

We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900

Open access books available

186,000

International authors and editors

200M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com



Pharmaceutical Development of Liposomes Using the QbD Approach

Alina Porfire, Marcela Achim, Cristina Barbalata, Iulia Rus, Ioan Tomuta and Cecilia Cristea

Abstract

Quality by Design (QbD) is a systematic, risk-based approach to pharmaceutical product and manufacturing development, which uses quality-improving scientific methods upstream in the research, development, and design phases, in order to assure that quality and safety are designed into product at as early stage as possible. This work focuses on the state-of-the-art applications of the QbD principles in the development of liposomes. The QbD approach has recently been proposed as a useful tool to obtain higher-quality liposomal products, as their development is a challenging task, involving intricate formulation and manufacturing processes. Thus, the current strategies to define the relationship between the critical material attributes or process parameters and product critical quality attributes and to establish the design space are overviewed. Additionally, the current characterization methodologies are described, as part of the control strategy required within the QbD paradigm.

Keywords: liposomes, Quality by Design, critical quality attributes, design space, quality control

1. Introduction

In the recent years, the Quality by Design (QbD) concept has gained importance in drug development and drug manufacturing. QbD is recommended by the drug regulatory agencies (FDA—Food and Drug Administration and EMA—European Medicines Agency) to improve the quality of pharmaceutical products.

For the pharmaceutical products, quality is regarded as a mandatory topic and must be assured for all. Pharmaceutical products' quality is ensured through the ability to get the therapeutic benefit mentioned on the label and through the absence of contamination [1, 2]. Product quality refers to performance, robustness, trustworthiness, and has to be built into it [3]. To ensure the quality of a product, scientific approaches such as QbD can be implemented. The concept of QbD was first defined a few decades ago by Dr. Joseph M. Juran, a well-known pioneer of quality, and emphasizes the design of a product and manufacturing process to reach a certain predefined quality [1, 2, 4]. According to Dr. Juran, quality should be built into a product, and the way in which a product is designed is accountable for most

issues related to its quality. Dr. Juran believed that the quality of a product could be planned and the most quality inadequacies originate from the way in which the quality of the product was planned [4, 5].

In the pharmaceutical field, there are several regulatory guidelines developed by ICH, FDA, and EMA, which offer the necessary information in understanding how the QbD concept may be implemented [2, 3, 6–8]. In ICH Q8—Pharmaceutical Development guideline, QbD is defined as “a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management” [9]. Such a scientific, risk-based, and pro-active approach from the development to manufacturing of a pharmaceutical product will provide the necessary knowledge and information to minimize the risk and ensure a predefined quality of the product [10]. So, QbD provides the tool to understand the way in which the quality of a pharmaceutical product is influenced by formulation, input materials’ characteristics, and process variables; therefore, the quality of the product can be ensured by controlling the formulation input materials and the manufacturing process key variables [2, 4, 11]. It involves designing a formulation and manufacturing process in such a way to obtain a pharmaceutical product with predetermined quality specifications [12]. QbD identifies characteristics that are critical to quality of the product, translates them into attributes that the drug should possess, and establishes how the critical formulation and process parameters can be varied to constantly produce a drug product with the desired characteristics [13, 14]. The goal of the QbD approach is in-depth understanding of the formulation and process variables, and of the relationship between them, in order to obtain a drug product with consistent desired characteristics [15, 16]. Practically, QbD helps establishing the critical quality attributes (CQAs) of the product and how the critical material attributes (CMAs) and critical process parameters (CPPs) can be modified to deliver a product with predetermined quality specifications [12].

A QbD approach includes several key steps, as follows: (1) defining the quality target product profile (QTPP); (2) performing a risk assessment, in order to identify which formulation, material, or process parameters can potentially influence the product’s quality attributes (CQAs); (3) studying the impact of the formulation variables, material attributes, and process parameters on the critical quality attributes (CQAs) of the drug product, and finding which of them are critical material attributes (CMAs) and critical process parameters (CPPs); (4) establishing a design space that ensures desired product specifications; and (5) designing and implementing a control strategy in order to ensure a continuous improvement [9, 17, 18]. In order to study the relationship between the CMAs and CPPs and their impact on CQAs in a mathematical form, the Design of Experiments (DoE) strategy is used. This method also allows to establish the design space by running a minimum number of experiments [2, 11, 19]. The design space for liposome preparation may be established by implementing the QbD strategy as a systematic approach in liposome development, in order to improve the product quality, by understanding and controlling formulation, materials, and manufacturing variables. The strategy is recommended by the drug regulatory agencies for the development of better-quality products and may be used in liposomal drug product development.

2. Defining the QTPP for liposomal products and identification of the CQAs

The most important element in using QbD concept to assist formulation and process design is to predefine the desired final product quality profile [9, 14, 20].

QTPP elements		Target
Dosage form		Nanoformulation
Dosage design		For targeted delivery
Administration route		Parenteral
Quality attributes of the liposomal product	Biocompatibility	Lack of hemolytic activity
	Microbiological quality	Sterile
	Bacterial endotoxins	Free
	Physical attributes (particle size and morphology, viscosity, zeta potential, osmolarity, appearance)	Must meet the standards resulted from the specifications of similar approved products or from the current scientific research
	Drug identification	
	Drug content	
	<i>In vivo</i> stability	
	Drug release	
	Degradation products	
	Residual solvents	

Table 1.
QTPP of liposomal products.

According to the definition of ICH, the QTPP is “a prospective summary of the quality characteristics of a drug product that ideally will be achieved to ensure the desired quality, taking into account safety and efficiency of the drug product” [9]. In order to establish the QTPP, the following considerations must be taken into account: route of administration, dosage form, dosage strength, delivery system, attributes affecting pharmacokinetic characteristics, stability, sterility, and drug release appropriate for the intended final product [14, 20].

QTPP is established based on prior scientific knowledge and appropriate *in vivo* relevance. According to the current flux of literature, a QTPP for liposomal products is presented in **Table 1** along with the targets for each element [21].

When designing a nanoformulation, the efficiency of the final product will be directly related to particle size. Particle size is the most important factor influencing biodistribution, circulation half-life, and cellular uptake. Due to their small size and large surface area, pharmaceutical nanosystems show enhanced bioavailability and additional ability to cross the biological membranes. Furthermore, in cancer therapy, smart nanoparticles deliver the drug into the tumor tissue and avoid normal tissues and organs [22]. After intravenous administration, liposomes accumulate in tumors by a passive or active targeting, for determining higher therapeutic efficiency and less side effects [23]. As a parenteral dosage form, liposomal products must be sterile, pyrogen free, and well tolerated [24]. The lack of hemolytic activity is also mentioned as requirement for liposome products [25].

The second step in a QbD approach is the identification of CQAs. According to ICH Q8 definition, 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 desired product quality.” They are either derived from the QTPP or defined based on regulatory requirements and review of the literature [9].

Based on these recommendations, for the liposomal products the following CQAs are usually identified: mean particle size, size distribution, and zeta potential (as physical CQAs); drug content (as chemical CQAs); *in vivo* stability and drug release (as biological CQAs); sterility (as microbiological CQA).

- a. Generally, for all nanoparticles, the mean particle size and particle size distribution are major CQAs, which play an important role in determining their *in vivo* distribution, drug loading, drug release, and targeting ability.

The biodistribution of nanoparticles circulating in the blood stream is considerably influenced by particle size. Smaller particles, of 20–30 nm, are eliminated by kidneys, while larger particles, of up to 300 nm, are taken up by reticuloendothelial system (RES) [26]. By decreasing particle size below 100 nm, RES uptake can be reduced [27]. According to other studies, the ideal nanocarriers should have particle size ranging between 10 and 100 nm in order to evade the kidney filtration and capture by the liver [28]. For example, in tumor targeting, the particles need to be smaller than the cut-off of the fenestrations in the tumor neovasculature [29]. The literature reveals that for an efficient enhanced permeability and retention (EPR) effect, the particle size must be, generally, between 50 and 100 nm, depending on the tumor type, its environment, and its localization [28]. Particle size less than 200 nm is also beneficial for the sterility of the liposomal product, since this size allows aseptic preparation and sterile filtration of the final product [30].

Particle size also influences the kinetics of drug release. As particle size gets smaller, their surface area-to-volume ratio gets larger. This would imply that more of the drug is closer to the surface of the particle compared to a larger particle. Being at or near the surface would lead to a faster drug release [23].

Polydispersity index, reflecting particle size distribution of liposomal products, is a physical parameter for which the target is to have reduced values, indicating a good homogeneity of the dispersion. Generally, polydispersity index values below 0.5 are reported to be acceptable [31].

- b. Zeta potential or particle charge is an important parameter in the evaluation of colloidal system's stability. Particles with a high negative or positive zeta potential value repel each other, indicating that the colloidal system is stable [32]. On the contrary, decreasing the zeta potential value to nearly neutral leads to liposomal aggregation [20]. The charge of nanosystems influences both systemic circulation time and the interactions with the target tissue. The presence of surface charge can alter the opsonization profile of the particles, its recognition by cells in the organs of the RES, and the overall plasma circulation profile [29]. Regarding the interaction with the target, it is known that cationic liposomes have affinity for negatively charged cancer cell membrane and higher selectivity than neutral or anionic liposomes. The intracellular uptake of cationic liposomes by tumor cells can be 14-fold higher than that of normal liposomes. Consequently, cationic liposomes enhance the safety of liposomal drug. The liposome charge can also influence drug loading, cationic liposomes exhibiting higher encapsulation efficiency for negatively charged drugs [32].
- c. Depending on drug distribution in liposomal dispersion, the drug content of liposomes can be expressed in three ways: drug concentration ($\mu\text{g/mL}$); entrapment efficiency (EE, the amount of drug contained inside liposomes compared with total amount of drug; %); and drug loading (the amount of drug contained relative to the amount of the lipid used; drug-to-lipid ratio) [14, 20].

High encapsulation efficiency is very important for both manufacturers and patients. A higher percentage of drug encapsulation could reduce the manufacturing cost and increase drug concentration in the final formulation allowing greater flexibility in dosing. Depending on the pharmacokinetics, higher drug concentration can result in increased dosing intervals and hence improved patient compliance [30].

Drug retention inside liposomes is also very important. Since liposomes are intended to deliver the drug to the target site, there should be no drug leakage until cellular uptake [30].

- d. In order to have a prolonged drug release and an efficient tumor accumulation by passive targeting, *in vivo* stability is essential. *In vivo* behavior of liposomes after intravenous administration is directly linked to their interaction with blood components, which depends on the hydrophobic/hydrophilic character of liposome surface. The more hydrophobic a nanoparticle is, the more likely it is to be cleared by phagocytosis, due to higher binding of blood proteins (opsonization) [22]. The improved surface properties are associated with the steric hindrance effect offered by hydrophilic polymers, which can prevent the surface-modified liposomes from being rapidly eliminated by RES ("stealth liposomes"). Hydrophilization of liposome surface prolongs circulation time (long-circulating liposomes), enhancing the therapeutic potential of the entrapped drug [33].
- e. Drug release kinetics has important implication for the therapeutic activity of liposomes. The drug must be delivered to the disease site at a level within its therapeutic window, at a sufficient rate, for a sufficient period, to have optimal therapeutic activity [34]. Drug delivery to the right site is related to the identification and the interaction with the target cells. Cell surface or blood vessel surface of tumor tissues has a series of specific and overexpressed receptors, which are closely related with tumor process [33]. Targeting ligands attached on liposomes' surface bind the corresponding receptors and, as a result, the liposomes are internalized by endocytosis. These ligands can be monoclonal antibodies, fragments of antibodies, peptides, proteins, nucleic acids, carbohydrates, or small molecules [29].

3. The critical material attributes and critical process parameters: identification and linking to CQAs

All the material attributes (MAs) and/or process parameters (PPs) that can affect the desired critical quality attributes (CQAs) of the final/intermediate product are identified through risk assessment. After risk identification, risk analysis evaluates the impact of the identified MAs and PPs on the CQAs. Further, through risk evaluation, a qualitative or quantitative scale is used for risk estimation of each identified factor on the desired CQA [35]. After the risk evaluation of potential MAs and PPs, only few of them will become potentially critical for quality attributes of the final drug product. In this case, identified MAs will become critical material attributes (CMAs), which must possess certain characteristics or should be chosen in an appropriate range to assure CQAs of the intermediate/final drug product [21]. Critical process parameters (CPPs) are those PPs that should be monitored and controlled in order to obtain the desired CQAs of the intermediate/final drug product [9]. Many tools are used for risk assessment, but the most used ones are Ishikawa or fish-bone diagram for risk identification and failure mode and effect analysis for risk evaluation [36]. Risk assessment should be done in the first step of the drug product development, and analysis of these risk factors is recommended to be reconsidered during different stages of the drug development [37].

To establish the CMAs, the main components of liposomes are evaluated, that is the active substance, the lipids, and others like buffer solution [38]. Each active substance has different physiochemical properties and can influence the desired CQAs [9, 39]. Depending on its solubility, it can be entrapped in the aqueous core

or in the lipid bilayer [40]. In order to achieve a greater therapeutic effect, more than one active substance may be encapsulated in the same product, so different physiochemical properties of one may influence the other/others drug entrapment process [40, 41]. Besides this, their octanol/buffer partition must also be taken into consideration because a low partition will lead to a prolonged release of the drug and vice versa [42].

Regarding the lipids, their physicochemical characteristics are also important. For example, lipids that contain unsaturated fatty acids are predisposed to degradation reactions like oxidation or hydrolysis and those which contain saturated fatty acids have a higher transition temperature (T_m) [43]. Another specific characteristic of lipids is their chain length. Usually, a shorter chain length will contribute to a thinner lipid bilayer and a larger internal volume for drug encapsulation. However, comparing three lipids with a difference of two carbon atoms between them, the lipid bilayer thickness obtained did not differ with more than 1 nm and a very small difference in liposomes' size and EE was observed [30]. Lipid properties can have a great impact on liposomes' membrane fluidity, permeability, or charge [44]. In this regard, cholesterol increases liposomal stability, reduces membrane fluidity, and, consequently, contributes to an increased EE [45].

Different compounds used for surface modification, in order to obtain a prolonged blood circulation or modified drug release, must be evaluated during risk identification and analysis. For example, when chitosan was used for coating, its concentration, solubility, and molecular weight influenced liposomal size [46]. In the case of polyethylene glycols (PEGs), the molecular weight and the density on liposomal surface influenced the biodistribution and size [47].

Besides the nature of liposomal components, their concentration and ratios between them are also critical. Some of these ratios, identified in risk assessments of different studies, are: organic phase-to-aqueous phase ratio; cholesterol-to-lecithin ratio; chloroform-to-methanol ratio; phospholipids-to-cholesterol molar ratio; and drug-to-lipid ratio [40, 46, 48, 49].

Regarding the preparation process, many techniques and methods like film hydration, emulsification, and reverse phase evaporation were developed but the most used one remains the film hydration method [37]. For this method to be efficient from the viewpoint of EE, parameters like temperature or rotation speed in the evaporation and hydration steps are critical and must be optimized [41, 49]. Film hydration method's great disadvantage is that the obtained liposomes are in the vast majority of micron size with a multilamellar structure, being characterized by a high percentage of lipids and a reduced internal volume. Thus, a reduction in particle size is mandatory, to increase their internal volume and to have a controlled size and narrow particle size distribution [30, 37]. For reduction of liposomal size, several techniques like sonication, freeze-thaw cycling, or extrusion may be used. Comparing these size-reduction methods through a screening experimental design, they were found to be critical for particle size distribution but also for EE [30]. When extrusion through membranes is used for size reduction, membrane pore size, temperature, and applied pressure are important CPPs because these factors define the final particle size and can seriously influence the final EE [30]. Sonication process also needs optimization as regards its time in order to achieve the desired liposomal size [46]. For the freeze-thaw cycling process, the number of the cycles might be taken into consideration for further optimization in order to get the desired particle size [30].

After the risk assessment, next step in QbD development is linking the identified CMAs and CPPs to CQAs by using Design of Experiments (DoE). The greatest advantage of using DoE is that it can track the interactions between the studied factors, CMAs and CPPs, and it can establish a quantitative relationship between the

identified variables and the results [36]. For a better understanding of this concept in liposomes development, the most important CQAs will be discussed further, and how CMAs and CPPs can influence them according to different studies.

3.1 Drug content

The drug content is most frequently optimized in terms of EE than drug concentration as EE reflects better the preparation process performance and robustness. According to published data, the most influential factors for EE are: the amount of drug and phospholipids; cholesterol concentration; the nature of lipids and drug; the interactions between different components; the lipid-to-drug ratio; and several process parameters. Among these, several studies established, through DoE approaches, that lipid molar ratio and lipid-to-drug ratio are the most critical parameters for EE optimization. Using a great amount of lipids for liposome preparation favors the formation of many vesicles with a significant internal volume for drug encapsulation and, consequently, the EE of hydrophilic drugs increases [30, 45]. Including cholesterol in liposome formulations increases not only their stability but also the drug content, due to the so-called “pocket” theory, presuming that cholesterol can generate different size pockets inside the lipid bilayer where API can be entrapped [45]. The use of unsaturated lipids was shown to have a similar effect, the unsaturated lipids forming pockets inside the lipid bilayer where lipophilic drugs can be entrapped [50]. By increasing lipid-to-drug ratio from 1:1 to 10:1, the EE of ritonavir, a lipophilic drug, was doubled from 45 to 90% [43]. The ratio between phospholipid concentration and drug concentration was demonstrated to have a significant impact on EE in the case of simvastatin. Thus, at high simvastatin concentration and low concentration of phospholipids, EE decreased [51].

In the case of drugs with pH-dependent solubility like doxorubicin, the EE can be improved by the use of active loading method at specific pH range, the pH of hydrating buffer and of external buffer being critical parameters [52].

Regarding the drug concentration, several studies concluded that the total amount of drug used for encapsulation has a breaking point from which the EE cannot be further increased [41, 46].

EE may also be influenced by PPs. A fractional factorial design was used to establish the link between PPs like hydration temperature and the number of freeze-thaw cycles during preparation of liposomes loaded with FK50, an immunosuppressant. An interaction between these PPs was highlighted. It was observed that by using an increased number of cycles, and high hydration temperature, EE decreased, because repetition of the freeze-thaw cycle at high temperature might induce leakage of FK506 from the membrane of the liposomes [39]. In another study, it was observed that PPs like temperature during film hydration and rotation speed during solvent evaporation are critical. Using high temperature, above T_m , the concentration of the encapsulated drug increases because at high temperature, the lipid bilayer is more fluid and permits the entrapment of more drug. Also, using a high rotation speed at the film formation can have a great impact on EE because this CPP leads to formation of a thinner lipid bilayer which can easily be hydrated [53].

3.2 Particle size and size distribution

Maintaining a controlled particle size and low PDI is one of the main challenges when preparing liposomes, and optimization of these parameters is improved through the use of DoE. There are many critical factors influencing these parameters, both formulation and process related. For example, the influence of phospholipid concentration, active substances (curcumin and doxorubicin) concentration,

working temperature, buffer pH, and phospholipid-to-cholesterol ratio, on liposomal size, was examined through a screening experimental design. Out of the studied factors, only phospholipid concentration and phospholipid-to-cholesterol ratio significantly influenced the size, while the concentration of the drugs and the working conditions were not critical for particle size. Noteworthy, none of the studied parameters influenced particle size distribution [41].

Regarding the effect of lipid concentration, different studies showed contradictory results on the size in relation to this factor [43, 46]. Usually, on increasing lipid concentration, liposomal size increases, simultaneously with PDI values [54]. Another observation was that increasing lipid concentration over a certain value leads to smaller size, probably due to lipid bilayer rearrangement into a bigger number of liposomes with smaller size and better size distribution [55]. Depending on the lipid type, a different influence on liposomal size was observed [56].

The active substances influence liposomal size depending on their physico-chemical properties. It was observed that quercetin, a lipophilic compound, might replace some lipids in the lipid bilayer causing a reduction in size when it is used in high concentrations [55]. On the other hand, high concentrations of pravastatin, a hydrosoluble compound, led to a small size of liposomes due to an interaction between the active substance and the lipids [53].

3.3 Zeta potential

This parameter is influenced by formulation factors like ionic strength, pH, bilayer composition, or charged lipids and PPs like sonication time [48, 57]. In order to modulate the ZP values, different stabilizers, such as stearylamine and diacetyl phosphate, or modified lipids, such as poly(2-ethyl-2-oxazoline) (PEtOz) or PEG, can be incorporated in lipid bilayer. The concentration of these excipients may be optimized such as to obtain optimal stability [50, 57]. In conventional liposomes, cholesterol-to-lecithin ratio influences the zeta potential value [48]. Through DoE, several papers established the critical parameters influencing the zeta potential values. For example, a screening design study was used to determine which of the formulation factors (lipid concentration, cholesterol concentration, chitosan concentration, drug concentration, organic phase/aqueous phase ratio) and process parameters (temperature, stirring speed, sonication time) had a significant influence on zeta potential of chitosan-coated liposomes. As expected, chitosan concentration was a critical parameter, along with the temperature, which favored the coating process through reduction of vesicles size [46].

3.4 In vivo stability and drug release

By choosing a suitable lipid bilayer composition, a higher stability in vivo might be achieved. In order to sustain this, it was observed that using saturated lipids or cholesterol in formulations, lipid bilayer stability is increased and liposomal uptake by mononuclear phagocyte system will be reduced. Another used pathway is incorporation of different excipients, such as ganglioside GM1, phosphatidylinositol or PEG-lipids, creating a steric barrier which prevents their clearance from the system [42]. The organs in which liposomes accumulate for being eliminated are lungs, liver, spleen, and kidneys. It was observed that those liposomes with negative surface charge present a higher uptake in tumor cells and a slower uptake in liver cells [58]. In a study, four liposome formulations were compared regarding their *in vivo* stability, by tracking their accumulation in spleen. Results showed that the molecular weight of PEG attached to their surface as well as particle size were CMAs influencing the accumulation in the spleen [47].

Depending on the lipophilic or hydrophilic character of the active substance, the kinetics of release is different because the diffusion through liposomal membrane is influenced by its physiochemical properties [41]. This behavior was shown when the release of two different lipophilic drugs and a hydrophilic drug from the same liposomal system was studied *in vitro*. The lipophilic agent displayed prolonged release and a smaller total drug release in comparison with the hydrophilic one due to their different characteristics [40].

For pH-sensitive liposomes, the objective in terms of *in vitro* drug release is to have a very good stability of the encapsulated drug under physiological conditions and triggered drug release at certain pH values. To achieve this, the use of excipients having a membrane-destabilizing effect and their concentration are critical parameters [52, 56].

4. Defining the product and process design space

The design space (DS) is a multidimensional combination and interaction of the input variables, such as material attributes, and process parameters that have been shown to assure quality [9]. Thus, the advantage of DS determination is that it establishes the operating region which ensures consistent product qualities between different batches. Working within the DS, the product will meet the specifications mentioned in the QTPP, while moving out of it is considered a change that would normally require a regulatory approval in the case of authorized products [9].

Determination of the DS is based on multivariate analysis, considering the main effects of factors as well as their interaction, which helps in determining an operational region based on a predefined confidence level. The DS includes the product design space and process design space. The product DS is established with product's CQAs as dimensions, while the process design space is exhibited as CQAs with respect to CPPs [59].

One approach to establish the DS for liposomal products is to take simultaneously into consideration both formulation factors and CPPs. This method was used for the determination of the DS for lyophilized liposomes with simvastatin. Thus, the cholesterol concentration, the PEG proportion, the cryoprotectant-to-phospholipids molar ratio, and the number of extrusions through polycarbonate membranes were selected as the most influential factors for lyophilized liposome CQAs. Their variation range was determined, in which the established quality requirements of the product are met: reduced particle size, maximized drug retention during lyophilization, reduced change in phospholipid transition temperature, low residual moisture content. The validity of the DS was confirmed by determining the CQAs of a formulation corresponding to the robust set point, that is, the formulation for which the prediction errors are lowest. Thus, defining the DS was found to be a useful strategy for the development of stable lyophilized liposomes having predictable quality [60]. The same approach was used to generate the DS for preparation of prednisolon-loaded long-circulating liposomes at laboratory scale. In this case, the selected formulation parameters were prednisolon concentration and the PEG proportion in lipidic membrane, and the process parameters were the extrusion temperature and the rotation speed at the hydration of the lipid film. The DS was developed such as to ensure high liposomal drug concentration, high EE, and controlled size [49].

When process parameters are not found as critical for the quality of the product, or their impact on quality is easily controlled by fixing a certain operating level, the DS is proposed as a function of formulation variables. This approach has been used in several studies, the most studied formulation variables being the active substance

concentration and the composition of the lipid membrane. For example, the DS for liposomes with tenofovir was constructed with respect to phospholipid, concentration, cholesterol concentration, and drug concentration, with a focus to obtain high drug encapsulation efficiency, as this was considered the most difficult property to predict and control for liposomes containing hydrophilic drugs. Other quality attributes considered critical in the study, particle size and stability, were controlled by the pore size of the extrusion membrane and by storing the samples at low temperature, respectively [61]. In another paper, the DS for chitosan-coated liposomes was established as a function of drug concentration, chitosan concentration, and the organic phase-to-aqueous phase ratio during liposome preparation by ethanol injection technique. These variables were found to be the only significant factors affecting the CQAs of the product, although other formulation and process parameters were evaluated through a screening study. The composite desirability function based on constraints was used to determine the conditions that would result in an optimal formulation design, in terms of particle size, encapsulation efficiency, and coating efficiency. Target values were selected for the mentioned quality attributes, and, on the basis of these target values, the optimum values for each variable or processing parameter were obtained. In an additional step, the robustness of the DS was analyzed and the results showed that the selected CPPs may help minimize the variations in QAs that might arise due to the variability of the raw materials [46].

The development of liposomal systems entrapping more than one active substances is more complex, because the properties of each will influence the CQAs of the product and their stability in the processing conditions will impact the process parameters. In this regard, a group of authors established and evaluated the DS for long-circulating liposomes co-encapsulated with curcumin and doxorubicin. DS development was based on a previous screening study, which revealed the critical parameters, that is, phospholipid concentration, the phospholipid-to-cholesterol molar ratio, doxorubicin concentration, and curcumin concentration. The purpose was to obtain the variation range of these factors for which the size of the liposomes is minimized and the encapsulation efficiencies of both drugs are maximized. The DS was established as the region within which the prediction of the CQAs is made with a probability of failure of less than 1%. Moreover, a DS hypercube was set out as a restricted zone in the DS where factors' values can vary independent of each other, without influencing the quality of the product [41].

The incomplete understanding of the manufacturing process is a major barrier in liposomal products' industrial production and clinical application. The destabilization of their structure during long-time storage as aqueous dispersions revealed the need for complex fabrication processes, involving drying steps such as lyophilization and spray drying. The key to the successful design and preparation of optimal liposomal dry powder formulations is an understanding of the significance of the drying process parameters [62]. This aim was achieved by several authors by determining the DS for lyophilization or spray drying process.

The DS for the freeze-drying process of pravastatin-loaded long-circulating liposomes was established as a function of the freezing rate and shelf temperature during primary drying. The two process parameters were found to have a great impact on product's CQAs, along with the presence of an annealing step. The conditions to obtain freeze-dried liposomes with the desired characteristics were generated using the combination trehalose-mannitol as cryoprotectant and by including an annealing step. A series of limitations and target values were applied for the critical quality attributes of the lyophilized product. Thus, the DS was constructed such as to ensure high drug retention after lyophilization, particle size below a certain value, low zeta potential, low residual moisture content, and a short duration of the primary drying. Out of the DS, an optimal formulation was selected and testing this

formulation confirmed that the process delivers the desired quality of the product, as long as it is operated within the DS [63].

A process design space for spray drying of liposomes was developed such as to get a product that met the criteria for all CQAs of an inhalable powder formulation. When developing an inhalable product, the mass median aerodynamic diameter (MMAD) is the most important CQA, as this characteristic influences the deposition in the conducting airway. Besides this, the size stabilization, relative moisture content, and process yield were identified as CQAs. The DS was plotted by imposing restrictions especially for MMAD, which should be within the range 4.5–5.5 μm , but also for liposome size ratio (before/after drying) and the yield of the process, while for moisture content, no restrictions were set, as acceptably low values were obtained for all the experiments. First, an optimal operating space (OOS) was identified with a high feed flow rate, a low outlet temperature, a medium aspirator rate and in the area of low feedstock concentration and high atomizing airflow. The MMAD was the QA restricting the entire ODS to the low feedstock concentration and high atomizing airflow, whereas the other CQAs met the imposed criteria in a larger space [64].

5. The control strategy

Due to the great success of liposomal systems, not only in pharmaceutical formulation but also in cosmetics and food industry, there is a huge demand on developing and standardizing analytical and bioanalytical methods for liposome complete characterization, as well as for their detection in blood and tissues. Official guidance regarding the manufacturing and controls recommend several methods for lipid components as well as for the drug products [65]. According to FDA guideline, “liposome structures and integrity are important physicochemical properties and they reflect the ability of the liposome drug formulation to contain the drug substance and to retain the drug substance within the appropriate liposome structure” [65].

A key issue in the control of liposomes is closely related with the quality control of the lipid components, including modified lipids, which could dramatically influence the properties and performances of liposome drug product [65]. The quality of the final product is influenced by the source of lipids and also by the type of the lipids: synthetic, semi-synthetic, or natural.

All types of separation methods, such as gas chromatography (GC) or liquid chromatography (LC), capillary electrophoresis (CE), gel electrophoresis, or electrochromatography have proved their value in the analysis of lipid components of liposomes [66–68]. In order to evaluate the chemical stability of the liposomes, it is mandatory to assess the chemical stability of the lipid components in the final drug formulation, taking into account that some lipids could be degraded by oxidation or hydrolysis. From the practical point of view, most of the separation methods offer important information on this matter. The broad versatility, high selectivity, efficiency, and low time of analysis are making them a good choice.

While the analysis of lipids using liquid chromatography does not need a long stage of pretreatment, the lipids analyzed using gas chromatography have to be derivatized in order to obtain more volatile compounds which are not turning into degradation products at their boiling point [69]. For fatty acids, the most used derivatization method consists in the esterification of the acids and their transformation in methyl esters. The esterification takes place under various conditions of temperature, mixing process, using different catalysts and for various periods of time. There are plenty of studies among scientific literature about derivatization

methods of fatty acids for their GC analysis, researchers being still interested in improving the pretreatment of these compounds to obtain an efficient and rapid process [70].

Even though the literature abounds in examples of separation techniques applied for lipid detection and quantification, some drawbacks are obvious: poor solubility, poor absorbance properties, the need for derivatization, and laborious sample preparations. Nevertheless, the hyphenation between techniques could overcome the drawbacks and offers promising results.

Regarding the control of the final product, the following properties are generally determined to characterize a liposome drug formulation: morphology; surface charge (expressed as zeta potential); particle size (average diameter) and polydispersity index; encapsulation efficiency; the amount of drug relative to the amount of lipids; phase transition; residual solvents; in vitro and in vivo drug release [71]. Variability in these properties may lead to changes in the quality of the liposomal drug products, including leakage of the drug from the liposomes. The QAs monitored for liposomes are presented in **Table 2**, together with the currently employed methods of analysis.

Particle size is one of the crucial parameters for further in vivo application of liposomes. The required size is usually in the range 20–250 nm. When using microscopy techniques, one will obtain a high-resolution three-dimensional profile of the vesicle surface under study. For instance, AFM permits liposomes’ visualization without alteration of their native form, given that the requisite surface immobilization does not adversely affect the sample and that the force of the probe itself does not have deleterious effects on the vesicles [75]. AFM is a rapid, powerful, and relatively non invasive technique and compared to TEM, does not requires complicated sample preparation and removal of liposomes from their native environment. Additionally, TEM provides information on the size distribution and shape of vesicles. Unfortunately, liposomes can suffer from structure perturbations triggered by the high vacuum conditions and the staining process.

The investigated property	Methods	Ref.
Morphology	UV-vis spectroscopy Spectrofluorimetry RMN Angle X-ray scattering (SAXS) Freeze fracture technique with subsequent transmission electron microscopy	[71–73]
Net charge (zeta potential)	ELS	[74]
Particle size	Microscopy techniques (TEM, AFM, SEM) SEC Field-flow fractionation and static DLS	[74, 75]
Drug encapsulation efficiency	Spectroscopy LC	[75]
In vitro drug release	Spectroscopy LC	[75]
In vivo drug release	Radiolabeling, fluorescence labeling, MRI, CT, MS	[75]

AFM, atomic force microscopy; TEM, transmission electron microscopy; SEM, scanning electron microscopy; ELS, electrophoretic light scattering; LC, liquid chromatography; SEC, size-exclusion chromatography; DLS, dynamic light scattering; MRI, magnetic resonance imaging; CT, computed tomography; MS, mass spectrometry.

Table 2.
The quality attributes monitored for liposomal products.

To measure particle size and size distribution, three types of techniques could be used: all together, separation, and particle-by-particle counting. In the first case, multi-angle (static) and dynamic light scattering (MALS and DLS, respectively) techniques permit the calculation of the average particle size and charge from the signal generated by multiple particles within the sample. Even though these techniques are fast, they have low resolution and do not provide the particle concentration. Separation techniques, such as disk centrifuge and field-flow fractionation, have the advantage of improving size analysis resolution by using differences in the sample properties, typically sedimentation rates, to pre-separate the sample prior to light-based (absorbance or scattering) analysis. The separation techniques do not measure particle concentration or charge, and they often suffer from separation-based issues. More effective are particle-by-particle counting techniques, such as tunable resistive pulse sensors (TRPSs). The main advantage is the possibility to measure the properties of individual liposomes, offering a direct measurement of the particle concentration as well as high resolution and more accurate analysis of the particle size and charge (zeta potential) distribution. This ability to simultaneously measure the distribution of both the size and zeta potential represents a new and effective means of analyzing liposome properties [76].

To measure the zeta potential, ELS technique is currently used. It consists in using heterodyne scattering methods in which a fraction of the laser beam is split away by a mirror before reaching the sample, and is directed to the detector where it is combined with scattered light from particles diffusing in the sample. The fraction of redirected light is referred to as the “local oscillator” and, unlike the light scattered by the sample, does not fluctuate. It is used as a reference beam and must be much larger than the average intensity of the scattered light produced by the diffusing particles. To determine the zeta potential, the electrophoretic mobility must first be ascertained [77].

The surface modification of liposomes is sometimes performed in order to increase their *in vivo* drug-delivery performances. As mentioned earlier, several ways of modifying the surfaces were reported, like the addition of polyethylene glycol (PEG) chains, or the attachment of antibodies and cellular receptor recognition molecules (e.g., the RGD peptide) as molecular targeting probes. Tracking the successful modification of liposomes is made by measuring the change in their electrophoretic mobility, in fact the modification of their zeta potential arising from the change in the number of charged surface groups. Another method for measuring the zeta potential of liposome particles is via ensemble light scattering techniques, which use a similar principle as DLS [78].

Within the control strategy, process analytical technology (PAT) has been evidenced lately as a significant tool for measuring parameters and attributes related to the active substance, the finished product as well as the processing conditions. Among the PAT tools, in the field of liposomes, near-infrared spectroscopy (NIRs) has been shown to be useful for the chemical characterization of liposomes in terms of the composition of the lipid membrane, as well as for the simultaneous quantification of excipients and active substance [79, 80].

Besides the large-scale production, which is a major challenge, the standardization of analysis procedures easily scalable is another important goal in practical application of liposomes. The liposome production is done in small size batches compared with other pharmaceutical products which are produced in large batches [71]. However, the possible application of liposomes and the increasing number of clinical trials involving liposomes prove the fact that this field of research is very dynamic and the synthesis and analysis methods become more effective, encouraging their application in the development of new drug carriers.

6. Conclusions

The implementation of the QbD approach in the pharmaceutical industry is intended to enhance the quality of pharmaceutical products through identifying, analyzing, and controlling all factors that could alter their quality, and, consequently, its efficacy and safety. Currently, an increasing number of papers describe the development of liposomal products based on this risk-based approach, although only few are following all the steps recommended by the regulatory guidelines. Finally, this strategy could be useful to promote liposomes from laboratory into authorized products.

Acknowledgements

The authors are grateful for the financial support from the Romanian National Authority for Scientific Research and Innovation, CNCS/CCCDI-UEFISCDI; project number PN-III-P1-1.2-PCCDI2017-022159PCCDI/2018 (IMPROVE), within PNCDI III.

Conflict of interest


The authors declare that there is no conflict of interest.

Author details

Alina Porfire*, Marcela Achim, Cristina Barbalata, Iulia Rus, Ioan Tomuta and Cecilia Cristea
Faculty of Pharmacy, University of Medicine and Pharmacy “Iuliu Hatieganu”, Cluj Napoca, Romania

*Address all correspondence to: aporfire@umfcluj.ro

IntechOpen

© 2019 The Author(s). Licensee IntechOpen. This chapter is distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/3.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. 

References

- [1] Rayaprolu BM. Quality by design: A brief introduction. *Journal of Pharmacovigilance*. 2015;**3**(4):e142
- [2] Tefas LR, Rus LM, Achim M, Vlase L, Tomuta I. Application of the quality by design concept in the development of quercetin-loaded polymeric nanoparticles. *Farmácia*. 2018;**66**(5):798-810
- [3] Sangshetti JN, Deshpande M, Zaheer Z, Shinde DB, Arote R. Quality by design approach: Regulatory need. *Arabian Journal of Chemistry*. 2017;**10**:S3412-S3425
- [4] Lionberger RA, Lee SL, Lee L, Raw A, Yu LX. Quality by design: Concepts for ANDAs. *The AAPS Journal*. 2008;**10**(2):268-276
- [5] Yu LX, Amidon G, Khan MA, Hoag SW, Polli J, Raju GK, et al. Understanding pharmaceutical quality by design. *The AAPS Journal*. 2014;**16**(4):771-783
- [6] The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Guidelines [Internet]. 2019. Available from: <https://www.ich.org/products/guidelines/quality/article/quality-guidelines.html>
- [7] FDA Guidances [Internet]. 2019. Available from: <https://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances%20/default.htm>
- [8] EMA Reflection Paper: Chemical, pharmaceutical and biological information to be included in dossiers when Process Analytical Technology (PAT) is employed [Internet]. 2006. Available from: http://www.ema.europa.eu/docs/en_GB/document%20library/Other/2009/10/WC500004890.pdf
- [9] ICH harmonised tripartite guideline. Pharmaceutical development Q8(R2). 2009. Available from: https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q8_R1/Step4/Q8_R2_Guideline.pdf [Accessed 01-2019]
- [10] Raman NVVSS, Mallu UR, Bapatu HR. Analytical quality by design approach to test method development and validation in drug substance manufacturing. *Journal of Chemistry*. 2015;**2015**:1-8
- [11] Pramod K, Tahir MA, Charoo NA, Ansari SH, Ali J. Pharmaceutical product development: A quality by design approach. *International Journal of Pharmaceutical Investigation*. 2016;**6**(3):129-138
- [12] Chowdary KPR, Shankar KR, Kumar PS. Recent research on QbD approach in formulation development – A review. *International Journal of Computer Science Trends and Technology*. 2014;**4**(1):282-292
- [13] Yu LX. Pharmaceutical quality by design: Product and process development, understanding, and control. *Pharmaceutical Research*. 2008;**25**(4):781-791
- [14] Sylvester B, Porfire A, Muntean DM, Vlase L, Banciu M, Luput L, et al. Optimzation of prednisolone-loaded long-circulating liposomes via application of Quality by Design (QbD) approach. *Journal of Liposome Research*. 2018;**28**(1):49-61
- [15] Shirsat AE, Chitlange SS. Application of quality by design approach to optimize process and formulation parameters of rizatriptan loaded chitosan nanoparticles. *Journal of Advanced Pharmaceutical Technology & Research*. 2015;**6**(3):88-96
- [16] De Beer TR, Wiggenhorn M, Hawe A, Kasper JC, Almeida A, Quinten T,

- et al. Optimization of a pharmaceutical freeze-dried product and its process using an experimental design approach and innovative process analyzers. *Talanta*. 2011;**83**(5):1623-1633
- [17] Eon-Duval A, Valax P, Solacroup T, Broly H, Gleixner R, Strat CL, et al. Application of the Quality by Design approach to the drug substance manufacturing process of an Fc fusion protein: Towards a global multi-step design space. *Journal of Pharmaceutical Sciences*. 2012;**101**(10):3604-3618
- [18] Sangshetti JN, Deshpande M, Zaheer Z, Shinde DB, Arote R. Quality by design approach: Regulatory need. *Arabian Journal of Chemistry*. 2014;**101**:1-14
- [19] Mishra V, Thakur S, Patil A, Shukla A. Quality by design (QbD) approaches in current pharmaceutical set-up. *Expert Opinion on Drug Delivery*. 2018;**15**(8):737-758
- [20] Xu X, Khan MA, Burgess DJ. A quality by design (QbD) case study on liposomes containing hydrophilic API: II. Screening of critical variables, and establishment of design space at laboratory scale. *International Journal of Pharmaceutics*. 2012;**423**:543-553
- [21] Beg S, Rahman M, Kohli K. Quality-by-design approach as a systematic tool for the development of nanopharmaceutical products. *Drug Discovery Today*. 2019;**24**(3):717-725
- [22] Rizvi SAA, Saleh AM. Applications of nanoparticle systems in drug delivery technology. *Saudi Pharmaceutical Journal*. 2018;**26**:64-70
- [23] Gabizon AA. Stealth liposomes and tumor targeting: one step further in the quest for the magic bullet. *Clinical Cancer Research*. 2001;**7**:223-225
- [24] European Pharmacopoeia Online 9.3 [Internet]. 2018. Available from: <http://online6.edqm.eu/ep903/> [Accessed 01-02-2019]
- [25] Sesarman A, Tefas L, Sylvester B, Licarete E, Rauca V, Luput L, et al. Co-delivery of curcumin and doxorubicin in PEGylated liposomes favored the antineoplastic C26 murine colon carcinoma microenvironment. *Drug Delivery and Translational Research*. 2019;**9**(1):260-272
- [26] Gaumet M, Vargas A, Gurny R, Delie F. Nanoparticles for drug delivery: The need for precision in reporting particle size parameters. *European Journal of Pharmaceutics and Biopharmaceutics*. 2008;**69**:1-9
- [27] Fukumori Y, Ichikawa H. Nanoparticles for cancer therapy and diagnosis. *Advanced Powder Technology*. 2006;**17**(1):1-28
- [28] Aftab S, Shah A, Nadhman A, Kurbanoglu S, Ozkan SA, Dionysiou DD, et al. Nanomedicine: An effective tool in cancer therapy. *International Journal of Pharmaceutics*. 2018;**540**:132-149
- [29] Bertrand N, Wu J, Xu X, Kamaly N, Farokhzad OC. Cancer nanotechnology: The impact of passive and active targeting in the era of modern cancer biology. *Advanced Drug Delivery Reviews*. 2014;**66**:2-25
- [30] Xu X, Khan MA, Burgess DJ. A quality by design (QbD) case study on liposomes containing hydrophilic API: I. Formulation, processing design and risk assessment. *International Journal of Pharmaceutics*. 2011;**419**:52-59
- [31] Amasya G, Badilli U, Aksu B, Tarimci N. Quality by design case study 1: Design of 5-fluorouracil loaded lipid nanoparticles by the W/O/W double emulsion—Solvent evaporation method. *European Journal of Pharmaceutical Sciences*. 2016;**84**:92-102
- [32] He K, Tang M. Safety of novel liposomal drugs for cancer treatment:

Advances and prospects. *Chemico-Biological Interactions*. 2018;**295**:13-19

[33] Li M, Du C, Guo N, Teng Y, Meng X, Sun H, et al. Composition design and medical application of liposomes. *European Journal of Medicinal Chemistry*. 2019;**164**:640-653

[34] Allen TM, Cullis PR. Liposomal drug delivery systems: From concept to clinical applications. *Advanced Drug Delivery Reviews*. 2013;**65**:36-48

[35] ICH harmonised tripartite guideline. Quality Risk Management Q9 [Internet]. 2005;(November):1-23. Available from: <http://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:Quality+Risk+Management+Q9#0%5Cnhttp://www.cls.co.at/downloads/ich-q9---quality-risk-management.pdf> [Accessed 01-2019]

[36] Zhang L, Mao S. Application of quality by design in the current drug development. *Asian Journal of Pharmaceutical Sciences*. 2017;**12**(1):1-8

[37] Simões A, Veiga F, Figueiras A, Vitorino C. A practical framework for implementing Quality by Design to the development of topical drug products: Nanosystem-based dosage forms. *International Journal of Pharmaceutics*. 2018;**548**(1):385-399

[38] U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research. Guidance for industry: Liposome drug products: chemistry, manufacturing, and controls; human pharmacokinetics and bioavailability; and labeling documentation. *Pharmaceutical Quality* [Internet]. 2018. Available from: <https://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm064979.htm>

[39] Toyota H, Asai T, Oku N. Process optimization by use of design

of experiments: Application for liposomalization of FK506. *European Journal of Pharmaceutical Sciences*. 2017;**102**:196-202

[40] Lin J, Wang X, Wu Q, Dai J, Guan H, Cao W, et al. Development of Salvianolic acid B-Tanshinone II A-Glycyrrhetic acid compound liposomes: Formulation optimization and its effects on proliferation of hepatic stellate cells. *International Journal of Pharmaceutics*. 2014;**462**(1-2):11-18

[41] Tefas LR, Sylvester B, Tomuta I, Sesarman A, Licarete E, Banciu M, et al. Development of antiproliferative long-circulating liposomes co-encapsulating doxorubicin and curcumin, through the use of a quality-by-design approach. *Drug Design, Development and Therapy*. 2017;**11**:1605-1621

[42] Abu Lila AS, Ishida T. Liposomal delivery systems: Design optimization and current applications. *Biological & Pharmaceutical Bulletin*. 2017;**40**(1):1-10

[43] Patel GM, Shelat PK, Lalwani AN. QbD based development of proliposome of lopinavir for improved oral bioavailability. *European Journal of Pharmaceutical Sciences*. 2017;**108**:50-61. DOI: 10.1016/j.ejps.2016.08.057

[44] Zununi Vahed S, Salehi R, Davaran S, Sharifi S. Liposome-based drug co-delivery systems in cancer cells. *Materials Science and Engineering: C*. 2017;**71**:1327-1341

[45] Xu X, Costa AP, Khan MA, Burgess DJ. Application of quality by design to formulation and processing of protein liposomes. *International Journal of Pharmaceutics*. 2012;**434**(1-2):349-359

[46] Pandey AP, Karande KP, Sonawane RO, Deshmukh PK. Applying quality by design (QbD) concept for fabrication of

- chitosan coated nanoliposomes. *Journal of Liposome Research*. 2014;**24**(1):37-52
- [47] Sieber S, Grossen P, Uhl P, Detampel P, Mier W, Witzigmann D, et al. Zebrafish as a predictive screening model to assess macrophage clearance of liposomes in vivo. *Nanomedicine*. 2019;**17**:82-93
- [48] Sahu AK, Jain V. Screening of process variables using Plackett–Burman design in the fabrication of gedunin-loaded liposomes. *Artificial Cells, Nanomedicine, and Biotechnology*. 2017;**45**(5):1011-1022
- [49] Sylvester B, Porfire A, Muntean DM, Vlase L, Lupu L, Licarete E, et al. Optimization of prednisolone-loaded long-circulating liposomes via application of Quality by Design (QbD) approach. *Journal of Liposome Research*. 2018;**28**(1):49-61
- [50] Ingle SG, Pai RV, Monpara JD, Vavia PR. Liposils: An effective strategy for stabilizing Paclitaxel loaded liposomes by surface coating with silica. *European Journal of Pharmaceutical Sciences*. 2018;**122**:51-63
- [51] Porfire A, Tomuta I, Muntean D, Luca L, Licarete E, Alupe MC, et al. Optimizing long-circulating liposomes for delivery of simvastatin to C26 colon carcinoma cells. *Journal of Liposome Research*. 2015;**25**(4):261-269
- [52] Rehman AU, Omran Z, Anton H, Mély Y, Akram S, Vandamme TF, et al. Development of doxorubicin hydrochloride loaded pH-sensitive liposomes: Investigation on the impact of chemical nature of lipids and liposome composition on pH-sensitivity. *European Journal of Pharmaceutics and Biopharmaceutics*. 2018;**133**(74):331-338
- [53] Sylvester B, Porfire A, Muntean DM, Vlase L, Tomuța I. Formulation optimization of pravastatin loaded long-circulating liposomes using a design of experiments. *Farmácia*. 2016;**64**(3):449-458
- [54] Achim M, Tomuța I, Muntean D, Porfire A, Tefas LR, Patras L, et al. Optimization and in vitro evaluation of 5-fluorouracil—loaded long—circulating liposomes. *Farmácia*. 2017;**65**(1):82-91
- [55] Tefas LR, Muntean DM, Vlase L, Porfire AS, Achim M, Tomuța I. Quercetin-loaded liposomes: Formulation optimization through a D-optimal experimental design. *Farmácia*. 2015;**63**(1):26-33
- [56] Xu H, Hu M, Yu X, Li Y, Fu Y, Zhou X, et al. Design and evaluation of pH-sensitive liposomes constructed by poly(2-ethyl-2-oxazoline)-cholesterol hemisuccinate for doxorubicin delivery. *European Journal of Pharmaceutics and Biopharmaceutics*. 2015;**91**(3):66-74
- [57] Smith MC, Crist RM, Clogston JD, McNeil SE. Zeta potential: a case study of cationic, anionic, and neutral liposomes. *Analytical and Bioanalytical Chemistry*. 2017;**409**(24):5779-5787
- [58] Agrahari V, Agrahari V. Facilitating the translation of nanomedicines to a clinical product: challenges and opportunities. *Drug Discovery Today*. 2018;**23**(5):974-991
- [59] Li J, Qiao Y, Wu Z. Nanosystem trends in drug delivery using quality-by-design concept. *Journal of Controlled Release*. 2017;**256**:9-18
- [60] Porfire A, Muntean DM, Rus L, Sylvester B, Tomuta I. A quality by design approach for the development of lyophilized liposomes with simvastatin. *Saudi Pharmaceutical Journal*. 2017;**25**:981-992
- [61] Xu X, Khan MA, Burgess DJ. A quality by design (QbD) case study on liposomes hydrophilic API: II. Screening

of critical variables, and establishment of design space at laboratory scale. *International Journal of Pharmaceutics*. 2012;**423**:543-553

[62] Ingvarsson PT, Yang M, Nielsen HM, Rantanen J, Foged C. Stabilization of liposomes during drying. *Expert Opinion on Drug Delivery*. 2011;**8**(3):375-388

[63] Sylvester B, Porfire A, Achim M, Rus L, Tomuta I. A setp forward towards the development of stable freeze-dried liposomes: a quality by design approach (QbD). *Drug Development and Industrial Pharmacy*. 2018;**44**(3):385-397

[64] Ingvarsson PT, Yang M, Mulvad H, Nielsen HM, Rantanen J, Foged C. Engineering of an inhalable DDA/TDB liposomal adjuvant: a Quality by Design approach towards optimization of the spray drying process. *Pharmaceutical Research*. 2013;**30**:2772-2784

[65] Center for Drug Evaluation and Research, Food and Drug Administration. Liposome Drug Products. Guidance for Industry [Internet]. 2018. Available from: <https://www.fda.gov/downloads/drugs/guidances/ucm070570.pdf>

[66] Hübschmann HJ. Handbook of GC/MS Fundamentals and Applications. 2nd ed. Weinheim: Wiley-VCH; 2008. <https://www.wiley.com/en-us/Handbook+of+GC+MS%3A+Fundamentals+and+Applications%2C+3rd+Edition-p-9783527334742>

[67] Fanali S, Haddad PR, Poole C, Riekkola M-L. *Liquid Chromatography*. Volume 2, Applications. 2nd ed. Amsterdam: Elsevier; 2017

[68] Landers JP. Handbook of capillary and microchip electrophoresis and associated microtechniques. 3rd ed. Boca Raton: CRC Press;

2007. <https://www.crcpress.com/Handbook-of-Capillary-and-Microchip-Electrophoresis-and-Associated-Microtechniques/Landers/p/book/9780849333293>

[69] Dettmer-Wilde K, Engewald W. *Practical Gas Chromatography*. Heidelberg: Springer; 2014

[70] Saliu F, Orlandi M. In situ alcoholysis of triacylglycerols by application of switchable-polarity solvents. A new derivatization procedure for the gas-chromatographic analysis of vegetable oils. *Analytical and Bioanalytical Chemistry*. 2013;**405**:8677-8684

[71] Laouini A, Sfar S, Charcosset C, Fessi H, Lyon CPE, Bat G, et al. Preparation, characterization and applications of liposomes: State of the art. *Journal of Colloid Science and Biotechnology*. 2012;**1**(2):147-168

[72] Subramanian A, Veler WH. Dot-blot analysis of the degree of covalent modification of proteins and antibodies at amino groups. *Journal of Immunological Methods*. 1997;**203**:45-53

[73] Mukherjee S, Raghuraman H, Dasgupta S, Chattopadhyay A. Organization and dynamics of N - (7-nitrobenz-2-oxa- 1 , 3-diazol-4-yl) -labeled lipids: a fluorescence approach. *Chemistry and Physics of Lipids*. 2004;**127**:91-101

[74] Baxa U. Imaging of liposomes by transmission electron microscopy. In: *Characterization of Nanoparticles Intended for Drug Delivery Methods in Molecular Biology*. New York: Human Press; 2018

[75] Teschke O. Liposome structure imaging by atomic force microscopy: Verification of improved liposome stability during adsorption of multiple

aggregated vesicles. *Langmuir*. 2002;**18**(17):6513-6520

[76] Kozak D, Anderson W, Vogel R, Chen S, Antaw F, Trau M. Simultaneous size and zeta-potential measurements of individual nanoparticles in dispersion using size-tunable pore sensors. *ACS Nano*. 2012;**6**(8):6990-6997

[77] Electrophoretic Light Scattering (ELS) [Internet]. 2019. Available from: https://www.malvernpanalytical.com/en/products/technology/light-scattering/electrophoretic-light-scattering?utm_source=MaterialsTalks&utm_medium=Blog&utm_campaign=Liposome&utm_term=22724

[78] Ying, B. and R. Campbell, Polyethylene-glycol (PEG) enhances siRNA delivery and reduces toxic effect of cationic lipids under optimized experimental conditions. *Proceedings of the American Association for Cancer Research Annual Meeting*; 2009;**50**:153-154

[79] Porfire A, Tomuta I, Tefas L, Leucuta SE, Achim M. Simultaneous quantification of L-alpha-phosphatidylcholine and cholesterol in liposomes using near infrared spectrometry and chemometry. *Journal of Pharmaceutical and Biomedical Analysis*. 2012;**63**:87-94

[80] Porfire A, Muntean D, Achim M, Vlase L, Tomuta I. Simultaneous quantification of simvastatin and excipients in liposomes using near infrared spectroscopy and chemometry. *Journal of Pharmaceutical and Biomedical Analysis*. 2015;**107**:40-49