs) of the targeted product, critical material attributes (CMAs), and critical process parameters (CPPs), which are related to the selected production method. According to the definition of the ICH guideline, a CQA is a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the targeted product quality. CQAs are generally

associated with the drug substance, the excipients, the intermediates (in-process materials), and the drug product [33]. A CPP is a process parameter that variability has an impact on the CQAs and therefore should be monitored or controlled to ensure that the process produces the targeted quality [33].

(3) Risk assessment (RA): RA is a valuable science-based process that is used to identify and rank the parameters on the basis of their impact on the CQAs of the product. Risk assessment is typically performed as the first step during an early phase of the pharmaceutical development processes and is evaluated again when more information becomes available and higher knowledge is obtained [32,33]. The current experimental knowledge obtained from the former practical studies have to be aligned with information from the relevant literature. To perform a successful RA, first, the research team has to define the precise target product (QTPP) and then has to select the critical factors and estimate the interdependence of the critical factors, ranking them by the severity of their impact. The team members estimate the level of the interactions between the parameters occurring during the formulation process (production settings, materials, etc.). All the elements applied in the RA (QTPP elements, CQAs, CMAs, and CPPs) are defined and selected by the research group; therefore, their knowledge strongly impacts this selection process. Risk is defined as the combination of the probability of the occurrence of harm and the severity of that damage. The RA is a systematic process to evaluate the necessary information for the support of the risk-defining step within the risk management process. It means the identification of hazards and the analysis of risks [31]. The quality risk management tools provide systemic and reproducible methods based on up-to-date knowledge to rate the probability, severity, and sometimes detectability of the risk. These methods can be qualitative or quantitative. Once the risk is expressed quantitatively, a numerical scale is assigned for evaluation [33]. The numeric score of the evaluated risks could arise from the multiplications of the severity and occurrence (or probability) values, or sometimes from the severity, occurrence, and detectability if the same scale was used for the estimation of all of these parameters. The RA software can help in this process, but even during the software-supported assessments, the identification of the risks and the estimations of the severity and the occurrence are the task and responsibility of the research group. The software only makes the calculations and provides the data assessment and visualisation of the final results. These results are the basis of the design of experiments (DoE).

(4) Design space (DS) development: DS is a multidimensional combination and interaction of the input variables (e.g., material attributes) and the process parameters that have been demonstrated to assure quality.

(5) Definition of the control strategy.

(6) Life cycle management.

For better understanding, the schematic structure of the QbD approach is presented in Figure 1.

This paper aimed to collect and evaluate the parameters that influence the manufacturing process of a liposomal pharmaceutical product in order to help the researchers and the professionals in the pharmaceutical industry in the QbD-based new liposome design and development. The authors aim to present a wide range of potential QTPP and CQA elements and their characteristics to highlight the potential decision and target points. It was also intended to give an example of how to use RA to rank the influencing parameters. For this illustration, the thin-film hydration method [35], the most common liposome production process (Table 1), was chosen, as the authors have practical experience and knowledge about this technique from their previous studies [27]. This method was described for the first time and used to prepare the first liposomes by Alec Douglas Bangham and his colleagues in 1965 [35]. Several modified versions of the original technique exist (Table 1), however, the basic steps of the process are mutual [36]: (1) preparation of the lipid film from phospholipids and cholesterol, (2) hydration of the thin film with a hydration medium, and (3) modification of the numbers of layers and the size of vesicles.

Figure 1. Schematic structure of the quality by design (QbD) approach.

Table 1. Potential methods to prepare liposomes.

Preparation Methods	Subtypes	Comments
Mechanical dispersion methods	probe or bath sonication	– the critical parameters vary on the basis of the selected preparation method; therefore, the definition of the production technique has to be the first step of every liposome formulation process – the properties of the liposomes (e.g., number of lamellas, size, and distribution of vesicles)
	French pressure cells—extrusion	
	freeze-thawed liposomes	
	membrane extrusion	
	lipid film hydration techniques	
	hydration of proliposomes	
	micro emulsification, coalescence of small vesicles	
	dual asymmetric centrifugation	
	heating method, Mozafari method	
	electro-formation	
Solvent dispersion methods	ether injection	
	ethanol injection	
	reverse-phase evaporation	
	solvent spherule method	
Detergent removal methods	dialysis	
	detergent removal of mixed micelles	
	gel-permeation chromatography	
Novel methods	microfluidisation	
	supercritical-assisted method	
	freeze-drying of double emulsions	
	membrane contractor method	
	curvature-tuning	
	biometric reaction for vesicular self-assembly	

2. Methods

The LeanQbD software (QbD Works LLC, Fremont, CA, USA) was used for the RA procedure. The first element of this procedure was the interdependence rating between the QTPPs and the CQAs, and the CQAs and the CPPs. A three-level scale was used to describe the relation between the

parameters: "high" (H), "medium" (M), or "low" (L). In the software, the qualitative three-level scale, used for the estimation, is linked to a selectable numeric scale (0–10, or 0–100), which gives, at the end, the severity scores of the evaluated risk factors on the basis of mathematical calculations. In this study, the 0–10 scale was used. After the categorisation of the interdependence, a risk occurrence rating of the CPPs (or probability rating step) was made, applying the same three-grade scale (H/M/L) for the analysis. As the output of the initial RA evaluation, Pareto diagrams [37] were generated by the software, presenting the numeric data and the ranking of the CQAs and the CPPs according to their potential impact on the aimed final product (QTPP). The Pareto charts not only show the differences of the CMAs and the CPPs by their effect but also help to select the factors of a potential experimental design.

3. Results

Table 2 summarises the potential QTPP elements collected by the authors. Potential CQAs are collected and presented in Table 3.

Table 2. Collection of the possible factors of the quality target product profile (QTPP) for a liposome-based formulation.

QTPP Factors	Details	Comments
Indication/therapeutic effect	based on the API	its characteristics may necessitate the use of liposomes
	not important for empty liposomes	empty liposomes are used, e.g., in cosmetology
Target patient population	based on the indication	applicable for each age group in the suitable dosage form
Route of administration	the composition may differ on the basis of the target	can be determined by the API and the target patient population
Site of activity/target	based on the indication	targeted delivery
	based on the API	
Dosage strength	based on the API	differs even in the same pharmaceutical subgroup
	based on the target patient population	needed dose changes with age and health condition
	based on the indication	appear in the case of preparation with a wide range of indications
	based on the administration route	e.g., in the case of nasal application, the needed dose is less than per os
Dosage form/appearance	liposomes in aqueous solution	transparent, light scattering liquid (vesicles in colloid size)
	lyophilised powder	solid powder; colour based on the API and the excipients
Viscosity	based on the administration route	sign of stability; maintains efficient drug release; higher viscosity indicates a smaller size, a narrow PdI, slower drug release, and lower clearance rate
Osmolarity	based on the administration route	be tolerable, ideally 300 ± 30 mOsm/kg
Physical attributes of the liposomes	morphology, particle size, and zeta potential	change with the adjustment of the composition
Pharmacokinetics	liberation, adsorption, distribution, metabolism, elimination	necessary mostly for API-loaded liposomes
Safety	complement activation-related pseudoallergy (CARPA)	all types of intravenous liposomes can cause CARPA; enhanced by increasing size in the 70–300 nm range; more than 71 mol% cholesterol; PEG-PE insertion
	chemical/biological decomposition	needs to be investigated
	degradation products	concentration must be under the legal limit
Sterility	based on the administration route	sterile and pyrogen-free or aseptic preparation is not needed
Stability	in aqueous solution	needs to be stable; duration of stability is decisive
	in freeze-dried powder form	
Solubility/dissolution	in aqueous solution	media: non-toxic, non-irritable
	in freeze-dried powder	immediate release
Homogeneity	homogenous formulation	sign of stability
Drug release	based on the treatment	site and timing can be modified

Table 3. Collection of the possible factors of critical quality attributes (CQAs) of liposomes.

CQAs	Details	Comments
Type of liposomes	conventional liposomes	neutral or negative phospholipids
	immune liposomes	antibodies, antibody fragments
	cationic liposomes	positive phospholipids
	magnetic liposomes	metal particles
	bioresponsive liposomes	thermosensitive (37 °C < Tm)
		pH-sensitive (acidic milieu)
		LiPlasome (secretory phospholipase A2)
Number of lamellas	more layers	multilamellar (>0.5 μm)
		oligolamellar (0.1–1.0 μm)
	one layer	unilamellar
Size of vesicle	small unilamellar vesicle (SUV)	20–100 nm
	medium-sized unilamellar vesicle (MUV)	between SUV and LUV, >100 nm
	large unilamellar vesicle (LUV)	>100 nm
	giant unilamellar vesicle (GUV)	>1 μm
Surface modifications	no modification	rapid elimination
	polyethylene glycol (PEG) chains (stealth liposomes) (quality and quantity of the chains)	steric exclusion (decreased opsonisation and phagocytosis); prolonged circulation
	monoclonal antibodies, antibody fragments, peptides, nucleic acids, carbohydrates, small molecules	provide targeted delivery by biding to the targeted receptors
Morphology of liposomes	spherical vesicles	self-organised structure
	concentric layers	multi-layered vesicles
	spherical with multiple non-concentric lipid vesicles inside	multivesicular liposome (MVL)
Particle size and size distribution	d(0.1), d(0.5), d(0.9), span, surface weighted mean (D[3,2]), volume weighted mean (D[4,2])	mean particle size should be under 200 nm; ideal around 100 nm
Polydispersity index (PdI)	indicating polydispersity of the system	below 0.5 is acceptable
Specific surface area (SSA)	influences drug release	smaller vesicles maintain higher surface area-to-volume ratio than the larger particles
Zeta potential	indicating stability	stable formulation around ±30 mV
Phase transition temperature (T_m)	influences drug release	determined by the composition of the liposome; cholesterols reduce the value
Empty liposomes/API content	modifies the physical attributes of the liposomes	the characteristics of the API determine its position
Position of the API	hydrophilic API	in the hydrophilic aqueous centre
	lipophilic API	in the lipophilic double membrane
	surface-bounded	monoclonal antibodies, antibody fragments, peptides, nucleic acids, carbohydrates, small molecules
Encapsulation efficiency (EE)	higher EE% is the goal to increase the drug concentration in the final formulation	manufacturing costs can be reduced, and more flexible dosing can be provided by higher EE%
Permeability targeted drug delivery	semi-permeable membrane target specificity	the highest permeability is at T_m; increases effectiveness
Drug release profile	maintains therapeutic activity	site and timing can be modified
Sterility	if necessary	even for the materials in the case of aseptic preparation
Stability	chemical, biological, microbiological	characteristic values must remain in the recommended ranges until use

As the preparation method (Table 1) defines the CPPs of the liposome formulation process, a production technique that provides the target CQAs need to be selected prior to the investigation of CMAs and CPPs. The API can be added to the formulation via passive or active loading techniques [3]. Mechanical dispersion [3,19,38,39], solvent dispersion [3,38,39], and detergent removal [3,38,39] methods belong to the passive loading techniques, in which methods the lipid films are prepared via

different techniques, hydrated to obtain liposomes, and the drug is captured during the manufacturing process [3,39]. In case of active loading, the API is incorporated into the already prepared liposomes via gradient loading techniques using buffers or ammonium sulphate gradients [39]. Besides the conventional preparation methods, there are also numerous approaches that have been recently developed to produce liposomes [39,40]. In this paper, the thin-film hydration method-related factors are presented. The potential CMAs and CPPs of the technique are systemised in a flow chart in Figure 2. The steps of the thin-film hydration method [36] are shown in the middle of the figure, while the related material attributes (MAs) and process parameters (PPs) are presented on the two sides of the chart.

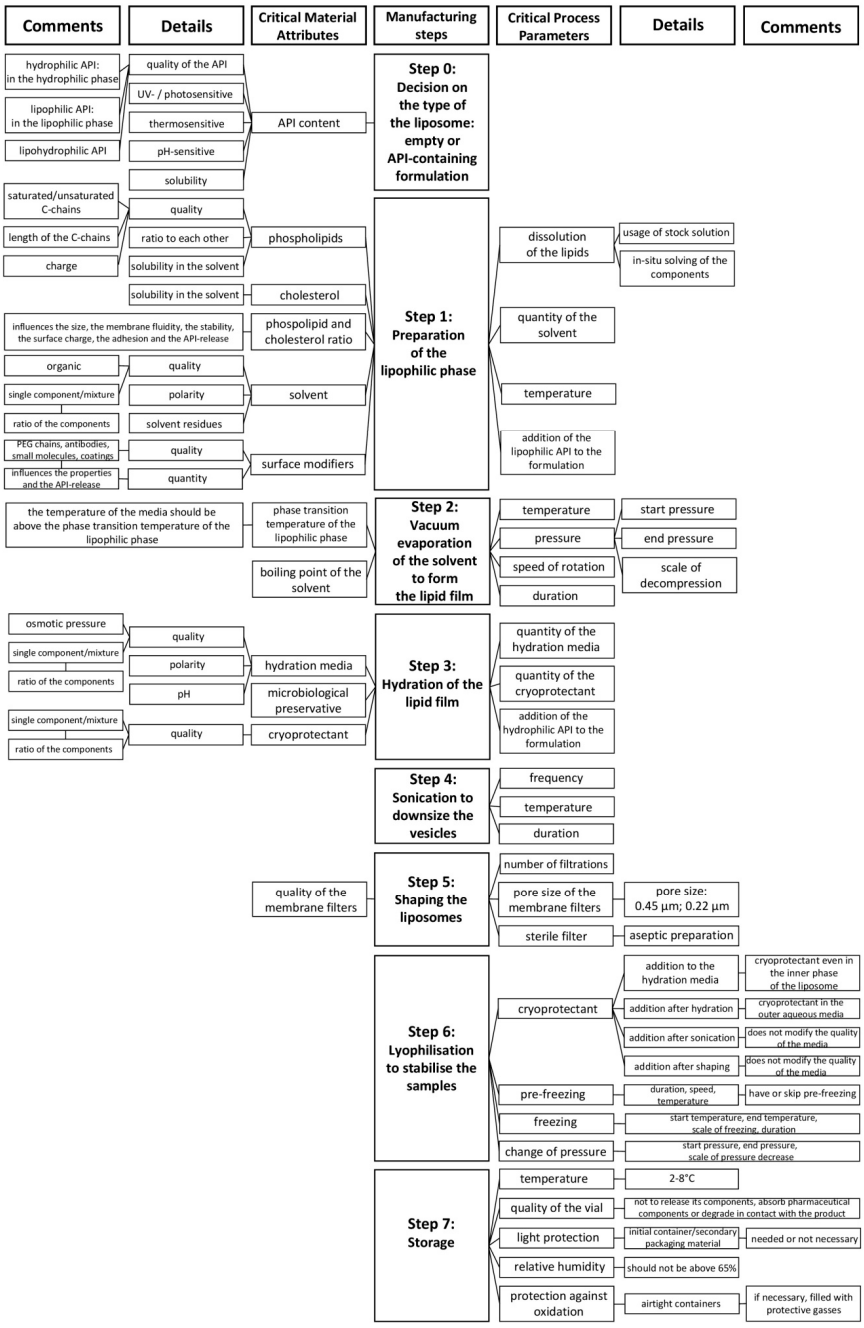

Figure 2. Collection of the properties of the liposome components (material attributes (MAs)) and the preparation method (process parameters (PPs)) that affect the result of the thin-film hydration liposome manufacturing technique.

The general criticality of the presented factors was investigated in a RA, and the rankings of the elements of CQAs, illustrated with Pareto charts for better understanding, are shown in Figure 3, while CMAs and CPPs are shown in Figure 4.

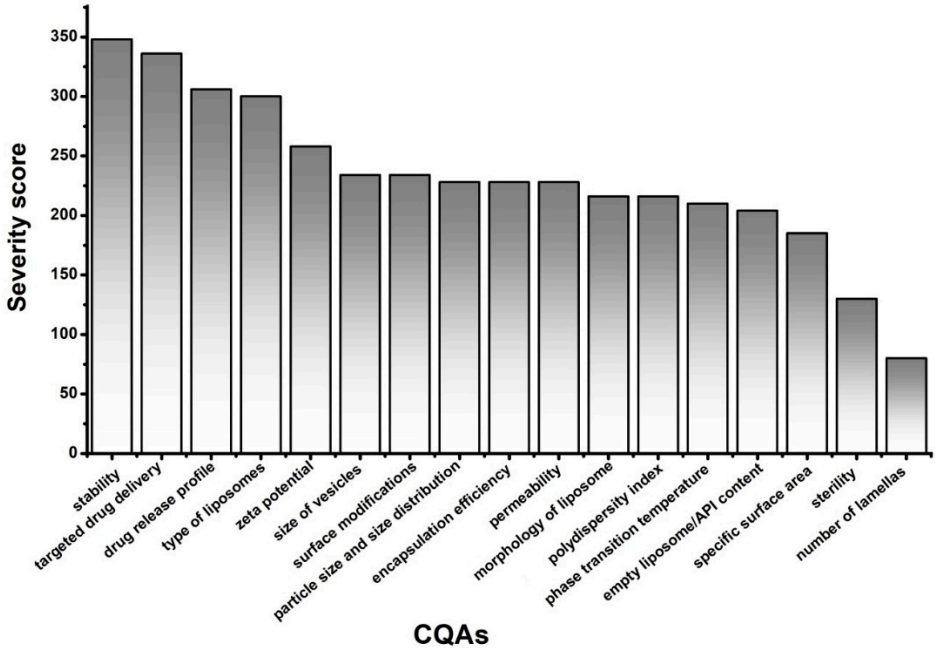

Figure 3. Rankings of the critical quality attributes (CQAs) of the liposomes.

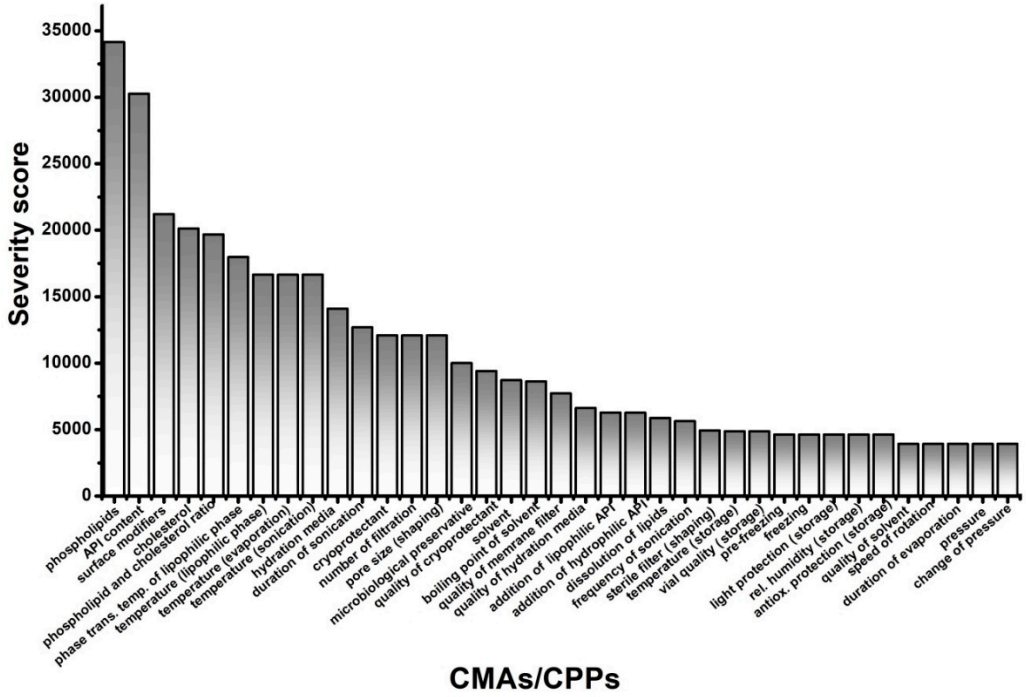

Figure 4. Rankings of the critical material attributes (CMAs) of the liposome components along with the critical process parameters (CPPs) of thin-film hydration.

4. Discussion

The QTPP (Table 2) depends mainly on the therapeutic/clinical aims and requirements, as well as the characteristics of the drug substance, and it is always unique. For instance, QTPP may be a nano-sized liposome-containing injection for cancer therapy with a proper dose of drug and drug

release dedicated to the therapeutic needs. Those quality attributes that are critically related to the QTPP are the CQAs. That is the reason why the CQAs are also always unique and depend on the QTPP. The potential CQAs (Table 3) are, e.g., the type of the liposome, its lamellar structure, vesicle size, size distribution, sterility, viscosity, and stability, or the dissolution profile of the formulation. The API encapsulation efficiency is also a critical attribute for the liposomes, in addition to the zeta potential, which refers to the stability of the vesicles. PdI is one further potential CQA for lipid-based nanocarrier systems such as liposomes.

The application of a quality management visualisation tool, such as a fishbone diagram, process mapping, or a flow chart, is always useful for the identification of the CMAs and the CPPs of the aimed liposomal product. In this case, to show the systemic collection and presentation of the potential CMAs and CPPs, we built a flow chart (Figure 2). In the middle of the figure, the steps of the production process, which in this case was the thin-film hydration liposome preparation method, are presented. The left side of the flow chart contains the material attributes (MAs), and the right side shows the process parameters (PPs). These MAs and PPs can affect the result of the thin-film hydration-based liposome manufacturing process. The critical ones have to be selected and labelled as CMAs and CPPs. To make this figure and the tables of QTPP and CQAs, prior knowledge, previous experimental experience, and a thorough literature background survey of the field [31,41–48] were necessary. Although, the main points of the tables and figures are shreds of evidence from the literature mixed with practical experiences, the systemic collection of all the relevant factors and data in one paper is the novelty of the work. The demonstration of the CMAs and the CPPs parallelly enhances the transparency of their relationships. In the following step, RA can be performed among the elements of the QTPP, the CQAs, and the CMAs and the CPPs. Several tools are suitable for an RA, e.g., the support of an RA software can help to achieve proper and quick implementation. In the presented case, the LeanQbD (QbD Works LLC, Fremont, CA, USA) RA software was applied. The interdependence rating among the elements was made on a three-grade scale, as the interaction is low (L), medium (M), or high (H). This process was made step by step for each pair of factors on the basis of the prior experimental and literary knowledge. The results of the RA are presented in Pareto charts generated by the software (Figures 3 and 4). Figure 3 shows the theoretical ranking of the CQAs of the liposomes according to the initial general RA made by the authors. It may also vary in other cases on the basis of the QTPP. Figure 4 presents the general ranking of the CMAs and the CPPs depending on their severity for the liposomal product. It may vary on the basis of the QTPP and the CQAs. According to the RA, the most influential CMAs, organised in descending order, are the phospholipids, the API content [27], the surface modifiers, the cholesterol content, the ratio between the phospholipids and the cholesterol, the phase transition temperature of the lipids, and the quality of the hydration media and the cryoprotectant, while the CPPs are the working temperature, the duration of the sonication, and the number of filtrations. The effect of the CMAs/CPPs can be accurately investigated if some of the values are set on the same level, while the ones under the scope of the study are changed according to the DoE.

Xu et al. performed a risk analysis study on liposomes prepared using the thin-film hydration technique and loaded with superoxide dismutase via a freeze–thaw cycling technique. They analysed those factors that affect the size, the encapsulation efficiency, and the stability of the liposomes. For this evaluation, they checked the properties of the formulation, the process, the analytical method, and the instrumentation reliability. They found that the "analytical method" and the "instrument reliability" categories can be well-controlled; therefore, the factors of these two categories are not critical. However, the factors of the "analytical method" and the "instrument reliability" are non-negligible for the selection and settings of the characterisation methods. Their findings, namely, the influencing role of the lipid concentration, the cholesterol ratio, and the quality of the phospholipids are consistent with our results [49]. Porfire et al. provided a general overview of the QbD approach for liposomes without defining a production process and described methodologies for liposome characterisation as a control strategy in detail. Their reasonable considerations were built into the tables of this paper with

our additions. The facts above draw attention to the low number of studies following the steps of the QbD recommended by the regulatory authorities [31]. Our presented work fits well into this scientific research area; it extends the previous knowledge and gives a detailed overview of the QbD application. The systemised and structured form of the facts and information may help researchers in designing and planning their future studies of liposomes.

5. Conclusions

This work aimed to collect and systemise all the relevant factors of the liposome formulation development via the QbD technique. The application of the QbD approach is a regulatory requirement in the pharmaceutical submissions, and in these applications, RA is the key step. In this study, the theoretical method was presented, the potential QTPP elements of the liposome-based formulations were determined, and the potential CQAs of the liposomes were also collected. The potential critical material attributes and process parameters that need to be considered during the formulation design of the thin-film hydration liposome preparation method were listed and evaluated. The method of screening was also presented to identify the most critical factors. The phospholipids, the API content, the surface modifiers, the cholesterol content, the ratio between the phospholipids and the cholesterol, the phase transition temperature of the lipophilic phase, and the quality of the hydration media and the cryoprotectant were found to be the CMAs of highest influence. Furthermore, the working temperature, the duration of the sonication, and the number of filtrations were identified to be essential CPPs. The authors believe that the presented concept may help researchers to establish and perform studies on liposomes with less effort and more success.

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Abbreviations

API	active pharmaceutical agents
CARPA	complement activation-related pseudoallergy
CMAs	critical material attributes
CPPs	critical process parameters
CQAs	critical quality attributes
D[3,2]	surface-weighted mean
D[4,2]	volume-weighted mean
DoE	design of experiments
DS	design space
EE	encapsulation efficiency
GUV	giant unilamellar vesicle
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IV	intravenous
ISO	International Organisation for Standardisation
LUV	large unilamellar vesicle

MUV	medium-sized unilamellar vesicle
MVL	multivesicular liposomes
PdI	polydispersity index
PE	phosphatidylethanolamine
PEG	polyethylene glycol
QbD	quality by design
QTPP	quality target product profile
RA	risk assessment
RES	reticuloendothelial system
SSA	specific surface area
SUV	small unilamellar vesicle
T_m	phase transition temperature

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III.

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An Updated Risk Assessment as Part of the QbD-Based Liposome Design and Development

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Abstract: Liposomal formulation development is a challenging process. Certain factors have a critical influence on the characteristics of the liposomes, and even the relevant properties can vary based on the predefined interests of the research. In this paper, a Quality by Design-guided and Risk Assessment (RA)-based study was performed to determine the Critical Material Attributes and the Critical Process Parameters of an "intermediate" active pharmaceutical ingredient-free liposome formulation prepared via the thin-film hydration method, collect the Critical Quality Attributes of the future carrier system and show the process of narrowing a general initial RA for a specific case. The theoretical liposome design was proved through experimental models. The investigated critical factors covered the working temperature, the ratio between the wall-forming agents (phosphatidylcholine and cholesterol), the PEGylated phospholipid content (DPPE-PEG_{2000}), the type of the hydration media (saline or phosphate-buffered saline solutions) and the cryoprotectants (glucose, sorbitol or trehalose). The characterisation results (size, surface charge, thermodynamic behaviours, formed structure and bonds) of the prepared liposomes supported the outcomes of the updated RA. The findings can be used as a basis for a particular study with specified circumstances.

Keywords: Quality by Design; initial risk assessment; updated risk assessment; critical factors; "intermediate" liposome formulation; thin-film hydration method; liposome characterisation

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1. Introduction

According to the European Medicine Agency (EMA), "liposomes are classically described as artificially prepared vesicles composed of one or more concentric lipid bilayers enclosing one or more aqueous compartments" [1]. These vesicles can be described as "microscopic phospholipid bubbles with a bilayered membrane structure" and an aqueous media in the centre [2]. In this way, liposomes provide a suitable delivery system for both the hydrophobic drugs (in the membrane) and the hydrophilic compounds (in the central part).

A. D. Bangham developed the first liposomes in the early 1960s [3]. Since that time, liposomes have been proved to be successful nanocarriers for a targeted gene and drug delivery; however, as the amount of information and improvements on liposomes increases, the scale of the challenges in the field raises as well. To get a high-quality product, knowledge of medical, pharmaceutical, chemical, biological and physical sciences should be used and mixed with technological experiences [4]. All this information needs to be considered, organised and evaluated to achieve a successful liposome-based formulation development [5].

The Quality by Design (QbD) concept is a knowledge-, and risk assessment-based quality management approach, used mainly in the pharmaceutical industrial production process [6,7]; however, it also can be extended and applied in the early pharmaceutical research and development (R&D) phase [5,8,9]. Nowadays, including the QbD elements during the submissions of the marketing authorisation documents is a regulatory requirement. The QbD is a holistic and systemic way of improvements, where the primary focus is on the profound preliminary target product design. Thus, the theoretical design phase is extended based on prior knowledge (from literature and previous research) and risk estimation. This accurate design, especially the implementation of the Risk Assessment(s) (RA), helps correctly set up the practical experiments.

The whole method and the elements of the QbD are described in the guidelines of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) [10–12].

A QbD method-guided development has several steps that are specified in the guidelines mentioned above. The first step is the definition of the Quality Target Product Profile (QTPP), which contains the essential parameters of the formulation from the patient's point of view and the requirements from the clinical field. The QTPP is a prospective summary of the quality characteristics of the product that ideally will be achieved. It is related to the quality, safety and efficacy of the product, considering, for example, the route of administration, the dosage form, the bioavailability, the strength and the stability. [10]. The QTPP selection is followed by the design of the product and the manufacturing process according to the predefined quality profile, which means selecting those parameters that have a critical influence on the QTPP. These are the Critical Quality Attributes (CQAs), which are related to the safety and efficacy of the product. The CQAs are those physical, chemical, biological or microbiological properties or characteristics that should be within an appropriate limit range or distribution to ensure the targeted product quality [10]. The potential CQAs of the drug product are derived from the QTPPs, and prior knowledge guides the product and process development [10]. Other crucial factors are the Critical Material Attributes (CMAs) related to the materials and the Critical Process Parameters (CPPs) associated with the selected production method. The CPPs are those factors that should be monitored or controlled to ensure the process maintains the aimed quality [10]. The key element of a QbD-guided development is the RA (initial, recurrent/updated or finalised) [6]. This process results in the CQAs/CPPs ranked by their critical effect on the targeted product quality. Then, the Design of Experiments (DoE) [10] can be set up based on the results of the RA, which means that the practical experiments are planned and carried out according to the most relevant influencing factors (CMAs and CPPs). In the next step, the determination of the Design Space (DS) [13] of the product can be performed. The DS has remarkable regulatory benefits because the alterations in the production parameters in the DS do not require modifications during the submission. The following steps of the QbD method are the application of the Control Strategy and the planning of the Continuous Improvement, which have relevance from the perspective of the pharmaceutical industry. The QbD-, and RA-based development and screening have several advantages; thus, the experiments could be more effective in practice, and it can be especially useful in the early pharmaceutical developments of complex or sensitive drugs or systems with special considerations [14–22].

The requirements for the liposomal formulations vary depending on the chosen medical need and the selected route of administration. The proper liposome formulation design is assigned to the therapeutic needs. Furthermore, identifying and collecting those factors that impact the final product is an essential step. The factors that critically influence the quality and characteristics of the liposomes require the most significant attention during the development process. The critical parameters affecting the liposomes were collected and evaluated both in general and with particular attention to the process parameters of the thin-film hydration method in a previous work from our research group (initial RA) [5] to extend the QbD method to the early development phase of the liposome-related

pharmaceutical researches. A general overview of the QbD approach for liposomes without a defined production process completed with characterisation methodologies is available, owing to the work made by Porfire et al. [23]. Xu et al. performed a risk analysis study on liposomes gained from the thin-film hydration technique and loaded with superoxide dismutase via a freeze-thaw cycling technique [24]. Their evaluation involved the analytics and the instrumentation reliability as well. The findings of the previously mentioned studies were built into our former theoretical article [5]. Ahmed et al. combined the QbD tools with process analytical technology to support the development of transdermal glimepiride liposomal films [25]. The results of the risk analysis were studied according to the design by Plackett–Burman. Factorial design-based RA results were utilised to evaluate the formulation variables of an early development phase, nose-to-brain, lipophilic API-containing liposome preparation by Pallagi et al. [26]. Chitosan-coated ghrelin-containing liposomes were developed for intranasal delivery following the RA steps by de Barros et al. [27] to determine the optimal active pharmaceutical ingredient (API) and chitosan concentrations applying the thin-film hydration method. Pandey et al. applied the QbD method in the development of chitosan-coated, hydrophilic API-enclosing liposomes prepared via a modified ethanol injection method [28]. Merlo-Mas et al. presented the use of the QbD tools and risk analysis in the case of the α-galactosidase-loaded nanoliposomes preparation through the DELOS-susp (depressurisation of an expanded liquid organic solution into aqueous solution) method, a compressed fluid-based technique that results in reproducible and scalable nanovesicular systems [29]. As the presented list about the diversified applications of the QbD approach shows, this quality management method is useable when complex fields need to be combined to meet the development, therapeutic, authorisation and patient-centred requirements.

This present research aimed to investigate the critical parameters highlighted in the initial RA [5] from new perspectives, carry out a comparative characterisation study and determine the general effects of the selected CMAs and CPPs on the properties of the liposomes. Accordingly, an evaluation to perform an updated RA was targeted for "intermediate" API-free liposomal formulations to get a practical decision-making system that enables the change of the liposome properties according to possible predefined goals (i.e., requirements of the API, the dosage form and the administration route) in the future.

2. Materials and Methods

2.1. Materials

Two different compositions were used to form liposomes, with modifications in the phospholipid and cholesterol ratios according to the goal of the investigations. One of the compositions was a phosphatidylcholine- and cholesterol-based simple formulation (hereinafter: PC-CH, Table 1 and Table 3), while the other one contained PEGylated phosphatidylethanolamine as well (hereinafter: PC-CH-PEG, Tables 2 and 3).

The following materials were used as liposomal wall-forming excipients (in an alcoholic solution): cholesterol (CH) (Molar Chemicals Kft., Budapest, Hungary), L-α-phosphatidylcholine (PC) (Sigma-Aldrich Chemie GmbH, Munich, Germany) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DPPE-PEG_{2000}) (Avanti® Polar Lipids Inc., Alabaster, Alabama, AL, USA), solved in ethanol 96% (Molar Chemicals Kft., Budapest, Hungary). The excipients were used in different ratios (Tables 1 and 2).

Phosphate-buffered saline pH 7.4 (PBS pH 7.4) and pH 5.6 (PBS pH 5.6) and sodium chloride physiological solution (saline solution) pH 5.5 [30] were used as hydration media. The composition of these solutions are the followings: PBS pH 7.4: 8.0 g/L NaCl, 0.20 g/L KCl, 1.44 g/L $Na_2HPO_4 \times 2\ H_2O$, 0.12 g/L KH_2PO_4; PBS pH 5.6: 0.65 g/L K_2HPO_4, 8.57 g/L KH_2PO_4; saline solution: 9.0 g/L NaCl dissolved in distilled water. The materials used to make these hydration media are the following: sodium chloride (NaCl) (Molar Chemicals Kft., Budapest, Hungary), potassium chloride (KCl) (Molar Chemicals Kft., Budapest, Hungary), disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \times 2\ H_2O$)

(Spektrum-3D Kft., Debrecen, Hungary), dipotassium phosphate (K_2HPO_4) (Spektrum-3D Kft., Debrecen, Hungary) and potassium dihydrogen phosphate (KH_2PO_4) (Molar Chemicals Kft., Budapest, Hungary). Three carbohydrates were used as cryoprotectants for lyophilisation, such as D-glucose (Hungaropharma Zrt., Budapest, Hungary), D-sorbitol (Hungaropharma Zrt., Budapest, Hungary) and D-trehalose (Sigma-Aldrich Chemie GmbH, Munich, Germany). The amount of the chosen cryoprotectant was 5% of the phospholipid mass in every case, solved in the hydration media [31]. None of the formulations contained active pharmaceutical ingredients (API).

Table 1. Phosphatidylcholine and cholesterol-based (PC-CH) compositions.

Compositions	**Phosphatidylcholine–Cholesterol Liposomes**				
	Phospholipid:Cholesterol Mass Ratio				
	100:0	**90:10**	**80:20**	**70:30**	**60:40**
PC (*w*/*w*%)	100	90	80	70	60
cholesterol (*w*/*w*%)	-	10	20	30	40
	solvent of the stock solution				
EtOH 96%	+				
	hydration media				
saline solution (mL)	100				

Table 2. Phosphatidylcholine, PEGylated phosphatidylethanolamine and cholesterol-based (PC-CH-PEG) compositions.

Compositions	**PEGylated Liposomes**								
	PC:DPPE-PEG$_{2000}$:Cholesterol Mass Ratio								
	55:5:40			**50:10**			**40:20:40**		
PC (*w*/*w*%)	55			50			40		
DPPE-PEG$_{2000}$ (*w*/*w*%)	5			10			10		
cholesterol (*w*/*w*%)	40			40			40		
	solvent of the stock solution								
EtOH 96%	+								
	hydration media								
saline solution (mL)	100	-	100	100	-	100	-	100	-
PBS pH 5.6 (mL)	-	100	-	-	-	-	-	-	100
PBS pH 7.4 (mL)	-	-	-	-	100	-	100	-	-
	cryoprotectant								
glucose (%)	5	5	-	5	5	5	5	-	5
sorbitol (%)	-	-	5	-	-	-	-	-	-
trehalose (%)	-	-	-	-	-	-	-	5	-

Table 3. Nomenclature of the samples presented in the article.

(A) Sample Name	**Composition (m/m%)**		**Hydration Media**	**Cryoprotectant (5% of Total PPL. Mass)**
	PC	**CH**		
PPL-CH-60-40/50-SS	60	40	saline solution	-
PPL-CH-60-40/60-SS	60	40	saline solution	-
PPL-CH-70-30/60-SS	70	30	saline solution	-
PPL-CH-80-20/60-SS	80	20	saline solution	-
PPL-CH-90-10/60-SS	90	10	saline solution	-
PPL-CH-100-0/60-SS	100	0	saline solution	-
PPL-CH-60-40/70-SS	60	40	saline solution	-

Table 3. *Cont.*

(B) Sample Name	Composition (m/m%)			Hydration Media	Cryoprotectant (5% of Total PPL. Mass)
	PC	DPPE-PEG_{2000}	CH		
PPL-CH-55-5-40/60-SS+G	55	5	40	saline solution	glucose
PPL-CH-55-5-40/60-PBS7.4+G	55	5	40	pH 7.4 PBS	glucose
PPL-CH-50-10-40/60-SS+G	50	10	40	saline solution	glucose
PPL-CH-50-10-40/60-PBS7.4+G	50	10	40	pH 7.4 PBS	glucose
PPL-CH-40-20-40/60-SS+G	40	20	40	saline solution	glucose
PPL-CH-40-20-40/60-PBS7.4+G	40	20	40	pH 7.4 PBS	glucose
PPL-CH-40-20-40/60-SS+T	40	20	40	saline solution	trehalose
PPL-CH-40-20-40/60-PBS5.6+G	40	20	40	pH 5.6 PBS	glucose
PPL-CH-55-5-40/60-SS+S	55	5	40	saline solution	sorbitol

2.2. Methods

2.2.1. Elements of the QbD Design

Development of the Knowledge Space and Determination of the QTPP

Determining the QTPP of the aimed formula is the essential first step in the QbD-guided development process. For this purpose, a primary knowledge space development [15] must be carried out, which means collecting and systematising all the relevant information regarding the aimed product and the production. Besides the definition of the QTPP, this step can help identify the potential critical factors of the formulation development. In this case, an "intermediate" API-free liposomal product was targeted as the QTPP, with the following requirements: spherical, large unilamellar vesicles (LUVs) in stable, monodisperse systems. Homogeneity was the requirement for the liposome formulations in an aqueous solution form and a dry solid phase for the lyophilised powders.

Determination of the CQAs, the CMAs and the CPPs

The nomination of a factor as a CQA, CMA or CPP always depends on the predefined goals, the expected quality of the product, the therapeutic needs and the selected production process. The stability, the zeta potential, the size of the vesicles, the number of lamellas, the polydispersity index, the surface modifications (in this study: PEGylation) and the phase transition temperature were identified as the CQAs, and the following factors were enumerated in the CMAs/CPPs group: the quality of the phospholipids and the cholesterol derivatives, the ratio between the phospholipids and the cholesterol, the surface modifiers, the phase transition temperature of the lipids, the quality of the solvent, the hydration media and the cryoprotectants, the working temperature and the process settings

Risk Assessment

After identifying the risks, the LeanQbD® software (QbD Works LLC, Fremont, CA, USA) was used for the RA process. The first element of this procedure was the interdependence rating between the QTPPs and the CQAs and the CQAs and CMAs/CPPs. A three-level (1-3-9) scale was used to describe the relationship between the parameters as "high" (H), "medium" (M) or "low" (L) and the results presented in Risk Estimation Matrices. Then, a risk occurrence rating (or probability rating step) was made for the CMAs/CPPs, using the same three-grade scale (H/M/L) for the analysis. The scoring was done for each parameter pair individually. After the scoring, the combination of the information provided a risk evaluation transforming the established risk levels into numerical scorings [32]. As the output of the RA evaluation, Pareto diagrams [33] were generated by the software presenting the numeric data and the ranking of the CQAs and CMAs/CPPs according to their potential impact on the aimed final product (QTPP). Due to that generated origin of the severity scores (which is influenced by the level number of the scale used for the analysis), the relative position of the factors should be considered

instead of their value. The Pareto charts show the differences between the effects of the CMAs and the CPPs and help select the experimental design factors.

Design of the Experiments

Based on the results of the RA, the DoE was built up. Five variables were identified as CMAs/CPPs: the working temperature, the phosphatidylcholine:cholesterol mass ratio, the PEGylated phospholipid content, the quality of the hydration media and the quality of the cryoprotectants. Each factor was investigated at different levels (Table 4). The effect of the working temperature and the phosphatidylcholine:cholesterol mass ratio on the PC-CH composition was investigated. Using the information obtained from these early studies, the effect of the PEGylated phospholipid content, the quality of the hydration media and the type of the cryoprotectant were investigated under improved conditions (pre-set temperature (60 °C) and phospholipid:cholesterol mass ratio (60:40)) on the PC-CH-PEG formulations. Three parallel samples were made and checked for each measurement.

Table 4. Critical factors and their levels investigated in the liposome formulation processes.

	Critical Factors	Investigated Levels or Parameters				
CPP	**working temperature**	50 °C		60 °C		70 °C
CMA	**Phosphatidylcholine:cholesterol mass ratio**	100:0	90:10	80:20	70:30	60:40
	PEGylated phospholipid content	5%		10%		20%
	PC:DPPE-PEG2000:cholesterol mass ratio	55:5:40		50:10:40		40:20:40
	quality of hydration media	saline solution		PBS pH 5.6		PBS pH 7.4
	pH	pH 5.5		pH 5.6		pH 7.4
	ionic strength	0.15 M		0.40 M		0.16 M
	quality of cryoprotectants	glucose		sorbitol		trehalose

2.2.2. Preparation of Liposomes and Process of Lyophilisation

The preparation of the liposome samples was based on the thin-film hydration method [34]. This method ensures a stable and straightforward way for liposome preparation [35] and, according to previous experiences [26], can be easily adapted for liposome studies. The alcoholic solutions of the wall-forming agents were used in the optimised concentrations regarding the chosen formulations (PC-CH or PC-CH-PEG). The ethanol was evaporated in a water bath under decreasing pressure with the Rotavapor® R-210/215 (BÜCHI Labortechnik AG, Flawil, Switzerland) rotary evaporator. The rotation speed was 25 rpm. Firstly, the temperature of the water bath was investigated, and the preparations were done at 50 °C, 60 °C and 70 °C; then, 60 °C was chosen and used for the later formulations. The decrease in air pressure was gradual. The pressure was decreased with steps of 100 mbar and kept at the lowest value (100 mbar) while an entire film has formed. The dried lipid film was hydrated with selected hydration media. The formation of the vesicles was supported by ultrasonication (Elmasonic S 30 H ultrasonic bath, Elma Schmidbauer GmbH, Singen, Germany). The sonication was performed at the investigated temperature for 30 min. The shaping of the liposomes happened in two steps via vacuum membrane filtration using a 0.45 µm (nylon membrane disk filter 47 mm, Labsystem Kft., Budapest, Hungary), then a 0.22 µm membrane-filter (Ultipor® N66 nylon 6.6 membrane disk filter 47 mm, Pall Corporation, New York, NY, USA). The vacuum was created by a vacuum pump (Rocker 400 oil-free vacuum pump, Rocker Scientific Co., Ltd. New Taipei City, Taiwan). The prepared liposome samples were immediately investigated for vesicle size, polydispersity and zeta potential; some samples were stored in liquid state and retested, but all were lyophilised for further investigations. The lyophilisation was done via SanVac CoolSafe freeze dryer (LaboGeneTM, Lillerød, Denmark) at normal atmospheric pressure, gradually decreasing the temperature from +25 °C to −40 °C. The vacuum was created when the temperature of the samples reached the chosen value, reducing the pressure to

0.01 atmosphere where the samples were stored for 8–10 h. After this period, the temperature of the tray was increased manually step by step from −40 °C to +25 °C until the pressure reached the normal atmospheric value. The lyophilised samples were stored in closed vials at 2–8 °C.

2.2.3. Characterisation of the Liposomes

Vesicle Size and Zeta Potential Analysis

Dynamic light scattering (DLS) technique was used to determine the vesicle size (expressed in Z-average) and the polydispersity index (PdI), referring to the heterogeneity or uniformity of the particles in the investigated samples. For the lipid-based nanocarrier systems, PdI values less or equal to 0.3 are considered the indicator of a monodisperse distribution [28]. The studied samples were accepted as a suitable formulation around or below this value. 1 mL was investigated from each sample in folded capillary zeta cells (Malvern Panalytical Ltd., Malvern, Worcestershire, UK). Zeta potential is the potential difference between the investigation media and the stationary fluid layer adsorbed to the surface of the particles, and among others, describes the stability of a formulation. Low zeta potential values indicate the aggregation of the dispersed particles, while higher potentials refer to a more stable formulation [36]. Vesicles with a charge less or equal to 10 mV are considered negatively, more or equal to 10 mV as positively charged, while between these two values as neutral liposomes [37]. These values were measured via the Malvern Zetasizer Nano ZS system (Malvern Panalytical Ltd., Malvern, Worcestershire, UK), equipped with a 633 nm wavelength laser.

Differential Scanning Calorimetry (DSC) and Thermogravimetric Analysis (TGA) Investigations

The thermodynamic state of the liposomes was studied in the temperature range of 25–300 °C via differential scanning calorimetry (DSC) technique (TA Instruments DSC Q20, TA Instruments, New Castle, Delaware, DE, USA). The number of the possible phase transitions (T_m) and the gel to liquid-crystalline phase transition temperature ($T_{c)}$ were determined using a 10 °C/min heating rate. Freeze-dried samples of 6–10 mg were studied and placed into hermetically sealed aluminium sample pans in dry nitrogen gas. During the thermogravimetric (TGA) measurements, the lyophilised samples are heated to a defined temperature and investigated for mass changes. The Setaram Labsys TG-DTG-DTA analyser (SETARAM Instrumentation, Caluire, France) was used for the investigations. The studies were done in a nitrogen atmosphere in the temperature range of 25–300 °C with a 10 °C/min heating rate from 8–10 mg freeze-dried samples.

Fourier-Transform Infrared (FT-IR) Spectroscopy Measurements

The interactions between the compounds of the liposome products were measured via an Avatar 330 FT-IR Thermo Nicolet spectrometer (Thermo Electron Corporation, Waltham, MA, USA) equipped with an infrared light source and optics. The measurements were made from freeze-dried powder samples in 4000–400 cm^{-1} wavelength range with 4 cm^{-1} spectral resolution in absorbance mode. For sample preparation, the lyophilised powders were mixed with potassium bromide (KBr), pulverised and pressed to form pellets. KBr pellets were used as references.

Atomic Force Microscopy (AFM) Measurements

In this investigation, one drop of the formulation was applied on a freshly cleaved mica surface (Muscovite mica, V-1 quality, Electron Microscopy Sciences, Washington, DC, USA) to obtain AFM images under normal ambient conditions using the tapping mode of an NT-MDT SolverPro Scanning Probe Microscope (NT-MDT, Spectrum Instruments, Moscow, Russia). AFM tips type PPP-NCHAuD-10 (thickness: 4.0 μm, length: 125 μm, width: 30 μm) (NanoWorld AG, Neuchâtel, Switzerland) was applied with 2 nm nominal

radius of curvature and 15 µm length. The non-contact silicon cantilevers had a typical force constant of 42 N/m and a resonance frequency of 330 kHz.

Residual Ethanol Measurements via Gas Chromatography-Mass Spectrometry (GC-MS)

The determination of the residual ethanol content of the samples was carried out using a Shimadzu GCMS-QP2010 SE gas chromatograph-mass spectrometer (Shimadzu Corporation, Kyoto, Japan). A total of 50 mg from the freeze-dried samples were sonicated in 1 mL toluene, settled, decanted and filtered through a 0.22 µm polytetrafluoroethylene syringe filter (Thermo Fisher Scientific Inc., Waltham, MA, USA). A sample of 1 µL was investigated. The oven program was as follows: initial temperature 80 °C for 2 min, increased at 20 °C/min to 180 °C, held at 180 °C for 2 min. The mass spectrometer measured from 0.5 min to 1.6 min, and from 25 m/z to 46 m/z with continuous scan.

2.2.4. Statistical Analysis

Data analysis, statistics and graphs were performed from the experimental data via Microsoft® Excel® (Microsoft Office Professional Plus 2013, Microsoft Excel 15.0.5023.100, Microsoft Corporation, Redmond, WA, USA), OriginPro® 8.6 software (OriginLab® Corporation, Northampton, MA, USA) and JMP® 13 Software (SAS Institute, Cary, NC, USA). The significance of the difference between a pair of investigated formulation groups was calculated via a one-way analysis of variance (ANOVA) with post-hoc Tukey test in Minitab® 17.1.0 software (Minitab, LLC, State College, Pennsylvania, PA, USA) with $p < 0.05$ as a minimal level of significance. Results were expressed as the mean value ± standard error. Three independently prepared parallel samples were made and studied in the investigations.

3. Results

3.1. Development of the Knowledge Space, the Definition of the QTPP and the Identification of the CQAs, CMAs and CPPs

Based on the quality-concerned requirements of the liposomal formulations [5], the relevant properties were narrowed down, and a stable, LUV-containing, monodisperse and homogeneous, API-free formulation was determined as the QTPP of the "intermediate" liposomal products that can provide a base for later carrier systems (Table 5). The elements and factors that can be identified as the CQAs of the aimed liposome preparation are shown in detail in Table 6. These specified CQAs include the size of the vesicles, the number of the lamellas, the polydispersity index, the zeta potential, the stability, the surface modifications (PEGylation) and the phase transition temperature of the liposomes. The CMAs and the CPPs are the quality of the phospholipids and the cholesterol derivatives, the ratio between the wall forming agents, the surface modifiers, the phase transition temperature of the lipids, the quality of the solvent, the hydration media, the cryoprotectants and further additives, the working temperature, the sterility requirements and the settings of the thin-film hydration method (dissolution, vacuum evaporation, sonication, filtration, lyophilisation and storage).

Table 5. QTPP elements of the "intermediate" liposomes designed as optimal carrier systems.

QTPP Factors	Details	Comments/Justifications
dosage form/ appearance	lyophilised powder	- stable solid powder - the dosage form can affect the potential administration routes and clinical application manners
physical attributes	morphology, large unilamellar structured, liposomes (LUV), optimal particle size, proper zeta potential	- the structure and the size of the vesicle is critically related to the potential dosage forms, dosage strength and administration routes, which can affect the incorporation of an API - critically related to excipients(e.g., surface modifiers and additives) - vesicles with a size of 100–200 nm can be suitable for several application methods
stable structure	in aqueous solution in freeze-dried powder form	- stability is a quality requirement; the duration of the stability is important - the stability of the formulation influences the safety, efficacy and quality profile of the product
homogeneoussystem	homogenous formulation	- critically related to the quality of the later product - influenced by the polydispersity of the system

Table 6. CQAs of the "intermediate", API-free liposomal formulation.

CQAs	Details	Comments/Justification
type of liposomes	conventional, cationic, immune, bioresponsive, magnetic	determine the quality of the lipids
targeted delivery compatibility	knowledge about the possible administration route	formulation needs to be suitable for the requirements of the later API
size of the vesicles	mean particle size: 100–200 nm	large vesicles (LUV)
number of lamellas	1 lamella	unilamellar vesicles (LUV)
morphology	shape and structure	spherical unilamellar vesicles
polydispersity index (PdI)	acceptable: below: 0.3	monodisperse system
zeta potential	the higher in absolute value, the more stable the formulation	indicates stability
surface modifications	attachment of polyethylene glycol (PEG) chains, monoclonal antibodies, antibody fragments peptides, nucleic acids, carbohydrates or small molecules	maintain targeted delivery
specific surface area	surface area-to-volume ratio	determines the properties of the later drug release

Table 6. *Cont.*

CQAs	Details	Comments/Justification
phase transition temperature (T_m)	working temperature is recommended to be higher than T_m	different value for each composition
sterility	meets the microbiological requirements	depends on the chosen administration route
stability	stable under given circumstances	in aqueous solution/in freeze-dried powder form

3.2. Risk Assessment

After the profound and careful knowledge space development and the determination of the QTPP and the CQAs of the potential liposome carriers presented above, the classification of the CMAs and CPPs by their criticality was performed during the risk analysis. A research group-level brainstorming utilising the prior experiences and the literature knowledge supported the three-grade scaled interdependence rating between the items of the QTPP elements and the CQAs and between the CQAs and CMAs/CPPs, helping to determine the severity of the risks what the factors mean to each other (Figure 1). The sonication has a strong impact on the lamellarity and the size of the liposomes (interdependence evaluated as "high"), while it does not influence the phase transition temperature of the lipid formulation (effect estimated as "low") (see in Figure 1). Based on the Risk Estimation Matrices and the results of the occurrence rating of the factors, the software transformed the given data into numerical information and calculated the overall severity of the risks. The generated Pareto charts show the ranking of the critical factors, presented in Figures 2 and 3.

According to the RA process, the CMAs/CPPs are the followings organised in descending order based on their criticality: quality of phospholipids, quality and quantity of surface modifiers, ratio between the phospholipids and the cholesterol, cholesterol content, phase transition temperature, working temperature, quality of the hydration media, settings of sonication, quality and quantity of cryoprotectants, properties of filtration, sterility, quality of solvent, addition of additives, dissolution of lipids, storage conditions, settings of lyophilisation and vacuum evaporation. The settings of the thin-film hydration process were kept in formerly set stable values, except the working temperature, which was chosen for further investigation based on its high severity score. The phospholipid:cholesterol ratio, the effect of a PEGylated phospholipid as the equivalent of the surface modifications in this study and the quality of the hydration media and the cryoprotectant were investigated from the relevant CMAs. The quality of the solvent was set as ethanol 96% in all the experiments. The factors of the CMAs/CPPs were studied according to the DoE (Table 4).

A

CQAs \ QTPP	dosage form/ appearance	physical attributes	stable structure	homogen-ous system
type of liposomes	high	high	medium	medium
targeted delivery compatibility	high	high	high	medium
size of the vesicles	high	high	medium	medium
number of lamellas	low	high	low	low
morphology	medium	high	medium	medium
monodispersity	high	medium	low	high
zeta potential	high	medium	high	medium
surface modifications	high	high	medium	medium
specific surface area	medium	high	low	low
phase transition temperature	high	low	high	low
sterility	high	low	medium	low
stability	high	medium	high	high

B

Process	Preparation of the lipophilic phase						Vacuum evaporation of the solvent		
CQAs \ CMAs/CPPs	phospho-lipids	surface modifiers	cholesterol	PPL-CHOL ratio	solvent	dissolution of lipids	phase transition temperature	working temperature	settings of vacuum evaporation
type of liposomes	high	high	high	medium	low	low	high	high	low
targeted delivery compatibility	high	high	medium	medium	medium	low	high	low	low
size of the vesicles	high	high	high	medium	low	low	medium	medium	low
number of lamellas	high	low	low	low	low	low	medium	medium	low
morphology	high	medium	medium	medium	low	low	medium	high	low
monodispersity	high	medium	medium	medium	medium	low	medium	medium	low
zeta potential	high	medium	high	high	medium	low	medium	medium	low
surface modifications	high	high	low	medium	low	low	medium	medium	low
specific surface area	high	medium	medium	medium	medium	low	medium	high	low
phase transition temperature	high	high	medium	high	medium	high	high	high	low
sterility	high	low	high	low	high	medium	low	low	low
stability	high	high	high	medium	low	medium	medium	low	low

Process	Hydration of the lipid film		Sonication	Shaping of vesicles		Lyophilisation		Storage
CQAs \ CMAs/CPPs	hydration media	addition of additives	settings of sonication	properties of filtration	sterility	cryo-protectant	settings of lyophili-sation	storage
type of liposomes	low	low	low	low	low	low	low	low
targeted delivery compatibility	medium	low	low	low	high	medium	low	low
size of the vesicles	high	medium	high	high	low	medium	low	low
number of lamellas	low	low	high	medium	low	low	low	low
morphology	medium	medium	high	medium	low	medium	low	low
monodispersity	medium	low	medium	high	low	medium	low	low
zeta potential	high	medium	medium	low	low	medium	low	low
surface modifications	low	low	medium	low	low	low	low	low
specific surface area	medium	medium	high	high	low	medium	low	low
phase transition temperature	medium	medium	low	low	low	medium	low	low
sterility	high	medium	low	medium	high	high	low	medium
stability	medium	high	medium	low	high	high	medium	medium

Figure 1. Results of the interdependence investigations between the elements of the Quality Target Product Profile (QTPP) and the Critical Quality Attributes (CQAs) (**A**), and between the CQAs and the Critical Material Attributes (CMAs) and the Critical Process Parameters (CPPs) (**B**).

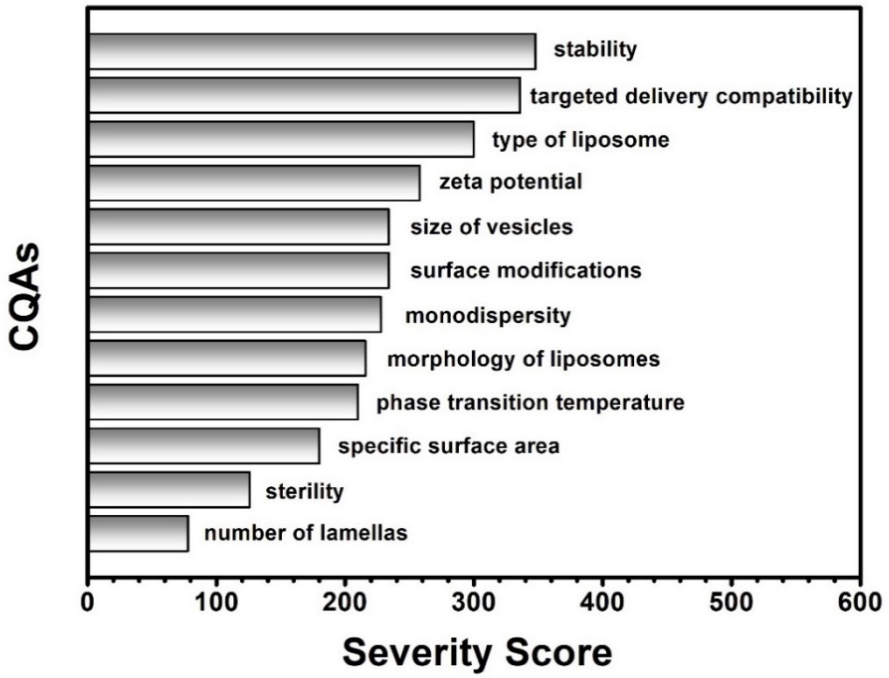

Figure 2. List of the Critical Quality Attributes (CQAs) of the liposomes ranked by their calculated severity scores.

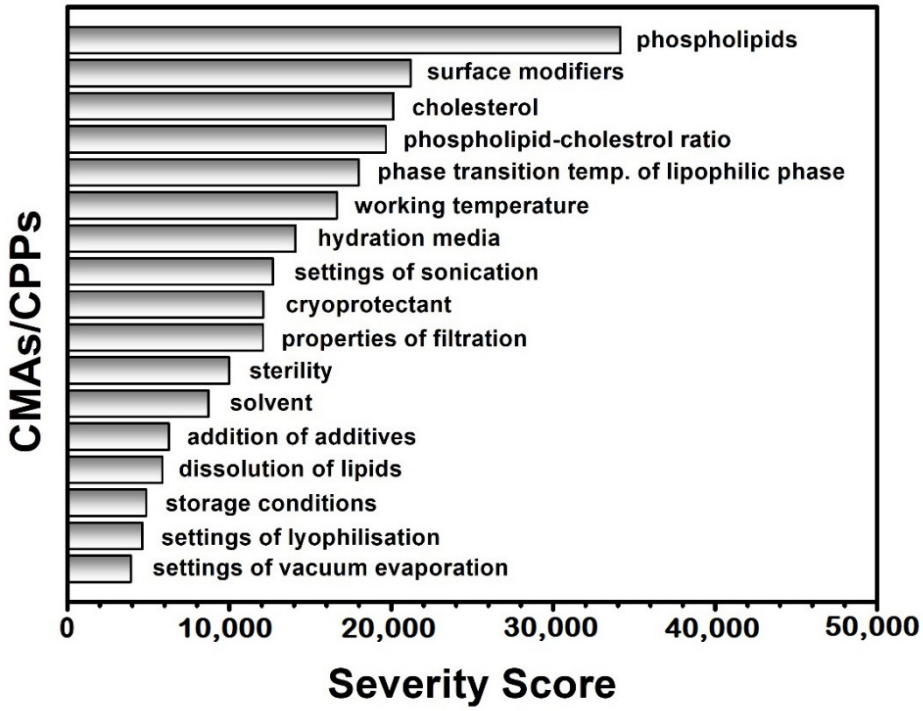

Figure 3. List of the Critical Material Attributes (CMAs) of the liposome components and the Critical Process Parameters (CPPs) of the thin-film hydration preparation method ranked by their calculated severity scores.

3.3. Characterisation Results of the Liposomal Products

The following data show how the changes in some of the CPPs (working temperature) and CMAs (phospholipid:cholesterol mass ratio, addition of PEGylated phospholipids, quality of the hydration media and quality of the cryoprotectants) affect the characteristics

of the API-free liposomal products. Different derivatives of the two basic formulations (PC-CH and PC-CH-PEG) were used for the investigations and characterised.

3.3.1. Effects of Using Different Temperature Values

The effect of the working temperature is a little-investigated factor regarding the size of the liposomes. In this research, phosphatidylcholine–cholesterol (mass ratio: 60:40) vesicles were prepared at 50, 60 and 70 °C. The mean vesicle size values were measured as 154–166 nm with no significant difference (Table 7, Figure 4). The polydispersity indexes were under the acceptance limit of 0.30 in all cases, showing homogenous formulations. However, the formulation prepared at 60 °C showed significantly more negative zeta potential (−10.3 ± 1.8 mV) than the one at 70 °C (−8.1 ± 1.6 mV) (*, $p < 0.05$), while it did not differ from the 50 °C one (Figure 4A,B). Based on these data, 50–60 °C can be the design space for the liposomes with PC origin. According to this observation, 60 °C was chosen as the working temperature for the further studies presented in the article.

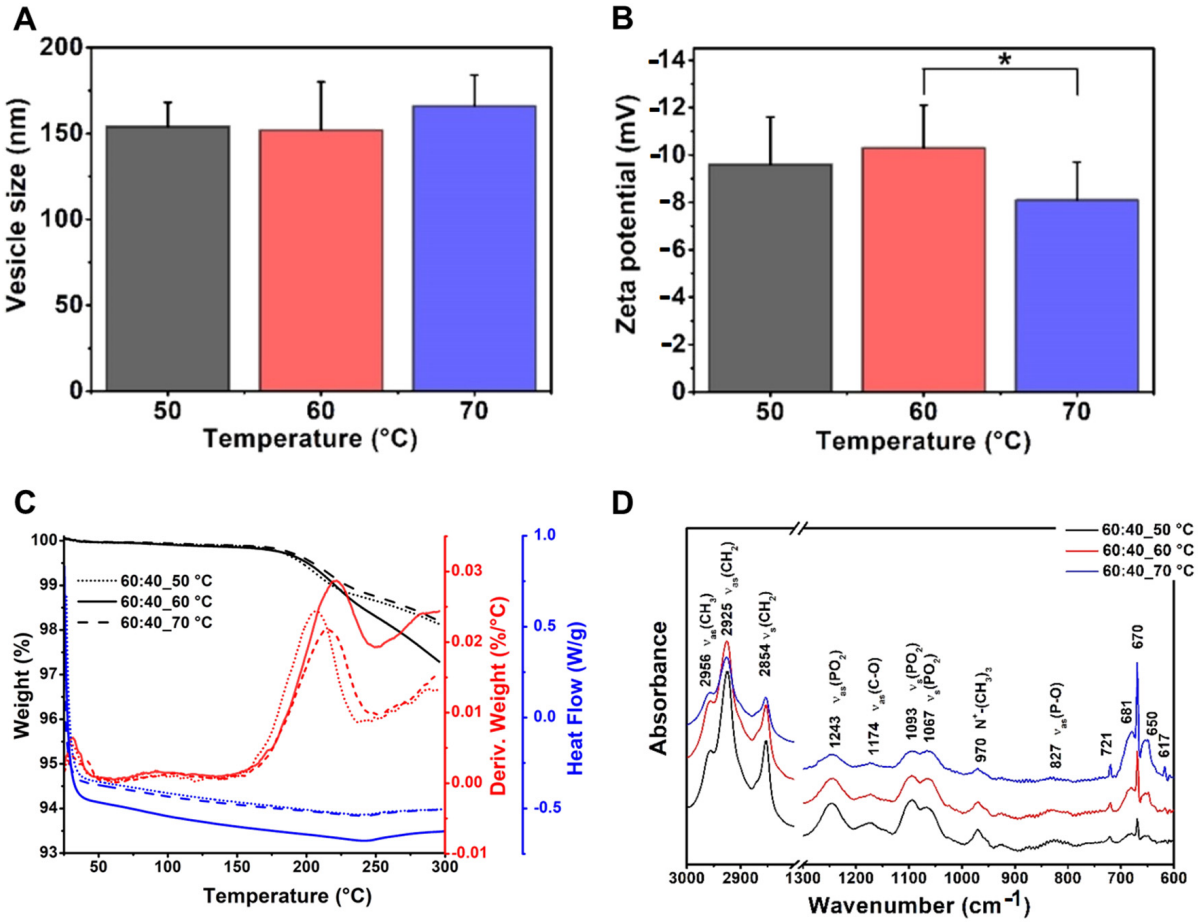

Figure 4. Characteristic features of the liposome samples prepared at different temperature values (50, 60 and 70 °C) from PC-CH 60:40 mass ratio composition, and hydrated with saline solution, presenting the results of the investigations: vesicle size and zeta potential analysis (**A**,**B**), differential scanning calorimetry and thermogravimetric analysis (**C**) and Fourier-transform infrared spectroscopy (**D**). *: $p < 0.05$.

Table 7. Measurement results of the liposome samples prepared at different temperature values (50, 60 and 70 °C) from PC-CH 60:40 mass ratio composition and hydrated with saline solution.

Compositions	Phosphatidylcholine–Cholesterol Liposomes (Mass Ratio: 60:40)					
	50 °C		60 °C		70 °C	
	mean	SD	mean	SD	mean	SD
vesicle size (nm)	154	14	152	28	166	18
PdI	0.24	0.02	0.18	0.08	0.21	0.04
zeta potential (mV)	−9.6	2.0	−10.3	1.8	−8.1	1.6
TG%	2		3		2	
sample name	PPL-CH-60-40/50-SS		PPL-CH-60-40/60-SS		PPL-CH-60-40/70-SS	

The DSC and TGA measurements of the liposome samples prepared from the same PC-CH composition at different temperatures (50, 60 and 70 °C) have resulted in the following curves: TG (black lines), dTG (red lines) and DSC (blue lines) diagrams. The dotted, full and dashed lines refer to the samples made at 50, 60 and 70 °C, respectively (Figure 4C). The results are congruent with the previous findings described in the literature. Based on the TG and dTG curves, the desorption of the physisorbed water content has been completed at around 100 °C for all the samples. The gel to liquid-crystalline phase transition temperatures (T_c) of the samples made from the same compositions at different temperatures was ~30–32 °C, as the DSC measurements proved as well. Below the T_c value, the presence of the cholesterol makes the chains more mobile in the liposomes, preventing the hydrocarbon chains from crystallisation, which modifies the T_m and causes a separation before the phase transition—which is the reason why the curve is smooth—while above the Tc value it maintains the rigidity of the membrane [38]. Another change occurs at 200–225°, representing the molecular changes happening at this temperature range [39]. A total of 2–3% of the weight of the samples is lost during the heat treatment, which continued to 300 °C [40,41].

3.3.2. Effects of Using Different Ratios of Wall-Forming Agents

The effect of using different phospholipid and cholesterol ratios was investigated in the PC-CH compositions prepared at 60 °C (Table 8, Figure 5). The size of the liposomes decreased with the reduction of the cholesterol ratio as the mass ratios changed from 70:30 to 100:0. The only PC-containing sample had significantly smaller vesicles (135 ± 24 nm) than the 20 *w/w*% (176 ± 30 nm) or 30 *w/w*% (200 ± 34 nm) CH-containing ones (**, $p < 0.01$). Applying lower proportions of phospholipids leads to larger vesicles until the 70:30 PC:CH mass ratio; however, the investigation of the PC:CH 60:40 vesicles indicated a decreased particle size (152 ± 20 nm). The lowest polydispersity index (0.18 ± 0.08) was measured in the same case as well. Our results strengthen the statement that the zeta potential values decrease by reducing the cholesterol concentration from the 30 *w/w*% content. This phenomenon may appear due to the presence of a higher number of phosphatidylcholine on the vesicle surface [42]. The most negative zeta potential value (−10.3 ± 1.8 mV) was measured in the case of the PC:CH 60:40 mass ratio liposomes, which significantly differs from the 80:20 samples (−8.7 ± 1.5 mV), (*, $p < 0.05$). Our results correlate with the work from López Pinto et al.; namely, an increment in the cholesterol concentration increases the size of the vesicles [43]. However, the cholesterol can maintain the rigidity of the liposomal membrane and improve its mechanical strength and packing density, thereby decreasing the permeability of water and small molecules through the membrane, as Magarkar et al. maintained in their work [44]. Based on these facts, cholesterol usage is recommended in the liposomal formulations to stabilise them; thus, in our case, the other investigations were carried out on cholesterol-containing compositions.

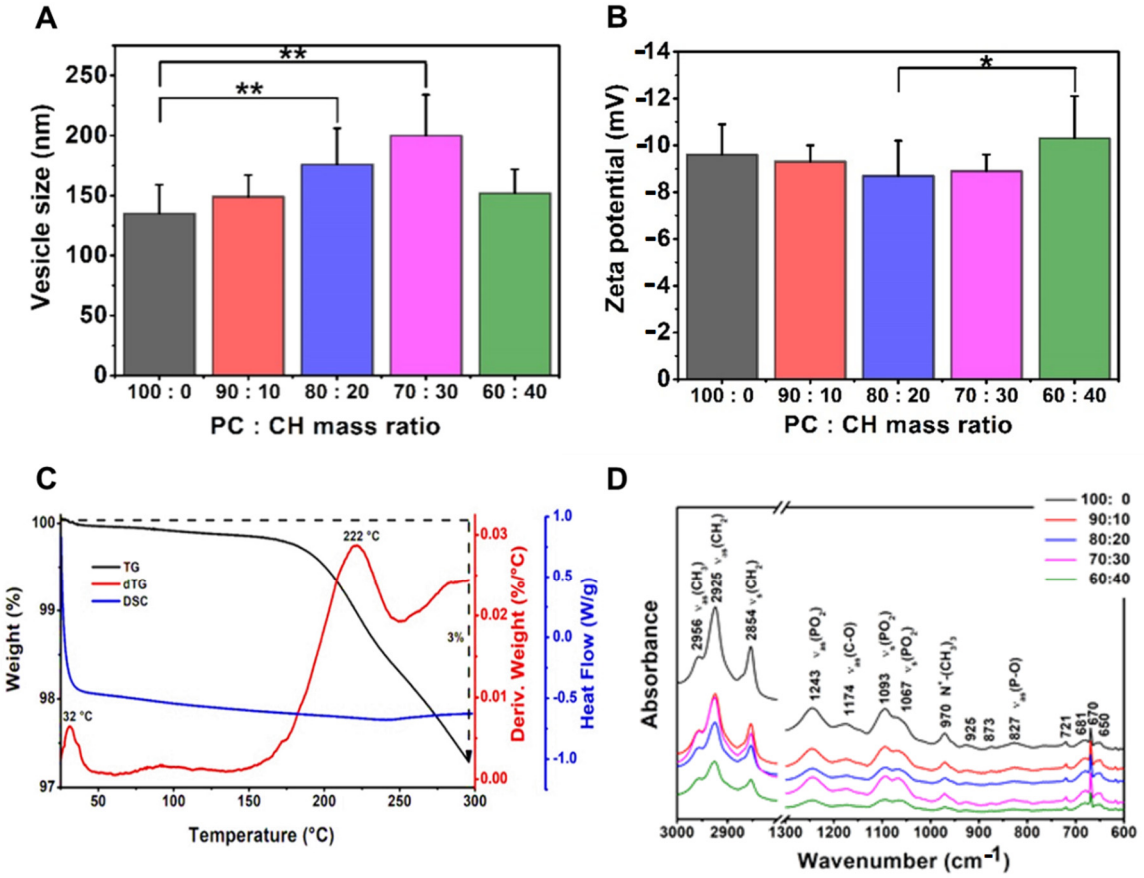

Figure 5. Characteristic features of the liposome samples prepared from different ratios of phosphatidylcholine (PC) and cholesterol (CH) at 60 °C, and hydrated with saline solution, presenting the results of the investigations: vesicle size and zeta potential analysis (**A**,**B**), differential scanning calorimetry and thermogravimetric analysis (**C**) and Fourier-transform infrared spectroscopy (**D**). *: p <0.05; **: p < 0.01.

Table 8. Measurement results of the liposome samples prepared from different phosphatidylcholine (PC) and cholesterol (CH) ratios at 60 °C and hydrated with saline solution.

Compositions	Phosphatidylcholine–Cholesterol Liposomes (60 °C)									
	Phosphatidylcholine:Cholesterol Mass Ratio									
	100:0		90:10		80:20		70:30		60:40	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
vesicle size (nm)	135	24	149	18	176	30	200	34	152	20
PdI	0.24	0.02	0.25	0.03	0.26	0.03	0.30	0.08	0.18	0.08
zeta potential (mV)	−9.6	1.3	−9.3	0.7	−8.7	1.5	−8.9	0.7	−10.3	1.8
TG%	4		3		4		2		3	
sample name	PPL-CH-100-0/60-SS		PPL-CH-90-10/60-SS		PPL-CH-80-20/60-SS		PPL-CH-70-30/60-SS		PPL-CH-60-40/60-SS	

Figure 6 illustrates the AFM images of the PPL-CH-60-40/60 (Figure 6A) and PPL-CH-80-20/60 (Figure 6B) samples proving homogeneous size distribution and 120–150 nm of mean vesicle size consistently with the DLS results.

Figure 5C presents the DSC and TGA curves of the PPL-CH-60-40/60 liposome sample. As in all cases, the end of the physisorbed water content desorption was detected around 100 °C on the TG and dTG curves. The T_c temperature of the sample was 33 °C

based on the DSC measurement result. The endotherm curve broadens with the rise of the cholesterol mole percentage; thus, the phase transition decreases [45,46]. Molecular alteration indicating changes are detected at the 200 225° range [39]. The thermal treatment causes a 3% mass loss in the samples until 300 °C [40,41].

The results of the FT-IR investigations made on the liposome samples prepared at 60 °C from different PC-CH compositions are shown in Figure 5D. The FT-IR spectra differ based on the type of the used lipids. In the PC-CH compositions, phosphatidylcholine (PC) is the wall-forming lipid, and the FT-IR figures showed two separate regions concerning the already known information about PC [47]. The so-called fingerprint region is at ~900–600 cm^{-1}. The 3000–2800 cm^{-1} wavenumber domain shows the C-H stretching vibrations originated mainly from the hydrocarbon chains. The lower wavenumber region of the spectrum (below 1800 cm^{-1}) belongs to the polar head groups of the phospholipids. The shape of the measured spectra was the same as those gained from the PC-CH 60:40 formulations produced at different temperatures (Figure 4D). At 827 cm^{-1} asymmetric ν_{as}(P-O), at 970 cm^{-1} N+-$(CH_3)_3$, at 1067 cm^{-1} and 1093 cm^{-1} symmetric $\nu_s(PO)_2$, at 1174 cm^{-1} asymmetric ν_{as}(C-O) and at 1243 cm^{-1} wavelength asymmetric $\nu_{as}(PO_2)$, stretchings are detected, which is typical for the polar head groups [48]. The symmetric stretchings $\nu_s(CH_2)$ at 2854 cm and the asymmetric ones $\nu_{as}(CH_2)$ at 2925 and 2956 cm^{-1} are the characteristics of the apolar hydrocarbon chains [47]. The traces of the FT-IR curves were consistent despite the different sample production temperatures; all the investigated samples contained similar bonds.

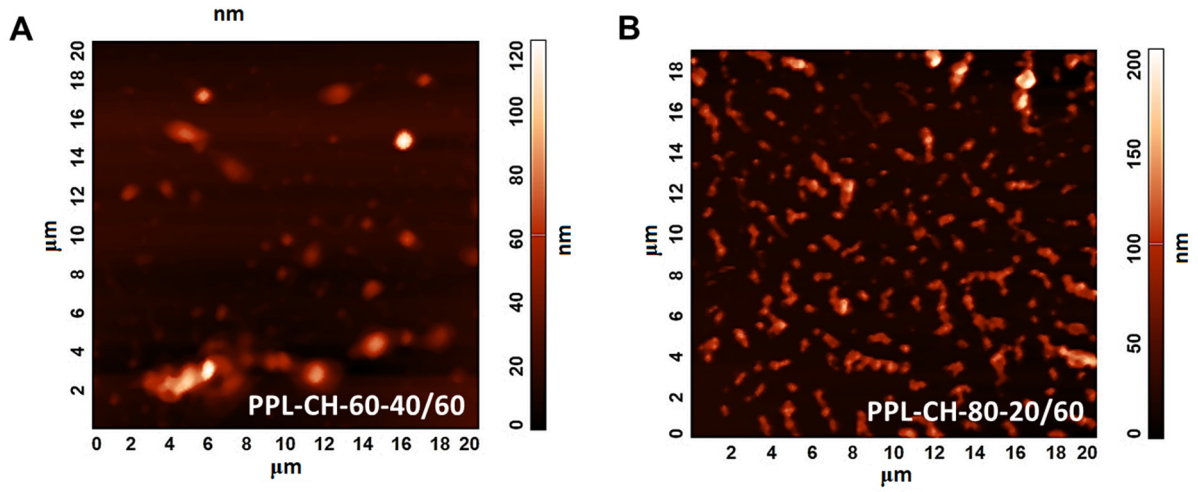

Figure 6. Atomic force microscopy images taken of the liposome samples prepared from PC-CH 60:40 (**A**) and 80:20 (**B**) mass ratio compositions at 60 °C, and hydrated with saline solution.

3.3.3. Effect of Using Different Concentrations of PEGylated Phospholipid

The samples were made at 60 °C and hydrated with different media (saline solution and PBS pH 7.4) to study the effect of using different concentrations of PEGylated phosphatidylethanolamine. A non-linear relationship can be detected between the phospholipid ratios and the vesicle size (Table 9, Figure 7). The increase in the concentration of the PEGylated phospholipid from 55:5:40 mass ratio meant first larger vesicles (50:10:40 mass ratio), then a decrease in the size (40:20:40 mass ratio). The significantly largest particle size was measured in the case of the formulations made with PC:DPPE-PEG_{2000}:cholesterol 55:10:40 mass ratio for both hydration media (saline solution: 152 ± 44 nm; PBS pH 7.4: 138 ± 23 nm) (**, $p < 0.01$) (Figure 6A,B). Increasing the amount of the PEGylated phospholipids to this certain ratio enlarges the size of the vesicles. However, further addition causes a sharp decrease in the mean size value due to the formation of PEGylated phospholipid-based micelles, as Garbuzenko et al. described for disteroylphosphoethanolamine (DSPE)–PEG_{2000}-containing vesicles [49]. Our results show the same

phenomenon for DPPE-PEG_{2000} as well. The polydispersity index was measured as the lowest in the 55:5:40 case (0.25 ± 0.05; 0.20 ± 0.02). Thus, this formulation is the best regarding the particle size and uniformity, providing vesicles around 100 nm with uniform size. Even the zeta potential values were significantly more negative in case of the 55:5:40 ratios (-2.5 ± 0.5 mV; -3.6 ± 1.1 mV) than of the 40:20:40 ones (saline solution: *, $p < 0.05$; PBS pH 7.4: **; $p < 0.01$). Although the zeta potential values were negative, the highest was the used proportion of the DPPE-PEG_{2000}; the least negative was the measured zeta potential value. Using PBS pH 7.4 resulted in moderately larger and more negative liposomes than those hydrated with saline solution.

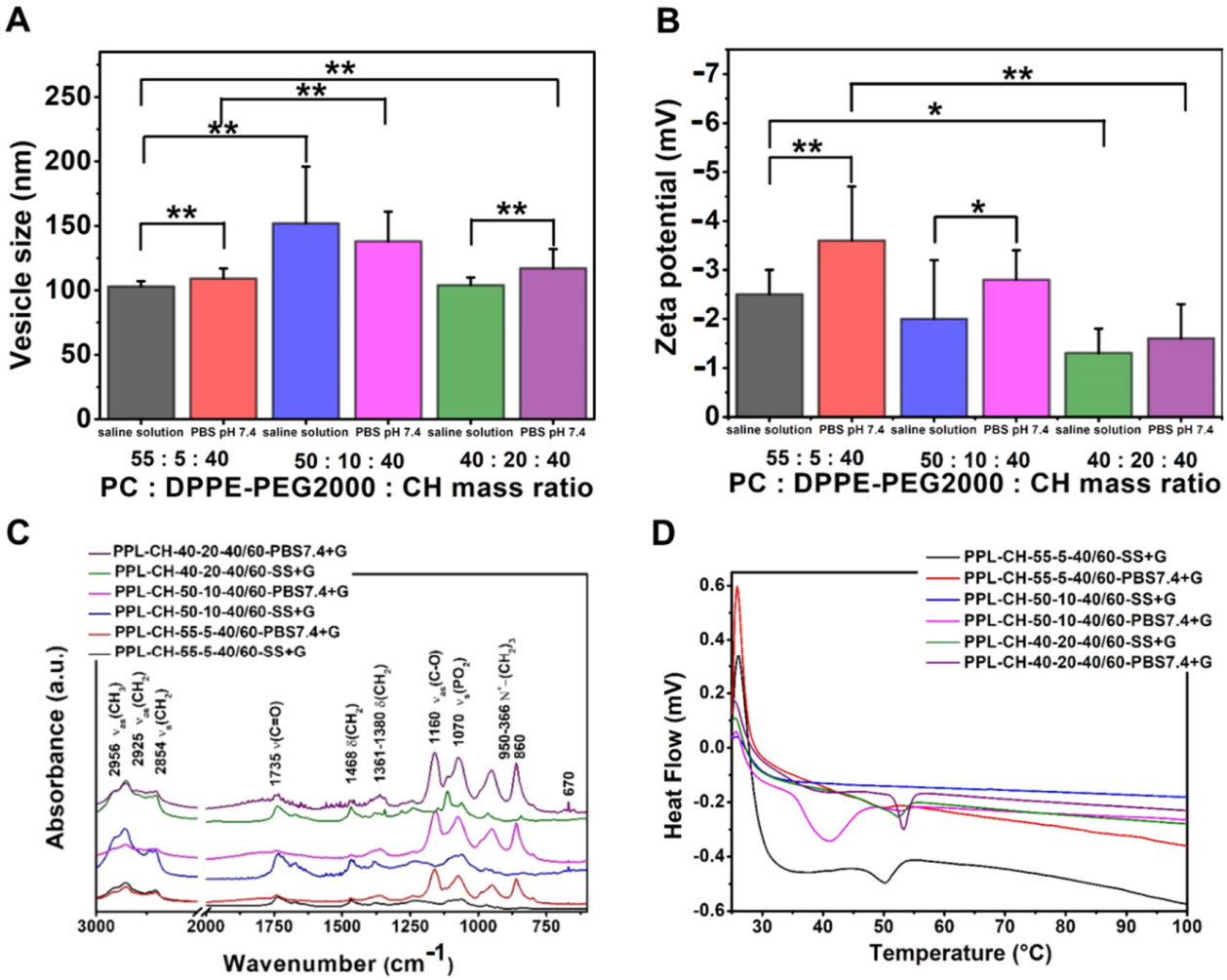

Figure 7. Characteristic features of the liposome samples prepared from different ratios of phosphatidylcholine (PC), PEGylated phosphatidylethanolamine (DPPE-PEG_{2000}) and cholesterol (CH) at 60 °C, hydrated with saline solution or PBS pH 7.4, and lyophilised with glucose as cryoprotectant, presenting the results of the investigations: vesicle size and zeta potential analysis (**A**,**B**), differential scanning calorimetry (**C**) and Fourier-transform infrared spectroscopy (**D**). *: $p < 0.05$; **: $p < 0.01$.

Table 9. Measurement results of the liposome samples prepared from different phosphatidylcholine (PC), PEGylated phosphatidylethanolamine (DPPE-PEG_{2000}) and cholesterol (CH) ratios at 60 °C, hydrated with saline solution or PBS pH 7.4 and lyophilised with glucose as cryoprotectant.

Compositions	Cryoprotectant: Glucose; 60 °C											
	PC:DPPE-PEG_{2000}:Cholesterol Mass Ratio											
	55:5:40				50:10:40				40:20:40			
	Hydration Media											
	Saline Solution		PBS pH 7.4		Saline Solution		PBS pH 7.4		Saline Solution		PBS pH 7.4	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
vesicle size (nm)	103	4	109	8	152	44	138	23	104	6	117	15
PdI	0.25	0.05	0.20	0.02	0.27	0.01	0.27	0.07	0.29	0.07	0.26	0.06
zeta potential (mV)	−2.5	0.5	−3.6	1.1	−2.0	1.2	−2.8	0.6	−1.3	0.5	−1.6	0.7
TG%	4		4		5		6		5		7	
sample name	PPL-CH-55-5-40/60-SS+G		PPL-CH-55-5-40/60-PBS7.4+G		PPL-CH-50-10-40/60-SS+G		PPL-CH-50-10-40/60-PBS7.4+G		PPL-CH-40-20-40/60-SS+G		PPL-CH-40-20-40/60-PBS7.4+G	

Figure 7C presents the DSC measurements results of the PEGylated phospholipid-containing liposome. Remarkable phase transition can be observed in the case of the samples hydrated with PBS pH 7.4. This phase transition was detected at 52 °C for the PPL-CH-55-5-40/60-PBS7.4+G, at 40 °C for the PPL-CH-50-10-40/60-PBS7.4+G and at 50 °C in case of the PPL-CH-40-20-40/60-PBS7.4+G samples. The observed increase in the phase transition temperature originated from the decreasing lateral pressure as the hydrocarbon chains of PC and DPPE-PEG_{2000} became growingly mismatched as the membrane enriched with the PEGylated phospholipid [50].

The TG analysis (Figure 7C) resulted in curves resembling the previously described samples. The weight loss happened in two steps: first at 75–80 °C, then between 200–250 °C. The samples lost ~5% of their mass until 300 °C were achieved.

Figure 7D presents the results from the FT-IR measurements. The spectra were the same in the samples hydrated with PBS and saline solution independently from the composition ratios. Two different regions can be distinguished. The C-H stretching vibrations appeared in the 3000–2800 cm^{-1} wavenumber domain [47], while peaks typical to the polar head groups emerged below 1800 cm^{-1} in the lower wavenumber region: ester ν(C=O) at 1735 cm^{-1}, δ(CH_2) at 1468 cm^{-1}, δ(CH_3) between 1361–1380 $cm^{-1,}$ ν(C-O) at 1160 cm^{-1} and ν(PO_2) stretchings at 1070 cm^{-1} wavelength [48]. The lipid hydrocarbon chains can be detected in various spectral regions; however, the most significant ones appear between 3050 and 2800 cm^{-1}. C-H stretching bands from different vibrational modes ($\nu_{as}(CH_2)$ at ~2917 cm^{-1} and $\nu_s(CH_2)$ at ~2850 cm^{-1}) belong to this region. Some overlaps with other vibrations can be detected in this part of the spectra. Usually, these vibrational modes are uncoupled from the other modes; thus, they are not influenced by the lipid head groups but are sensitive to the structure of the chains [47].

Comparing the PEGylated phospholipid-containing liposomal formulations with the non-PEGylated ones (Table 10, Figure 8) led to the following observations. The addition of the PEGylated phosphatidylethanolamine to the PC-CH formulation (samples prepared at 60 °C and hydrated with saline solution) significantly decreased the size of the vesicles (**, $p < 0.01$), slightly increased the polydispersity of the samples and resulted in a significantly less negative zeta potential value (**, $p < 0.01$). Changing a part of the PC content to phosphatidylethanolamine (PE) decreases the size of the liposomes, as Akizuki and Kaneko showed in their work [51]. Our results show that even the usage of PEGylated PE could decrease the vesicle size. The reason behind this size-decreasing ability, according to Li et al., is that the bilayer structure can be stabilised by the application of non-bilayer lipids, such as the unsaturated PE [52]. Our finding that the addition of PEGylated phospholipids

can decrease the size of the formulated vesicles agrees with the report by Tsermentseli et al. for DSPE-PEG$_{2000}$ [53]. The significant decrease in the zeta potential value (**, $p < 0.01$) after the addition of the DPPE-PEG$_{2000}$ is due to the positive charge of the PEGylated phospholipid [54].

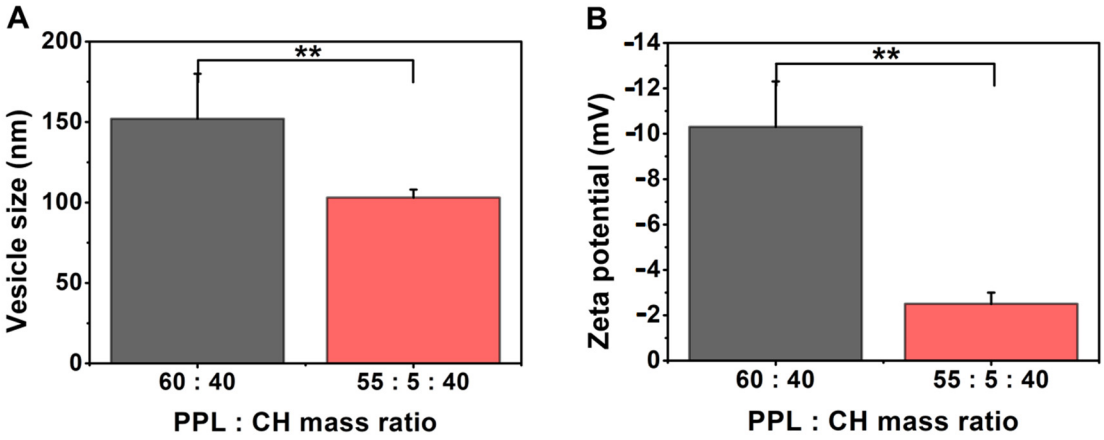

Figure 8. Characteristic features of the liposome samples prepared from phospholipid (PC only or PC and DPPE-PEG$_{2000}$) and cholesterol (CH) at 60 °C, hydrated with saline solution or PBS pH 7.4 and lyophilised with glucose as cryoprotectant, presenting the results of the investigations: vesicle size and zeta potential analysis (**A**,**B**). **: $p < 0.01$.

Table 10. Measurement results of the liposome samples prepared from phospholipid (PC only or PC and DPPE-PEG$_{2000}$) and cholesterol (CH) at 60 °C, hydrated with saline solution or PBS pH 7.4 and lyophilised with glucose as cryoprotectant.

Compositions	Phospholipid:Cholesterol Mass Ratio 60:40 Liposomes; Saline Solution; 60°			
	PC: Cholesterol 60:40		PC:DPPE-PEG$_{2000}$: Cholesterol 55:5:40	
	Mean	SD	Mean	SD
vesicle size (nm)	152	28	103	5
PdI	0.18	0.08	0.25	0.05
zeta potential (mV)	−10.3	2.0	−2.5	0.5
TG%	3		4	
sample name	PPL-CH-60-40/60-SS		PPL-CH-55-5-40/60-SS+G	

3.3.4. Effect of Using Different Types of Hydration Media

The effect of the quality of the hydration media on the PC-CH-PEG 40:20:40 mass ratio formulations made at 60 °C was studied (Table 11, Figure 9). The size of the particles increased in the order saline solution (104 ± 7 nm) < PBS pH 5.6 (110 ± 5 nm) < PBS pH 7.4 (117 ± 15 nm), with a significantly larger size in case of the PBS pH 7.4 than the saline solution (**, $p < 0.01$). The polydispersity of the formulations is mainly not influenced by the quality of the hydration media (0.29 ± 0.07; 0.33 ± 0.05; 0.26 ± 0.06), while a decrease could have been detected in the zeta potential values according to the following order: saline solution (−1.3 ± 0.5 mV) > PBS pH 7.4 (−1.6 ± 0.7 mV) > PBS pH 5.6 (−2.3 ± 1.2). Vesicles made with PBS pH 5.6 had significantly higher zeta potential than those hydrated with saline solution (*; $p < 0.05$). The ionic strength of the hydration media influences the value of the zeta potential; the higher the ionic strength is, the more compact the ion layer formed around the vesicles, and due to this phenomenon, the higher the zeta potential, as Tefas et al. described in their work [42]. In the presented case, the ionic strengths of the hydration media were: saline solution (0.15 M) < PBS pH 7.4 (0.16 M) < PBS pH 5.6 (0.40 M), which prove that the increasing ionic strength of the media increases the value of

the zeta potential for the hydrated lipid vesicles. Therefore, not only its pH but also the ionic strength of the chosen solution should be determined for liposomal preparation.

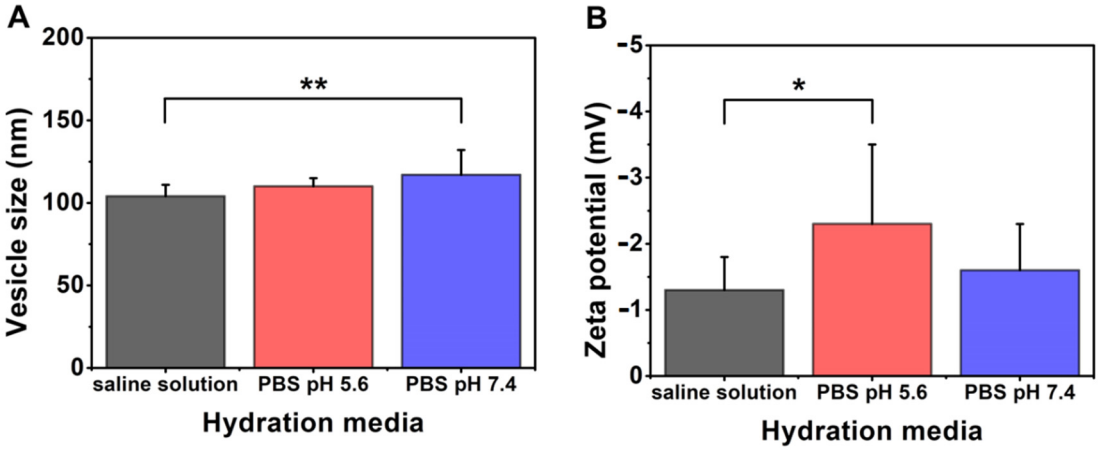

Figure 9. Characteristic features of the liposome samples prepared with different hydration media (saline solution, PBS pH 5.6 and 7.4) from PC-DPPE-PEG_{2000}-CH 40:20:40 mass ratio composition at 60 °C, and lyophilised with glucose as cryoprotectant, presenting the results of the investigations: vesicle size and zeta potential analysis (**A**,**B**). *: $p < 0.05$; **: $p < 0.01$.

Table 11. Measurement results of the liposome samples prepared with different hydration media (saline solution, PBS pH 5.6 and 7.4) from PC-DPPE-PEG2000-CH 40:20:40 mass ratio composition at 60 °C, and lyophilised with glucose as cryoprotectant.

Compositions	PC:DPPE-PEG_{2000}:Cholesterol Mass Ratio 40:20:40; Cryoprotectant: Glucose; 60 °C					
	Hydration Media					
	Saline Solution 0.154 M		PBS pH 5.6 0.40 M		PBS pH 7.4 0.16 M	
	Mean	SD	Mean	SD	Mean	SD
vesicle size (nm)	104	7	110	5	117	15
PdI	0.29	0.07	0.33	0.05	0.26	0.06
zeta potential (mV)	−1.3	0.5	−2.3	1.2	−1.6	0.7
TG%	5		15		7	
sample name	PPL-CH-40-20-40/60-SS+G		PPL-CH-40-20-40/60-PBS7.4+G		PPL-CH-40-20-40/60-PBS5.6+G	

Table 9 and Figure 7 show the compared results between saline solution and PBS pH 7.4 in the case of the two other liposome mass ratio compositions, PC-CH-PEG 55:5:40 and 50:10:40. The application of PBS pH 7.4 led to significantly larger vesicles not only for the 40:20:40 but for the 55:5:40 composition as well (**, $p < 0.01$). The zeta potential became significantly more negative after hydration with PBS pH 7.4 than with saline solution (55:5:40: **, $p < 0.01$; 50:10:40: *, $p < 0.05$).

Figure 10 represents the AFM measurement results in the case of the PPL-CH-55-5-40/60-SS+G (Figure 10A) and the PPL-CH-55-5-40/60-PBS7.4+G (Figure 10B) samples, which differ only in the quality of the hydration media. The images show homogeneous size distribution and vesicles with 100–120 nm size, supporting the results of the DLS measurements.

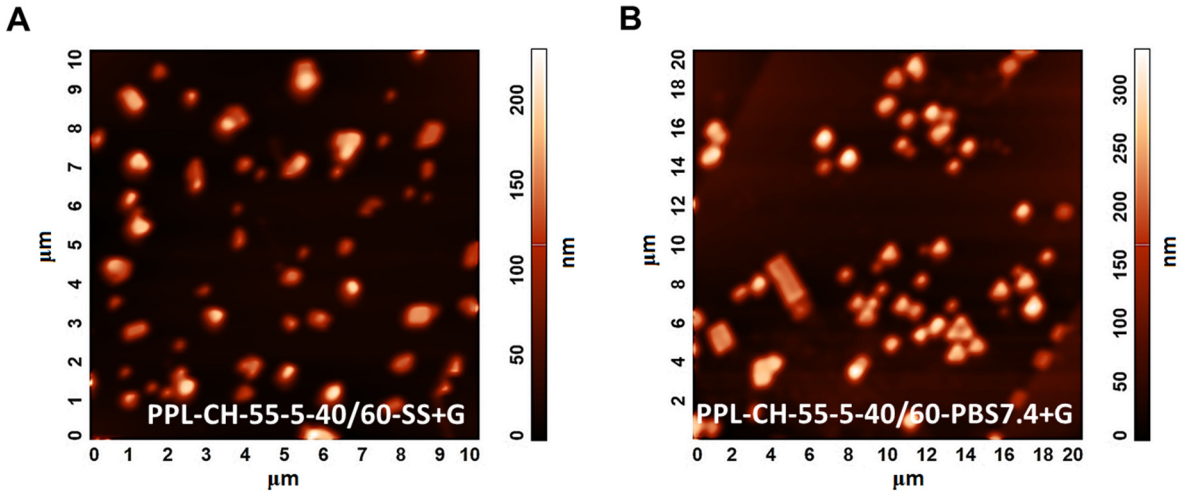

Figure 10. Atomic force microscopy images taken of the liposome samples prepared with different hydration media (saline solution (**A**), PBS pH 7.4 (**B**)) from PC-DPPE-PEG$_{2000}$-CH 55:5:40 mass ratio composition at 60 °C, and lyophilised with glucose as cryoprotectant.

3.3.5. Effect of Using Different Types of Cryoprotectants

In total, 5% of the whole mass of the phospholipids was given to the hydration media from glucose, sorbitol or trehalose to investigate the difference between the effects of different cryoprotectants (Table 12, Figure 11). The PC-CH-PEG formulations made at 60 °C were used for this purpose. Both the 55:5:40 and the 40:20:40 PC:DPPE-PEG$_{2000}$:cholesterol mass ratios were used for the study. When trehalose was used instead of glucose, the size of the vesicles was almost the same (103 ± 4 nm, 104 ± 7 nm, respectively). At the same time, sorbitol significantly increased the size of the liposomes (130 ± 5 nm and 103 ± 4 nm) (**, $p < 0.01$). The higher the ratio is of the DPPE-PEG$_{2000}$ in the formulation, the less negative the zeta potential; however, it could have been determined that by using these cryoprotectants, the zeta potential values became more negative in the following order: glucose (−1.3 ± 0.5; −2.5 ± 0.5) > trehalose (−3.2 ± 0.9) > sorbitol (−4.1 ± 0.8). The sorbitol and the trehalose increased the zeta potential significantly compared to the glucose-containing formulations (**, $p < 0.01$ in both cases).

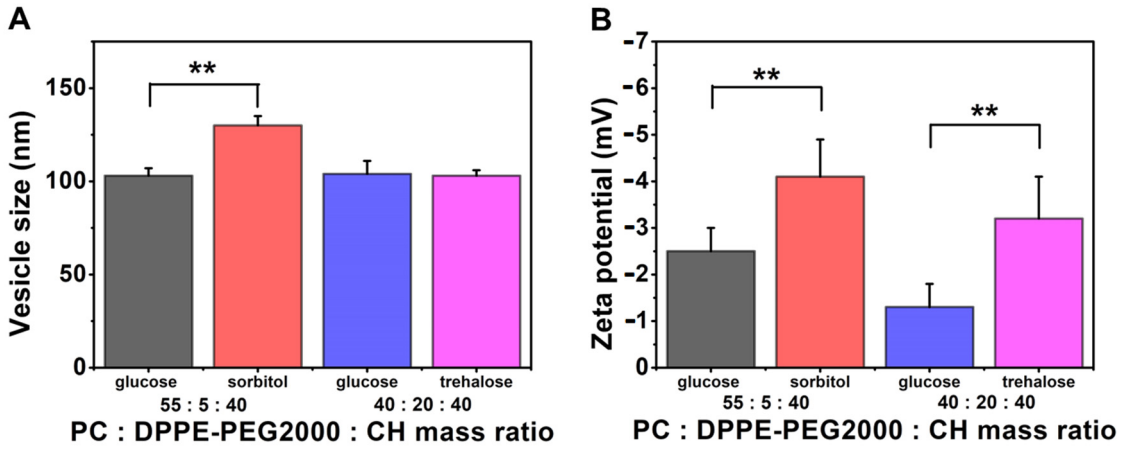

Figure 11. Characteristic features of the liposome samples lyophilised with different cryoprotectants prepared from PC-DPPE-PEG$_{2000}$-CH 55:5:40 or 40:20:40 mass ratio composition at 60 °C and hydrated with saline solution, presenting the results of the investigations: vesicle size and zeta potential analysis (**A**,**B**). **: $p < 0.01$.

Table 12. Measurement results of the liposome samples lyophilised with different cryoprotectants prepared from PC-DPPE-PEG_{2000}-CH 55:5:40 or 40:20:40 mass ratio composition at 60 °C and hydrated with saline solution.

Compositions	Hydration Media: Saline Solution; 60 °C							
	PC:DPPE-PEG_{2000}:Cholesterol Mass Ratio							
	55:5:40				40:20:40			
	Glucose		Sorbitol		Glucose		Trehalose	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
vesicle size (nm)	103	4	130	5	104	7	103	3
PdI	0.25	0.05	0.30	0.05	0.29	0.07	0.29	0.03
zeta potential (mV)	−2.5	0.5	−4.1	0.8	−1.3	0.5	−3.2	0.9
TG%	4		25		5		6	
sample name	PPL-CH-55-5-40/60-SS+G		PPL-CH-55-5-40/60-SS+S		PPL-CH-40-20-40/60-SS+G		PPL-CH-40-20-40/60-SS+T	

3.4. Residual Ethanol Measurements via Gas Chromatography-Mass Spectrometry (GC-MS)

Ethanol as a solvent has low toxic potential on the patients' health according to the guideline of the ICH [55]. Class 3, to which ethanol belongs, includes no solvent known as a human health hazard at the commonly accepted levels in pharmaceuticals. A total of 50 mg per day or less of these residual solvents (corresponding to 5000 ppm or 0.5%) would be acceptable without justification. Even higher amounts can be accepted if they are realistic with regards to the manufacturing capability and good manufacturing practices. All the investigated samples (PPL-CH-60-40/60, PPL-CH-55-5-40/60-SS+G, PPL-CH-55-5-40/60-PBS7.4+G, PPL-CH-40-20-40/60-PBS5.6+G) contained ethanol under the limit of detection, showing safety towards the possible patients and the formulation itself.

4. Discussion

4.1. Development of the Knowledge Space, the Definition of the QTPP and the Identification of the CQAs, CMAs and CPPs

As a knowledge space development, the possible factors that can build up the quality target product profile (QTPP) for a liposome-based formulation, the general critical quality attributes (CQAs) of the liposomes were collected and the properties of the liposomes components and the thin-film hydration liposome preparation method were surveyed in a previous article [5]. This study narrowed the broad approach, and a stable, LUV-containing, monodisperse and homogeneous, API-free formulation was established as the QTPP of the "intermediate" liposomal products that can later provide suitable drug carrier systems (Table 5). The relevant CQAs, similar to the physical and chemical characteristics of the liposomes, were listed in Table 6. The CMAs and the CPPs regarding the indicated formulations were checked and compressed to assist the RA.

4.2. Risk Assessment and Design of Experiment

The collected knowledge was transformed in the LeanQbD® software (QbD Works LLC, Fremont, CA, USA, www.qbdworks.com) into risk factors demonstrated with the severity scores during the RA. As the output, the former general liposomal RA has been refreshed, and the "intermediate" product-representing CQAs and CPPs were initiated with a numeric ranking reflecting their potential impact on the quality of the API-free formulations (Figures 2 and 3). In the following, a DoE was set up to optimise the formulation process and inspect the correctness of the results. For this process, the factors with the highest risk severity scores were chosen from the CMAs and CPPs for experimental investigation. Five variables were identified, the working temperature (CPP), the phosphatidylcholine–cholesterol weight ratio (CMA), the PEGylated phospholipid content (CMA), the quality of the hydration media (CMA) and the quality of the cryoprotectants (CMA). A non-PEGylated (PC-CH, Table 1) and a PEGylated (PC-CH-PEG, Table 2) basic

formulation were made up (Table 3) and then modified according to the levels of the investigated factors (Table 4). The effect of the working temperature and the mass ratio of the wall-forming agents was studied on the PC-CH composition. Based on the information obtained from these experiments, the effect of the PEGylated phospholipid content, the quality of the hydration media, and the cryoprotectant type were investigated under improved conditions (60 °C, PC-CH mass ratio 60:40) on the PC-CH-PEG formulations.

Our results, that the quantity and quality of the phospholipids and the cholesterol has the most significant impact on the final quality of the product, agrees with the findings in the work from Ahmed et al. [25], who investigated the question on glimepiride-containing phosphatidylserine-cholesterol liposomes. In the case of drug-loaded formulations, the API itself has a significant role in the liposomal properties, as it changes the structure of the liposome system [5,26]. Besides the drug-lipid ratio, Dhoble et al. studied the effect of the hydration and the sonication time on erlotinib-carrying 1,2-dipalmitoyl-n-glycerol-3-phosphocholine- and cholesterol-based liposomes [56]. They found that not only the composition but the sonication time has a significant impact on the entrapment efficiency.

4.3. Characterisation Results of the Liposomal Products

The working temperature is a rarely investigated parameter in the liposome preparation field. Our work showed that the size of the phosphatidylcholine–cholesterol (mass ratio 60:40) formulation is not affected by the temperature change; however, at 60 °C, significantly more negative zeta potential was reached than at 70 °C. The data suggest 50–60 °C as the optimal temperature for the preparation of these liposomes through thin-film hydration. The general practice suggests preparing liposomes above their transition temperature to receive efficient dispersion. The phase transition temperature of PC ranges between 50–55 °C [28,57]. Pandey et al. studied the formulation process of liposomes prepared via the ethanol injection technique in the temperature range of 40–70 °C. Their optimal working temperature range was 55–70 °C. In their case, the increase in the temperature resulted in insignificantly smaller particle size, which suggests that the formulation settings should be determined case-by-case for the different preparation methods. The effect of the changing phospholipid-cholesterol weight ratio was investigated at 60 °C, decreasing the CH content from 40% to zero. The size was decreasing with the reduction of the CH concentration. The size of the only PC-formed liposomes was significantly smaller than the 20% and 30% containing ones. López-Pinto et al. found the same correlation, that increasing the cholesterol concentration increases the size of the vesicles when investigated dipalmitoylphosphatidylcholine–cholesterol liposomes prepared according to the hydration technique [43]. After the concentration-based increase in the CH-content, significantly higher absolute zeta potential values were reached in the 40% CH-containing samples than in the 20% case, in parallel with some size reduction, which can strengthen the statement about the increasing effect of the cholesterol on the surface charge made by Sahu et al., who were screening this influence in the 20–50% of CH ratio [58]. As the CH maintains the mechanical strength of the membrane, it is suggested to complete the liposome compositions with cholesterol. In the following, 5–10–20% of the formulation weight was changed from PC to DPPE-PEG_{2000} to investigate the effect of the PEGylated phospholipid on the characteristics of the liposomes. The addition of 5% DPPE-PEG_{2000} to the PC-CH formulation caused a significant decrease in the size and the zeta potential of the vesicles due to the stabilised bilayer and the positive charge of the PEGylated phospholipid. Increasing the DPPE-PEG_{2000} concentration to 10% significantly enlarged the vesicle size; however, with further addition, the size dropped. A descending tendency was observed regarding the surface charge as well with the rise of the DPPE-PEG_{2000} concentration. Size and charge reduction were observed as well when DPPE-PEG_{2000} was added to linolenate-linolenic acid vesicles by Teo et al. [59]. The quality of the applied hydration media affects the characteristics of the liposomes as well. Vesicles made with PBS pH 7.4 (0.16 M) are significantly larger than those hydrated with saline solution. The zeta potential of the liposomes parallelly increases with the ionic strength of the hydration media due to the

forming ion layer around the vesicles. In the investigated cases, hydration with PBS pH 5.6 (0.40 M) significantly enhanced the zeta potential of the vesicles in contrast to the saline solution (0.15 M). A similar tendency was noticed by Tefas et al. applying PBS pH 4.5 and 5.0 in the preparation process of curcumin-doxorubicin-hydrochloride co-encapsulating liposomes made from with 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, N-(carbonyl-methoxypolyethyleneglycol-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine and cholesterol following the thin film hydration method [42]. In conclusion, the ionic strength of the hydration media should be considered when it is chosen for the formulation besides the required pH. Generally, 5% of the phospholipid mass used to be added to the formulations from carbohydrates as cryoprotectants. In this study, sorbitol caused a significant increase in the mean vesicle size compared to glucose and trehalose, while both sorbitol and trehalose increased the surface charge significantly. Hau et al. made quantitative observations about cryoprotectants on PC-CH liposomes [60]. The protective effect of various cryoprotectants is different. They suggest using 5% glucose, 10% sucrose, 15% mannitol, or 10% trehalose to achieve the best protective effect, as the smallest diameters for the vesicles were measured in these cases. They found that the mean vesicle diameter of liposomes prepared with glucose was the biggest, with trehalose as the smallest of these four carbohydrates. Sylvester et al. applied the QbD concept to understand the freeze-drying process more and found that combining the cryoprotectants (trehalose + mannitol) leads to better results [61]. Thus, consideration of the quality is suggested even for the cryoprotectants.

The thermal analysis has an important role in the characterisation of the liposomes due to the phase transitions of the phospholipids. The phase transition temperature is typical for the wall-forming lipids. Below the T_c value, the cholesterol mobilises the hydrocarbon chains hindering them from crystallisation that modifies the T_m and indicates a separation before the phase transition. In contrast, above the T_c value, cholesterol maintains the rigidity of the bilayer membrane [38]. Increasing the cholesterol content broadens the endotherm curve; thus, the observed phase transition lessens [45,46].

The physisorbed water content desorption ended around 100 °C in the case of all the investigated samples concluded from the TG and dTG curves. Another change could be detected in the shape of the curves at 200–225°, representing the molecular changes [39]. In total, 3–5% of the sample weight were lost during the heat treatment up to 300 °C [40,41].

The FT-IR spectra differ from type to type of wall-forming lipids. In the presented formulations, PC was the main component; thus, the FT-IR curves demonstrated two separate regions in the spectra that are typical for the phosphatidylcholine [47]: the fingerprint region was observed at ~900–600 cm^{-1}, while the C-H stretching vibrations originated mainly from the hydrocarbon chains appeared in the 3000–2800 cm^{-1} wavenumber domain. The lower wavenumber region of the spectra (below 1800 cm^{-1}) represented the polar head groups of the phospholipids. Differences among the FT-IR spectra of the samples hydrated with different hydration media were well-detectable. The typical $\upsilon_{as}(PO_2)$ and $\upsilon_s(PO_2)$ stretchings appeared in the case of the liposomes hydrated with the PBS solutions. The differing ionic strengths can cause the differences between the spectra of the samples hydrated with PBS of different pH.

5. Conclusions

This QbD-guided and RA-based study aimed to determine the CMAs and the CPPs of an "intermediate", API-free liposome formulation prepared via the thin-film hydration method and show the process of how to tighten a general initial RA for a specific case. The theoretical liposome design was followed by experimental modelling to prove the concept. The QTPP elements of these liposomal products were determined as spherical, large unilamellar vesicles (LUVs) in stable, homogeneous, monodisperse systems. The necessary CQAs that must be ensured to maintain the targeted QTPP were also collected parallel to the CMAs and the CPPs that need to be considered during the formulation design. How the screening of the factors should be done to find the most critical one is

also presented. The quality of phospholipids, the quality and quantity of surface modifiers, the ratio between the phospholipids and the cholesterol, the type of the cholesterol, the phase transition temperature, the working temperature, the quality of the hydration media, the settings of the sonication, the quality and quantity of cryoprotectants, the properties of filtration, the sterility of the formulation, the quality of the solvent, the possible addition of other additives, the dissolution step for the lipids, the storage conditions, the settings of the lyophilisation and the vacuum evaporation were found as the most highly influencing CMAs/CPPs in descending order according to their severity scores. This RA result led to an effective practical experimental design to investigate the effect of the five riskiest parameters for an intermediate formulation (the working temperature, the phosphatidylcholine–cholesterol weight ratio, the PEGylated phospholipid content, the quality of the hydration media and the cryoprotectants). The prepared liposomes were investigated via the most typical analysation techniques. The characterisation findings (vesicle size, PdI, zeta potential, DSC, TGA and FT-IR) can support and complete the results of the updated RA. A working temperature of 50–60 °C might be ideal for the investigated PC-based formulations. Applying higher proportions of phospholipids leads to smaller vesicles. The increase in the ratio of PEGylated phospholipids enlarges the size of the liposomes, but further addition causes a decrease. From the investigated compositions, the PC-DPPE-PEG_{2000}-CH 55:5:40 mass ratio fits the most to the formerly established criteria. The pH and ionic strength of the hydration media, as well as the type of the cryoprotectant, impact the liposomal formulation quality. The applied levels of the investigated factors and the number of the analytical techniques can be widened, and the compositions may be changed or completed even with APIs to meet the requirements of studies with more specific circumstances. In these cases, newly updated RAs should be established. RAs can be repeated and finalised in the later phases of the development cycle, when further or better information is available to assess the actual risk. This article aimed to provide a base for a practical decision-making system that facilitates modifying the features of the liposomes according to previously defined target values or criteria. The results of this study may help the researchers to perform a successful RA-based liposome design, set up the DoE and later a DS.

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Abbreviations

AFM	atomic force microscopy
API	active pharmaceutical ingredient
CH	cholesterol
CMAs	Critical Material Attributes
CPPs	Critical Process Parameters
CQAs	Critical Quality Attributes
DLS	dynamic light scattering
DoE	Design of Experiments
DPPE-PEG2000	1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt)
DS	Design Space
DSC	differential scanning calorimetry
DSPE–PEG_{2000}	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt)
dTG	derivative thermogravimetry
EMA	European Medicine Agency
FT-IR	Fourier-transform infrared spectroscopy
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
LUV	large unilamellar vesicles
PBS	phosphate-buffered saline
PC	L-α-phosphatidylcholine
PdI	Polydispersity Index
PE	phosphatidylethanolamine
PEG	polyethylene glycol
QbD	Quality by Design
QTPP	Quality Target Product Profile
R&D	Research and Development
RA	Risk Assessment
T_c	gel to liquid-crystalline phase transition temperature
T_g	glass transitions temperature
TGA	thermogravimetric analysis
T_m	phase transition temperature

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IV.

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Article

Quality by Design-Driven Zeta Potential Optimisation Study of Liposomes with Charge Imparting Membrane Additives

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Abstract: Liposomal formulations, as versatile nanocarrier systems suitable for targeted delivery, have a highly focused role in the therapy development of unmet clinical needs and diagnostic imaging techniques. Formulating nanomedicine with suitable zeta potential is an essential but challenging task. Formulations with a minimum ±30 mV zeta potential are considered stable. The charge of the phospholipid bilayer can be adjusted with membrane additives. The present Quality by Design-derived study aimed to optimise liposomal formulations prepared via the thin-film hydration technique by applying stearylamine (SA) or dicetyl phosphate (DCP) as charge imparting agents. This 3^2 fractional factorial design-based study determined phosphatidylcholine, cholesterol, and SA/DCP molar ratios for liposomes with characteristics meeting the formulation requirements. The polynomials describing the effects on the zeta potential were calculated. The optimal molar ratios of the lipids were given as 12.0:5.0:5.0 for the *SA-PBS pH 5.6* (optimised sample containing stearylamine) and 8.5:4.5:6.5 for the *DCP-PBS pH 5.6* (optimised sample containing dicetyl phosphate) particles hydrated with phosphate-buffered saline pH 5.6. The *SA-PBS pH 5.6* liposomes had a vesicle size of 108 ± 15 nm, 0.20 ± 0.04 polydispersity index, and +30.1 ± 1.2 mV zeta potential, while these values were given as 88 ± 14 nm, 0.21 ± 0.02, and −36.7 ± 3.3 mV for the *DCP-PBS pH 5.6* vesicles. The prepared liposomes acquired the requirements of the zeta potential for stable formulations.

Keywords: liposome; zeta potential; factorial design; optimisation; stearylamine; dicetyl phosphate

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1. Introduction

1.1. Liposomal Formulations

Liposomal formulations, lipid bilayer-built up nanocarriers, provide a modern and innovative way for drug delivery. Liposomal administration of the active pharmaceutical ingredients (API) can reduce potential side effects and provide more favourable pharmacokinetic profiles and targeted therapy [1]. The typically negatively charged or neutral, nonpolar carbon chains of the wall forming phospholipids are oriented towards each other, while the polar heads condense into a layer along with the outer and inner aqueous phases. Due to this structural design, the vesicles can encapsulate hydrophilic and lipophilic drugs [1–3]. The stability of the phospholipid bilayer is frequently enhanced by the addition of cholesterol [4]. To increase the circulation time, liposomes are also commonly PEGylated, i.e., polyethene glycol (=PEG) chains are attached to the phospholipid surface thereby inhibiting immune response phagocytosis [5]. Recent studies have also focused on immunoliposomes that bind to antibodies or antibody fragments on their surface, cationic liposomes composed of positively charged phospholipids, and stimu-

li-responsive vesicles sensitive to local environmental conditions [6,7]

Although more and more liposomal products are entering the phase of clinical trials and registration, the regulation of this area is not complete yet [8]. The liposome-based products belong to the group of non-biologically complex drugs (NBCDs). Due to the complexity and diverse clinical use of NBCDs, it is not possible to establish a general regulatory procedure for these systems; only product-specific guidelines are available [9]. The guideline of the European Medicines Agency [10] provides information on relevant clinical and non-clinical data required for the authorisation of intravenous liposomal products; however, it does not specify concrete analytical and testing strategies or criteria systems, only general principles for the evaluation of the traditional intravenous liposomal products. As even small changes in liposomes significantly affect the result parameters, a well-defined manufacturing process and optimal process control are required to ensure that the quality of the product meets the quality requirements at all times. Creating a surface charge of high absolute value, and thus the production of a long-term stable formulation and recovering the original quality of the freeze-dried samples during reconstitution, are challenging. Although liposome research has a nearly 60-year-old history [11], the proportions of compositions in the literature are still based on traditions, and the liposome recipes have not been optimised in comprehensive studies to date [12,13]. The applied compositions, the chosen production methods, and the opted parameters greatly influence the experimental results. The reason why those formulations were previously studied and how the circumstances were selected is essential for further utilisation of the results. Finding the most appropriate compositions for the purposes and achieving the best results is one of the challenges of this time.

1.2. Quality by Design-Based Design and Development

The Quality by Design (QbD) approach [14–17] and its elements are described in the guidelines of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) [18–20]. Briefly, the steps of a QbD-guided study are to determine the Quality Target Product Profile (QTPP) that describes the essential parameters of the product from the viewpoint of the patient, the clinics and the industry and that in the ideal case should be achieved. The Critical Quality Attributes (CQAs) mean the definitive list of characteristics in the formulation derived from the QTPP and related to the safety and efficacy of the product. The Critical Material Attributes (CMAs) and the Critical Process Parameters (CPPs) are related to the chosen materials and the selected production method. The results of the Risk Assessment (RA) assign the core points of the Design of Experiments (DoE), and the evaluation of the experimental findings leads to the development of the Design Space (DS). The key step of the QbD-driven development process is the RA, which assists in ranking the CQAs and CPPs based on the criticality of their impact on the targeted product quality.

The effects of factors related to the manufacturing process on product quality are known from a prior study performing an RA. The properties of the liposomes made via thin-film hydration are influenced by the presence and quality of the API, the type and proportion of the wall-forming compounds, the quality of the cryoprotectant and the hydration media, and they are affected by the applied temperature, pressure and settings of the filtration [21]. Based on the results, recommendations are available on the QTPP of an API-free liposomal carrier system [22]; however, the zeta potential needs to be investigated to characterise liposome stability further. The influencing factors on liposome properties as results of the RAs are summarised in Figure 1. The four main sections, i.e., material properties, preparation process-, carrier system-, and liposomal formulation-related factors, correspond to CMAs, CPPs, QTPP, and CQAs, respectively.

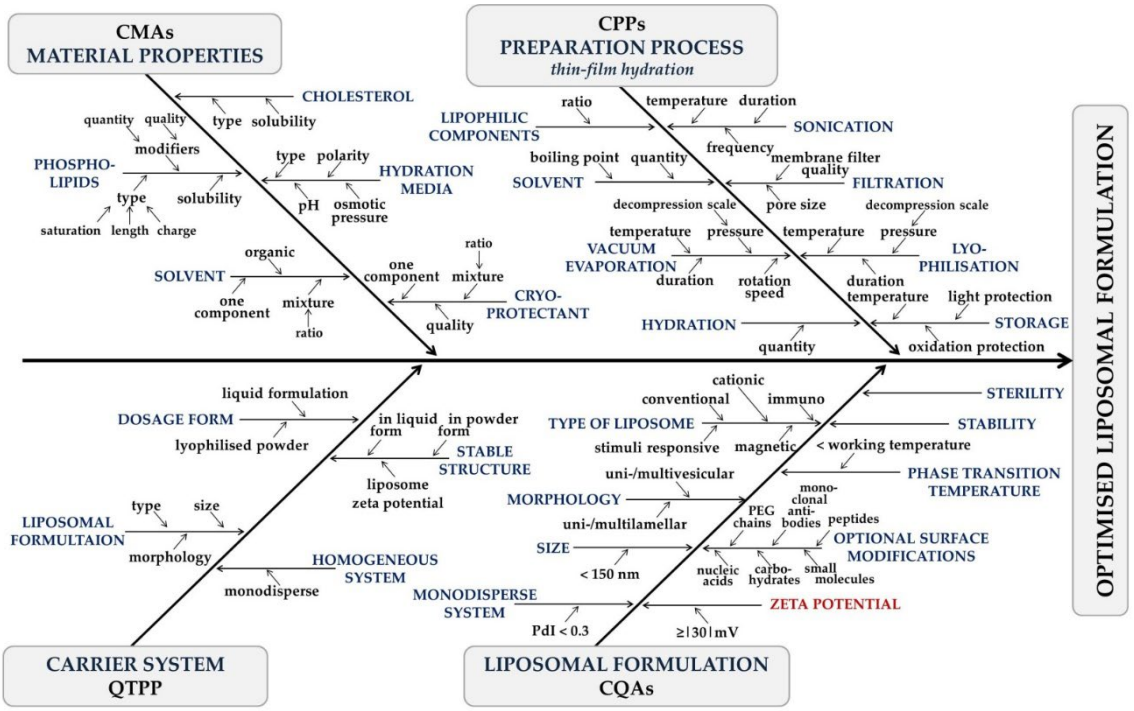

Figure 1. Factors impacting the quality of liposomes made via the thin-film hydration method. Zeta potential plays a crucial role in liposomal formulation through stability criteria. (CMAs = Critical Material Attributes, CPPs = Critical Process Parameters, QTPP = Quality Target Product Profile, CQAs = Critical Quality Attributes).

1.3. Importance of Zeta Potential

Solid surfaces can possess a non-zero surface charge due to dissociative surface groups, specifically adsorbed ions, even without an external potential bias. This so-called surface potential is neutralised by ions of opposite charge attracted to the first layer and in the surroundings of the solid, where the rigid inner Stern layer proceeds in a looser diffuse region. The boundary layer that separates the species attached to the surface and the mobile medium is the slip(ping) plane, generally ~0.2 nm from the surface [23–26]. The electrokinetic potential, i.e., the zeta potential (ζ) for a colloid system, is the electric potential at the slipping plane relative to a point in the bulk medium away from the surface. It is thus the average electrostatic potential at the hydrodynamic plane of shear [27,28]

Zeta potential characterises the electrical double layer and the nanoparticle, the colloidal formulation itself. It gives information about the stability, circulation time, protein interactions, permeability, and biocompatibility of the nanoparticles [23,25]. Since zeta potential is influenced by temperature, solvent viscosity, pH, ionic strength, and surface characteristics, even minor parameter variations can significantly change its absolute value [23]. The magnitude of the zeta potential can predict the stability of a nanoformulation. High values show highly charged particles that prevent aggregation and ensure redispersion due to repulsive electric forces, while at low zeta potential coagulation may form [29–31]. As a general rule, $\zeta \geq 30$ mV and ≤ 60 mV in absolute value is considered good and excellent stability, respectively [29,30,32]. Zeta potential ≥ ±30 mV indicates monodisperse formulations without aggregates [26], while ζ ~±20 mV are prone to have only short-term stability, and $\zeta < 5$ mV tends to aggregate rapidly [30]. Nevertheless, the zeta potential value is not the absolute sign of nanoparticle stability. These observations are made for electric stabilisation and low molecular weight surfactants only.

Furthermore, the cellular uptake of nanoparticles is influenced by their shape, size and charge, as their zeta potential affects the cell and tissue binding processes. Higher

zeta potentials lead to stronger membrane bindings and a higher level of cellular uptake [28,33]. Moreover, the protein adsorption of the nanoparticles is influenced by electrostatic interactions. Particles with positive zeta potential were found to adsorb well to proteins, while negatively charged ones did not show a significant level of binding. Protein-binding can be influenced by changing the surface charge [30].

The properties of the nano-delivery systems, i.e., circulation, release, and absorption, are also regulated by the characteristics of the nanoparticles [28]. At the liposome–cell interaction, the vesicle wall can adsorb into and fuse with the cell membrane, degrade; and then the released content can diffuse to the cytoplasm. The mechanism of the liposome–cell interaction depends on the features and charge of the liposome surface. From a maximum diameter of 150 nm vesicles, the drug content can be transported into the cell by receptor-mediated endocytosis [7]. Due to the negatively charged endothelial cell surface, tumour cells take up positively charged nanoparticles and retain longer than negative or neutral ones. Other studies showed that particles with a slightly negative zeta potential and a vesicle size of 150 nm are prone to accumulate in tumours. The electrostatic interactions between the nanocarriers and the cell membrane can be utilised for transportation through the blood-brain barrier (BBB). The negatively charged BBB cell membrane attracts particles with positive zeta potential. Reaching a suitable zeta potential is essential for effective nanomedicine, as it affects the targeted therapy, stability and drug release profile [28,30].

1.4. Modification of Zeta Potential

Phospholipids are the major components of liposomes and cell membranes. The lipid bilayer is formed due to its amphiphilic property: a hydrophilic 'head' (including a phosphate group) and a hydrophobic 'tail' (two fatty acid chains) connected via a glycol molecule. Phosphatidylcholine (PC), the most common neutral phospholipid in biological membranes, has a choline molecule in its structure as the 'head' group. The stability of the liposomes depends on the lengths and the saturation of the fatty acid chains. The more saturated chains build up the bilayer, the more stable the liposome is. The integrity of the membrane originates from its cholesterol (CH)-content [34]. Zeta potential can be modified by many factors, such as the liposome composition, charged lipids, the pH and the ionic strength of the hydration media, and the production parameters. Charged liposomes can be formed from cationic and anionic phospholipids completing the neutral lipids and causing electrostatic repulsion between the layers [35]. By incorporating various charge-inducing agents into the phospholipid bilayer of the liposome (stearylamine (octadecylamine, SA) or dicetyl phosphate (dihexadecyl phosphate, DCP)), the absolute value of the zeta potential and thus the stability of the vesicles can be increased due to electrostatic interactions [35,36]. SA gives the vesicles a positive/cationic, while DCP a negative/anionic charge, thus preventing aggregation [37]. Experimental results demonstrated the oxidative stability-enhancing effect of these substances as well. Adding cholesterol, SA, and DCP to the composition of the nanocarriers is one of the best practices to improve the stability of the formulations due to the physical stabilisation of the lipid layers [38]. Cationic, synthetic lipids can incorporate positive charges into the liposome membranes and are thus commonly used in nucleic acid delivery [34,39]. SA contains an ionisable nitrogen atom with a positive charge on physiological pH [40]. It distributes asymmetrically in the lipid bilayer, located mainly on the outer surface of the liposomes [41]. Studies on SA-nanoparticles showed increased stability, minimised drug leakage, and a controlled release profile [42]. However, cytotoxicity limits the clinical use of SA as the hydrophilic nitrogen 'head' group of the molecule interacts with certain enzymes [36,42]. Other works reported apoptosis induced by SA generating reactive oxygen species, activating protein kinase C, or enhancing the release of apoptosis-dependent proteins, and hemolysis arising from the interaction between the molecule and the negatively charged erythrocyte membrane [43,44]. Human red blood cells are less sensitive to SA; thus, the addition of small amounts can be safe [45]. DCP is a safe cosmetic ingredi-

ent, even if it has lower skin permeability than SA due to the negatively charged mammalian skin [36]. Intracerebrally administered SA-liposomes led to respiratory failure and brain damage, while DCP caused epileptic seizures and rapid death in mice [46,47]. A review study made on the immunological and toxicological effects of liposomes concluded that relatively low mol% of cholesterol and PEG is recommended for intravenous application of chemotherapeutic agents. Liposomes with zeta potential less than 30 mV should be considered for gene delivery to minimise toxicities [47]. The toxicity of the formulations can differ based on compositions, delivery routes, and the applied models; thus, they should be evaluated individually in relevant circumstances.

A detailed literature study on previously reported liposomal formulations was performed, and findings on SA and DCP-containing systems were collected in Table 1; Table 2, respectively. The applied ratios and the results varied mainly from study to study, justifying the importance of a time- and material-saving experimental design-based liposome development to optimise the necessary amount of SA and DCP.

Table 1. Composition, size, polydispersity index (PdI) and zeta potential of SA-containing formulations (PC = L-α-phosphatidylcholine: EPC = from egg yolk, SPC = from soybean, CH = cholesterol, SA = stearylamine, Span 60 = sorbitan monostearate, Tween 20 = sorbitan monolaurate/Polysorbate 20, DSPC = 1,2-distearoyl-sn-glycero-3-phosphocholine, DOPC = 1,2-dioleoyl-sn-glycero-3-phosphocholine, DOPE = 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, DSPE-PEG2000 = 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy(polyethyleneglycol)-2000], GDNF = glial cell-line derived neurotrophic factor).

Composition	Molar Ratio	Drug	Size (nm)	PdI	Zeta Potential (mV)	Source
EPC:CH:SA	1:1:3.85	ketorolac tromethamine	7060 ± -	0.43 ± -	-	Mehanna et al. [48]
SPC:CH:SA:Span 60	1:1:0.15:1	flucytosine	135 ± 12	0.27 ± -	+42.5 ± 2.1	Salem et al. [49]
SPC:SA:Tween 20	20:6.3:2.4	curcumin	252 ± 52	0.17 ± 0.01	+34.0 ± 0.6	Ternullo et al. [50]
SPC:SA:Tween 20	20:6.3:2.4	curcumin	232 ± 68	0.22 ± 0.04	+33.7 ± 1.1	Ternullo et al. [51]
EPC:CH:SA	5.5:1.0: 1.5	butamben	240 ± 65	0.22 ± -	+30.2 ± 3.9	Mura et al. [52]
EPC:CH:SA	6.6:10.3:11.13	sumatriptan	349 ± 100	0.28 ± 0.24	+37.9 ± 3.7	Villasmil-Sánchez et al. [35]
SPC:CH:SA	7:3:1.5	amphotericin B	940 ± 40	-	+28.4 ± 0.3	Soni et al. [53]
SPC:SA	2:0.5	amphotericin B	140 ± 4	0.24 ± 0.04	+32.0 ± 0.2	Mishra et al. [39]
SPC:SA	1:0.5	amphotericin B	202 ± 6	0.39 ± 0.03	+63.0 ± 0.4	Mishra et al. [39]
SPC:CH; SA	9.13:1; 5.18 mg	paclitaxel	193 ± 2	0.17 ± 0.03	+38.2 ± 3.5	Ingle et al. [37]
SPC:CH; SA	7:2; 5.00 mg	resveratrol	146 ± 10	-	+38.0 ±9.1	Jagwani et al. [41]
PC:SA	7:2	doxorubicin	148 ± -	-	+43.1 ± -	De et al. [54]
DSPC:CH:SA	7.5:2.5:0.5	prednisolone, methotrexate	159 ± 2	0.09 ± -	+6.3 ± 0.4	Verma et al. [55]
EPC:CH:SA	7.8:2.6:2.9	pemetrexed disodium	220 ± 5	0.23 ± 0.02	+22.2 ± 0.5	He et al. [45]
SPC:CH:SA	8:1:2	risperidone	209 ± 16	-	+22.4 ± 1.5	Narayan et al. [56]
SPC:CH:SA	8:1:0.25	risperidone	99 ± 7	-	+15.6 ± 1.4	Narayan et al. [56]
SPC:CH:SA	7:3:1.1	monensin	121 ± 20	0.25 ± 0.01	+43.9 ± 0.9	Rajendran et al. [57]
DOPC:CH:SA	10:6:1	GDNF	149 ±11	-	+30.0 ± 3.0	Migliore et al. [58]
DOPC:CH:SA	10:6:1	ovalbumin	299 ± 26	-	+19.0 ± 1.5	Migliore et al. [59]
DOPE:SPC:CH:SA	10:45:29:16	-	95 ± 9	0.24 ± 0.03	+52.8 ± 3.7	Vhora et al. [44]
SPC:CH:SA:DSPE-PEG$_{2000}$	11:7:0.6:1.4	-	209 ± 2	-	+48.7 ± 4.3	Tran et al. [60]
SPC:CH:SA	7:3:1.1	-	77 ± 2	0.21 ± -	+32.9 ± 2.1	Sharma et al. [61]
SPC:SA	7.3:1	-	81 ± 6	0.24 ± 0.02	+17.5 ± 1.8	Caddeo et al. [62]
PC:SA	7:2	-	146 ± -	0.20 ± -	+52.0 ± -	De et al. [63]
SPC:SA	3:1	-	140 ± 49	-	+11.4 ± 0.4	Lotosh et al. [64,65]
EPC:CH:SA	12:5:5	-	108 ± 15	0.20 ± 0.04	+30.1 ± 1.2	***SA-PBS pH 5.6***

Table 2. Composition, size, polydispersity index (PdI) and zeta potential of DCP-containing formulations (PC = L-α-phosphatidylcholine: EPC = from egg yolk, SPC = from soybean, CH = cholesterol, DCP = dicetylphosphate, Span 60 = sorbitan monostearate, Tween 80 = sorbitan monooleate/Polysorbate 80, DSPE-PEG2000 = 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy(polyethyleneglycol)-2000], DPPE = 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine, FITC = fluorescein isothiocyanate).

Composition	Molar Ratio	Drug	Size (nm)	PdI	Zeta Potential (mV)	Source
EPC:CH:DCP	1:1:3.85	ketorolac tromethamine	8350 ± -	0.45 ± -	-	Mehanna et al. [48]
PC:CH:DCP	1:1:0.7	tretinoin	318 ± 3	0.43 ± -	−41.2 ± 1.2	Rahman et al. [66]
SPC:CH:DCP	6:1:1.5	silymarin	756 ± -	0.61 ± -	−77.3 ± -	Kumar et al. [67]
EPC:CH:DCP	6.6:10.3:5.49	sumatriptan	549 ± 10	0.37 ± 0.09	−68.1 ± 0.4	Villasmil-Sánchez et al. [35]
SPC:CH:DCP:Span 60	1:1:0.1:1	flucytosine	159 ± 5	0.26 ± -	−59.1 ± 1.7	Salem et al. [49]
SPC:CH:DCP:Tween 80	9:3:1:1	5-fluorouracil	108 ± 11	0.31 ± 0.05	−16.3 ± 1.5	Alomrani et al. [68]
EPC:CH:DCP:DSPE-PEG$_{2000}$: DPPE	7:2:1:1:0.025	FITC-dextran	116 ± -	0.12 ± -	−29.0 ± -	Togami et al. [69]
EPC:CH:DCP:DSPE-PEG$_{2000}$: DPPE	7:2:1:1:0.025	rhodamine B	125 ± -	0.09 ± -	−32.0 ± -	Togami et al. [69]
SPC:CH:DCP: DSPE-PEG$_{2000}$:	11:7:1.4:0.6	-	191 ± 4		−45.1 ± 2.5	Tran et al. [60]
SPC:CH:DCP	15:8:1	-	195 ± 5	0.28 ± -	−47.0 ± 1.0	Calvo et al. [70]
SPC:CH:DCP	10:4:1	-	146 ± 6		−18.6 ± 0.5	Ethemoglu et al. [71]
EPC:CH:DCP	7:2:1	-	134 ± 4	0.12 ± 0.03	−49.4 ± 3.5	Togami et al. [72]
EPC:CH:DCP	8.5:4.5:6.5	-	88 ±1	0.21 ± 0.02	−36.7 ± 3.3	***DCP-PBS pH 5.6***

In this research, the 3^2 fractional factorial design was chosen as the material- and time-effective approach to improve vesicle stability through zeta potential optimisation. The goal was to develop formulations with vesicle size under 150 nm, polydispersity index lower than 0.30 and absolute zeta potential higher than 30 mV. For this outcome, parameters determining zeta potentials were identified. Based on the preliminary risk assessment, the ratios between the wall-forming agents (PC, CH) and the charge imparting membrane additives (SA, DCP) affected the liposomal charge and thus were chosen as independent variables.

2. Materials and Methods

2.1. Materials

Liposomes were made from the following materials: cholesterol (CH) (from Molar Chemicals Kft., Budapest, Hungary); L-α-phosphatidylcholine (1,2-diacyl-sn-glycero-3-phosphocholine) (PC) from egg yolk (60 *w*/*w*% purity); and octadecylamine (= stearylamine, SA), or dihexadecyl phosphate (= dicetylphosphate, DCP) (all purchased from Sigma-Aldrich Chemie GmbH, Munich, Germany). The lipids were dissolved in ethanol 96% (*v*/*v*) (Molar Chemicals Kft., Budapest, Hungary). Phosphate-buffered saline pH 7.4 (PBS pH 7.4) (ionic strength: 0.16 M), pH 5.6 (PBS pH 5.6) (ionic strength: 0.40 M) and sodium chloride physiological solution (saline solution) (ionic strength: 0.15 M, pH 5.5) were used as hydration media. The composition of these solutions are the followings: *PBS pH 7.4*: 8.0 g/L NaCl, 0.20 g/L KCl, 1.44 g/L $Na_2HPO_4 \times 2 H_2O$, 0.12 g/L KH_2PO_4; *PBS pH 5.6*: 0.65 g/L K_2HPO_4, 8.57 g/L KH_2PO_4; *physiological saline solution*: 9.0 g/L NaCl dissolved in purified water. The undermentioned materials were used to make these hydration media: sodium chloride (NaCl), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4) (Molar Chemicals Kft., Budapest, Hungary), disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \times 2 H_2O$), and dipotassium phosphate (K_2HPO_4) (Spektrum-3D Kft., Debrecen, Hungary).

2.2. Methods

2.2.1. Factorial Design-Based Experiment Design for Zeta Potential Optimisation

After getting a profound knowledge of the main factors influencing the quality of the liposomal products [24,25], we aimed to prepare stable formulations with zeta potential above 30 mV in absolute value. We have chosen to apply two membrane additives, SA and DCP, and experimentally determine the optimal ratios. The 3^2 fractional factorial design was used as an experimental design to optimise the zeta potential values. The selected independent variables were the molar quantities of the liposome components: PC, CH, and SA/DCP. As shown in Table 3, these experimental factors were systematically varied at 3 levels and 9 runs in the design. The molar value of PC ranged from 7.5 to 12.5 mmol, of CH from 3.5 to 5.5 mmol, while the amounts of the membrane additives (SA/DCP) were adjusted between 3 and 9 mmol. The optimal component ratios were further investigated by altering the quality of the hydration media. Each composition was prepared in triplicate for parallel measurements. The effects of these independent factors on the vesicle size (Z-average), polydispersity index (PdI) and zeta potential were investigated before lyophilisation. In the case of zeta potential, one-one quadratic response surface was investigated, and the second-order polynomial models were constructed using TIBCO Statistica® 13.4 software (Statsoft Hungary, Budapest, Hungary). The relationship between the variables in the response could be analysed using this second-order Equation:

$$Y = \beta_0 + \beta_1 x_1 + \beta_{11} x_1^2 + \beta_2 x_2 + \beta_{22} x_2^2 + \beta_3 x_3 + \beta_{33} x_3^2, \quad (1)$$

where Y is the response variable; β_0 is a constant; β_1, β_2, and β_3 are linear coefficients; and β_{11}, β_{22}, and β_{33} are quadratic coefficients. The one-way analysis of variance (ANOVA) statistical analysis was carried out to evaluate the significance of the variables. The results were evaluated according to their p-value when variables with p less than 0.05 at a 95% confidence level were considered significant. Response surface plots for zeta potential were plotted according to the regression model for SA/DCP.

Table 3. Composition of the 3^2 fractional factorial design with the molar ratio of PC = L-α-phosphatidylcholine, CH = cholesterol, SA = stearylamine or DCP = dicetyl phosphate.

Run	Composition (Molar Ratio)		
	PC	**CH**	**SA/DCP**
1	7.5	3.5	3.0
2	7.5	4.5	9.0
3	7.5	5.5	6.0
4	10.0	3.5	9.0
5	10.0	4.5	6.0
6	10.0	5.5	3.0
7	12.5	3.5	6.0
8	12.5	4.5	3.0
9	12.5	5.5	9.0

2.2.2. Preparation of Liposomes

The liposomes were prepared via the thin-film hydration method [11] with modifications based on our prior findings [21]. The ethanol was evaporated from the alcoholic compositions (Table 3) at 150 mbar and 60 °C in a Rotavapor® R-210/215 (BÜCHI Labortechnik AG, Flawil, Switzerland) rotary evaporator at 25 rpm rotation speed. The lipid film was hydrated, and the formulations were subjected to a 30-min ultrasonication (Elmasonic S 30 H ultrasonic bath (Elma Schmidbauer GmbH, Singen, Germany). The liposomes were vacuum filtered (Rocker 400 oil-free vacuum pump, Rocker Scientific Co., Ltd. New Taipei City, Taiwan) using a 0.45 μm (nylon membrane disk filter 47 mm, Labsystem Kft., Budapest, Hungary), then a 0.22 μm membrane-filter (Ultipor® N66 ny-

lon 6.6 membrane disk filter 47 mm, Pall Corporation, New York, NY, USA). The obtained samples were immediately investigated for vesicle size, polydispersity and zeta potential in a liquid state, then lyophilised for further investigations (SanVac CoolSafe freeze dryer, LaboGene™, Lillerød, Denmark). During lyophilisation, the temperature was gradually decreased from +25 °C to −40 °C at atmospheric pressure, and then the pressure was reduced to 0.01 atm. The samples were dried for 8–10 h before the temperature, and the pressure was increased step by step to +25 °C and 1 atm, respectively. The lyophilised samples were stored at 2–8 °C.

2.2.3. Characterisation of Liposomes

Vesicle Size and Zeta Potential Analysis

The vesicle size (expressed in Z-average) and the polydispersity index (PdI) of the liquid liposome formulations were measured using the dynamic light scattering (DLS) technique. The measurements were carried out using a Malvern Zetasizer Nano ZS system (Malvern Panalytical Ltd., Malvern, Worcestershire, UK) equipped with a 633 nm wavelength laser from 1 mL of samples in folded capillary zeta cells (Malvern Panalytical Ltd., Malvern, Worcestershire, UK) at 25 °C. DLS measurements (size, PDI and zeta potential) were performed before lyophilisation and after filtration in all cases.

Atomic Force Microscopy (AFM)

Atomic force microscopy (AFM) images of liposomes were obtained under ambient conditions using the tapping mode of an NT-MDT SolverPro Scanning Probe Microscope (NT-MDT, Spectrum Instruments, Moscow, Russia) from one drop of the formulations applied on a freshly cleaved mica surface (Muscovite mica, V-1 quality, Electron Microscopy Sciences, Washington, DC, USA). AFM tips (type PPP-NCHAuD-10, thickness: 4.0 μm, length: 125 μm, width: 30 μm, nominal radius of curvature: 2 nm; NanoWorld AG, Neuchâtel, Switzerland) were applied for the measurements. The non-contact silicon cantilevers had a typical force constant of 42 N/m and a resonance frequency of 330 kHz.

Transmission Electron Microscopy (TEM)

The size of the liposomes was determined by transmission electron microscopy (TEM). The TEM images were made with an FEI Tecnai G^2 X-Twin HRTEM microscope (FEI, Hillsboro, OR, USA) using an accelerating voltage of 200 kV. The TEM measurements were performed after the lyophilisation. Suspensions were prepared from the formulations with ethanol and then dropped onto a carbon film-coated 3 mm diameter copper grid.

Thermal Analysis

Differential scanning calorimetry (DSC) measurements (Mettler-Toledo DSC 3^+ $Star^e$ System DSC analyser, Mettler-Toledo International Inc., Columbus, OH, USA) were performed to study the thermodynamic state of the liposomes in the temperature range of 10–65 °C at 2 °C/min heating rate. Phase transition (T_m) and glass transition (T_g) temperatures were determined using 6–10 mg of the freeze-dried samples in hermetically sealed aluminium sample pans under a 150 mL/min constant argon flow. Thermogravimetric analysis (TGA) was done in a Mettler-Toledo TGA/DSC 1 thermogravimetric analyser (Mettler-Toledo International Inc., Columbus, OH, USA). In each run, 8–10 mg of the lyophilised samples was heated in aluminium pans at a temperature range of 25–300 °C at a 10 °C/min heating rate, and the mass changes were recorded under dry nitrogen. Empty aluminium pans were used as a blank, and data were normalised to the weight of the sample. The DSC and TGA curves were evaluated using the $STAR^e$ 9.30 software (Mettler-Toledo International Inc., Columbus, OH, USA).

Fourier Transform Infrared Spectroscopy (FT-IR)

The interactions between the compounds of the liposome were investigated by mid-infrared (MIR) spectroscopy using a Thermo Nicolet Avatar 330 FT-IR spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Spectra were recorded on the freeze-dried powder samples in the 4000–400 cm^{-1} wavenumber range with 4 cm^{-1} spectral resolution in absorbance mode. Samples were prepared using a hydraulic tablet press by compressing the lyophilised powders into pellets with potassium bromide (KBr) powder at 10 kN for 2 min (Specac Ltd., Orpington, UK). Pure KBr pellets were used as references.

Raman Spectroscopy

Raman spectra were recorded using a Bruker Senterra II Raman microscope (Bruker Scientific Instruments, Billerica, MA, USA) in 180° reflection geometry in the 400–2000 cm^{-1} Raman shift region at 1.5 cm^{-1} resolution. The 785 nm excitation source operated at 50 mW. In each measurement, 5 spectra were averaged with 10 s integration time.

Residual Ethanol measurement via Gas Chromatography-Mass Spectrometry (GC-MS)

The amount of residual ethanol in the samples was determined using a Shimadzu GCMS-QP2010 SE gas chromatograph-mass spectrometer (Shimadzu Corporation, Kyoto, Japan) equipped with a Zebron ZB-5MSi column (Phenomenex, Torrance, CA, USA). The initial oven temperature was 80 °C for 2 min, which was then increased to 180 °C at 20 °C/min and held at 180 °C for 2 more minutes. The mass spectra were recorded in continuous scans from 0.5 to 1.6 min in the 25–46 m/z region. For the measurements, 1 mg/L sample solutions were made in toluene, and 5 µL aliquots were injected in each run. The system was calibrated using a 0.01 mmol/L ethanol solution in toluene.

2.2.4. Physical Stability Studies

The liquid samples were investigated for stability issues via DLS measurements and zeta potential analysis weekly for a month. The physical stability of the freeze-dried nanoparticles was investigated according to the circumstances of the storage conditions described in the ICH Q1A (R2) guideline [73] for accelerated stability tests. The presented results refer to the samples stored at 40 ± 2 °C and 75 ± 5% relative humidity for 3 months. DSC, TGA, FT-IR and Raman studies were done on the stored samples.

2.2.5. Statistical Analysis

Data analysis and graphs were made in Microsoft® Excel® (Microsoft Office Professional Plus 2013, Microsoft Excel 15.0.5023.100, Microsoft Corporation, Redmond, WA, USA), OriginPro® 8.6 (OriginLab® Corporation, Northampton, MA, USA) and JMP® 13 (SAS Institute, Cary, NC, USA). One-way ANOVA statistical analysis was performed using the TIBCO Statistica® 13.4 software (Statsoft Hungary, Budapest, Hungary). All experiments were performed in triplicates, and the corresponding mean and standard deviations were indicated.

3. Results

3.1. Factorial Design-Based Experimental Design for Zeta Potential Optimisation

After thoroughly reviewing the available literature on the charge impairing membrane additives (Tables 1 and 2), the 3^2 fractional factorial design was chosen for the liposome production optimisation. The molar ratio between the wall-forming lipids (PC, CH) and the special additives (SA/DCP) was examined in the optimisation study. The liposome samples were prepared via the thin-film hydration method with three independent parallels and investigated for the primary outcomes: vesicle size (Z-average), polydispersity index, and zeta potential (collected in Table 4). Only the samples hydrated

with PBS pH 5.6 reached the primary set goals; thus, those results are presented in detail (*SA-PBS pH 5.6* and *DCP-PBS pH 5.6* samples).

Table 4. Responses of the 3^2 fractional factorial design studied on liposomes hydrated with PBS pH 5.6 (PC = L-*α*-phosphatidylcholine, CH = cholesterol, SA = stearylamine, DCP = dicetyl phosphate). Results are expressed in mean ± standard deviation from three independent parallels.

Run	Composition (Molar Ratio)			Responses		
	PC	CH	SA	Vesicle Size (nm)	Polydispersity Index	Zeta Potential (mV)
1	7.5	3.5	3.0	121 ± 28	0.22 ± 0.02	+22.0 ± 7.8
2	7.5	4.5	9.0	106 ± 21	0.23 ± 0.03	+17.6 ± 3.4
3	7.5	5.5	6.0	116 ± 14	0.23 ± 0.02	+24.6 ± 1.4
4	10.0	3.5	9.0	93 ± 6	0.22 ± 0.03	+25.0 ± 3.5
5	10.0	4.5	6.0	113 ± 16	0.23 ± 0.06	+25.8 ± 3.7
6	10.0	5.5	3.0	112 ± 7	0.16 ± 0.01	+26.6. ± 2.7
7	12.5	3.5	6.0	111 ± 6	0.19 ± 0.03	+26.3 ± 1.2
8	12.5	4.5	3.0	109 ± 7	0.17 ± 0.03	+26.6 ± 0.8
9	12.5	5.5	9.0	100 ± 17	0.17 ± 0.01	+27.1 ± 2.8
Run	**Composition (molar ratio)**			**Responses**		
	PC	**CH**	**DCP**	**Vesicle Size (nm)**	**Polydispersity Index**	**Zeta Potential (mV)**
1	7.5	3.5	3.0	98 ± 11	0.20 ± 0.03	−29.9 ± 1.6
2	7.5	4.5	9.0	82 ± 16	0.24 ± 0.02	−29.6 ± 3.4
3	7.5	5.5	6.0	108 ± 9	0.21 ± 0.04	−32.5 ± 6,5
4	10.0	3.5	9.0	87 ± 16	0.24 ± 0.03	−32.6 ± 2.7
5	10.0	4.5	6.0	93 ± 23	0.23 ± 0.03	−29.7 ± 6.2
6	10.0	5.5	3.0	119 ± 25	0.21 ± 0.07	−29.7 ± 3.3
7	12.5	3.5	6.0	95 ± 8	0.18 ± 0.03	−29.2 ± 3.2
8	12.5	4.5	3.0	104 ± 25	0.18 ± 0.02	−27.6 ± 1.3
9	12.5	5.5	9.0	105 ± 2	0.18 ± 0.02	−17.7 ± 3.1

The results were analysed by the TIBCO Statistica® 13.4 software, and polynomial equations were generated to individually describe the main and the interaction effects of the independent variables on the dependent factor. The relationships between the variables were investigated and described on the zeta potential (Y) according to the ANOVA and regression analysis of the data. As all the size and PdI results of the compositions hydrated with PBS pH 5.6 fulfilled the acceptance criteria of 150 nm and 0.3 PdI, respectively, the impact of the experimental factors was analysed only on the surface charge of the liposomes. Due to the limitations of the factorial plan, the equations provide only approximate results. The material quality limits the nominal maximum point of the response surface curve; thus, the experimental results are expectedly under the predicted values. The importance is in the effect of the coefficients indicating the changes in the responses.

The relationship of the variables on the zeta potential (Y) in the case of the SA-containing formulations could be described with the following Equation:

$$Y(SA) = 24.622 + 2.633x_1 + 0.883x_1^2 + 0.833x_2 - 0.967x_2^2 - 0.917x_3 + 0.708x_3^2 \quad (2)$$

The regression coefficient $R^2 = 0.920$ showed a good correlation for the surface plot. The molar ratio between PC (x_1), CH (x_2) and SA (x_3) has no significant effect on the surface charge ($0.05 < p$). Zeta potential increases with positive coefficients (x_1, x_1^2, x_2, x_3^2) of the independent variables in Equation (2), while negative coefficients (x_2^2, x_3) have the opposite effect. Liposomes with SA and DCP have positive and negative zeta potential, respectively. The zeta potential (Y) in the DCP-containing formulation is given as follows:

$$Y(DCP) = -29.833 + 1.250x_1 - 0.625x_1^2 + 0.300x_2 + 0.650x_2^2 - 0.450x_3 - 0.475x_3^2 \quad (3)$$

A high correlation ($R^2 = 0.984$) was found in this case as well. As with the SA formulation, we found no significant effect of the PC (x_1), CH (x_2) and DCP (x_3) molar ratios on zeta potentials ($0.05 < p$). As the DCP liposomes possess a negative charge, the negative coefficients (x_1^2, x_3, x_3^2) have favourable effects on the outcome values. Positive coefficients (x_1, x_2, x_2^2) decrease the absolute zeta potential.

3D response surface plots visualise the main and interaction effects of two factors at fixed values of the others. The contour plots in Figure 2 show the effect of the PC:SA (A) and PC:DCP (B) molar ratios on the vesicle zeta potential by fixing one variable at a certain level. There is no factor with linearly or quadratically significant effects on zeta potential, and the optimised compositions were deduced from the contour plot of the design space for the SA (dark red) and the DCP (dark green) containing samples: PC:CH:SA = 12.0:5.0:5.0 molar ratio for SA, and PC:CH:DCP = 8.5:4.5:6.5 molar ratio for DCP-liposomes. The resulted liposome forming agent concentrations yielded liposomes where size (<150 nm), polydispersity (PdI < 0.3), and absolute zeta potential ($|\zeta| > 30$ mV) fall in the required parameter regime for the samples hydrated with PBS pH 5.6. The detailed characterisation was done on these optimised formulations.

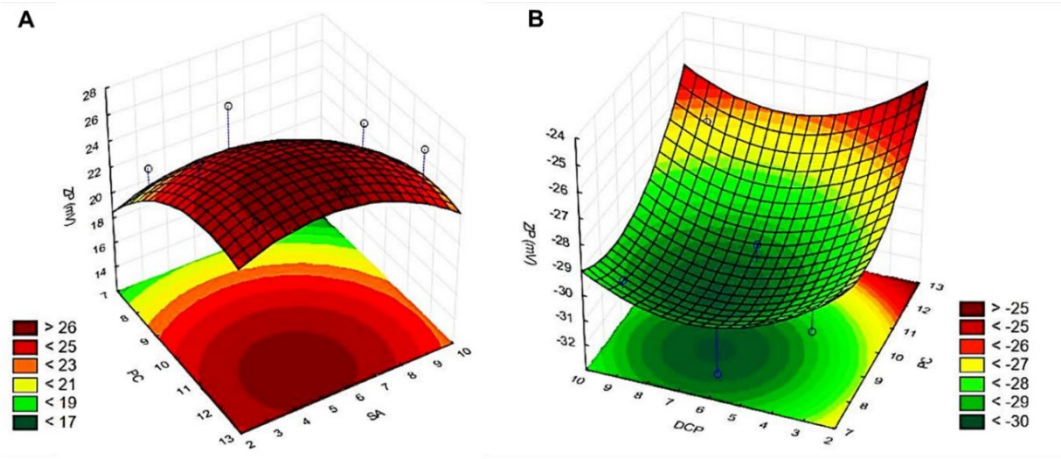

Figure 2. Three-dimensional surface plots of the effect of independent variables on the zeta potential in the 3^2 fractional factorial design for the compositions made with the membrane additives: stearylamine (**A**) and dicetyl phosphate (**B**) and hydrated with PBS pH 5.6 (PC = L-α-phosphatidylcholine, SA = stearylamine, DCP = dicetyl phosphate).

3.2. Characterisation of Liposomes

3.2.1. Vesicle Size and Zeta Potential Analysis

Among the CQAs, the average vesicle size, the polydispersity index and the zeta potential have the highest impacts on the quality of a stable liposome formulation. The optimised ratios between the liposome components were studied by applying PBS pH 5.6, pH 7.4 and saline solution as hydration media (Table 5). The acquired SA-containing samples hydrated with PBS pH 5.6 (*SA-PBS pH 5.6*) and saline solution (*SA-saline sol.*) were significantly smaller ($p < 0.05$ in both cases) than the one made with PBS pH 7.4 (*SA-PBS pH 7.4*) (108 ± 15 nm; 105 ± 18 nm; and 134 ± 24 nm, respectively). Significantly smaller-sized ($p < 0.05$) vesicles were found as well in the case of the DCP-based samples hydrated with PBS pH 5.6 (*DCP-PBS pH 5.6*) (88 ± 14 nm) than with saline solution (*DCP-saline sol.*) (120 ± 10 nm). The uniformity of the vesicles is in the acceptable range for a monodisperse formulation in the case of the lipid-based nanocarrier systems when the PdI value is less or equal to 0.30 [74] and was met in all cases. The zeta potential values were in the acceptance range, higher than 30 mV in absolute value, only in the case when the samples were hydrated with PBS pH 5.6. The measurements indicated significantly

more positive ($p < 0.01$ in both cases) zeta potential for the *SA-PBS pH 5.6* sample (+30.1 ± 1.2 mV) than for the *SA-saline sol.* (+13.6 ± 5.3 mV) or the *SA-PBS pH 7.4* (+5.2 ± 2.2) ones. A significant difference ($p < 0.05$) was detected between the zeta potential values presented by the *SA-saline sol.* and the *SA-PBS pH 7.4* samples as well. These values were significantly more negative ($p < 0.01$) in the case of the *DCP-PBS pH 5.6* (−36.7 ± 3.3 mV) sample than in those made with the saline solution (−19.8 ± 2.0 mV) or the PBS pH 7.4 made ones (−19.8 ± 2.0 mV). These results prove the relation between the ionic strength of the hydration media and the zeta potential value of the produced liposomes: the absolute zeta potential value increases with the ionic strength [22,75]. The highest absolute zeta potentials were measured in the samples made with PBS pH 5.6 (ionic strength: 0.40 M).

Table 5. Results of the dynamic light scattering measurements of the optimised formulations (PC=L-*α*-phosphatidylcholine, CH = cholesterol, SA = stearylamine, DCP = dicetyl phosphate). Results are expressed in mean ± standard deviation from three independent parallels.

Sample	Composition (Molar Ratio)			Responses		
	PC	CH	SA/DCP	Vesicle Size (nm)	Polydispersity Index	Zeta Potential (mV)
SA-PBS pH 5.6	12.0	5.0	5.0	108 ± 15	0.20 ± 0.04	+30.1 ± 1.2
SA-PBS pH 7.4				134 ± 24	0.22 ± 0.07	+5.2 ± 2.2
SA-saline sol.				105 ± 18	0.20 ± 0.10	+13.6 ± 5.3
DCP-PBS pH 5.6	8.5	4.5	6.5	88 ± 14	0.21 ± 0.02	−36.7 ± 3.3
DCP-PBS pH 7.4				112 ± 11	0.22 ± 0.02	−23.5 ± 1.6
DCP-saline sol.				120 ± 10	0.21 ± 0.05	−19.8 ± 2.0

All of the critical product parameters of the liposomes, such as the vesicle size (108 ± 15 nm; 88 ± 14 nm for the *SA-PBS pH 5.6* and the *DCP-PBS pH 5.6* samples, respectively), the low PdI (0.20 ± 0.04; 0.21 ± 0.02) that indicated monodisperse size distribution and the zeta potential (+30.1 ± 1.2 mV; −36.7 ± 3.3 mV) met the requirements of the nanosized drug delivery systems in the case of the liposomes made with PBS pH 5.6 (Table 5). The positively charged vesicles were larger than the negatively charged counterparts, which can be explained by the spacing difference between the bilayers and the bulkiness of the charge imparting membrane additives [35,48,53]. The *SA-PBS pH 5.6* formulation had significantly larger ($p < 0.05$) vesicles than the *DCP-PBS pH 5.6* ones, while the DCP-containing formulation reached significantly higher ($p < 0.05$) zeta potential in absolute value.

Further conclusions could be drawn from examining the formulation compositions. The optimisation was done in molar ratio; using weight ratio allows the direct comparison with previous findings (Table 6). PC:CH = 60:40 and 80:20 weight ratios were found as bests during former research on the topic of optimal phospholipid-cholesterol ratio for liposome formation [21]. The weight ratios of PC in the PC:CH:SA = 12.0:5.0:5.0 and PC:CH:DCP = 8.5:4.5:6.5 molar ratio formulations were essentially the same, i.e., 60 weight units (59.9 and 60.3 in the PC:CH:SA and the PC:CH:DCP compositions, respectively), while 80 weight units were found for the PC:CH ratio alone (82.7 and 78.9 in the PC:CH:SA and the PC:CH:DCP compositions, respectively) in good agreement with the previous results. Investigating the samples made with PBS pH 5.6, the DLS measurements indicated that the addition of SA and DCP to the PC-CH compositions resulted in decreased vesicle size (Figure 3) along with increased zeta potential values: the Tukey inference was $p < 0.05$ for the *SA-PBS pH 5.6* PC-CH-60-40 relationship and $p < 0.01$ for the other relations between the *SA-PBS pH 5.6*, *DCP-PBS pH 5.6*, *PC-CH-60-40* and *PC-CH-80-20* samples. The absolute zeta potential increased significantly with $p < 0.01$ inference for all pairs.

Table 6. Results of the dynamic light scattering measurements of the SA-, and DCP-containing and SA-, and DCP-free formulations made with PBS pH 5.6 indicating their compositions (PC = L-α-phosphatidylcholine, CH = cholesterol, SA = stearylamine, DCP = dicetyl phosphate). Results are expressed in mean ± standard deviation from three independent parallels.

	SA-PBS pH 5.6	**DCP-PBS pH 5.6**	**PC-CH-60-40**	**PC-CH-80-20**
	PC : CH: SA	**PC : CH : DCP**	**PC : CH**	**PC : CH**
Molar ratio	12.0 : 5.0 : 5.0	8.5 : 4.5 : 6.5	1 : 1.32	2.01 : 1
Molar % (n/n%)	54.5 : 22.75 : 22.75	43.6 : 23.1 : 33.3	43.1 : 56.9	66.8 : 33.2
Weight ratio	**59.9** : 12.5 : 8.8	**60.3** : 16.1 : 32.8	**60.0** : 40.0	80.0 : 20.0
Weight % (*w*/*w*%)	73.8 : 15.4 : 10.8	55.2 : 14.7 : 30.1	60.0 : 40.0	80.0 : 20.0
PC:CH weight ratio	**82.7** : 17.3	**78.9** : 21.1	60.0 : 40.0	**80.0** : 20.0
Vesicle size (nm)	108 ± 15	88 ± 14	151 ± 28	172 ± 44
Polydispersity index	0.20 ± 0.04	0.21 ± 0.02	0.18 ± 0.07	0.24 ± 0.03
Zeta potential (mV)	+30.1 ± 1.2	−36.7 ± 3.3	−9.0 ± 2,4	−8.9 ± 1.3

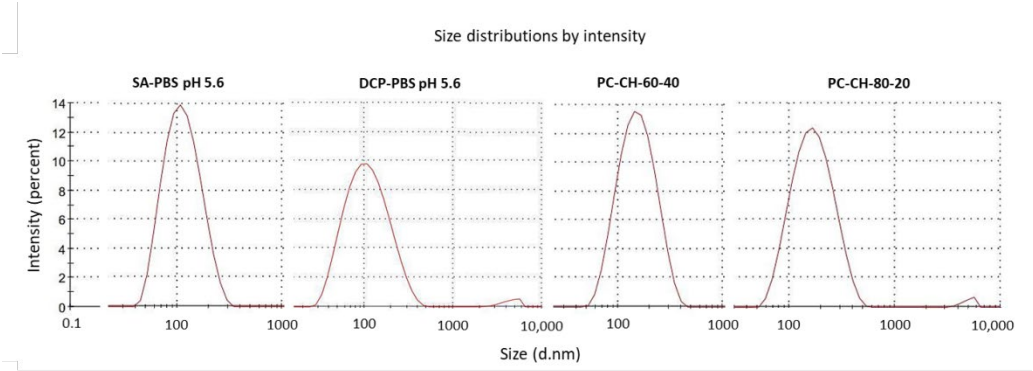

Figure 3. Size distribution results of the SA- and DCP-containing (*SA-PBS pH 5.6*; *DCP-PBS pH 5.6*) and SA- and DCP-free formulations (*PC-CH-60-40*; *PC-CH-80-20*) made with PBS pH 5.6 measured via the dynamic light scattering technique.

3.2.2. Physical Stability Studies

Clear formulations were obtained with a blueish opalescence indicating nanoscale colloidal systems. DLS measurements and zeta potential analysis were first done on fresh samples and then repeated weekly for a month. Measurement results are presented in Figure 4. The formulations kept their characteristics in the liquid state within two weeks (results are shown in Table 7); thus, we considered them stable for that period. By the end of the fourth week, the surface charge of the vesicles had significantly decreased ($p < 0.05$ for *SA-PBS pH 5.6* and $p < 0.01$ for *DCP-PBS pH 5.6*).

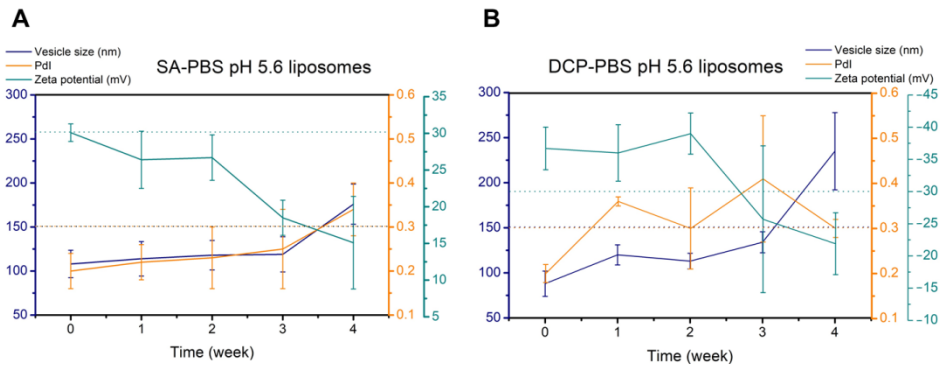

Figure 4. 4-week stability tests in the optimised formulations hydrated with PBS pH 5.6 measured by dynamic light scattering: *SA-PBS pH 5.6* (**A**) and *DCP-PBS pH 5.6* (**B**).. The changes in vesicle size, polydispersity and zeta potential were followed. Results are from three independent parallels.

Table 7. 2-week stability tests in the optimised formulations hydrated with PBS pH 5.6 measured by dynamic light scattering. Changes in vesicle size, polydispersity and zeta potential were followed. Results are from three independent parallels.

2-Week Stability	SA-PBS pH 5.6	DCP-PBS pH 5.6
Vesicle size (nm)	118 ± 17	113 ± 9
Polydispersity index	0.23 ± 0.07	0.30 ± 0.09
Zeta potential (mV)	+26.7 ± 3.1	−39.0 ± 3.2

Possible changes in the physical stability of the freeze-dried optimised samples made with PBS pH 5.6 were checked after 3 months of storage under the circumstances required for the accelerated stability tests (*SA-PBS pH 5.6-stab.*; *DSC-PBS pH 5.6-stab.*). The performed DSC, TGA, FT-IR and Raman studies indicated stable structures during the investigated period and were presented in detail in the corresponding subsections.

3.2.3. Transmission Electron Microscopy (TEM)

Representative TEM images are seen in Figure 5. The average liposome size in the optimised samples hydrated with PBS pH 5.6 is assessable as 100–120 nm.

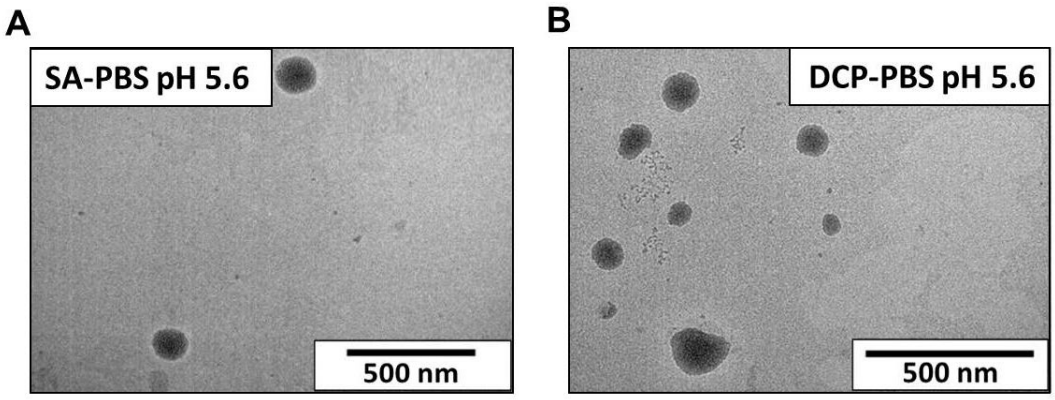

Figure 5. Transmission electron microscopy images of the optimised liposome samples *SA-PBS pH 5.6* (**A**) and *DCP-PBS pH 5.6* (**B**).

3.2.4. Atomic Force Microscopy (AFM)

The developed liquid liposomal formulations were converted into a solid phase product via lyophilisation. AFM measurements provided a three-dimensional surface profile of the optimised samples.

Figure 6 illustrates the AFM records of the *SA-PBS pH 5.6* (Figure 6A) and the *DCP-PBS pH 5.6* (Figure 6B) samples showing homogeneous size distribution with a mean vesicle size of around 100 nm. The pictures of the membrane additive-free PC- and CH-containing two samples, *PC-CH-60-40* (Figure 6C) and *PC-CH-80-20* (Figure 6D), show larger sizes between 150–180 nm. These images support the information obtained from the DLS measurements.

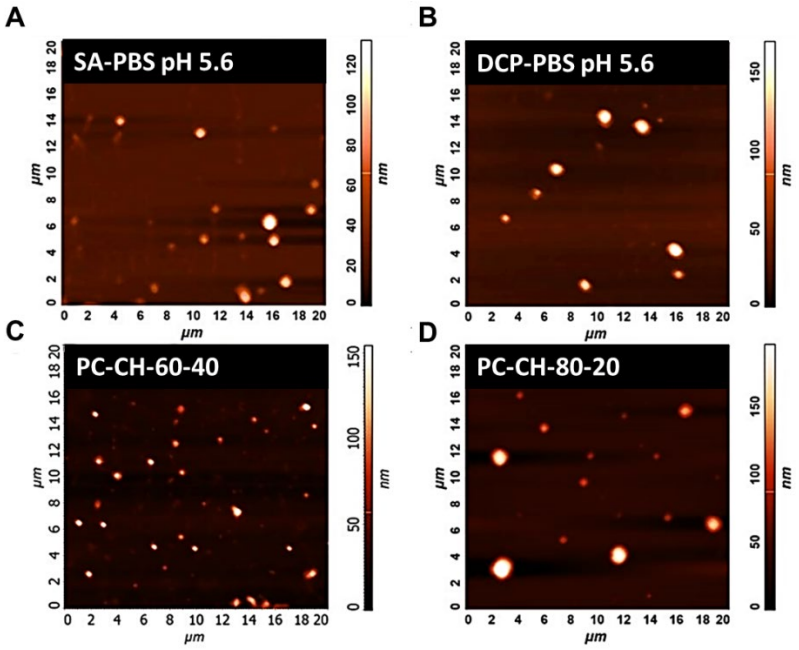

Figure 6. Atomic force microscopy images of the optimised liposome samples *SA-PBS pH 5.6* (**A**) and *DCP-PBS pH 5.6* (**B**) and the membrane additive-free compositions *PC-CH-60-40* (**C**) and *PC-CH-80-20* (**D**).

Based on the AFM measurement results of samples made from the optimal compositions prepared with different hydration media, it can be established that those liposomes are of ~100–110 nm in size with homogeneous size distribution for all samples. These measurement results are in accordance with the results from the DLS measurements.

3.2.5. Thermal Analysis

DSC studies provide information about, among others, the phase transition of the liposomes [76]. The so-called glass transition temperature (T_g) is an important parameter in characterising the stability of the lyophilised samples [77]. T_m and the corresponding enthalpy change (ΔH_m) influence not only the pharmacokinetics of the pharmaceuticals but the stability of the liposomes as well. High ΔH_m implies more rigid phospholipid bilayers [77], while similar transient enthalpies predict a bilayer phase with a similar structure [78]. The calorimetric results (Figure 7) show that the T_g for *SA-PBS pH 5.6* (black line) and *DCP-PBS pH 5.6* (red line) was 10 °C and 10.5 °C, respectively. For the membrane additive-free compositions (PC-CH), 10.2 °C (*PC-CH-60-40* (blue line)) and 10.6 °C (*PC-CH-80-20* (green line)) were found. The T_m values were 56.5 °C, 56.6 °C, 24.0 °C and 22.0 °C, respectively. The drop in T_m on DCP and SA addition is likely caused by the increased distance between the CH-chains due to the intercalation of the compounds, accompanied by the decreasing strength of the van der Waals interactions [79]. Similar ΔH_m of 21 J/g and 32 J/g were calculated for the *SA-PBS pH 5.6* and *DCP-PBS pH 5.6* systems, respectively. Compared with the PC-CH formulations (1.1 J/g and 1.0 J/g), these compositions formed more rigid bilayers.

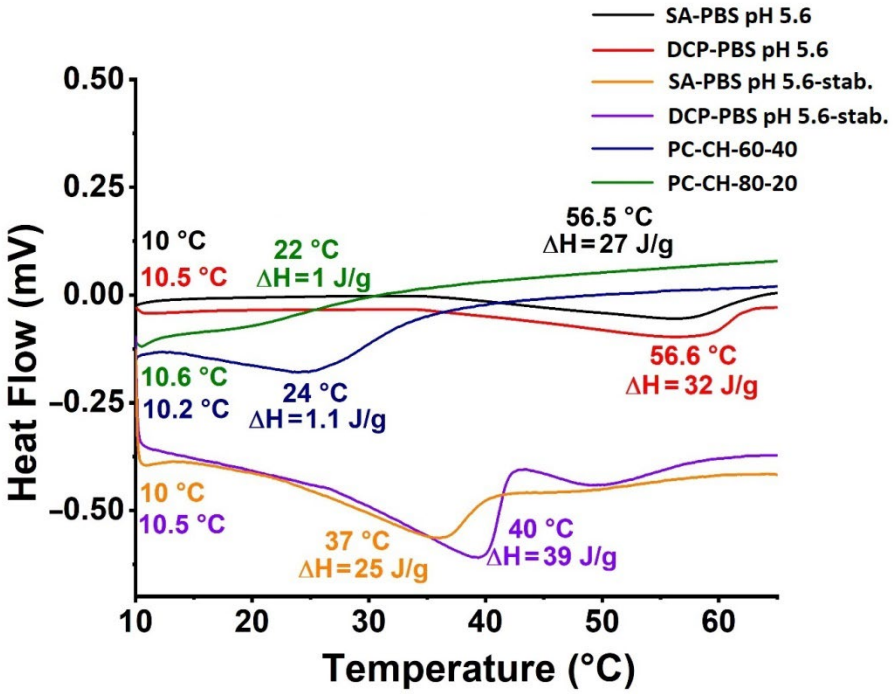

Figure 7. Differential scanning calorimetry results of the optimised liposome samples hydrated with PBS pH 5.6: *SA-PBS pH 5.6* (black) and *DCP-PBS pH 5.6* (red), their results after 3 months in accelerated stability testing circumstances: *SA-PBS pH 5.6-stab.* (yellow) and *DCP-PBS pH 5.6-stab.* (purple), and the membrane additive-free compositions *PC-CH-80-20* (green) and *PC-CH-60-40* (blue).

In the case of the stabilised samples, the T_g values are the same as those measured in the initial, freshly prepared samples: T_g for *SA-PBS pH 5.6-stab.* sample (yellow line) at 10 °C, for the *DCP-PBS pH 5.6* sample (purple line) at 10.5 °C were detected. While the T_m values decreased, the ΔH_m values remained in the same order of magnitude (25 J/g and 39 J/g), i.e., the stiffness of the double layers lasted for the three months in accelerated stability testing circumstances.

The thermal stability of the formulations was further investigated via TGA in the 0–300 °C temperature region. Similar gravimetric curves were recorded for both optimised liposomes (Figure 8), and the calculated weight losses are listed in Table 8. The weight loss took place in two steps: The first step at 75–80 °C, indicating the desorption of the physisorbed water content. Due to the minute amount of water in the lyophilised samples, weight losses at around the limit of detection of our system needed to be quantified. Hence the apparent weight gain in the TGA curves. The second step appeared at 200–225 °C, most likely due to molecular changes and chemical degradation in the structures. Both samples suffered ~4% weight loss during annealing. Since degradation occurred at high temperatures only, well above the limit of any practical applications, the optimised formulations are considered stable against temperature during production and storage.

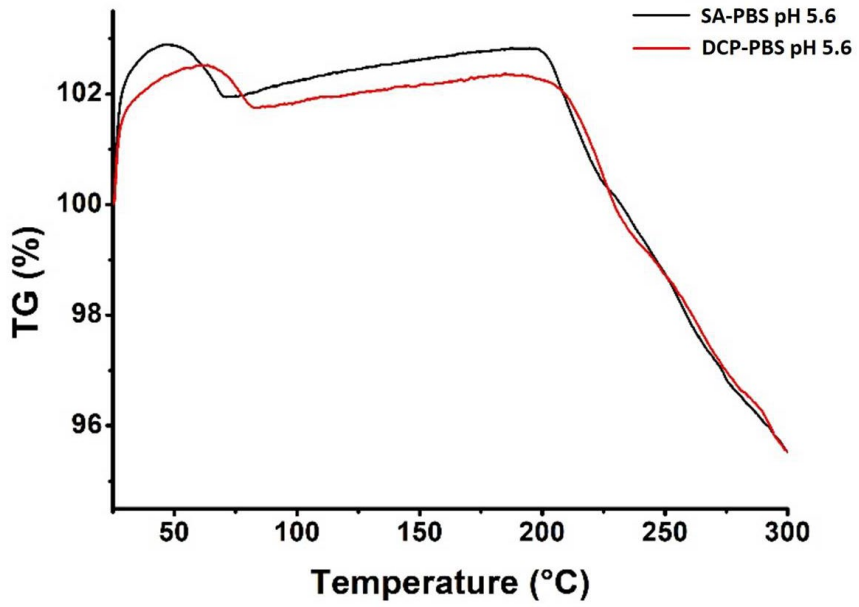

Figure 8. Thermogravimetric analysis of the optimised liposome samples *SA-PBS pH 5.6* (black) and *DCP-PBS pH 5.6* (red).

Table 8. Optimised formulations (SA = stearylamine, DCP = dicetyl phosphate).

Composition	Starting Point of Weight Loss (°C)	Maximal Weight Loss at 300 °C (%)
SA-PBS pH 5.6	75	4
DCP-PBS pH 5.6	80	4

3.2.6. Fourier Transformed Infrared Spectroscopy (FT-IR)

Similar FT-IR spectra were recorded for both optimised formulations (Figure 9). Since PC is the wall-forming lipid with the highest concentration in the compositions, its vibrational bands dominate the spectra. Multiple regions can be identified: from 3000 to 2800 cm^{-1} bonds from C-H stretching vibrations can be found, whereas the ~900–600 cm^{-1} regime is the fingerprint region [80]. The former mainly originate from the hydrocarbon chains, while bands corresponding to the vibrations of the polar phospholipid head groups appear at lower wavenumbers (<1800 cm^{-1}). At 864 $cm^{-1,}$ the asymmetric ν_{as}(P-O), at 922 cm^{-1} the $\nu_{as}(N^{+}\text{-}(CH_3)_3)$, at 980 cm^{-1} the $\nu_{s}(N^{+}\text{-}(CH_3)_3)$, at 1090 cm^{-1} the symmetric $\nu_{s}((PO)_2)$, while at 1296 cm^{-1} the asymmetric $\nu_{as}(PO_2)$ stretching can be seen [81]. The symmetric $\nu_{s}(CH_2)$ stretchings of the apolar hydrocarbon chains appear at 2850 cm^{-1}, while its asymmetric counterpart, $\nu_{as}(CH_2)$, is seen at 2923 cm^{-1} [80]. Neither the bands corresponding to SA nor DCP can unambiguously be identified since peaks at 2900–2960, and 2850 cm^{-1} originate from DCP, while vibrations from SA appear at 2920 and 2850 cm^{-1}. Thus FT-IR spectra are not conclusive in detecting these charge imparting agents in the formulations.

The FT-IR spectroscopy measurements made on the 3-month accelerated stability test samples (*SA-PBS pH 5.6-stab.* (yellow line); *DCP-PBS pH 5.6-stab.* (purple line)) resulted in the same spectra as the starting samples (*SA-PBS-pH 5.6* (black line); *DCP-PBS pH 5.6* (red line)); no structural change took place.

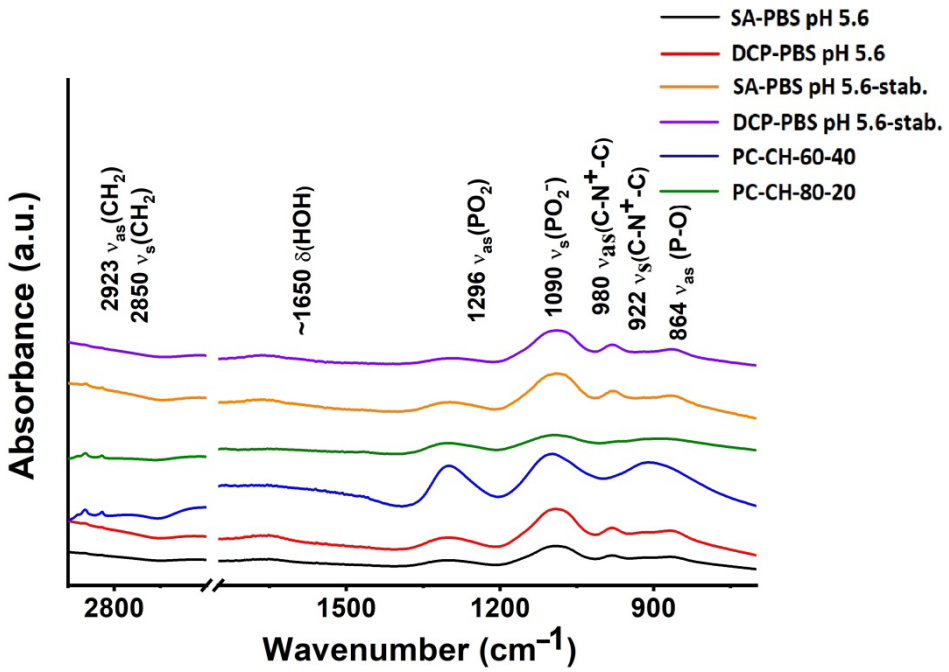

Figure 9. FT-IR spectra of the optimised liposome samples *SA-PBS pH 5.6* (black) and *DCP-PBS pH 5.6* (red), the same samples after 3-month accelerated stability testing *SA-PBS pH 5.6-stab.* (yellow) and *DCP-PBS pH 5.6-stab.* (purple), and the membrane additive-free compositions *PC-CH-60-40* (blue) and *PC-CH-80-20* (green).

3.2.7. Raman Spectroscopy

The Raman spectra of the optimised liposomes *SA-PBS pH 5.6* and *DCP-PBS pH 5.6* are compared to those of the membrane additive-free compositions *PC-CH-60-40* and *PC-CH-80-20* in Figure 10. The weak characteristic features of the components match with literature results [82]; however, a new peak at around 914 cm^{-1} Raman shift appears in each spectrum. It might stem from a hitherto unreported interaction between liposome constituents or an unwanted effect of some sample preparation step. To the best of our knowledge, there is no record of such a band in previous studies on PC-CH systems, and hence the investigation of its origin is ongoing.

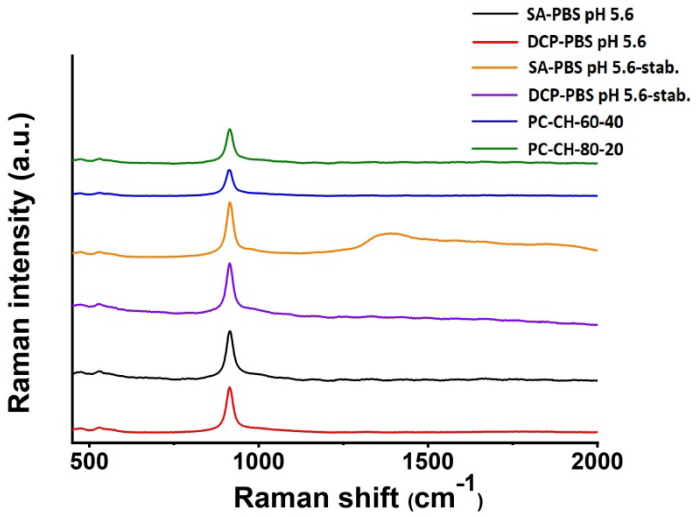

Figure 10. Raman spectra of the optimised liposome samples *SA-PBS pH 5.6* (black) and *DCP-PBS pH 5.6* (red), the same samples after 3-month accelerated stability testing *SA-PBS pH 5.6-stab.* (yellow) and *DCP-PBS pH 5.6-stab.* (purple), and the membrane additive-free compositions *PC-CH-60-40* (blue) and *PC-CH-80-20* (green).

The Raman spectroscopy measurements made on the 3-month accelerated stability test samples (*SA-PBS pH 5.6-stab*. (yellow line); *DCP-PBS pH 5.6-stab.* (purple line)) showed no difference in the spectra compared to the starting samples (*SA-PBS-pH 5.6* (black line); *DCP-PBS pH 5.6* (red line)), indicating no structural change during the period.

3.2.8. Residual Ethanol Measurement via Gas Chromatography-Mass Spectrometry (GC-MS)

Ethanol is a Class 3 solvent (solvents with low toxic potential) in the ICH guidelines, as it is regarded as less toxic and of lower risk for human health [83]. A daily intake of ≤ 50 mg, i.e., concentration of 5000 ppm (0.5%), is acceptable in pharmaceuticals. The ethanol residue was quantified via GC-MS, and ethanol concentrations of 11.1 and 23.9 μmol/L (0.51 and 1.10 ppb) were found in the *SA-PBS pH 5.6* and *DCP-PBS pH 5.6* samples, respectively. The amount of remaining ethanol in the samples of *PC-CH-60-40* and *PC-CH-80-20* was below the limit of detection in the current setup.

4. Discussion

We would like to point out here that only a few compositions reported in the literature (Tables 1 and 2) met the expectations of the formulations: vesicle size ≤ 150 nm, PdI ≤ 0.30 and ζ ≥ 30 mV. Mostly one of the three basic characteristic values was not investigated or was out of the acceptance criteria of this study; hence only formulations that lie in the acceptance range are included in this 'Discussion' section. Salem et al. and Vhora et al. reached higher zeta potentials than the *SA-PBS pH 5.6* and *DCP-PBS pH 5.6* samples but complemented their formulations with other surfactants and phospholipids. They reached +42.5 ± 2.1 mV and +52.8 ± 3.7 mV zeta potentials with SPC:CH:SA:Span 60 = 1:1:0.15:1 (+ flucytosine) and DOPE:SPC:CH:SA = 10:45:29:16 molar ratios, respectively [44,49]. Mishra et al. formulated only SPC- and SA-based (2:0.5 molar ratio) amphotericin B-liposomes without adding cholesterol to the compositions and reached +32.0 ± 0.2 mV with a vesicle size of 140 ± 4 nm [39]. Sharma et al. formulated liposomes with +32.9 ± 2.1 mV of zeta potential from SPC:CH:SA in a 7:3:1.1 molar ratio with a smaller vesicle size (77 ± 2 nm) than *SA-PBS pH 5.6* (108 ± 15 nm) [61]. Inserting monesin as an API into the same liposomes by Rajendran et al. resulted in both higher zeta potential (+43.9 ± 0.9 mV) and vesicle size (121 ± 20 nm) [57]. Salem et al. reached high zeta potential values (−59.1 ± 1.7) by adding Span 60 to the DCP-containing flucytosine liposomes with molar ratios of SPC:CH:DCP:Span 60 = 1:1:0.1:1 [49]. Calvo et al. formulated almost 200 nm large liposomes (195 ± 5 nm) with −47.0 ± 1.0 mV of zeta potential from SPC:CH:DCP = 15:8:1 [70]. Togami et al. reached approximately the same charge, i.e., −49.4 ± 3.5 mV, accompanied by a smaller vesicle size (134 ± 4 nm) in EPC:CH:DCP with a 7:2:1 molar ratio [72]. In evaluation, the modifying effect of the API needs to be considered where it was applied.

In this study, the effects of these charge imparting membrane additives were investigated in systems without the influence of an API. The necessary PC, CH and SA or DCP molar ratios were determined by applying a 3^2 fractional factorial design to get liposomal formulations with the predefined CQAs: vesicle size under 150 nm, PdI less than 0.30 and zeta potential higher than |30| mV. The regression models showed no independent variables with a significant effect on the zeta potential; however, the coefficients in the equations describing the relations between the independent variables and the magnitude of the zeta potential predict the changes in its value. The middle points of the design spaces were verified for compliance. The chosen formulations were optimal to meet the requirements of nano-drug delivery systems when the lipid films were hydrated with PBS pH 5.6. The vesicle size was significantly larger ($p < 0.05$) in the case of the *SA-PBS pH 5.6* (108 ± 15 nm) sample than in the *DCP-PBS pH 5.6* (88 ± 14) nm formulation. This phenomenon can be explained by the change in the spacing between bilayers and the bulkiness of the charge imparting membrane additives [35,48,53]. High zeta potentials, typically between 20 and 40 mV, ensure stable systems by decreasing aggregation and

increasing polydispersity due to high charge repulsion among liposomes [29]. The absolute zeta potential was significantly higher ($p < 0.05$) in the optimal DCP (−36.7 ± 3.3 mV) than in the SA preparation (+30.1 ± 1.2 mV). The higher repulsive forces between the vesicles resulted in two-week stability in an aqueous medium and up to 3 months in lyophilised form.

The presented results fit into the scientific research area and extend the knowledge on improving liposomal zeta potential. The presented concept helps to establish and perform liposome studies with less effort and more success, and the observations can provide a valuable base for further developments.

5. Conclusions

Optimised liposome compositions are vital to achieving highly stable systems for applications in, e.g., targeted drug delivery or diagnostic imaging. The present Quality by Design study is an extension of previous works on the Risk Assessment of liposomes made via the thin-film hydration method. It aims to optimise liposomal formulations through the improvement of the zeta potential of the vesicles, which was modified using stearylamine (SA) or dicetyl phosphate (DCP) charge imparting agents. The Knowledge Space was given about the optimal zeta potentials and membrane additives. A thorough review of the compositions reported in the literature showed that there is no best practice to determine the optimal ratios of the lipid components. Thus, we defined the PC, CH and SA or DCP molar ratios for liposomal formulations with optimised zeta potential and stability via carrying out two 3^2 fractional factorial designs. The molar ratio of the components was systematically varied 3 levels in 9 runs, and the effect on the vesicle size, PdI, and zeta potential was investigated. Quadratic response surfaces were drawn for the zeta potentials in the case of each charge imparting agent, and the second-order polynomial models describing the effects of the independent variables on the zeta potential were calculated. The optimal molar ratios of the lipids were derived from the contour plots: The optimised compositions for the SA (*SA-PBS pH 5.6*) and DCP (*DCP-PBS pH 5.6*) containing samples turned out to be PC:CH:SA= 12.0:5.0:5.0 and PC:CH:DCP = 8.5:4.5:6.5, respectively. Both formulations met the quality requirements of vesicle size (d(*SA-PBS pH 5.6*) = 108 ± 15 nm and d(*DCP-PBS pH 5.6*) = 88 ± 14 nm), PdI (PdI(*SA-PBS pH 5.6*) = 0.20 ± 0.04 and PdI(*DCP-PBS pH 5.6*) = 0.21 ± 0.02) and zeta potential (ζ(*SA-PBS pH 5.6*) = +30.1 ± 1.2 mV and ζ(*DCP-PBS pH 5.6*) = −36.7 ± 3.3 mV). The high absolute zeta potentials ($|\zeta| > 30$ mV) forecasted long-term stability by reducing vesicle aggregation, and indeed, optimised formulations were stable for up to two weeks in a liquid state. We pointed out that the optimal PC content was ~60 weight% in both *SA-PBS pH 5.6* and *DCP-PBS pH 5.6*, according to prior findings on the charge-inducing agent-free PC:CH system. Since QbD optimisation is an independent method, it supports the latter results on the optimal PC:CH ratio. Moreover, our work provides the parameters to be considered in a QbD-based design for producing liposomes with desired morphology and physical–chemical properties, such as the optimal zeta potential of the vesicles.

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Abbreviations

ΔH_m	enthalpy changes
AFM	atomic force microscopy
ANOVA	analysis of variance
API	active pharmaceutical ingredients
BBB	blood-brain barrier
CH	cholesterol
CMAs	Critical Material Attributes
CPPs	Critical Process Parameters
CQAs	Critical Quality Attributes
DCP	dicetyl phosphate
DLS	dynamic light scattering
DoE	Design of Experiments
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
DOPE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
DPPE	1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine
DS	Design Space
DSC	differential scanning calorimetry
DSPC	1,2-distearoyl-sn-glycero-3-phosphocholine
DSPE-PEG2000	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy(polyethyleneglycol)-2000]
EMA	European Medicines Agency
EPC	egg phosphatidylcholine
FITC	fluorescein isothiocyanate
FT-IR	Fourier transform infrared spectroscopy
GC-MS	gas chromatography-mass spectrometry
GDNF	glial cell-line derived neurotrophic factor
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
KCl	potassium chloride
K_2HPO_4	dipotassium phosphate
KH_2PO_4	potassium dihydrogen phosphate
MIR	mid-infrared spectroscopy
$Na_2HPO_4 \times 2 H_2O$	disodium hydrogen phosphate dihydrate
NaCl	sodium chloride
NBCDs	non-biologically complex drugs
PBS pH 5.6	phosphate-buffered saline pH 5.6
PBS pH 7.4	phosphate-buffered saline pH 7.4
PC	L-α-phosphatidylcholine

PdI	polydispersity index
QbD	Quality by Design
QTPP	Quality Target Product Profile
RA	Risk Assessment
RES	reticuloendothelial system
SA	stearylamine
Saline solution	sodium chloride physiological solution
Span 60	sorbitan monostearate
SPC	soy phosphatidylcholine
T_g	glass transition temperature
TG	thermogravimetric measurements
TGA	thermogravimetric analysis
T_m	phase transition temperature
Tween 20	sorbitan monolaurate/Polysorbate 20
Tween 80	sorbitan monooleate/Polysorbate 80

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