https://doi.org/10.31925/farmacia.2022.4.4 **ORIGINAL ARTICLE**

QUALITY BY DESIGN APPROACH FOR THE DEVELOPMENT OF SALINOMYCIN AND GEMCITABINE COMBINATION THERAPY LIPOSOMES FOR COLORECTAL CANCER

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Abstract

An ideal cancer treatment would eradicate all types of malignant cells, both rapidly replicating and quiescent stem-like cancer cells. Therefore, a combination of drugs capable of targeting all subtypes of cancer cells could offer a better outcome in cancer. Nano-delivery systems for combination therapy represent more secure and efficient platforms for the delivery of anticancer drugs to tumours. Nanocarrier drug development is a complex process which requires careful design and evaluation at various levels to ensure their safety and efficacy. In this sense, the Quality by Design (QbD) approach could help develop a robust, high-quality nanocarrier formulation by understanding the properties of the product and the manufacturing process. In this study, the QbD approach was used in the development of liposomes co-loaded with a combination of anticancer drugs, salinomycin (SAL) and gemcitabine (GEM) for colorectal cancer therapy. While GEM is a conventional anticancer drug, SAL has shown selectivity towards cancer stem cells. The implementation of the QbD approach involved the identification of the quality target product profile (QTPP) and critical quality attributes (CQAs) of the liposomes. Based on risk analysis, the Design of Experiments (DoE) methodology was employed to evaluate the impact of critical formulation factors on the liposomes' properties, in a time and cost-effective manner, in order to obtain SAL and GEM co-loaded liposomes with desired characteristics.

Rezumat

Tratamentul ideal al cancerului implică eradicarea atât a celulelor maligne care se multiplică rapid, cât și a celulelor stem pasive. De aceea, asocierea de substanțe care țintesc toate subtipurile de celule canceroase ar putea duce la efecte superioare în terapia cancerului. Nanosistemele pentru terapia combinată reprezintă sisteme mai sigure și mai eficiente pentru transportul substanțelor anticanceroase. Dezvoltarea nanosistemelor este un proces complex care necesită atenta formulare și evaluare pentru a conferi acestora siguranță și eficacitate. În acest sens, conceptul de Calitate prin Design (QbD) ar putea ajuta la dezvoltarea unei nanoformulări robuste și calitative prin înțelegerea proprietăților sistemelor de transport și a procesului de fabricație. În acest studiu, abordarea QbD a fost utilizată în dezvoltarea lipozomilor încărcați cu o asociere de substanțe anticanceroase, salinomicină (SAL) și gemcitabină (GEM) pentru terapia cancerului colorectal. În timp ce GEM este un agent anticanceros convențional, SAL a dovedit selectivitate față de celulele stem canceroase. Implementarea conceptului QbD a presupus identificarea profilului de calitate (QTPP) și a atributelor critice de calitate (CQA) ale lipozomilor. Pe baza analizei de risc, a fost utilizată metodologia planurilor experimentale (DoE) pentru a evalua impactul variabilelor critice de formulare asupra proprietăților lipozomilor, într-o manieră eficientă din punctul de vedere al timpului și al costurilor, pentru obținerea lipozomilor cu SAL și GEM care să posede caracteristicile dorite.

Keywords: liposomes, colorectal cancer, Quality by Design, combination therapy

Introduction

At global level, the second leading cause of death following cardiovascular diseases is cancer, accounting for more than 10 million cases in 2019 [1, 2]. Among malignancies, colorectal cancer (CRC) is a major cause of morbidity and mortality, its incidence steadily increasing in high-income and developing countries [3-5]. Unfortunately, most patients are in advanced stages of CRC when the disease is diagnosed. Even with surgery or neoadjuvant chemotherapy or radiation, the 5-year survival rate of patients with advanced CRC is limited. It has been reported that around half of the patients with CRC are likely to develop metastases at some point during the disease [6]. Liver metastases are the most common distant-organ spreads in late stages of CRC, with a 5-year survival rate of less than 15% [4, 6, 7]. When standard treatment has failed, new drugs need to be explored to expand the current therapy options. Gemcitabine (GEM) is currently used in clinical settings for several types of solid tumours. However, its unfavourable biopharmaceutical properties have raised challenges in ensuring the required therapeutic levels [8].

The phenotypic heterogeneity of tumours, particularly through quiescent and resistant cancer stem cells (CSCs), poses challenges for effective CRC treatment. Over

recent years, CSCs have been identified in several types of tumours, including CRC. CRC stem cells share traits with embryonic stem cells, such as self-renewal and multi-lineage differentiation ability. CSCs have been linked to metastasis development, therapy resistance and failure, and disease recurrence [9]. Since successful treatment is problematic, at least in part because of the resistance of CSCs, innovative or new therapeutic approaches are demanded.

Salinomycin (SAL) is a naturally occurring antibiotic found to selectively eliminate breast CSCs 100-fold more effectively than paclitaxel [10]. Its anticancer effects in CRC have been investigated in several studies [7, 11-13]. Several reports proved that SAL could enhance the cytotoxic effect of conventional anticancer agents, including 5-fluorouracil, cisplatin, paclitaxel, gemcitabine, etoposide, gefitinib, doxorubicin, tamoxifen, and overcome the acquired drug resistance [12,14-19]. Conceivably, a regimen for a more successful CRC treatment would involve the use of drugs that can effectively deplete both CSCs and the bulk tumour cells. Although SAL has demonstrated effectiveness in CRC models, it has poor aqueous solubility which is a major obstacle in its clinical use [20].

In order to ensure a suitable dose to the target tumour site, drug delivery can be facilitated through the use of nanocarriers. Nanocarriers, which can be tailored to possess different properties, provide precise and effective delivery of anticancer agents. Drugs with short half-life, rapid metabolic inactivation and/or poor bioavailability are excellent candidates for incorporation in nanocarriers [5]. Liposomes have been extensively explored for the encapsulation and delivery of both hydrophilic and hydrophobic drugs for cancer treatment [21].

Quality by Design (QbD) is a quality-improving approach that assists the formulation and process development in order to achieve predefined objectives and to improve product quality [22, 23]. Although nanocarriers such as liposomes may offer potential therapeutic benefits, their manufacturing raises certain issues such as complex preparation process, low reproducibility, high variability, mainly due to a lack of understanding of the effect of raw materials and process parameters on the product's characteristics, and inadequate regulatory guidelines related to development and characterization. The QbD approach could help get around these critical issues and gain more knowledge and understanding about the product in a rational and scientific manner [22, 24, 25]. The main objectives of the QbD approach are to increase manufacturing efficiency and process capability, and to reduce product variability. The QbD strategy starts with establishing the quality target product profile (QTPP) which takes into account the critical characteristics of the final product [26]. These product characteristics help in establishing the critical quality attributes (CQAs) that have a major impact on the product's performance [22, 24]. Prior knowledge about the product, and risk assessment help recognize critical process elements and their degree of impact on the product's quality. Accordingly, the development process should focus on the most influential variables [22].

In the present study, we investigated whether SAL can be used in combination with GEM for enhanced efficacy against CRC *via* liposomal drug delivery systems. The QbD approach was applied for determining the impact of several factors on the liposomal formulation's characteristics. According to their potential impact on the CQAs, the critical material attributes (CMAs) and critical process parameters (CPPs) were ranked through a risk estimation matrix (REM) based on general scientific knowledge. Furthermore, a multivariate design of experiments (DoE) was employed to investigate the effect of CMAs on the liposomes' CQAs.

Materials and Methods

Materials

Salinomycin sodium (SAL) was purchased from Glentham Life Sciences Ltd (Corsham, UK). Gemcitabine hydrochloride (GEM) was obtained from Farmhispania SA (Barcelona, Spain). Lipoid PC and Lipoid PE were procured from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol was purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals used in this study were of analytical grade.

Elements of Quality by Design (QbD)

The main elements of the QbD strategy are the following: (1) establishing the QTPP, (2) selecting the CQAs, (3) identifying the CMAs and CPPs through risk analysis, (4) determining the design space, (5) ensuring a quality control strategy and product life-cycle management. The definitions and protocols are indicated in the ICH guidelines Q8 (R2) on product development and ICH Q9 (R1) on quality risk management [27, 28].

Establishing the Quality Target Product Profile (QTPP) and Critical Quality Attributes (CQAs)

Defining the quality target product profile (QTPP) is the first step in the QbD approach to pharmaceutical development and consists in constructing a summary of the characteristics of the product [26]. In other words, the QTPP reflects the desired quality profile which will ensure the efficacy and safety of the product [29].

The critical quality attributes (CQAs) include physical, chemical, biological and microbiological properties, and are derived from the QTPP, and have to be within an appropriate range or limit. Therefore, in order to achieve desired CQAs, the CMAs and CPPs of a product have to be identified through risk assessment or prior knowledge [22, 24, 26]. While CMAs are the main properties of input materials, CPPs refer to manufacturing process parameters that affect the quality and performance of the product [30]. In order to obtain

the desired CQAs, the CMAs and CPPs should be defined and controlled [22, 26]. Screening and identifying the important CMAs and CPPs that impact the CQAs is considered a prerequisite to drug development. Preliminary data is also an important aspect that helps determine the importance of a CQA [31].

Risk assessment

The QbD risk assessment implies an interdependence rating established between the CMAs-CQAs and/or CPPs-CQAs, measuring the impact of each quality attribute on the final product [32, 33]. Risk estimation matrix (REM) is a risk rating tool constructed for CMAs and/or CPPs commonly using a three-grade scale (low, medium and high) to describe the criticality in relation to the CQAs [34]. In the present study, the developed REM classified the CMAs and CPPs into low-, medium- and high-risk, based on their potential impact on the chosen CQAs. The CMAs and CPPs with a high influence on the CQAs were selected as independent variables in the experimental design, to reduce the chance of risk. Whereas the parameters that had a medium or low risk were kept constant.

Design of Experiments (DoE)

The DoE was set up based on the results from the risk assessment. A Box-Behnken design with 3 factors and 3 levels was constructed to aid in the development of the liposomal formulations, using MODDE Pro 13 software (Sartorius Stedim Data Analytics AB, Umeå, Sweden). Based on the REM, three CMAs were chosen as independent variables in the experimental design, as follows: phospholipid concentration (X_1, mM), phospholipid: cholesterol molar ratio (X_2), SAL concentration (X_3, mM). The experimental design matrix consisting of 15 experimental runs is presented in Table I.

We evaluated the influence of these factors on the CQAs of the co-loaded liposomes: particle size, polydispersity index (PDI), zeta potential, entrapped drug concentration and entrapment efficiency (EE).

Table I

Experimental matrix for the Box-Behnken design

Experiment	CMA		
	Phospholipid concentration (X_1, mM)	**Phospholipid: cholesterol molar ratio (X_2)**	**SAL concentration (X_3, mM)**
N1	20	5	3.5
N2	40	5	3.5
N3	20	10	3.5
N4	40	10	3.5
N5	20	7.5	2
N6	40	7.5	2
N7	20	7.5	5
N8	40	7.5	5
N9	30	5	2
N10	30	10	2
N11	30	5	5
N12	30	10	5
N13	30	7.5	3.5
N14	30	7.5	3.5
N15	30	7.5	3.5

CMA – critical material attribute, SAL – salinomycin

Liposome preparation

First, we investigated the encapsulation of GEM into the liposomes by employing two loading methods described below.

The liposomes were prepared by the thin film hydration technique with modifications in the GEM loading method and conditions, after Tamam *et al.* and Ding *et al.* [35, 36]. In brief, Lipoid PC, Lipoid PE and cholesterol were dissolved in ethanol, and a thin film was obtained by rotary evaporation of the organic solvent at 45°C. The lipids were subsequently hydrated according to the protocols below.

For the passive-loading method, the thin film was hydrated with a 5 mM GEM solution at 45°C. The liposomes were afterwards extruded using a LiposoFast LF-50 extruder (Avestin Europe GmbH, Mannheim, Germany). In the active-loading method, the lipid film was hydrated with a 250 mM ammonium sulphate solution at 45°C. The resulting liposomes were extruded and dialyzed against a 154 mM sodium chloride solution for 3 h. After the purification step, the liposomes were incubated with a 5 mM GEM solution at either 45°C or 60°C for variable time periods (15 min, 30 min or 60 min).

For the liposomes containing both GEM and SAL, SAL was dissolved along with the lipids in the organic solvent. For the DoE, a 0.5 mM GEM solution was used in the liposomes' preparation.

Liposomes' characterization

The average size, polydispersity index (PDI) and zeta potential of the liposomes were measured on a ZS90 Zetasizer Nano from Malvern Panalytical (Malvern, UK) after dispersion in distilled water.

After removal of the unentrapped drugs by dialysis, the drug content was analysed by HPLC on an Agilent 1100 system (Agilent Technologies Inc., Santa Clara, CA, USA). For SAL, a pre-column derivatization with 2,4-dinitrophenylhydrazine [37] was carried out prior to chromatographic separation on a Luna C18(2) column (Phenomenex, Torrace, CA, USA; 5 µm, 150 x 4.6 mm), with a mixture of methanol and 1.5% acetic acid (92:8 v/v). The SAL derivative was detected at 354 nm. GEM was eluted with 0.1% phosphoric acid on a Zorbax SB C18 column (Agilent Technologies Inc., Santa Clara, CA, USA; 3.5 µm, 100 x 3 mm), and further detected at 275 nm. The entrapment efficiency (EE) was calculated according to previously reported calculation equations.

Statistical analysis

The statistical analysis was carried out using the statistical module of the MODDE Pro 13 software. The ANOVA was performed to estimate the best regression model based on the experimental data. Significance was found when $p < 0.05$.

Results and Discussion

Even though chemotherapy is highly efficient *in vitro*, the nonspecific organ distribution after *in vivo* administration leads to a series of side effects, some of which of increased severity [38]. The use of nano-particulate drug delivery systems could be a feasible way of reducing the toxicity of anticancer agents and improving colorectal tumour-targeting. Liposomes are the pioneers of nanotechnology-based drug delivery systems, and the first type of nanostructure to receive marketing approval both in Europe and the USA for cancer treatment [39]. They are spherical vesicles composed of phospholipids and cholesterol [38]. Because of their biodegradability, biocompatibility and lack of toxicity, liposomes are safe carriers for clinical applications [39]. In our study, we have developed stealth liposomes for the co-delivery of SAL and GEM for CRC therapy.

Effect of loading method on liposomal GEM content

First, we assessed the encapsulation of GEM into the liposomes by employing two loading methods: passive and active (or remote) loading.

Hydrophilic drugs can be passively incorporated into the liposomes during the hydration of the thin lipid film, while contained in the hydration medium. For base drugs, the entrapment can be enhanced by a liposomal transmembrane gradient using ammonium salts by forming an ionic complex with sulphate which has low diffusivity. This method, called active or remote method, has been reported efficient for doxorubicin loading [35].

Therefore, in our study, we investigated whether the active loading method could lead to higher entrapment of GEM. The GEM-loaded liposomes' characteristics are indicated in Table II.

Table II

GEM-loaded liposomes' characteristics after passive and active loading methods

Loading method	Incubation conditions	Liposome size (nm)	PDI	Zeta potential (mV)	GEM concentration (µg/mL)	GEM EE (%)
Passive loading method	45°C, 30 min	199.8 ± 1.88	0.081 ± 0.013	-41.5 ± 0.36	201.9 ± 5.02	13.45 ± 0.33
Active loading method	45°C, 15 min	202.3 ± 1.17	0.072 ± 0.002	-39.4 ± 1.10	171.87 ± 7.06	11.45 ± 0.46
	60°C, 15 min	178.9 ± 1.40	0.102 ± 0.011	-39.3 ± 0.66	137.74 ± 26.64	9.20 ± 1.64
	60°C, 30 min	181.1 ± 1.51	0.085 ± 0.002	-42.9 ± 0.52	125.36 ± 12.86	8.38 ± 0.86
	60°C, 60 min	307.0 ± 1.10	0.212 ± 0.010	-40.8 ± 0.60	204.97 ± 26.00	13.71 ± 1.73

PDI – polydispersity index, GEM – gemcitabine, EE – entrapment efficiency; *Data are expressed as mean values ± SD

We employed the film hydration method, which is the most employed technique in liposome manufacturing. This method was coupled with extrusion through poly-carbonate membranes in order to achieve a small size. Although the formulation can influence the size and polydispersity of the liposomes, they largely depend on the pore cut-off size of the extrusion membrane. The GEM-loaded liposomes showed adequate uniform size in the nanometer range. Interestingly, active loading of GEM over a longer incubation period at 60°C led to larger and more polydisperse liposomes. All liposomal samples had a negative, low zeta potential. Regarding the incorporation of GEM in the liposomes, comparable results in terms of entrapped GEM concentration and EE were obtained when employing the passive loading method and the active loading method at the highest temperature and incubation time.

Overall, we demonstrated that the passive method contributes to comparable or even higher GEM content than the active method, which we considered satisfactory. Even with the increase in incubation time and temperature in the remote method, no significant improvement in GEM loading has been achieved. Furthermore, prolonged heating of the liposomes can be detrimental to their quality and stability, by inducing lipid degradation [40]. GEM is a weak base that previously has been proven difficult to load in liposomes. Compared to doxorubicin, GEM does not ionize to the same extent and therefore does not generate a strong enough flux across the membrane to yield high loading [35]. Since the remote loading method is laborious and time-consuming, and, based on our results, did not lead to higher entrapment of GEM, we opted for the passive method to load

GEM in the SAL and GEM co-loaded liposomes in further experiments.

Quality by Design (QbD) approach

Establishing the Quality Target Product Profile (QTPP) and Critical Quality Attributes (CQAs)

The QbD approach is mainly applied in the pharmaceutical industry for which the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) have issued regulatory guidelines providing directions to integrate the principles of QbD at product development stage. However, lately, the QbD concept is being more and more used in research and development [25, 32]. A QbD-assisted approach for the development of liposomes for cancer therapy have been described previously [41].

The first step in our QbD-assisted development of liposomes was to determine the QTPP by collecting and structuring the relevant information about the product. A QTPP for the liposomal product was made based on literature survey, practical considerations, and relevance for the liposomal nanocarriers' manufacturing (Table III).

Table III

The quality target product profile (QTPP) of the liposomal product

QTPP	Target	Justification
Dosage form	Liposomes	To enhance drug efficiency, reduce side effects and toxicity, enable targeting ability of drugs with poor pharmacokinetics
Route of administration	Intravenous	To deliver the payload to the tumour site Immediate availability in the bloodstream
Physical form	Aqueous dispersion	For ease of administration
Particle size and size distribution	Nanometer range, up to 200 nm, with uniform size distribution	For adequate uptake, targeting and performance
Zeta potential	High absolute value	To ensure product stability, avoid agglomeration phenomena
Drug content	Maximized	To ensure a sufficient dose of drug available for absorption
Drug release	Sustained release	To ensure a continuous exposure to drugs for a longer period of time
Stability	Stable in aqueous medium	To maintain drug therapeutic activity
Purity	Physicochemically pure, sterile, pyrogen-free	To ensure patient safety

In this study, a QTPP was defined considering the following: a nanosized liposomal dispersion for intravenous administration, capable of delivering the double payload to the tumour site by passive targeting.

The next step of the QbD strategy was to select the CQAs based on the established QTPP and prior knowledge. Based on earlier reports, for liposomal products, the following CQAs are considered relevant: (1) size and size distribution, (2) zeta potential, (3) drug content, (4) drug release profile and (5) microbiological purity [41]. In our experiment, the following CQAs were identified: particle size and particle size distribution (polydispersity), zeta potential and drug content.

An essential requirement for liposomal products for cancer therapy is a nanoscale range for particle size and uniform size distribution. The size of the liposomes is directly related to drug loading, drug release and *in vivo* distribution. In general, small liposomes are cleared by renal filtration, while vesicles larger than 300 nm are engulfed by mononuclear cells. In order to escape capture by phagocytic cells and preferentially accumulate in the tumour by the enhanced permeability and retention (EPR) effect, the liposomes should have sizes between 50 and 200 nm [41, 42]. For colorectal cancer, the pore cut-off size has been shown to range between 400 and 600 nm [43]. Therefore, liposomes exhibiting sizes of 100 - 200 nm could extravasate through the above-mentioned passageways in the leaky vasculature and concentrate in the tumour. Furthermore, this size range would enable sterile filtration of the liposomal product [23]. The size distribution of the liposomes is another important attribute; for adequate homogeneity of the liposomal dispersion, generally, a PDI of less than 0.3 is considered acceptable [42]. Accordingly, in the present study, our goal was to obtain liposomes with sizes of 100 to 200 nm, and uniform size distribution reflected by a PDI value below 0.3.

The zeta potential is a parameter which describes the surface charge of liposomes and provides information regarding their stability and physiological behaviour (protein interaction, circulation time, cellular uptake). Prime factors which influence the zeta potential are, among others, ionic strength, and pH [44]. DPPC, which was used as building block for the liposomes in our study, is a zwitterionic lipid and, as suggested by Neunert *et al.*, the orientation of the phospholipid's polar head group produces changes in the zeta potential. At low ionic strength (i.e., saline solution), the zeta potential is expected to have a negative value [45].

Surface modifications with hydrophilic polymers is often required to improve the stability of the liposomes and bypass recognition and clearance by the mononuclear phagocyte system [38]. We have used a PEG-modified phospholipid for the outer coating of the

liposomes to avoid aggregation phenomena, since PEGylation has been proven reliable and efficient for marketed liposomal products [39]. The addition of a PEGylated lipid in concentrations of 0.2 - 10 mol% does not significantly affect the zeta potential of liposomes containing neutral lipids [44]. Therefore, our goal was to achieve a zeta potential of less than -30 mV, thus ensuring stability by electrostatic repulsion.

Regarding the drug content, there are several ways of expressing the amount of encapsulated payload: entrapped drug concentration, EE and drug loading, respectively [41]. A high drug content is recommended for ease of dosing and better patient compliance [23]. Consequently, our goal was to maximize drug content for both SAL and GEM.

Risk assessment

Identifying the CMAs and CPPs which impact the CQAs is a necessary step prior to product development [31]. The REM was constructed based on literature research, classifying the impact of different variables on the liposomal product's characteristics and performance, and assessing the ability to cause product failure. Table IV indicates several independent variables impacting the liposomes' CQAs. Based on the contribution of these variables to the variations in CQAs, three CMAs, namely the phospholipid concentration, the phospholipid: cholesterol molar ratio and the drug concentration, were considered high-risk factors. Due to their consequences on the liposomes' CQAs, the selected CMAs needed to be further investigated and controlled to ensure the desired quality of the product.

Table IV

Risk estimation matrix (REM) of critical material attributes (CMAs) and critical process parameters (CPPs) over the critical quality attributes (CQAs) of liposomes

CQA	CMA				CPP	
	Phospholipid concentration	**Cholesterol concentration**	**Phospholipid: cholesterol molar ratio**	**Drug concentration**	**Evaporation and hydration temperature***	**Stirring speed***
Particle size	High	High	High	Low	Medium	Medium
PDI	Medium	Medium	Medium	Low	Medium	Medium
ZP	Medium	Low	Medium	Low	Low	Low
Drug content	High	Low	Medium	High	Medium	Low

CQA – critical quality attribute, CMA – critical material attribute, CPP – critical process parameter, PDI – polydispersity index, ZP – zeta potential; *CPPs refer to the film hydration method

Design of Experiments (DoE)

In order to link the CMAs and CPPs to the CQAs, and understand the relation between them, often the DoE methodology is employed.

DoE is the strategy that enables the development and optimization of a product with the goal of achieving the desired quality, by measuring the relationship between the input variables and the characteristics of the product. It is increasingly employed in drug development because it expands knowledge about the product and process. Another advantage of the DoE is the potential of covering the entire multivariate experimental area with a minimum number of experiments, providing the predefined product quality in a time- and cost-effective manner [24].

In the present study, we have set up a Box-Behnken experimental design in which we have investigated the influence of three formulation factors or CMAs, namely the concentration of phospholipid, concentration of SAL and the phospholipid: cholesterol molar ratio, on the selected CQAs of the SAL and GEM co-loaded liposomes.

Table V (a and b) outlines the selected CQAs of the SAL and GEM co-loaded liposomes. The size of the liposomes before dialysis ranged from 177.2 nm to 201.3 nm, while the PDI varied from 0.036 to 0.072. After dialysis, the size and uniformity of the vesicles remained largely unchanged, with values varying between 180.1 nm and 199.9 nm for size, and between 0.052 and 0.089 for PDI, respectively. The zeta potential had negative values from -41.6 mV to -34.5 mV. Before dialysis, SAL concentration had values between 901.80 µg/mL and 2440.89 µg/mL, whereas, after dialysis, SAL concentration decreased dramatically (241.78 µg/mL to 1033.88 µg/mL). Regarding GEM, the concentrations ranged from 2.90 µg/mL to 24.73 µg/mL. With respect to EE, SAL presented values between 46.63% and 70.28% before dialysis, and between 6.67% and 55.77% after dialysis. Consistent with low loading, GEM EE ranged from 1.94% to 16.53%.

The experimental data were fitted by multiple linear regression, and the statistical parameters are shown in Figure 1. The multiple regression coefficient (R^2) and the prediction coefficient (Q^2) had values over 0.5 for all investigated CQAs. The model validity and reproducibility had values over 0.5 and 0.25, respectively, except for GEM concentration, for which a smaller model validity value was recorded. Overall, the experimental data model was considered adequate.

Table V-a

Results for liposomal CQAs according to the DoE

Exp.	Size before dialysis (nm)	Size after dialysis (nm)	PDI before dialysis	PDI after dialysis	Zeta Potential (mV)
N1	180.1 ± 4.22	181.9 ± 1.53	0.053 ± 0.01	0.070 ± 0.02	-36.6 ± 1.95
N2	192.3 ± 3.26	192.2 ± 1.24	0.036 ± 0.02	0.076 ± 0.01	-35.4 ± 2.49
N3	189.5 ± 0.92	190.2 ± 2.91	0.044 ± 0.03	0.081 ± 0.03	-36.4 ± 0.95
N4	194.7 ± 2.55	195.2 ± 1.98	0.044 ± 0.02	0.073 ± 0.01	-37.5 ± 2.25
N5	196.9 ± 1.86	199.9 ± 0.36	0.065 ± 0.01	0.062 ± 0.04	-38.3 ± 0.59
N6	201.1 ± 2.71	199.9 ± 1.29	0.051 ± 0.03	0.066 ± 0.03	-38.5 ± 0.15
N7	178.9 ± 2.50	180.1 ± 1.15	0.068 ± 0.02	0.076 ± 0.01	-37.2 ± 1.91
N8	187.2 ± 1.99	187.9 ± 0.70	0.072 ± 0.01	0.072 ± 0.01	-38.0 ± 0.40
N9	192.1 ± 2.32	193.7 ± 2.33	0.050 ± 0.02	0.052 ± 0.02	-36.9 ± 1.30
N10	196.5 ± 2.86	197.7 ± 1.27	0.046 ± 0.01	0.054 ± 0.01	-39.4 ± 0.87
N11	177.2 ± 0.95	180.5 ± 1.96	0.049 ± 0.02	0.089 ± 0.00	-38.4 ± 0.25
N12	181.9 ± 0.68	180.1 ± 1.23	0.071 ± 0.02	0.077 ± 0.00	-39.6 ± 0.96
N13	201.3 ± 3.56	193.0 ± 2.31	0.042 ± 0.04	0.058 ± 0.05	-35.0 ± 1.40
N14	195.6 ± 3.05	195.4 ± 2.96	0.039 ± 0.04	0.070 ± 0.02	-34.5 ± 2.79
N15	198.7 ± 2.57	188.6 ± 0.85	0.038 ± 0.03	0.065 ± 0.03	-41.6 ± 3.41

Exp. – experiment, PDI – polydispersity index, SAL – salinomycin, GEM – gemcitabine, EE – entrapment efficiency;
*Data expressed as mean values ± SD

Table V-b

Results for liposomal CQAs according to the DoE

Exp.	SAL conc. before dialysis (µg/mL)	SAL conc. after dialysis (µg/mL)	GEM conc. (µg/mL)	SAL EE before dialysis (%)	SAL EE after dialysis (%)	GEM EE (%)
N1	1378.52 ± 20.12	241.78 ± 1.15	4.47 ± 0.02	50.79 ± 0.74	8.91 ± 0.04	2.99 ± 0.02
N2	1565.88 ± 36.78	538.05 ± 1.72	7.04 ± 0.17	57.36 ± 1.35	19.71 ± 0.06	4.71 ± 0.12
N3	1894.67 ± 83.34	798.97 ± 22.42	8.75 ± 0.15	70.28 ± 3.09	29.64 ± 0.83	5.85 ± 0.10
N4	1543.12 ± 2.30	585.20 ± 7.47	4.80 ± 0.75	55.75 ± 0.08	21.14 ± 0.27	3.21 ± 0.50
N5	901.80 ± 8.05	277.13 ± 2.87	5.40 ± 0.10	58.56 ± 0.52	18.00 ± 0.19	3.61 ± 0.07
N6	1085.90 ± 1.72	500.66 ± 5.17	21.41 ± 0.10	69.34 ± 0.11	31.97 ± 0.33	14.31 ± 0.07
N7	1805.66 ± 13.79	258.44 ± 2.87	3.78 ± 0.10	46.63 ± 0.36	6.67 ± 0.07	2.53 ± 0.07
N8	2173.06 ± 0.00	826.20 ± 14.94	5.40 ± 0.20	56.18 ± 0.00	21.36 ± 0.39	3.61 ± 0.13
N9	993.24 ± 36.21	864.41 ± 170.13	14.75 ± 1.05	64.08 ± 2.34	55.77 ± 10.98	9.86 ± 0.70
N10	1007.06 ± 23.57	425.48 ± 10.35	14.91 ± 0.12	64.97 ± 1.52	27.45 ± 0.67	9.96 ± 0.08
N11	2060.49 ± 0.57	597.39 ± 0.57	5.79 ± 0.55	53.30 ± 0.01	15.45 ± 0.01	3.87 ± 0.37
N12	2440.89 ± 5.17	592.92 ± 8.05	2.90 ± 0.15	63.30 ± 0.13	15.38 ± 0.21	1.94 ± 0.10
N13	1777.62 ± 22.42	714.84 ± 4.60	7.39 ± 0.17	66.33 ± 0.84	26.67 ± 0.17	4.94 ± 0.12
N14	1756.89 ± 40.23	1033.88 ± 41.96	24.73 ± 0.40	63.89 ± 1.46	37.60 ± 1.53	16.53 ± 0.27
N15	1631.31 ± 52.30	683.55 ± 15.52	7.69 ± 0.15	60.51 ± 1.94	25.35 ± 0.58	11.39 ± 0.23

Exp. – experiment, PDI – polydispersity index, SAL – salinomycin, GEM – gemcitabine, EE – entrapment efficiency;
*Data expressed as mean values ± SD

Furthermore, according to the ANOVA test, the regression models were found to be significant ($p < 0.05$) with insignificant lack of fit ($p > 0.05$) for each CQA.

Regression coefficient plots have been constructed to graphically estimate the impact of the investigated CMAs on the SAL and GEM co-loaded liposomes' CQAs. These are shown in Figure 2.

Effects of the phospholipid concentration of the CQAs of the liposomes

The properties of the liposomes, especially their size, as well as their stability, are closely related to the type of lipids used for their manufacturing. Here, we have used DPPC, a neutral phospholipid with two saturated C16 acyl chains which contributes to the formation of homogeneous, ordered and more rigid liposomal membranes [46]. With respect to physical stability, to avoid changes in vesicle size and loss of entrapped API by aggregation or fusion, steric stabilization with PEG (by using a lipid with a PEGylated group) is frequently employed [39].

Except for two formulations, namely N6 and N13, all liposomal dispersions exhibited a mean vesicle size of up to 200 nm. There was no significant change in the size of the liposomes after dialysis. Thus, the predefined goal of achieving liposomes within the above-mentioned size range was possible by employing the thin film method followed by processing through polycarbonate membranes. Since DPPC is the main component of the lipid bilayer, using larger amounts in the preparation process usually results in the formation of larger vesicles which can entrap more drug [23, 47].

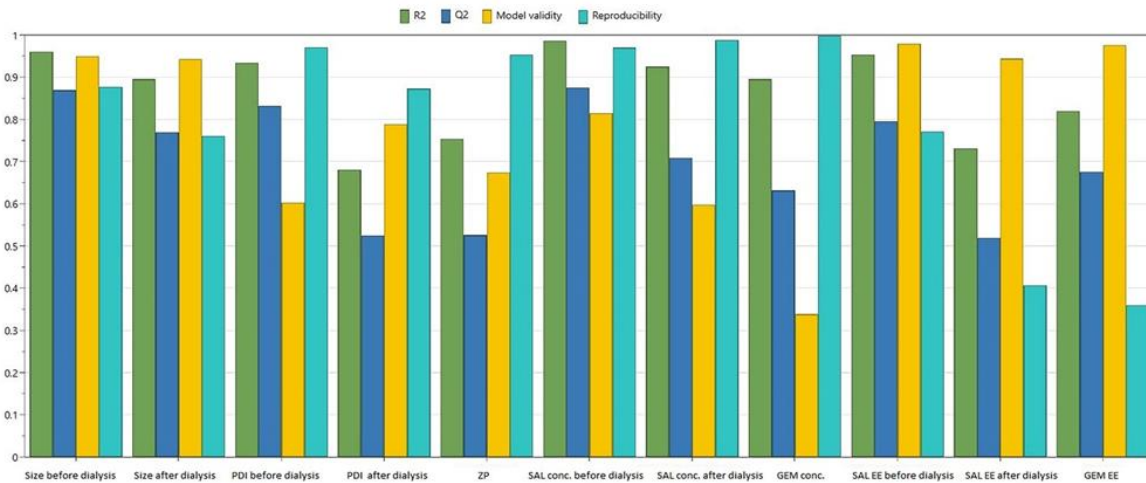

Figure 1.
Summary of fit plot

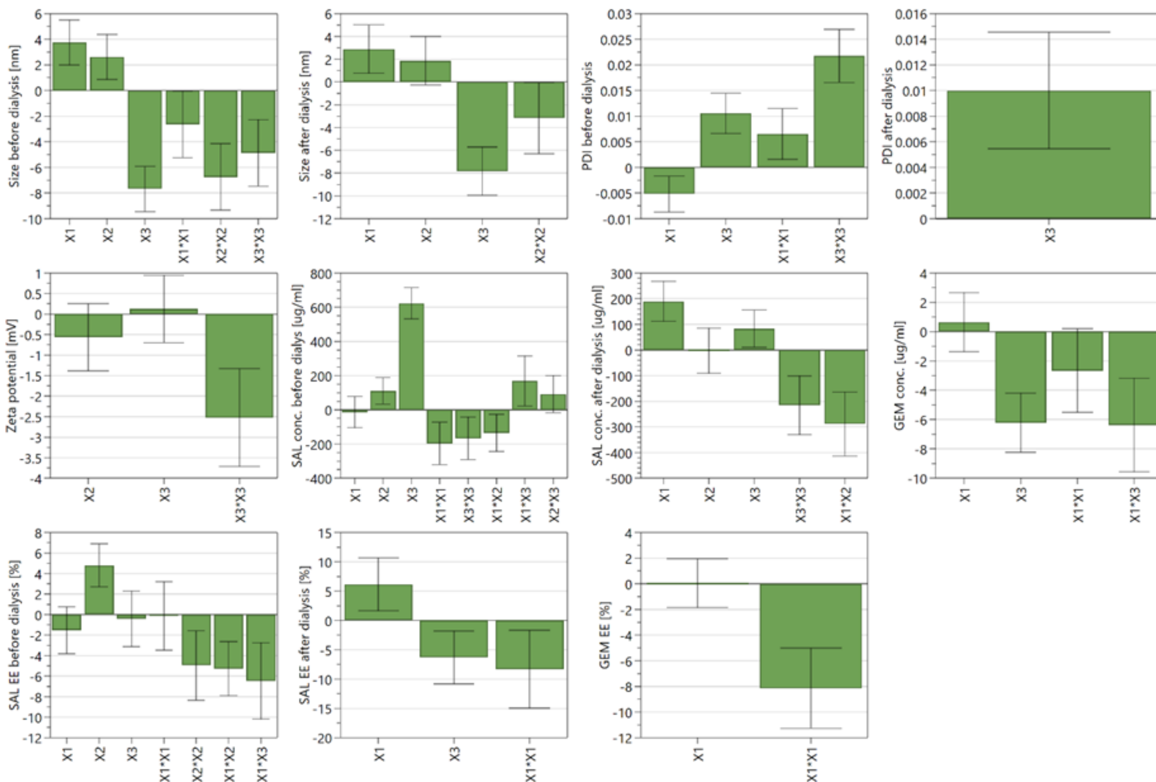

Figure 2.
Regression coefficients plots of dependent variables (CQAs)
X_1 – phospholipid concentration (mM), X_2 – phospholipid: cholesterol molar ratio, X_3 – SAL concentration (mM), PDI – polydispersity index, SAL – salinomycin, GEM – gemcitabine, EE – entrapment efficiency

According to Figure 2, the concentration of phospholipids had a significant impact on the mean size ($p = 0.0012$ and $p = 0.0127$ before and after dialysis, respectively) and size distribution ($p = 0.0089$) of the SAL and GEM co-loaded liposomes. The size of the vesicles increased with the concentration of phospholipid, both before and after the purification step by dialysis. Similar findings have been reported previously by our group [47].

Regarding the size distribution, a larger amount of phospholipid led to the formation of more monodispersed liposomes, as evidenced by a decrease in the PDI values.

The structural similarity of liposomes to biological membranes enables them to incorporate both hydro-

phobic and hydrophilic drugs [39]. While hydrophobic drugs, such as SAL, are incorporated in the lipid bilayer, hydrophilic drugs like GEM are usually entrapped in the aqueous core of the liposome [35]. The content of SAL after dialysis, in terms of concentration and EE, was positively influenced by the concentration of phospholipid ($p = 0.0010$ and $p = 0.0128$, respectively). Although the concentration of phospholipid had no obvious effect on the EE of GEM, the quadratic term X1 * X1 showed a significant negative influence ($p = 0.0003$) on this CQA. Usually, at a high phospholipid concentration, there is more drug-lipid interaction and higher internal-to-external volume ratio for drug encapsulation [23].

Effects of the phospholipid: cholesterol molar ratio on the CQAs of the liposomes

In addition to phospholipids, other components such as sterols are used to control liposome stability and drug release. Cholesterol modulates the fluidity of the liposomal membrane by inducing a denser packing of phospholipid molecules. It reduces the permeability of the bilayer, especially to hydrophilic molecules [39]. Also, it has been shown to increase vesicle size [34]. However, we did not observe such effects in our study. On the contrary, it appeared that cholesterol helped the down-sizing of the liposomes. As shown in Figure 2, the phospholipid: cholesterol molar ratio influenced the size of the liposomes, but the effect was significant only prior to their purification by dialysis ($p = 0.0091$). Apparently, a greater ratio between the phospholipids and cholesterol resulted in larger liposomes, but the effect was non-linear according to the quadratic factor X2 * X2.

The influence of the phospholipid: cholesterol ratio was also seen on the concentration ($p = 0.0138$) and EE ($p = 0.0020$) of SAL in the pre-dialyzed liposomes. Higher SAL concentrations and EE were obtained at a larger phospholipid: cholesterol molar ratio.

Interestingly, SAL entrapment in the liposomes was favoured by a lower amount of cholesterol. Since the liposomal membrane is less rigid at lower cholesterol concentrations, it may be that SAL molecules could intercalate more readily in the bilayer. Although expected, GEM loading was not sensitive to changes in cholesterol content.

Effects of the SAL concentration on the CQAs of the liposomes

It appears that the concentration of SAL had a significant effect on most of the investigated liposome CQAs, as can be seen in Figure 2. A higher concentration of SAL led to the formation of smaller liposomes. The effect was significant both before ($p = 8.23e^{-06}$) and after dialysis ($p = 9.39e^{-06}$) and had a similar magnitude on the CQA. However, the obtained liposomes were more polydispersed, as evidenced by an increase in the PDI with the concentration of SAL. Again, the effect was significant and comparable both prior to ($p = 0.0002$) and after the dialysis ($p = 0.0005$) of the liposomes.

Liposome drug encapsulation is highly affected by the amount of drug used in the manufacturing process. Accordingly, the concentration of SAL entrapped in the liposomes was directly influenced by the amount of SAL used in the preparation process. A higher concentration of SAL led to a higher SAL content, but the effect was more important before dialyzing the liposomes ($p = 1.09e^{-05}$ *vs.* $p = 0.0307$). In terms of EE, a negative effect was observed between the factor and the response, and the influence was significant only after the dialysis ($p = 0.0114$) of the liposomes. This could be because, beyond a point, any additional increase in the SAL concentration did not make a considerable change in the encapsulation. Xu et al have reported similar observations regarding tenofovir encapsulation in positively charged liposomes [23].

A significant influence was recorded regarding the concentration of entrapped GEM. Increasing the concentration of SAL led to an important decrease in the content of GEM ($p = 0.0001$) within the liposomes. Apparently, SAL hindered the entrapment of GEM in the liposomes, an observation previously made by our group for curcumin-loaded and doxorubicin-loaded liposomes [47].

Despite the lack of a detectable influence on the zeta potential, the quadratic term X3 * X3 showed a significant negative effect on the CQA ($p = 0.0009$).

Conclusions

In this work, we used the QbD approach in order to develop SAL and GEM co-loaded liposomes for CRC therapy and to achieve a product with the desired QTPP. We showed that due to physicochemical constraints, active loading of GEM in liposomes is limited. However, the passive loading method ensured satisfactory entrapment of GEM in the liposomes. The CMAs that affect the quality of liposomal formulations were identified by risk assessment: particle size, PDI, SAL and GEM concentration and EE.

We have demonstrated the successful application of DoE in developing liposomes as drug delivery systems for SAL and GEM. Moreover, the obtained liposomes have shown adequate characteristics that can ensure a uniform behaviour in terms of delivery properties. Overall, the present study demonstrated that the QbD approach provided a more time- and cost-effective development of liposomal formulations with SAL and GEM. Furthermore, the SAL and GEM co-loaded liposomes might have potential applications in CRC therapy. In conclusion, while further *in vitro* and *in vivo* studies are required for optimization, the liposomal formulations co-loading SAL and GEM have the potential to contribute to the development of novel and effective anti-CRC therapies.

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Conflict of interest

The authors declare no conflict of interest.

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