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Summary of Ph.D. Thesis

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

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1. INTRODUCTION

The pharmaceutical industry has complex areas evolving dynamically due to research and development trends, market dynamics, and regulatory requirements, leading to addressing new scientific and technical issues. 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 for the patient and a profitable plan for the industry should be provided. Regulatory requirements should also be considered even at the early stage of development. 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 these input requirements and incorporate them into the development plan. 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.

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. Liposomes have been proven to be successful nanocarriers for targeted gene and drug delivery since the discovery of liposomes made by Alec D. Bangham in 1965; however, the level of the challenges rises in parallel with 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

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Abbreviations: *ANOVA - Analysis of Variance; API - Active Pharmaceutical Ingredient; 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; DPPE-PEG$_{2000}$ - 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]; DS - Design Space; DSC - Differential Scanning Calorimetry; DSPE-PEG$_{3000}$ - 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-3000]; FT-IR - Fourier Transform Infrared Spectroscopy; MAs - Material Attributes; PBS - Phosphate-Buffered Saline; PC - L-α-phosphatidylcholine; PdI - Polydispersity Index; PEG - Polyethene glycol; PPL – Phospholipid; PPs - Process Parameters; QbD - Quality by Design; QTPP - Quality Target Product Profile; RA - Risk Assessment; SA – Stearylamine; saline solution - Sodium Chloride Physiological Solution; T_c - Gel to Liquid-Crystalline Phase Transition Temperature; T_g - Glass Transition Temperature; TGA - Thermogravimetric Analysis; T_m - Phase Transition Temperature; ZP - Zeta Potential*

material attributes (MAs, characteristics of the components) and process parameters (PPs, settings of the preparation process). 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. All this information needs to be considered, organized, and evaluated to develop a successful liposome-based formulation. Furthermore, the 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. One of these challenges is finding the most appropriate compositions for the purposes and achieving the best results. 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.

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. Adopting the QbD concept and the Risk Assessment (RA) method to the development phase of the liposomal formulations assists in optimizing the vesicles and rationalizing their design and study by classifying the formulation requirements, e.g. stability.

The electrokinetic potential for colloidal systems is defined as the zeta potential (ZP). ZP characterizes 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. 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. Generally, ZP above 30 mV in absolute value is considered a sign of good stability and indicates monodisperse formulations without aggregation. Many factors can modify ZP. By incorporating various charge-inducing agents into the phospholipid (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. The literature on SA- and DCP-containing liposomal formulations was accurately reviewed in the PhD work. The applied ratios and the results varied in the studies, justifying the importance of a time- and material-saving experimental design-based research and later development of liposomal formulations containing SA and DCP.

2. EXPERIMENTAL AIMS

The objective of my Ph.D. work was to develop and investigate liposomal formulations applying RA and the QbD 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 (QTPP).
2. Identifying the Critical Quality Attributes (CQAs), the potentially critical Material Attributes and Process Parameters.
3. Performing the RA, finding the Critical Material Attributes (CMAs) and Critical Process Parameters (CPPs), and setting up the Design of Experiments (DoE) based on results.
4. Determining the Design Space (DS).

In the studies, the requirements of liposome formulation prepared via the thin-film hydration preparation technique were investigated. After the universal initial RA, the highlighted critical parameters were investigated from new perspectives in an updated RA targeting 'intermediate' API-free liposomal formulations. 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 phosphatidylcholine (PC) – cholesterol (CH) weight ratio, the polythene glycol attached (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 characterized 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 (PdI) less than or equal to 0.30
- zeta potential higher than |30| mV

3. MATERIALS AND METHODS

3.1. Materials

Liposomes were made from the alcoholic solutions of the following materials 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 (=SA), or dihexadecyl phosphate (=DCP) (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 were used as hydration media made from the following materials: sodium chloride, potassium chloride, potassium dihydrogen phosphate (Molar Chemicals Kft., Budapest, Hungary), disodium hydrogen phosphate dihydrate, and dipotassium phosphate (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).

3.2. Methods

3.2.1. Application of the Quality by Design method

3.2.1.1. Knowledge Space development and definition of the QTPP

To define the target product profile of the aimed formula and its quality criteria, a knowledge space development was carried out by collecting the relevant information regarding the aimed product and the production.

The QTPP is a summary of the quality characteristics of the product that will ideally be achieved. 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.

3.2.1.2. Determination of the CQAs

The CQAs are those factors that 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 covering the physical, chemical, biological or microbiological properties that should reach an appropriate range or limit to ensure the constant end-product quality.

3.2.1.3. Determination of the CMAs and the CPPs

The CMAs and the CPPs 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.

3.2.1.4. Risk Assessment

The key step of the QbD-driven development process is the RA, which assists in identifying and ranking the CMAs and CPPs based on their impact on the CQAs of the product. The RA is typically performed as the first step of the pharmaceutical development process and is re-evaluated when more information becomes available. 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 on a three-level scale for each parameter pair individually. Pareto diagrams were generated, presenting the ranking of the CQAs and the CMAs/CPPs according to their potential impact on the final product (QTPP).

3.2.1.5. Design of Experiments

Based on the results of the initial RA, the DoE was built up. It 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 Design Space. The characterization 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 (***Table 2***). The effects of these independent

factors on the vesicle size, the polydispersity index 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 describing the relationships were constructed according to the followings:

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

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.

3.2.2. Preparation and lyophilisation processes of liposomes

The liposomes were prepared via the thin-film hydration method by evaporating the ethanol 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) 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). 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 (Labsystem Kft., Budapest, Hungary), then a 0.22 μm membrane-filter (Ultipor®, Pall Corporation, New York, NY, USA). The samples were immediately investigated for vesicle size, polydispersity and ZP in the liquid state and then lyophilized for further investigations (SanVac CoolSafe freeze dryer, LaboGene™, Lillerød, Denmark) at −40°C and 0.01 atm. The lyophilized samples were stored at 2-8°C.

3.2.3. Characterization of the liposomes

3.2.3.1. Vesicle size and zeta potential analysis

The vesicle size and the PdI of the liquid liposome formulations were measured using the dynamic light scattering (DLS) technique. The measurements were carried out via a Malvern Zetasizer Nano ZS system (Malvern Panalytical Ltd., Malvern, Worcestershire, UK) equipped with a 633 nm wavelength laser in folded capillary zeta cells (Malvern Panalytical Ltd., Malvern, Worcestershire, UK) at 25°C. Our acceptance criterion for liposome size was 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. Absolute ZP values above 30 mV indicate good formulation stability.

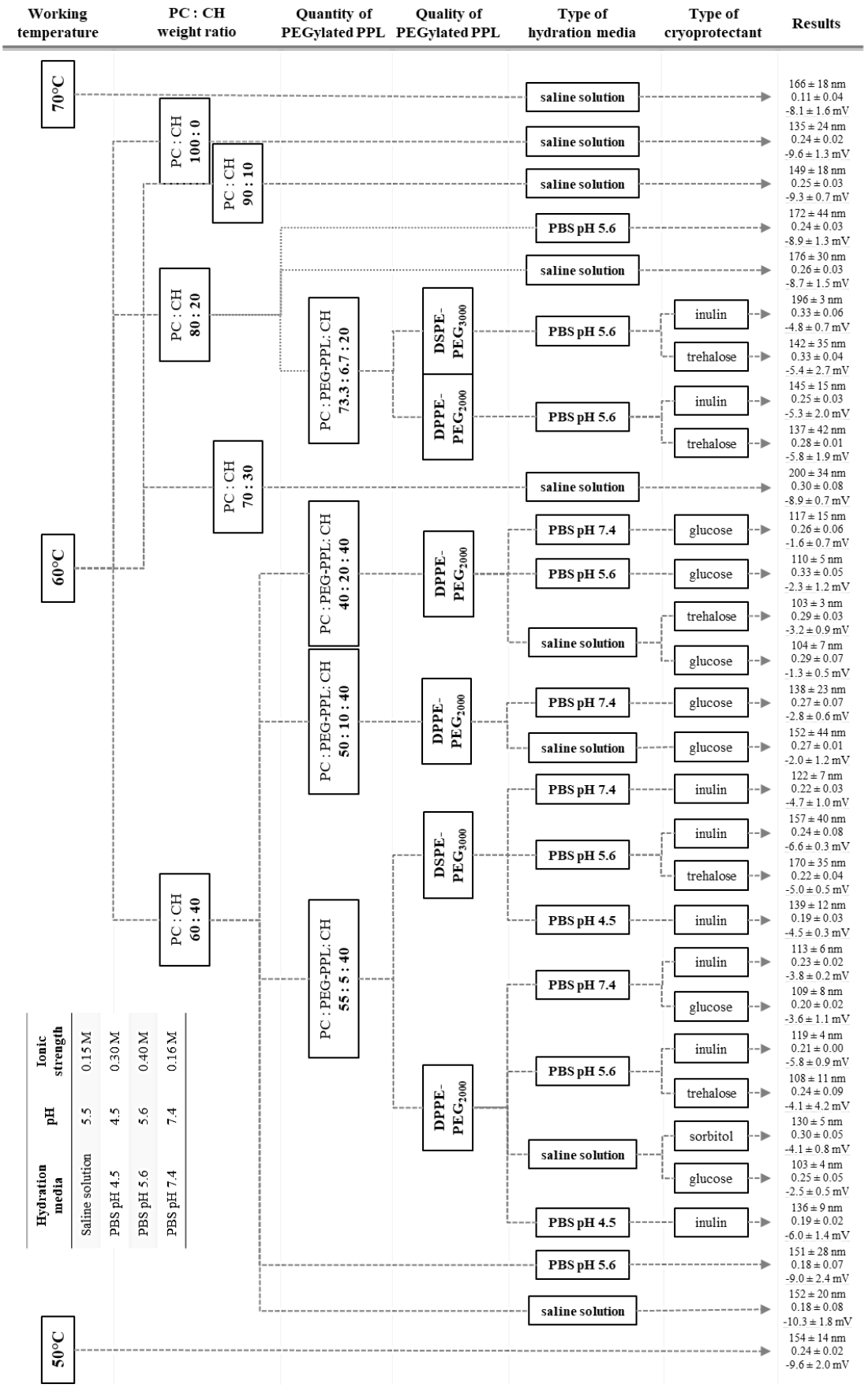

Figure 1. *A summary flowchart that combines the DOE presenting the investigated factors and applied combinations with the results of the liposome characterization studies.*

3.2.3.2. Thermal analysis

The thermodynamic state of the liposomes made for the RA studies was checked in the range of 25-300°C via differential scanning calorimetry (DSC) (TA Instruments DSC Q20, TA Instruments, New Castle, DE, USA). The possible phase (T_m) and glass (T_g) transition 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 lyophilized samples were heated up at a temperature range of 25-300°C at a 10°C/minute heating rate and investigated in the dry nitrogen gas using the Setaram Labsys TG-DTG-DTA analyzer (SETARAM Instrumentation, Caluire, France).

The DSC measurements were done with a Mettler-Toledo DSC 3^+ Star^e System DSC analyzer (Mettler-Toledo International, Inc., Columbus, OH, USA) in the range of 10-65°C at 2°C/min heating rate from 6-10 mg of the lyophilized samples under a 150 mL/min argon flow for the samples made for the ZP optimization study. 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 analyzer (Mettler-Toledo International, Inc., Columbus, OH, USA). The results were evaluated with the STAR^e 9.30 software (Mettler-Toledo International, Inc., Columbus, OH, USA).

3.2.3.3. 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). 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 from the lyophilized powders and pressed into pellets with potassium bromide.

3.2.4. 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, 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.

4. RESULTS AND DISCUSSION

4.1. Quality by Design-based liposome development

4.1.1. 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.

The factors that can describe a liposome formulation were collected, and a general QTPP was established. This universal selection was concretized for 'intermediate' liposomal products that can later provide an applicable base for carrier systems. Liquide formulation and stable lyophilized powder were accepted as the form of preparation. Vesicles in the 100-200 nm size range can be suitable for several application methods.

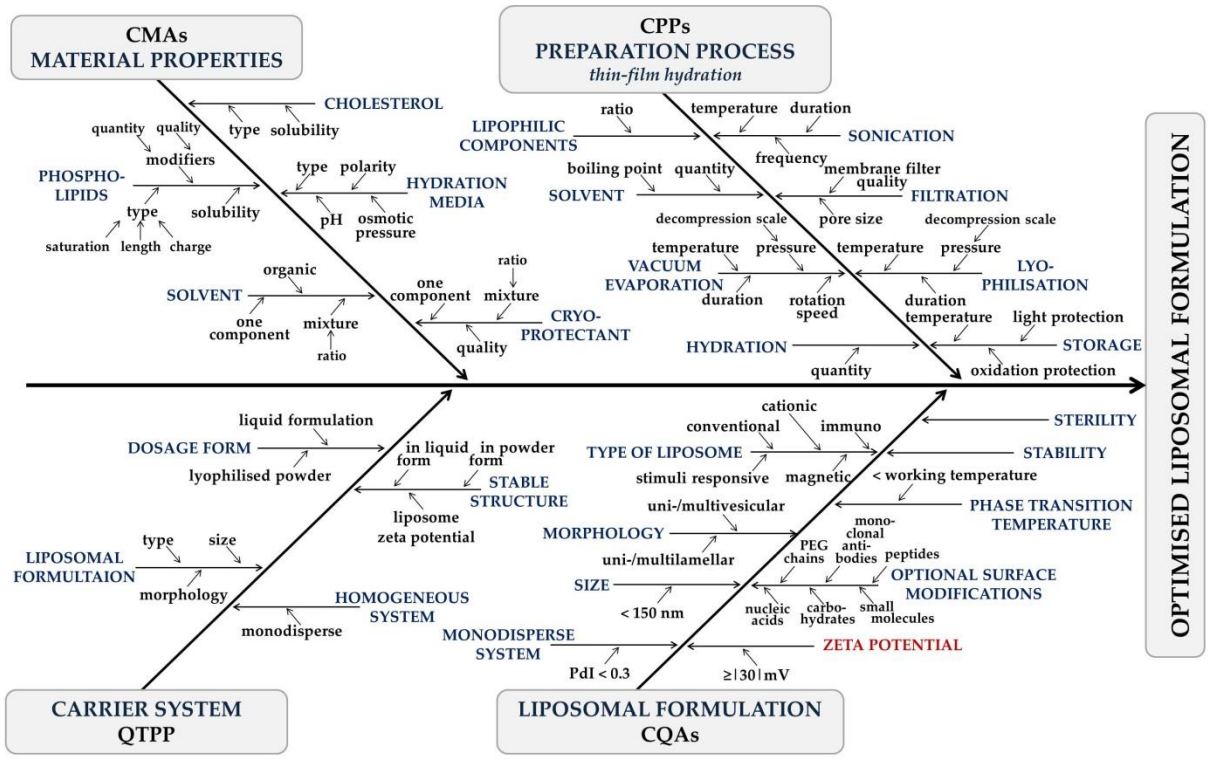

Figure 2. *Ishikawa diagram of the factors that impact the quality of the liposomes made in the thin-film hydration method.*

The stability of a formulation influences the safety, efficacy, and quality profile of the product. Thus a stable, large unilamellar vesicle-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.

4.1.2. Determination of the CQAs

The CQAs made up the list of those quality attributes that are critically related to the QTPP. 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: for 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 need to be pyrogen-free
- stability: vesicles with stable physical and chemical attributes, no aggregation

4.1.3. 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 was chosen for these studies. The critical Mas and PPs must be selected 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, lyophilization, and storage)

4.1.4. 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 interdependence rating between the items of the QTPP and the CQAs plus the CQAs and the CMAs/CPPs. The generated Pareto charts illustrated the ranking of the critical factors. The estimated criticality of the CMAs and CPPs obtained from the initial RA were verified in experiments, and the results were utilized in the updated RA.

In the case of an 'empty' delivery system, after neglecting the API-related factors, the stability, the targeted delivery compatibility, the type of the liposome, the zeta potential, the vesicle size, the surface modifications (PEGylated PPLs), 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 analyzed 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.

4.1.5. 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. 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 1***.

Table 1. *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

4.1.5.1. Vesicle size and zeta potential analysis

The results of the investigations are shown on the DoE map in ***Figure 1*** and illustrated in ***Figures 3 and 4***.

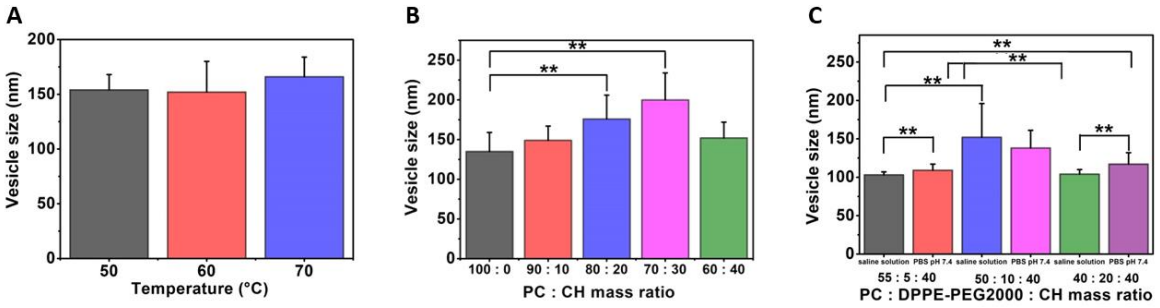

Figure 3. *Results of the DLS measurements in the case of the PC:CH = 60:40 samples made at different temperatures (50-70°C) (A), the different amount (0-40%) CH-containing samples made at 60°C and all hydrated with saline solution (B), the DPPE-PEG$_{2000}$-containing formulations made at 60°C and hydrated with saline solution or PBS pH 7.4.*

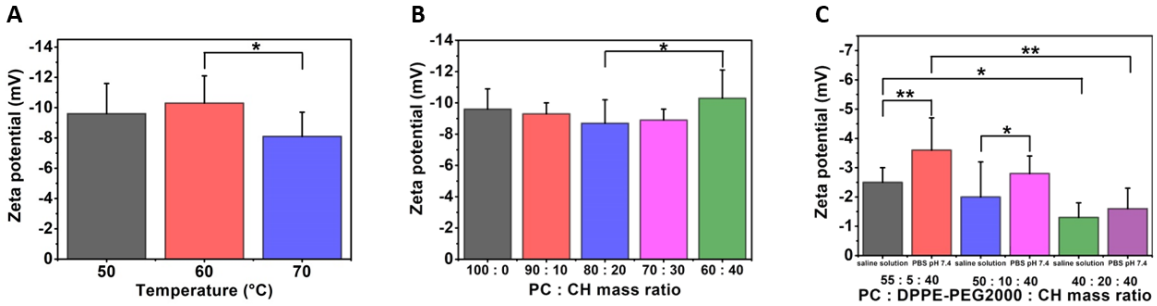

Figure 4. *Results of the ZP measurements in the case of the PC:CH = 60:40 samples made at different temperatures (50-70°C) (A), the different amount (0-40%) CH-containing samples made at 60°C and all hydrated with saline solution (B), the DPPE-PEG$_{2000}$-containing formulations made at 60°C and hydrated with saline solution or PBS pH 7.4.*

4.1.5.1.1. Effects of different working temperature

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 3-A***). 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 4-A***). Thus 60°C was chosen as the working temperature for further studies.

4.1.5.1.2. 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 decreased as the PC:CH ratio was changed from 70:30 to 100:0 (***Figure 3-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. With fewer than 30% CH, the ZP value decreases with the reduced CH% content (***Figure 4-B***) due to a higher number of PC on the vesicle surface. 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). Further

investigations were carried out on CH-containing compositions to keep the mechanical strength of the membrane.

4.1.5.1.3. Effect of different concentrations of PEGylated phospholipids

5-10-20% of the formulation weight of samples made at 60°C and hydrated with saline solution and PBS pH 7.4 was changed from PC to DPPE-PEG_{2000} to investigate the effect of the PEGylated PPL. A non-linear relationship was detected between the PPL ratios and the vesicle size (***Figure 3-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. The ZP values were significantly more negative in the 55:5:40 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 4-C**).

4.1.5.1.4. 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).

4.1.5.1.5. Effect of different hydration media

The hydration media quality (ionic strength) was studied on the PC:DPPE-PEG_{2000}:CH = 55:5:40 and 40:20:40 weight ratio formulations made at 60°C. The higher the ionic strength, the higher the ZP due to the more compact ion layer formed around the vesicles. 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. The application of the PBS pH 7.4 (117±15 nm) resulted in a significantly larger size ($p<0.01$) than 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) instead of saline solution (-1.3±0.5 mV) according to the 40:20:40 ratio had significantly higher ($p<0.05$) ZP. 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 strengths of the hydration media were increasing as: 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).

4.1.5.1.6. Effect of different cryoprotectants

5% of the PPL mass was applied from glucose, sorbitol, trehalose or inulin to investigate 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 (103±3 nm, 104±7 nm, respectively). Sorbitol (130±5 nm) caused a significant increase ($p<0.01$) in the mean vesicle size compared to glucose (103±4 nm). 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).

4.1.5.2. Thermal analysis

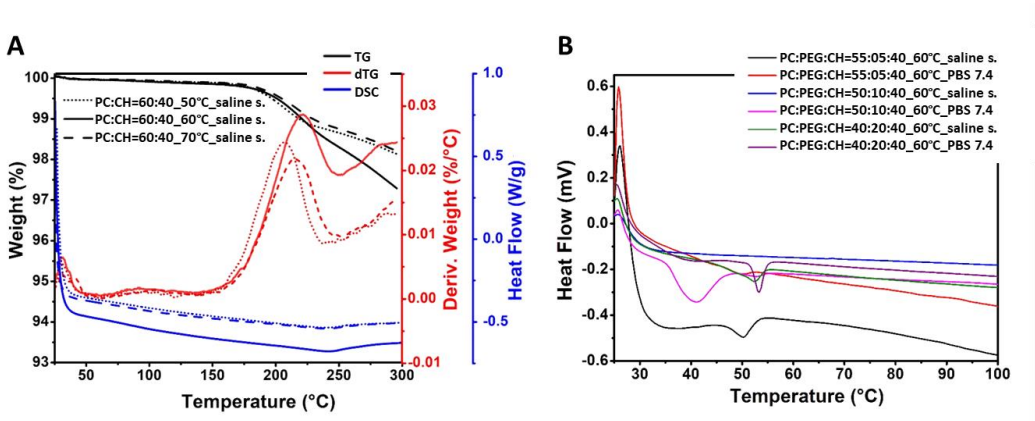

Figure 5. *TG, dTG, and DSC curves presenting the thermal behaviour of the PC:CH = 60:40 samples hydrated with saline solution prepared at different temperatures (50-70°C) (A), and DSC results of the 5%, 10% or 20% DPPE-PEG_{2000}-containing formulations made at 60°C and hydrated with saline solution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

The DSC and TGA measurement results (***Figure 5-A,-B***) were similar in the investigated cases. The gel to liquid-crystalline phase transition temperature (T_c) value of the samples made from the same compositions at different temperatures was ~30-33°C. A remarkable phase transition was observed in the case of the PEGylated PPL-containing vesicles 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, 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. 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. 2-5% of the weight of the samples was lost during the heat treatment up to 300°C.

4.1.5.3. Investigation of chemical bonding

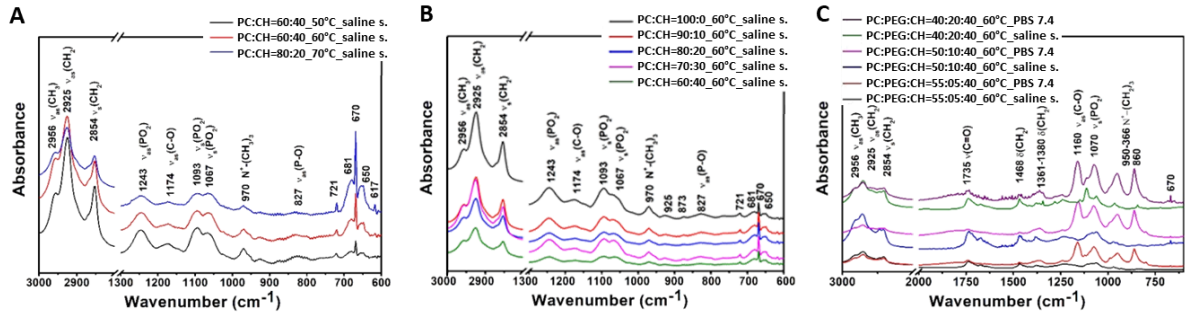

Figure 6. *FT-IR spectra of the PC:CH = 60:40 samples made at different temperatures (50-70°C) (A), the different amount (0-40%) CH-containing samples made at 60°C and all hydrated with saline solution (B), the 5%, 10% or 20% DPPE-PEG2000-containing formulations made at 60°C and hydrated with saline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

The FT-IR curves (***Figure 6-A,-B,-C***) demonstrated two distinct, separate regions in the spectra according to PC, the main component: 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 traces of the FT-IR curves were consistent despite the different sample production temperatures; however, differences among samples hydrated with different hydration media were well-detectable. Typical $\nu_{as}(PO_2)$ and $\nu_s(PO_2)$ stretchings appeared in the liposomes hydrated with the PBS solutions, and the different ionic strengths caused differences between the spectra. Overlaps with other vibrations were detected in the 3050-2800 cm^{-1} region.

4.1.6. Determination of the Design Space

The results led to the formation of the DS, which has regulatory benefits as if the alterations in the parameters are in the DS, the deviation does not require modifications in the marketing authorization documentation. Observations on the parameters of the thin-film hydration-based liposome preparation process are the following:

- 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 with PC:CH = 60:40 mass ratio.
- The ZP decreases as the amount of CH is reduced. Significantly higher 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.

- 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 significantly decreases ($p<0.05$) by applying larger DPPE-PEG_{2000} proportions.
- Changing the PEGylated PPL from DPPE-PEG_{2000} to DSPE-PEG_{3000} does not affect the ZP. Significant differences ($p<0.05$) were only measurable regarding the size increased by the larger PEG number.
- The size of the particles hydrated with saline solution and PBS pH 5.6 does not differ. The significantly largest ($p<0.05$) vesicles are from the usage of PBS pH 4.5.
- The ZP of the liposomes increases with the ionic strength of the hydration media due to the ion layer forming around the vesicles. 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 at adding 5 w/w% of PPL from trehalose and glucose as cryoprotectants, but 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 of the samples made with trehalose or inulin.

4.2. 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.

4.2.1. 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 and investigated for the primary outcomes: vesicle size, polydispersity index, and zeta potential. The investigated levels of the independent variables and the experimental results are shown in ***Table 2***.

Polynomial equations were generated from the results to describe the individual main and interaction effects of the independent variables on the dependent factors. As all of the compositions fulfilled the size and PdI acceptance criteria, the impact of the experimental factors was analyzed only on the ZP of the liposomes.

Table 2. *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

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 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:

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

The high regression coefficient (R^2=0.984) indicated a good correlation. 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 ones (x_1, x_2, x_2^2) decrease the absolute ZP values.

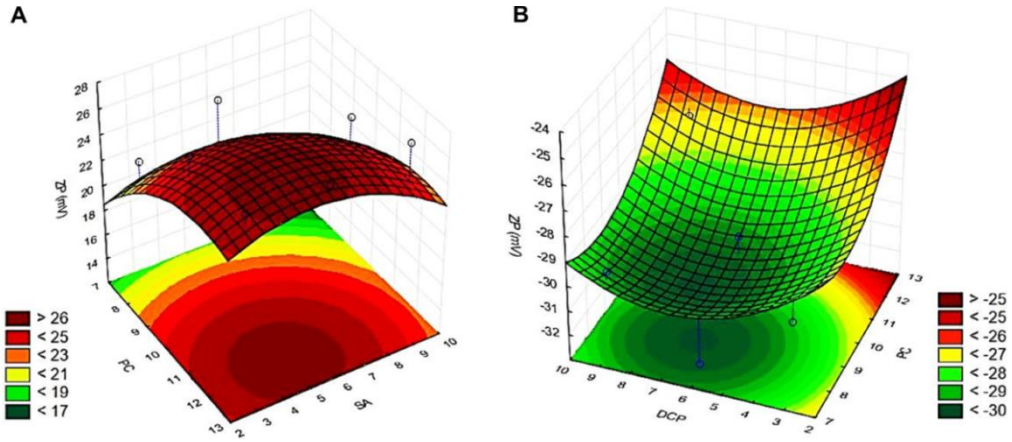

Figure 7. *Three-dimensional surface plots of the effect of independent variables on the ZP value in the 3^2 fractional factorial designs for the compositions made with the membrane additives: stearylamine (A) and dicetyl phosphate (B).*

The 3D response surface plots for the ZP values (Y(SA) – ***Figure 7-A*** and Y(DCP) – ***Figure 7-B***) were plotted by keeping one variable at a certain level. As the regression models show no factor with a significant effect on the ZP values, the optimized compositions were read from the DSs drawn out on the contour plots. The optimal formulation for the SA-containing liposomes (OPT-SA) was made from a 12.0:5.0:5.0 molar ratio of PC, CH, and SA, respectively. The optimized DCP-liposomes (OPT-DCP) contained PC, CH, and DCP in an 8.5:4.5:6.5 molar ratio.

4.2.2. Characterization of the zeta potential optimized formulations

4.2.2.1. Vesicle size and zeta potential analysis

All 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) and the ZP (+30.1 ± 1.2 mV; -36.7 ± 3.3 mV) met the requirements (***Table 2***), while only some of the literature-derived results of compositions applied by other research groups met the requirements we set. The OPT-SA and the OPT-DCP formulations reached the minimal requirements of the ZP based on the stability requirements with small vesicle sizes.

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. The weight ratio of PC in the optimized formulations was essentially the same, i.e. 60 weight units, while 80 weight units were found for the PC:CH ratio alone in good agreement with the previous results.

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. 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).

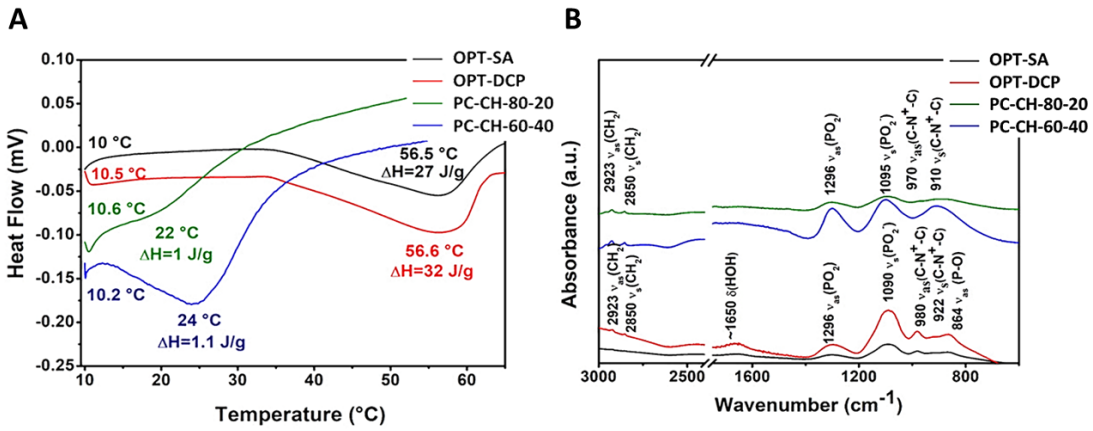

Figure 8. *DSC (A), and FT-IR (B)curves show the optimized liposome samples and the membrane additive-free compositions: PC-CH-80-20 and PC-CH-60-40*

4.2.2.2. Thermal analysis

DSC measurements were done in the 10-65°C range. The calorimetric results (***Figure 8-A)*** show that the T_g value for the OPT-SA sample was 10°C, and for the OPT-DCP sample was 10.5°C. For the membrane additive-free compositions (PC-CH) 10.6 °C (PC-CH-80-20) and 10.2 °C (PC-CH-60-40) were found. The T_m values were 56.5°C, 56.6°C, 22.0°C and 24.0°C, respectively.

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. 4-6% of the original weight was lost until 300°C 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, so the optimized formulations are considered stable against temperature during production and storage.

4.2.2.3. Investigation of chemical bonding

Similar FT-IR spectra were recorded for the optimized formulations (***Figure 8-B)***, indicating two separate regions according to the PC 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 originates from the hydrocarbon chains, while bonds corresponding to the vibrations of the polar PPL head groups appear at lower wavenumbers (<1800 cm^{-1}). 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.

5. SUMMARY

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, 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 impact of the selected CMAs and CPPs on the properties of the liposomes. Changes in the working temperature, 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 liposomes with the predefined CQAs: vesicle size under 150 nm, PdI less than 0.30 and ZP higher than |30| mV.

New findings and practical relevance of the work:

- This is the first QbD-based study successfully applying an updated RA to determine the critical factors of an 'intermediate', API-free liposomal formulation and identifying the CMAs and CPPs of the thin-film hydration 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 on the parameters of a thin-film hydration 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.
- 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 can be 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.

PUBLICATIONS RELATED TO THE SUBJECT OF THE THESIS

I. E. Pallagi, O. Jójárt-Laczkovich, **Zs. Németh**, P. Szabó-Révész, I. Csóka: Application of the QbD-based approach in the early development of liposomes for nasal administration; ***INTERNATIONAL JOURNAL OF PHARMACEUTICS 562 (2019) 11-22;*** **Q1, IF: 4.845**

II. **Zs. Németh**, E. Pallagi, D. G. Dobó, I.Csóka: A proposed methodology for a Risk Assessment-based liposome development process; ***PHARMACEUTICS 12 (2020) 1164;*** **Q1, IF: 6.321**

III. **Zs. Németh**, E.Pallagi, D. G. Dobó, G. Kozma, Z. Kónya, I. Csóka: An updated Risk Assessment as part of the QbD-based liposome design and development; ***PHARMACEUTICS 13 (2021) 1071;*** **Q1, IF: 6.525**

IV. **Zs. Németh**, I. Csóka, R. Semnani Jazani, B. Sipos, H. Haspel, G. Kozma, Z. Kónya, D. G. Dobó: Quality by Design-driven zeta potential optimization study of liposomes with charge imparting membrane additives; ***PHARMACEUTICS 14 (2022) 1798;*** **Q1, IF: 6.525 (2021)**

PUBLICATIONS NOT RELATED TO THE SUBJECT OF THE THESIS

I. Jójártné Laczkovich O., Bónis E., **Németh Zs.**, Szabóné Révész P.: Liposzómák átlagos vezikulaméretének befolyásolása búzacsíra olajjal; ***ACTA PHARMACEUTICA HUNGARICA 88. 9-16 (2018);*****Q4, IF:0.111**

II. **Németh Zs.**, Kiss L., Maléth J., Hegyi P., Szabóné Révész P., Jójártné Laczkovich O.: Liposzómális formulációk kutatása és fejlesztése akut pankreatitisz kezelése céljából; ***ACTA PHARMACEUTICA HUNGARICA 88. 215-226 (2018);*** **Q4, IF: 0.111**

III. **Németh Zs.**, Pallagi E., Csóka I.: Gyógyszertechnológiai és regulációs kihívások - Megállapítások a neurológiai kórképek kezelésére szánt nazális liposzómás rezveratrol-tartalmú készítmény fejlesztése kapcsán; ***GYÓGYSZERÉSZET 65 (2021) 724–734***

IV. **Zs. Németh**, E. Pallagi, D. G. Dobó, I. Csóka: How could QbD address the R&D challenges of ‘nose-to-brain’ liposomal resveratrol formulations?; ***PROCEEDINGS 78 (2021) 49***

V. D. G. Dobó, **Zs. Németh**; B. Sipos, M. Cseh, E. Pallagi, D. Berkesi, G. Kozma, Z. Kónya, I. Csóka: Pharmaceutical development and design of thermosensitive liposomes based on the QbD approach; ***MOLECULES 27 (2022) 1536;*** **Q1, IF: 4.927 (2021)**

PRESENTATIONS RELATED TO THE SUBJECT OF THE THESIS

Oral presentations

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

II. **Zs. Németh**, D. G. Dobó, E- Pallagi, I. Csóka: Basic research methods in the development process of the liposomal formulations; *I. Symposium of Young Researchers on Pharmaceutical Technology, Biotechnology and Regulatory Science, Szeged; Hungary (2019)*

III. **Zs. Németh**, D. G. Dobó, E. Pallagi, I. Csóka: Preparation and characterization of liposomes and the importance of their compositions; *XVI. János Szentágothai Multidisciplinary Conference and Student Competition, Pécs; Hungary (2019)*

IV. **Zs. Németh**, E. Palagi, O. Jójárt-Laczkovich, P. Szabó-Révész, I. Csóka: A QbD-based approach to develop a nasal formulation; *2nd Young Technologists' Forum 2019, Budapest, Hungary (2019)*

V. **Zs. Németh**, D. G. Dobó, E. Pallagi, I. Csóka: Quality by Design-based approach for liposomal development; *II. Symposium of Young Researchers on Pharmaceutical Technology, Biotechnology and Regulatory Science, Szeged, Hungary (2020)*

VI. **Zs. Németh**, D. G. Dobó, E. Pallagi, I. 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, Pécs, Hungary (2020)*

VII. **Zs. Németh**, D. G. Dobó, E. Pallagi, I. Csóka: Quality-focused formulation - QbD-based liposome design and development; *III. Symposium of Young Researchers on Pharmaceutical Technology, Biotechnology and Regulatory Science, Szeged, Hungary (2021)*

VIII. **Zs. 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, online (2021)*

IX. **Németh Zs.**, Sipos B., R. Semnani Jazani, Dobó D. G.: Liposzóma alapú nano-hordozórendszerek kockázatbecslésre épülő optimalizálása; *XIV. Clauder Ottó Emlékverseny, Budapest, Magyarország (2021)*

X. **Zs. Németh**, R. Semnani Jazani, B. Sipos, D. G. Dobó, E. Pallagi, I. Csóka: Risk-based optimization of liposome-based nanocarrier systems; *IV. Symposium of Young Researchers on Pharmaceutical Technology, Biotechnology and Regulatory Science, Szeged, Hungary (2022)*

Poster presentations

I. E. Pallagi, O. Jójárt-Laczkovich, **Zs. Németh**, P. Szabó-Révész, I. 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, Szeged, Hungary (2018)*

II. **Zs. Németh**, D. G. Dobó, E. Pallagi, I. Csóka: Analytical investigation techniques in the service of liposome development; *26th International Symposium on Analytical and Environmental Problems, Szeged, Hungary (2020)*

III. **Zs. Németh**, D. G. Dobó, I. Csóka: Quality by Design-based development process for the preparation of liposomal formulations; *12th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, online (2021)*

IV. **Zs. Németh**, R. Semnani Jazani, D. G. Dobó, I. Csóka: Factorial Design-based liposome optimization applying vesicle-modifying membrane additives; *13th Central European Symposium on Pharmaceutical Technology, Gdansk, Poland (2021)*

V. **Zs. Németh**, D. G. Dobó, I. Csóka: Updated Risk Assessment-based liposome design & development; *10th Anniversary pan-European Science Conference on QbD & PAT, online (2021)*

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