https://doi.org/10.31925/farmacia.2024.5.19 **ORIGINAL ARTICLE**

VINORELBINE-LOADED pH-SENSITIVE LIPOSOMES: DEVELOPMENT, CHARACTERIZATION AND *IN VITRO* EVALUATION

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Abstract

Abstract
Vinorelbine (NVB) is an anti-mitotic drug used to treat multiple types of cancers, demonstrating a high antitumor activity and low neurotoxicity. The pH-sensitive liposomes (PSL) undergo destabilization of the vesicular membranes in the acidic environment of tumours which triggers the release of active substance at the tumour site. The aim of this study was to design and evaluate the NVB-PSL formulations in non-small cell lung cancer (NSCLC) cell lines regarding their anti-tumour effect. The liposomes were obtained using the thin lipid film hydration method. An optimization study was performed using the Design of Experiments (DoE) methodology. The obtained liposomes were characterised in terms of particle size, homogeneity, surface charge, encapsulation efficiency and NVB content. The DoE analysis showed significant relation between the chosen factors (the ratio and concentration of lipids and NVB concentration) and the responses, which enabled the prediction of an optimum PSL formulation. We performed the *in vitro* release study at different pH values and the results demonstrated a pH-dependent release profile of the NVB-PSL optimal formulation. The *in vitro* evaluation of the optimal NVB-PSL formulation on two NSCLC cell lines revealed significant cytotoxicity of the liposomal formulations. In conclusion, this study reports the successful formulation and characterization of NVB-PSL as promising tool in NSCLC therapy.

Rezumat

Vinorelbina (NVB) este o substanță anti-mitotică utilizată pentru tratarea mai multor tipuri de cancer, demonstrând o activitate antitumorală ridicată și neurotoxicitate scăzută. Lipozomii cu cedare dependentă de pH (PSL) suferă destabilizarea veziculelor lipozomale în mediul acid al tumorilor care declanșează eliberarea NVB la țintă. Scopul acestui studiu a fost prepararea și evaluarea formulărilor NVB-PSL pe liniile celulare de cancer pulmonar non-microcelular (NSCLC) în ceea ce privește efectul antitumoral. Lipozomii au fost obținuți folosind metoda hidratării filmului lipidic. A fost realizat un studiu de optimizare folosind metodologia *Design of Experiments* (DoE). Lipozomii obținuți au fost caracterizați sub aspectul dimensiunii particulelor, omogenității, sarcinii de suprafață, eficienței încapsulării și conținutului de NVB. Analiza DoE a demonstrat existența unei relații semnificative între factorii aleși (raportul și concentrația de lipide și concentrația de NVB) și răspunsuri, ceea ce a permis predicția unei formulări optime de PSL. S-a efectuat studiul de cedare a NVB *in vitro* la diferite valori ale pH-ului, iar rezultatele au demonstrat un profil de eliberare dependent de pH a formulării optime. Evaluarea *in vitro* a formulării optime pe două linii celulare de NSCLC a demonstrat o citotoxicitate semnificativă a formulărilor lipozomale. În concluzie, acest studiu raportează formularea și caracterizarea cu succes a NVB-PSL ca instrument promițător în terapia NSCLC.

Keywords: vinorelbine, liposomes, Quality by design

Introduction

Vinorelbine (NVB) is a semi-synthetic vinca alkaloid, that has clinical efficacy for the treatment of non-small cell lung cancer (NSCLC) and for that of breast cancer in combination with other cancer chemotherapeutic drugs [6, 7, 10]. NVB was approved in 1989 for the treatment of bronchial cancer, under the brand name Navelbine® IV (vinorelbine bitartrate) and received approval for non-small cell lung cancer (NSCLC) in 1991 [6]. When compared with parent compounds vincristine and vinblastine, NVB has better antitumour activity and hematologic tolerance and reduced neurotoxicity [10, 16].

Liposomes are nanoparticles consisting of a hydrophilic core enclosed within a phospholipid bilayer membrane which have been widely studied as antitumour drug carries [3, 8, 16, 20]. The lipidic composition of liposomes provide them different characteristics in terms of rigidity, size, release rate and surface charge [3]. One of liposomes drawback is the slow drug release from its structure, therefore researchers investigated new potential carriers for cancer therapy as the pH-sensitive liposomes (PSL). It is well known that the tumour microenvironment has a lower pH than healthy tissues. To exploit this pH difference, PSL are designed

to be stable at physiological pH, but undergo the destabilization of the vesicular membranes in the acidic tumour microenvironment (pH 5 - 6.5), which triggers the release of the drug at the tumour site [3, 9]. The most common phospholipids used to form PSL are 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and Cholesteryl hemisuccinate (CHEMS) [1, 3, 13]. CHEMS self-assembles into bilayers in alkaline and neutral aqueous media [13] and has two functions in PSL composition: to protonate at weakly acidic pH, causing destabilization of lipid bilayer membranes in PSL, and to stabilize the bilayer structure at neutral pH [9, 13]. DOPE is used as a stabilizer for liposomes containing CHEMS with the role of preserving the bilayer structure of liposomes [25].

Quality by design (QbD) is a methodical, risk-based approach of the pharmaceutical formulation development described in the ICH guidelines that begins with pre-determined goals, focusing on the product, process understanding and control. The QbD approach assumes that quality should be ensured early in the research, development and design stages to consistently produce a product with the desired characteristics [11, 19, 24]. The QbD concept entails the identification of the characteristics that are critical to quality, and then transforms these characteristics into the attributes that the final product should have, and finally understands the sources of the variability assuring that quality is built into the product [11, 24]. Design of experiments (DoE) is an important tool that ensures the rational product development [17]. Applying the pharmaceutical QbD concept results in a better understanding of the product and process [19].

The aim of this study was to develop and optimize PSL for NVB delivery using the QbD methodology. This study focused on establishing a systematic and robust process to ensure consistent quality and the performance of the liposomal formulations. The drug release profile was studied in order to demonstrate the pH-dependent release of the NVB from the optimal PSL formulation. Furthermore, the cytotoxicity of NVB-PSL formulations was evaluated in NSCLC cell lines regarding their anti-tumour effect.

Materials and Methods

Vinorelbine (NVB) was purchased from Glentham Life Sciences (Corsham, Wiltshire, United Kingdom). DOPE was purchased from Lipoid GmbH (Ludwigshafen, Germany). CHEMS was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Methanol was purchased from International Laboratory (Titolchimica, Pontecchio Polesine, Italy).

Quality target product profile (QTPP) and critical quality attributes (QTA)

Identifying the formulation's quality target product profile (QTPP) and critical quality attributes (CQAs) are the first steps in the QbD concept [4, 11]. Clinical expectations, regulatory considerations, patient and industry needs all play a role in the QTPP selection process. QTPPs typically contain the dosage form, the administration method, the dose, its dissolution, or pharmacokinetic data [19].

The CQAs are the factors that have critical effects on the targeted product quality. These parameters are physical, chemical, biological or microbiological properties that must stay within a certain range to maintain the quality of the final product [19].

Risk assessment

Risk analysis is estimating the risk connected to the recognised hazards. Risk identification, risk analysis and risk evaluation are three primary components of the risk assessment outlined in the ICH Q9 document. These components are vital during the application of QbD principles in drug product development [12, 14, 17]. The Ishikawa or the cause-and-effect diagram helps gather and display the important parameters of the final product, enabling to anticipate the sources of variation for the next steps [19]. An Ishikawa diagram was constructed to identify the factors that could affect the most important CQAs of the NVB-PSL.

In order to identify and reduce any risks that could have an impact on the quality of the final product, risk assessment is essential. Throughout the development and manufacturing processes, possible risks can be identified, evaluated and prioritised using the risk management tools [5]. Failure mode effects analysis (FMEA) was performed to rank the effect of the input factors on the NVB-PSL CQAs. The risk priority number (RPN) was calculated for each factor by giving each parameter (severity, occurrence and detectability) scores from 1 to 5 [2].

Experimental design

Based on the risk analysis three process parameters were selected, namely CHEMS:DOPE ratio (X1), lipids concentration (X2; mM) and the NVB concentration (X3; μg/mL). An optimization study (central composite face design) was performed using the Modde 13 software (Sartorius Stedim Biotech, Göttingen, Germany). The DoE analysis showed significant relation between the chosen factors and the responses, which enabled the prediction of an optimum PSL formulation. Table I contains the DoE matrix with the factors, their level of variance, used for the NVB-PSL preparation.

Table I

Composition of liposomal formulations

Exp. name	Run order	X1	X2	X3
N1	11	1:1	10	1
N2	13	1:4	10	1
N3	10	1:1	40	1
N4	14	1:4	40	1
N5	2	1:1	10	8
N6	16	1:4	10	8
N7	7	1:1	40	8
N8	9	1:4	40	8
N9	12	1:1	25	4.5

Exp. name	Run order	X1	X2	X3
N10	1	1:4	25	4.5
N11	3	1:2.5	10	4.5
N12	15	1:2.5	40	4.5
N13	8	1:2.5	25	1
N14	6	1:2.5	25	8
N15	5	1:2.5	25	4.5
N16	17	1:2.5	25	4.5
N17	4	1:2.5	25	4.5

X1 – lipids (CHEMS:DOPE) molar ratio; X2 – lipids concentration (mM); X3 – NVB concentration (μg/mL); NVB – vinorelbine

Preparation of pH-sensitive liposomes

The NVB along with the appropriate amount of CHEMS:DOPE was mixed and dissolved using a 1:1 methanol:chloroform mixture. Subsequently, vacuum rotary evaporation was utilised to remove the organic solvent and to create a uniform film. The formed lipid film was hydrated with 200 mM phosphate buffer solution pH 8 by sonication in a water bath for 5 min to obtain the liposomal dispersion.

Characterization of NVB-PSL

Particle size, polydispersity index (PDI) and zeta potential (ZP) values were measured using a Nano ZS90 Zetasizer (Malvern Instruments Ltd, Malvern, UK). The NVB concentration in liposomal formulations was measured by HPLC with a UV detector (Agilent Technologies Inc., Cotati, CA, USA). The chromatographic conditions were as follows: Luna C18(2), 5 μm, 100 Å, 150 x 4.6 mm (PHENOMENEX, Torrance, USA), and the mobile phase ammonium acetate 80 mM (pH = 3, adjusted with HCl):acetonitrile 55:45, and the detected wavelength was 268 nm.

In vitro drug release study

The *in vitro* release of NVB from the PSL was performed using a six-cell automated sampling and collection platform Phoenix DB-6 Robotic Diffusion Station purchased from Teledyne Hanson (Chatsworth, CA, USA). A volume of 0.25 mL of NVB-PSL was placed on the membrane used for the release study (Spectra/Por™ 6 Pre-wetted Standard RC Dialysis Tubing, 3.5 kD MWCO, Thermo-Fisher Scientific, Waltham, MA, United States). The study was carried out at 37°C, in 10 mL phosphate buffer (pH 5.5 and 7.4) at a stirring rate of 200 rpm. The drug release study was performed in pH 5.5 and pH 7.4 conditions to evaluate the pH-dependent release simulating the physiological and acidic tumour microenvironment conditions.

For 48 hours, at regular time intervals, 200 μL of sample was collected from the donor compartment and the redrawn sample was replaced with the equivalent volume of fresh media at each time point. All the samples were analysed through the HPLC method described earlier. The study was performed in triplicate, and the results were expressed as mean ± standard deviation (SD).

Cell lines

The human lung adenocarcinoma cell line A549 and squamous cell carcinoma SKMES, both NSCLC cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). The A549 cell line was cultured in F12K medium supplemented with 10% Foetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin and the SKMES cell line was cultured in RPMI medium with 10% FBS and 1% Penicillin-Streptomycin. The culture medium and supplements were obtained from Sigma-Aldrich (St Louis, MO, USA).

Cell viability determination by MTT assay

1 x 10^4 cells/well were cultured in 96-well culture plates for 24 hours at 37ºC in 5% CO_2 atmosphere incubators. After 24 h incubation the cell cultures were treated with different doses of NVB, NVB-PSL and empty PSL. After 48 hours the medium was discarded and 100 μL 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) solution was added in every well. After 2 hours incubation at 37ºC, the MTT/ medium solution was removed, and the formazan crystals were solubilised with 100 μL DMSO (di-methyl sulfoxide). For the cell viability assay the absorbance was measured at 570/690 nm in a micro-plate reader (Synergy H1 Hybrid Reader Biotek, USA). All the experiments were performed in triplicate and mean data were presented.

Statistical analysis

The statistical analysis of the MTT cell viability assay was performed using GraphPad Prism version 10.2.3 (GraphPad Software, Boston, MA, USA). To statistically assess the effect of NVB-PSL on the A549 and SKMES cell lines, an unpaired t-test was applied to data obtained from the MTT assay. Significance was considered at values of $p < 0.05$ (ns, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). All data are expressed as mean ± standard deviation ($n = 3$).

Results and Discussion

Quality target product profile (QTPP) and critical quality attributes (QTA)

To facilitate a robust development of the NVB-PSL and to establish the relationship between the final product and the manufacturing process, the QTPP was determined. The most important QTPP elements identified for the NVB-PSL were dosage form, drug release, therapeutic indication, encapsulation efficiency (EE) and stability (Table II).

The selection of a factor as a CQA is based on existing knowledge and experience and is influenced by the predetermined objectives, the expected product quality and the therapeutic requirements. Table III contains the most important CQAs that could influence the NVB-PSL.

Table II

Quality Target Product Profile of NVB-PSL

Element	Target	Justification
Dosage form	Liposomes	To improve the efficacy, bioavailability and safety profile of NVB
Drug release	pH dependent drug release	To deliver the NVB in the tumour microenvironment
Therapeutic indication	NSCLC	NVB is associated with cisplatin in NSCLC treatment
Encapsulation efficiency	> 60 %	To ensure a high amount of encapsulated drug
Stability	Stable nanosuspension for at least one month	To maintain consistency in therapeutic effect

NVB – vinorelbine; NSCLC – non-small cell lung cancer

Table III

Critical quality attributes for NVB-PSL

CQA	Target	Is it critical?	Justification
Particle size	Less than 200 nm	Yes	To ensure the internalization of liposomes
PDI	Less than 0.2	Yes	Homogeneity/uniformity of the liposomal dispersion
Surface charge	Less than –50 mV		To ensure stability of the liposomal dispersion
EE	More than 60%	Yes	To ensure sufficient nano system drug loading
Aspect	White dispersion	No	To provide adequate aspect

CQA – critical quality attribute, PDI – polydispersity index, EE – entrapment efficiency

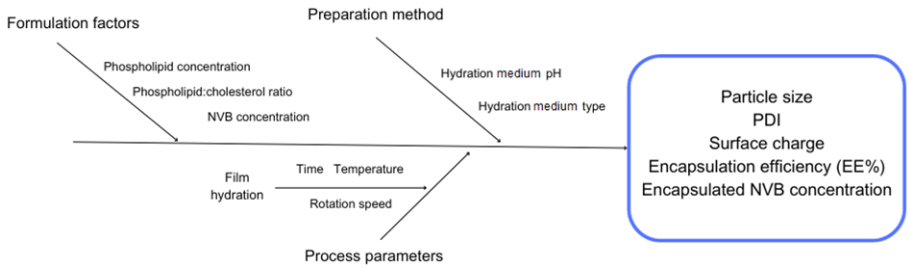

Figure 1.

Ishikawa diagram

NVB – vinorelbine; PDI – polydispersity index

The Ishikawa diagram shown in Figure 1 was created to identify the potential risks and corresponding causes for the chosen CQAs. The cause-and-effect diagram was divided in three major categories: formulation factors, preparation method and process parameters. From each category we identified several factors that could affect the most important CQAs of the NVB-PSL.

Risk assessment

Table IV contains the FMEA performed on each preparation step based on the risk severity, occurrence and detectability. After the analysis, the three variables that had a RPN higher than 40 were identified as factors with major impact for the CQAs. There is a high variety of CHEMS:DOPE ratios mentioned in literature: 1:2, 1:3, 1:4 [1, 9], 4:6 [21], 2:3 [1, 15]; therefore, we considered that lipids ratio is an important factor that could influence the observed results. Besides lipids ratio, lipids and NVB concentrations were also considered important factors and were included in the DoE.

Table IV

Failure mode effects analysis of NVB-PSL

Critical formulation factor or process parameter	Failure Mode	Failure effects	O	S	D	RPN
Lipids concentration	Improper lipids concentration	Particle size, PDI	4	4	4	64
NVB concentration	Improper NVB concentration	Efficiency	5	4	3	60
CHEMS:DOPE ratio	Improper CHEMS:DOPE molar ratio	Particle size, PDI, EE	3	4	3	48
Evaporation time	Short time	EE, stability	3	4	3	36
Sonication time	Short time	Particle size, PDI	3	3	4	36
Evaporation pressure	High pressure	EE, stability	3	3	3	27
Evaporation temperature	High temperature	EE, stability	4	3	2	24

S – severity; O – occurrence; D – detectability; RPN – risk priority number; NVB – vinorelbine; CHEMS – cholesteryl hemisuccinate; EE – encapsulation efficiency; PDI – polydispersity index

Experimental design analysis

The experimental results for all the observed results are listed in Table V. The particle size (Y1) for the NVB-PSL ranged between 142.7 nm for N15 and 888.5 nm for N7, while the PDI (Y2) ranged between 0.173 for N15 and 0.993 for N7. These variations can be explained by the higher amount of CHEMS, the overall lipids and NVB concentrations for N7 formulation, compared to N15. The zeta potential (Y3) had values from -79.4 mV for N5 to -59.8 mV for N11. This can be explained by the lower amount of CHEMS contained by the N11 formulation. In terms of EE (Y4) the results ranged between 69.14% for N10 and 93.94% for N15. These two formulations had the same NVB and lipids concentration, the only difference being the CHEMS:DOPE ratio. When compared, N10 had a higher amount of DOPE and lower amount of CHEMS, while N15 had a lower amount of DOPE and higher amount of CHEMS. For the last response, NVB concentration (Y5), the result varied from 0.695 µg/mL for N2 to 7.37 µg/mL for N6. These two formulations had the same values for X1 and X2 variables, meaning that the NVB concentration (factor X3) was the only variable that influenced this result.

Table V

The CQAs of NVB-PSL, evaluated as responses of the DoE

Exp. Name	Y1	Y2	Y3	Y4	Y5
N1	166.1 ± 1.457	0.259 ± 0.038	-70.2 ± 5.23	84.33 ± 6.61	0.843 ± 0.06
N2	159.8 ± 0.655	0.237 ± 0.013	-64.7 ± 2.86	69.57 ± 1.01	0.695 ± 0.01
N3	226.2 ± 7.012	0.312 ± 0.070	-74.7 ± 0.473	90.45 ± 2.03	0.904 ± 0.02
N4	159.5 ± 4.158	0.208 ± 0.025	-64.7 ± 6.48	87.93 ± 1.52	0.879 ± 0.01
N5	248.5 ± 23.50	0.514 ± 0.007	-79.4 ± 5.85	85.2 ± 0.50	6.816 ± 0.04
N6	163.9 ± 3.544	0.262 ± 0.010	-63.5 ± 1.37	92.13 ± 0.25	7.370 ± 0.02
N7	888.5 ± 225.8	0.993 ± 0.013	-77.3 ± 3.22	80.02 ± 1.33	6.401 ± 0.10
N8	160.3 ± 1.833	0.233 ± 0.022	-75.4 ± 3.92	75.57 ± 5.72	6.045 ± 0.45
N9	186 ± 2.616	0.244 ± 0.021	-77 ± 4.41	77.86 ± 8.59	3.503 ± 0.38
N10	151 ± 3.274	0.211 ± 0.024	-69.6 ± 1.75	69.14 ± 4.18	3.111 ± 0.18
N11	142.9 ± 2.053	0.206 ± 0.011	-59.8 ± 7.05	75.54 ± 5.31	3.399 ± 0.23
N12	173.5 ± 3.265	0.202 ± 0.024	-66.1 ± 1.77	82.82 ± 3.16	3.726 ± 0.14
N13	184.6 ± 3.383	0.192 ± 0.018	-73.6 ± 2.43	91.71 ± 3.05	0.917 ± 0.03
N14	154.1 ± 3.553	0.2 ± 0.013	-60.8 ± 0.819	90.46 ± 3.75	7.236 ± 0.30
N15	142.7 ± 6.358	0.173 ± 0.056	-62.8 ± 3.48	93.94 ± 0.79	4.227 ± 0.03
N16	156.8 ± 0.984	0.191 ± 0.031	-66.9 ± 4.40	87.94 ± 1.13	3.957 ± 0.05
N17	142.8 ± 3.137	0.176 ± 0.003	-64 ± 3.37	83.78 ± 1.81	3.770 ± 0.08

Y1 – particle size (nm); Y2 – polydispersity index; Y3 – zeta potential (mV); Y4 – encapsulation efficiency (%); Y5 – NVB concentration (µg/mL); NVB – vinorelbine

The partial least squares regression model summary of fit is presented in Figure 2. The model validity, reproducibility, determination coefficient (R^2) and prediction coefficient (Q^2) were assessed using the partial least squares fitting. R^2 and Q^2 had a score higher than 0.5, indicating a good fit and a good prediction power of the model. Other values that evidence that the results fit well with the proposed model are model validity and reproducibility with values higher than 0.5.

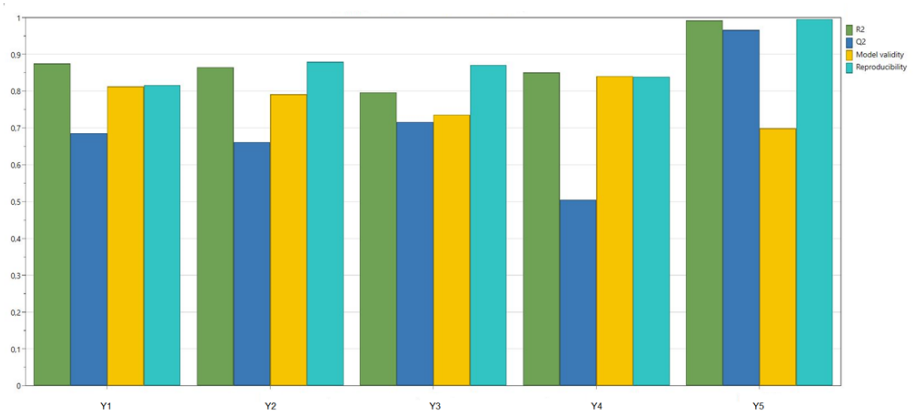

Figure 2.

Summary of fit

R^2 – determination coefficient; Q^2 – prediction coefficient; Y1 – particle size (nm); Y2 – polydispersity index; Y3 – zeta potential (mV); Y4 – encapsulation efficiency (%); Y5 – NVB concentration (µg/mL); NVB – vinorelbine

Figure 3 presents the regression model coefficient plots that shows the relation between the factors and the NVB-PSL attributes, and Figure 4 shows the contour plots of the studied responses. The factors that influenced the particle size (Y1) were the lipids concentration (X2) and the quadratic factors X1*X1 in a positive manner, while the interaction between the lipids concentration and NVB concentration (X2*X3) led to a decrease of this response. The factor with the greatest influence ($p = 0.0136$) was the lipids concentration, which led to an increase in particle size, as previously reported by our group in another study [22]. The increase of NVB concentration (X3) increased the NVB-PSL PDI (Y2), while the interaction between lipids ratio and NVB concentration (X1*X3) decreased the PDI. The lipids ratio (factor X1) and quadratic factor X1*X1 had a non-linear effect on this result. The lipids ratio (factor X1) had a non-linear influence on NVB-PSL ZP (Y3) according to the quadratic factor (X1*X1), while the lipids concentration (X2) led to a decrease of this response. EE (Y4) increased once the lipids concentration (X2) and quadratic factor X3*X3 were increased. Conversely, EE decreased with the increase in the quadratic factor X1*X1 and interaction between lipid's concentration and NVB concentration (X2*X3). The NVB concentration (factor X3) and quadratic factor X3*X3 had a non-linear influence on the NVB content (Y5) of NVB-PSL. The quadratic factor X1*X1 had a negative influence on this response, but with an inferior influence.

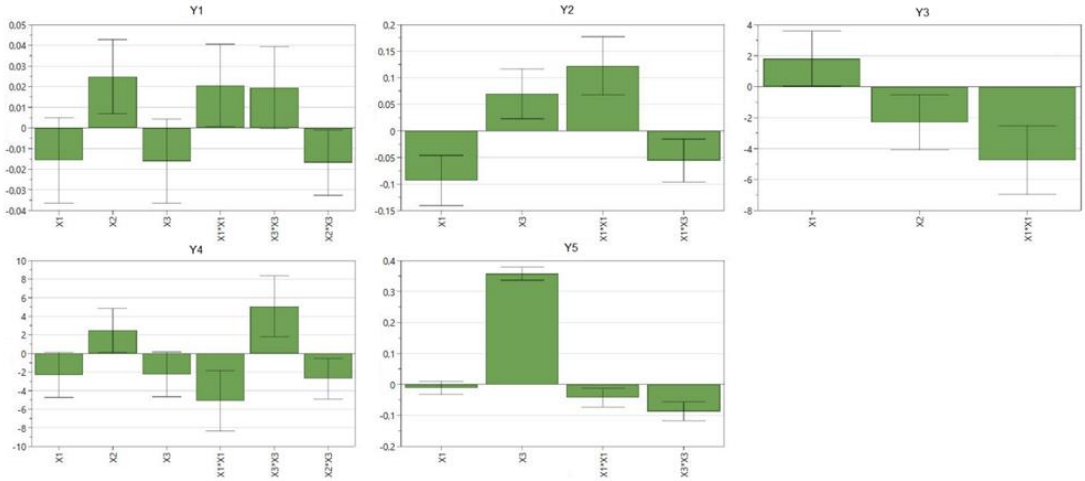

Figure 3.

Effect of investigated factors on the responses

Y1 – particle size (nm); Y2 – polydispersity index; Y3 – zeta potential (mV); Y4 – encapsulation efficiency (%); Y5 – NVB concentration (μg/mL); X1 – lipids ratio; X2 – lipids concentration (mM); X3 – NVB concentration (μg/mL); NVB – vinorelbine

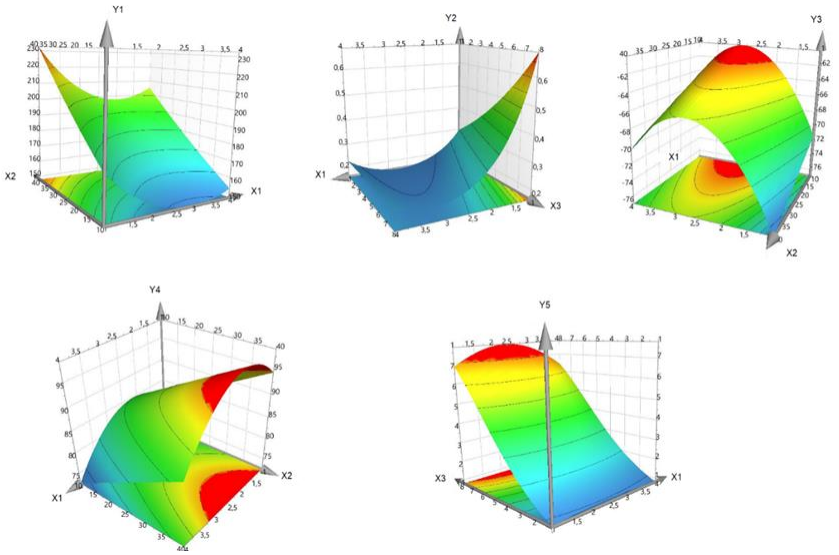

Figure 4.

Contour plots showing the effects of the investigated factors on the responses

Y1 – particle size (nm); Y2 – polydispersity index; Y3 – zeta potential (mV); Y4 – encapsulation efficiency (%); Y5 – NVB concentration (μg/mL); X1 – lipids ratio; X2 – lipids concentration (mM); X3 – NVB concentration (μg/mL); NVB – vinorelbine

Optimal formulation

The optimal formulation was defined after establishing the desired criteria of maximizing the last two responses, that is the EE (Y4) and NVB concentration (Y5). The following conditions were obtained for the NVB-PSL optimal formulation: lipids ratio 1:2, lipids concentration 10 mM and NVB concentration 8 µg/mL. The optimal formulation met the criteria established in the QTPP, being approximately 150 nm in size, with an acceptable PDI value (below 0.2), and encapsulated the active substance with an efficiency over 90%.

As shown in Table VI, the obtained experimental results for the responses were comparable to the predicted ones. The bias value shows the relative difference between the observed and the predicted value, providing valuable information about the accuracy of predictions. The obtained bias values for the particle size (Y1) and NVB concentration (Y5) were negative. This means that the particle size and EE of the obtained NVB-PSL were lower than the predicted ones. For responses Y3 (ZP) and Y4 (EE) the obtained bias had positive values, indicating that the observed values were higher than the predicted values. However, the bias results were lower than 5% in general, indicating quality of the develop models. For Y2 (PDI) the bias was higher, as the experimental value was much lower than the predicted one, which indicates better uniformity of particle size in practice, but both real and predicted are within the accepted range.

Table VI

Predicted results *vs*. experimental values for the NVB-PSL optimal formulation

Response	Y1	Y2	Y3	Y4	Y5
Predicted values	161.59	0.29	-61.90	90.25	7.72
Experimental values	154.8 ± 0.264	0.158 ± 0.009	-62.6 ± 2.05	91.59 ± 1.52	7.32 ± 0.12
Bias (%)	-4.2	45.51	1.13	1.48	-5.18

Y1 – particle size (nm); Y2 – polydispersity index; Y3 – zeta potential (mV); Y4 – encapsulation efficiency (%); Y5 – NVB concentration (µg/mL); X1 – lipids ratio; X2 – lipids concentration (mM); X3 – NVB concentration (µg/mL); NVB – vinorelbine

In vitro drug release study

The release profile of NVB from the optimal NVB-PSL formulation (Figure 5) indicated the pH-dependent release characteristic of the nano-system. In the first 6 hours there were no visible differences between the two release media (31.57% for pH = 5.5 and 26.92% for pH = 7.4). After 12 hours, the NVB percentage release in pH 5.5 medium began to increase (56.51%), compared to 34.92% for pH 7.4. The cumulative percentage of the NVB released after 48 hours at pH 5.5 was 89.51%, while at pH 7.4 the NVB released reached 53.84%.

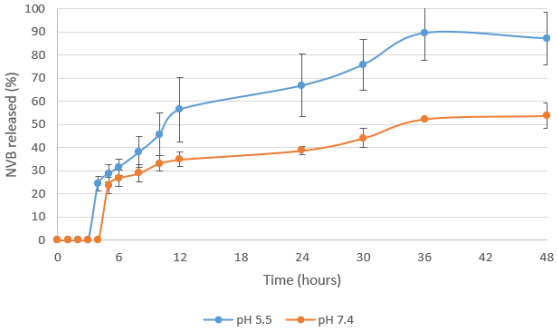

Figure 5.

In vitro NVB release in phosphate buffer (pH 5.5 and 7.4)

NVB – vinorelbine

Bahadori *et al.* developed vinorelbine sterically stabilised micelles, which showed a release of about 25% in 50 hours in PBS pH 7.4 [6]. Wang *et al.* developed polymeric micelles with cisplatin or vinorelbine, and co-loaded liposomes with cisplatin and vinorelbine. The amount of vinorelbine released after 48 hours in PBS was around 70% from the polymeric micelles and 56.96% from the liposomes, respectively [23]. Similarly, Nunes *et al.* showed that the amount of irinotecan released after 30 hours from pH-sensitive liposomes was over 80% [18]. Our results demonstrated a pH-dependent release, and furthermore the amount of NVB released from the NVB-PSL optimal formulation was similar to the literature mentions.

In vitro cytotoxicity analysis

The half maximal inhibitory concentration (IC_{50}) of NVB was evaluated for both A549 and SKMES cell lines before the MTT assay. The results showed that the A549 cell line was more sensitive to NVB than SKMES cells, with IC_{50} values of 8.73 nM and 12.43 nM, respectively (data not shown).

Both cell lines were treated with the optimal NVB-PSL formulation at different NVB concentrations (IC_{50}/2, IC_{50}, IC_{50} x 2). To observe the effect of the lipids used in the NVB-PSL formulation on cell viability, the two cell lines were also treated with the optimal formulation without NVB (empty PSL). The concentrations of lipids from the empty liposomal formulation were equivalent to those in PSL that contained ½ of the IC_{50} of NVB, IC_{50} of NVB, respectively IC_{50} x 2, of NVB (Figure 6).

For the A549 cell line, it can be observed that a significant decrease in cell viability appeared at a NVB concentration equivalent to IC_{50} x 2 ($p = 0.0079$). For SKMES cell line, significant cytotoxicity was observed even at concentration equal to IC_{50}/2 ($p = 0.0011$) and that increased while the NVB concentration increase (for NVB concentrations equivalent to IC_{50} and IC_{50} x 2 $p < 0.0001$). It can be observed that the empty liposomal formulation exhibited significant

cytotoxicity on the SKMES cell line (for L_2, p = 0.0203, for L_3, p = 0.008), but the cytotoxicity was lower compared to the optimal NVB-PSL formulation cytotoxicity.

Similar to this study, Wang *et al*. developed polymeric micelles with NVB and liposomes with NVB and cisplatin and studied their cytotoxic effect in A549 cell line. The results showed a decreased cell viability up to 20% at 100 µg/mL of polymeric micelles and co-loaded liposomes. The IC50 value of NVB was 7.451 microg/mL. At 10 microg/mL, the cell viability was around 55% proofing that the delivery systems were not more toxic than free NVB [23].

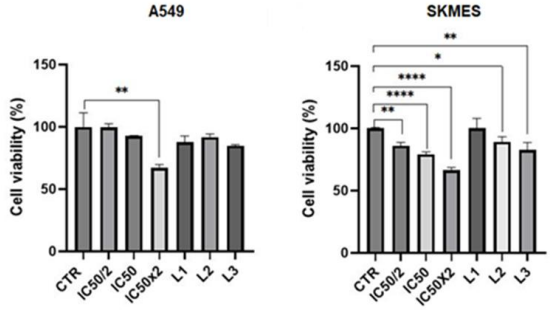

Figure 6.
Cell viability of A549 and SKMES cells evaluated by MTT assay for NBV-PSL and empty PSL
CTR – control, L_1 – empty PSL equivalent to PSL containing NVB $IC_{50}/2$, L_2 – empty PSL equivalent to PSL containing NVB IC_{50}, L_3 – empty PSL equivalent to PSL containing NVB IC_{50}x2

Conclusions

Liposomes have become one of the most significant delivery systems undergoing considerable advances regarding their structure and their scope. In this work, PSL have been developed to improve the release and therapeutic efficacy of NVB. The QbD framework allowed for a systematic development of the NVB-PSL and their formulation factors impact evaluation. The optimal NVB-PSL formulation demonstrated a pH-dependent drug release, confirming the potential of the PSL as a promising drug delivery system for tumour microenvironment-targeted cancer therapy.

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Conflict of interest

The authors declare no conflict of interest.

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