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Scientific paper

Design, Development and Optimization of Carmustine-Loaded Freeze-Dried Nanoliposomal Formulation Using 3^2 Factorial Design Approach

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Abstract

The objective of the current study was to develop and optimize a novel lyophilized liposomal formulation of anticancer agent carmustine, or bis-chloroethyl nitrosourea (BCNU) for prolonged release that could overcome the dose-dependent side effects and improve its bioavailability at the site of action. The optimization was done using a 3^2 factorial design approach wherein soya phosphatidylcholine (SPC) and cholesterol (CH) as independent variables. The optimized formulation (F4) exhibited high entrapment efficiency (81.57%) with an average vesicle size of 141.7 nm and a –22.6 mV Zeta potential. *In-vitro* drug release studies from all formulations revealed that the BCNU was released for up to 36 hours following the Higuchi matrix release model. The TEM, FTIR, DSC, PXRD, and SEM analyses confirm the formation of liposomes. BCNU-loaded nanoliposomal formulation demonstrated prolonged release, suggesting that it could be used to supplement cancer therapy efficiently with a reduction in dose-dependent side effects.

Keywords: Carmustine; Nanoliposomes; 3^2 Factorial design; Release kinetics; Freeze-drying.

1. Introduction

Despite the fact that cancer has been the second leading cause of mortality in the 21st century (besides cardiac ailments), it is plausibly the most complex disease and a serious health threat to people.[1,2] Currently, to treat cancer, physicians use chemotherapy, hormone treatment, gene therapy, surgery, and radiation therapy. Usually, cancer is treated with chemotherapy. On the other hand, high doses of chemotherapy drugs have undesired side effects and can be harmful to the body.[3] In comparison to conventional chemotherapy, the nanocarrier-targeted drug delivery system offers the advantage that it reduces drug exposure to healthy tissues and the risk of organ and tissue damage, which reduces the development of multi-drug resistance and improves bioavailability.[4–7] Moreover, a nanocarrier drug delivery system can also reduce toxicity and chemotherapy costs while achieving a long biological half-life and controlled drug release of chemotherapeutic drugs. Over time, a variety of nanocarriers have been developed for the delivery of tumor-specific drugs, including micelles, liposomes, inorganic nanoparticles, polymeric nanoparticles, nanorods, and others.[8,9]

Liposomes might be one of the most promising drug delivery systems. It consists of one or more concentric phospholipid bilayers formed from synthetic or natural phospholipids that surround an aqueous core. They can include both hydrophilic and lipophilic molecules while yet being dispersed in water as a result of a phospholipid bilayer. These features make liposomes a special nano-carrier for the delivery of biological therapeutics.[10,11] Furthermore, because liposomes are comprised of naturally oc-

curring substances found in biological membranes, they offer the advantages of being biodegradable and non-toxic. Currently, liposomes are a desirable delivery system because of their flexibility, structure, and colloidal size.[12] Liposomes have been produced using a variety of manufacturing techniques and lipid compositions in sizes ranging from nanometres to micrometres. More flexible liposomes can be created by altering the bilayer elements, which produce hard, impermeable, or porous and stable vesicles.[13] For their improved solubility, precise drug targeting, and controlled release of different formulations, liposomes are widely preferred nowadays.[14] According to the results of numerous experimental studies, cancer cells prefer nanoparticles up to 500 nm due to their enhanced penetration and retention effects (EPR). Nanoparticles as small as 500 nm can extravasate because the blood arteries in tumor cells are more permeable than those in healthy tissue.[15,16]

The sole FDA-approved chemotherapeutic drug to treat high-grade gliomas (HGG) is carmustine or BCNU.[17] It is a non-specific, alkylating antineoplastic drug that is used to treat many malignant neoplasms, including brain tumors.[18] Multiple pathways are used by BCNU to cause tumor cytotoxicity, and it frequently disrupts DNA transcription and replication.[19] In addition, BCNU binds to and alters (carbamoylates) glutathione reductase enzyme leading to cell death.[20]

BCNU's short half-life of about 15 to 30 minutes and high toxic side effects (lung fibrosis and bone marrow suppression) limit its efficacy in treating glioma; these are among its most significant disadvantages. Furthermore, it has poor bioavailability due to hepatic metabolism.[21–23] Therefore, an advanced novel prolonged-release formulation is needed for the efficient delivery of BCNU to the brain and other related malignancies, which may help reduce the dose as well as any dose-related side effects.

Therefore, the current work sought to evaluate the effects of polymer concentration and other process variables to create and optimize a nanoliposomal formulation with the desired size range, high entrapment efficiency, and prolonged release of BCNU, an anticancer drug.

2. Materials and Methods

2. 1. Materials

BCNU was received as a gift sample from Emcure Pharmaceuticals Ltd., Pune, Maharashtra, India. SPC was provided by the German company lipoid GmbH as a gift sample. CH, chloroform, and methanol were purchased from Loba Chemie Pvt. Ltd. Mumbai, Maharashtra, India. The other solvents and materials employed were of an analytical standard.

2. 2. Optimization of the Solvent System

The solvent system for the lipid phase was optimized using several combinations of organic solvents, specifically, methanol and chloroform and the homogeneity of the film was assessed as depicted in Table 1.

Table 1. Optimization of the solvent system

Trial	Chloroform (mL)	Methanol (mL)	Observation
1	**3**	**0**	**Uniform, transparent film**
2	3	1	Non-uniform sticky flocks
3	3	2	Non-uniform sticky flocks
4	3	3	Non-uniform sticky flocks
5	0	3	Non-uniform sticky flocks

2. 3. Optimization of Process Parameters for Preparation of Liposomes

Using chloroform as an organic solvent, preliminary optimization of the speed of rotation and hydration medium for uniform film formation and maximal drug entrapment efficiency of liposomes was investigated. To create a thin and uniform film, which controls the liposomal preparation process's result, the speed of rotation was changed from 30 revolutions per minute (rpm) to 90 rpm during film deposition under vacuum as depicted in Table 2. The drug's ability to become entrapped in liposomes depends on the pH of the phosphate buffer. Entrapment efficiency was calculated after the pH of the hydration buffer was changed to levels closer to the drug's pKa using phosphate buffer saline (PBS) solution pH 5.0, 6.8, and 7.4 as depicted in Table 2.

Table 2. Optimization of process parameters

Parameters	Variable	Observation
Speed of (rpm) rotary evaporator	**30**	**A thin and uniform film**
	60	A non-uniform film with flocks at the centre of round bottom flask (RBF)
	90	A non-uniform film with flocks at the centre of RBF
The pH of Hydrating medium	5.0	F4 (38.48 %)
	6.8	F4 (57.59 %)
	7.4	**F4 (81.57 %)**

2. 4. Preparation of Liposomes

A small modification to the thin film hydration process was used to produce blank and BCNU-loaded liposomes. SPC and CH were dissolved in chloroform as an organic phase at various molar ratios, along with BCNU (5 mg), to obtain a 60 mg/mL lipid phase concentration in a 250 mL rotary flask. The flask was attached to a rotary evaporator (Aditya Scientific, Hyderabad) that revolved at 30 rpm while immersed in a water bath that was maintained at 40 °C temperature and vacuumed for an hour to form the film.[10,11] Table 3 depicts the components of the liposomal formulation.

After the organic phase had evaporated, the flask was placed in a desiccator overnight to remove any remaining organic solvent residues from the film. The following day, a liposome with a 10 mg/mL lipid concentration was produced by thoroughly hydrating the thin film with PBS solution, pH 7.4, for one hour at a constant rotation of 160 rpm. To transform the produced liposomes from multilamellar to unilamellar vesicles, they were subjected to Ultra Turrax (IKA T25) at 7000 rpm for 15 min. Then they were passed through a high-pressure homogenizer (HPH) (GEA Lab, Panda PLUS 1000) at 200 bar pressure for 50 cycles to reduce particle size and obtain uniform sized- liposomes at the required nanometre size. The produced nanoliposomes were stored at 4 °C for further use.

Table 3. Optimization of BCNU-loaded liposomal formulation using a 3^2-factorial design

Formulation code	Factors [A:B] (mg)	SPC:CH Molar Ratio	Lipid: Drug ratio (mg)
F1	60(−1):20(−1)	1:0.67	16:1
F2	60(−1):40(0)	1:1.33	20:1
F3	60(−1):60(+1)	1:2	24:1
F4	70(0):20(−1)	1:0.57	18:1
F5	70(0):40(0)	1:1.14	22:1
F6	70(0):60(+1)	1:1.71	26:1
F7	80(+1):20(−1)	2:1	20:1
F8	80(+1):40(0)	1:1	24:1
F9	80(+1):60(+1)	1:1.5	28:1

2. 5. Full Factorial Design

The BCNU-loaded liposomes were developed using a 3^2 factorial design. In this approach, the quantities of SPC (A) and CH (B) were evaluated as independent variables. The fixed responses used were vesicle size (Y_1) and percent drug entrapment (PDE) (Y_2). By taking each control variable at three distinct levels nine alternative combinations were made, as depicted in Table 3. Later, the best-fit model derived from fit summary and analysis of variance (ANOVA) was used to examine the impact of various control variables on dependent variables. Design-Expert® software point prediction method was used to achieve the predicted formulation and verify optimization.

2. 6. Characterization of BCNU Loaded Liposome

2. 6. 1. Particle Size

The mean vesicle size and size distribution of blank and BCNU-loaded liposomes were measured using a device based on the dynamic light scattering method (HORIBA scientific SZ-100). The liposomal dispersion was diluted with distilled water (1:100 v/v ratio, dispersant viscosity 0.896 mPa.s) using an ultrasonicator for 15 minutes to obtain a stable suspension. A portion of the suspension was transferred to a quartz cuvette (four openings). Size analysis was performed using a 90° angle of detection for 120 seconds at room temperature. Analysis was performed in triplicates.[3]

2. 6. 2. Zeta Potential

Using Zetasizer (HORIBA scientific SZ-100), the surface charge of liposomes was measured. Before being positioned in measuring cells (cuvette with the carbon electrode, 6 mm), all compositions were diluted with distilled water (1:100 v/v). The measurement of average zeta potential and charge on the liposomes was done by subjecting the formulation for 60 seconds run time. Analysis was performed in triplicates.[3]

2. 6. 3. Entrapment Efficiency

To calculate the total quantity of drug (A) present in the formulation, 2 mL of the liposomal formulation was suspended in 2 mL of methanol to break up the liposomal matrix. This mixture was then centrifuged at 10,000 rpm at 1 °C temperature using a cooling centrifuge (REMI CM-12 Plus) for 30 minutes. The produced pellet was rinsed by overtaxing with a 1 mL PBS solution (pH 7.4) to remove the free drug deposited on the liposome's surface. The resultant dispersion was mixed with 10 ml of PBS solution (pH 7.4) and filtered using a 0.2-micron microsyringe filter. Using a UV/visible spectrophotometer (Shimadzu 1800, Japan), the absorbance was measured at 229 nm to determine the quantity of BCNU in the filtrate.[3] For the determination of free drug concentration (B), 2 mL of a drug-loaded liposomal mixture was centrifuged at 10,000 rpm at 1 °C for 30 minutes using a cooling centrifuge. The supernatant was discarded and diluted it with 10 mL of PBS solution (pH 7.4). The resultant solution was filtered through a microsyringe filter (0.2 μm), and absorbance was measured at 229 nm using a UV/visible spectrophotometer.[3] The entrapment efficiency was calculated by using a formula-

$$\mathrm{PDE} = \frac{\mathbf{A-B}}{\mathbf{A}} \times 100$$

Where 'A' is the total amount of drug and 'B' is the free drug concentration.

2. 6. 4. Transmission Electron Microscopy

TEM images were used to examine the structural integrity of BCNU-loaded liposomes (using Hitachi S-7500). A few drops of diluted liposomal dispersion were applied to a 200-mesh carbon-coated copper grid and photographed at 30,000x magnification and 100 kV.[10]

2. 6. 5. *In-vitro* Drug Release Study

An *in-vitro* drug release study of optimized liposomal formulation (F4) and pure drug (BCNU) was carried out by the diffusion method using a dialysis bag. The treated cellophane membrane (molecular weight cutoff [MWCO] 12 kDa, Thermo Fisher Scientific) was tied at both ends after filling the liposomal sample (equivalent to 5mg of BCNU) in it and placed into the 100 mL beaker containing 50 mL of PBS solution pH 7.4 as a dissolution medium. A magnetic stirrer was used to agitate the dissolving media at 100 rpm while maintaining the temperature at 37 ± 1 °C. 2 mL samples were taken from the receiver at periodic intervals up to 36 h and replaced with equal quantities of fresh dissolving liquid. Using a UV/Visible spectrophotometer, a spectrometric analysis was performed at 229 nm to obtain drug content. Three separate recordings of each reading were taken.[24]

2. 6. 6. Kinetic Modeling of Release Profiles

Several kinetic models, including zero order, first order, Higuchi matrix, Korsmeyer-Peppas, and Hixson-Crowell, were used to fit the data from *in-vitro* drug release studies of liposomal formulations. The best-suited model was chosen, based on the correlation coefficient with the highest value.[3]

2. 6. 7. Physical Stability of Liposomal Formulation

As per the ICH guidelines, stability experiments were carried out for the optimized formulation (F4) to evaluate the physical stability. The liposomal formulation (F4) was stored at room temperature (25±2 °C / 60±5 %RH) and in the refrigerator (4±2 °C) for three months. The samples were collected at predetermined intervals of initial, 30, 60, and 90 days to assess their physical appearance, mean vesicle sizes, size distributions, and amounts of drug entrapment as previously mentioned.[10,25]

2. 6. 8. Optimization of Cryoprotectant and Freeze-Drying Process

The cryoprotectant concentration and formulation parameters that are most likely to affect the freeze-drying cycle and the quality of the finished product were studied. A drug-loaded liposomal sample (F4) was centrifuged for 30 minutes at 10,000 rpm (REMI CM-12 Plus). The supernatant was discarded after centrifugation, and the sediment was collected in glass vials for freeze-drying. Along with the liposomal formulations, the cryoprotectant mannitol was used in various concentrations (lipid: mannitol 1:0w/w, 1:5w/w, 1:10w/w, and 1:15w/w). To produce homogenous ice nucleation, the above mixture was frozen overnight at –50 °C (1 °C/min) in a deep freezer. After that, it was freeze-dried using Christ, Alpha 1-2 LDplus. The aqueous solvent was then sublimated by maintaining the sample at –50 °C and 0.011 mBar for 12 h. The temperature and pressure were then raised to –20 °C (1 °C/min) and 1.0 mBar for 6 h. Secondary drying was done to remove bound water. For this, the shelf temperature was raised by 1°C every minute and maintained at 20°C and 1.6 mBar for almost 3 h. After the process was completed, the vials were sealed with rubber caps and kept at 4 °C for further analysis.[26]

2. 6. 9. Moisture Content

The Karl Fisher method was used to calculate the remaining moisture (RM) in the freeze-dried cake. 0.1 g of the sample was transferred to the titration cell. The water content was determined using a Metrohm 870 KF Titrino plus KF titrator.

2. 6. 10. Compatibility Studies

Using an FTIR spectrometer (Bruker Alpha II), the FTIR spectra of pure BCNU, physical mixtures, and freeze-dried formulation were recorded and analyzed between the wavelengths of 4000 and 650 cm^{-1}.

2. 6. 11. Differential Scanning Calorimetry

Using the Mettler Toledo DSC 822e instrument, DSC analysis of pure BCNU and a freeze-dried formulation were carried out to check the compatibility. Zinc and indium were used as standards to calibrate the temperature and enthalpy scales. Samples were heated in hermetically sealed aluminium containers at a constant rate of 10 °C/min from –60 to 200 °C. Liquid nitrogen was used at a flow rate of 40 mL/min to create an inert atmosphere.

2. 6. 12. Powder X-ray Diffraction

PXRD is a crucial method for determining whether a substance is crystalline or amorphous. Using a powder X-ray diffractometer (AXS D8 Advances, Bruker Ltd., Germany) diffractograms of a pure drug and formulation were obtained with tube anode Cr spanning the range of 10–70°/2θ employing copper as the X-ray target and a 1.54 Å wavelength.

2. 6. 13. Scanning Electron Microscopy

A scanning electron microscope (JSM-6360, Jeol Instruments, Japan) was used to examine the surface morphology of the BCNU-loaded freeze-dried liposomal formulation. With a 15 kV accelerating voltage, photomicrographs were taken of the sample while it was mounted on a double-faced gold-coated adhesive tape.[27]

3. Results and Discussion

3. 1. Development of the Solvent System

This system used organic solvents to dissolve the lipid phase and form a thin, uniform, and non-sticky film. Since the nature of the film affects the liposomal size and entrapment efficiency. Different compositions of chloroform and methanol were assessed for film formation. From the blend of organic solvents, a thick and sticky film was observed at the centre of the RBF, while chloroform alone produced a thin, uniform, and non-sticky film at the sides of the RBF. The results are depicted in Table 1.

3. 2. Optimization of Process Parameters for Preparation of Liposomes

For the preparation of liposomes, process parameters like the speed of rotation and pH of the hydrating medium were studied for thin, uniform non-sticky film formation and entrapment efficiency, respectively. From the observations, it was found that at slow speed, RBF (30 rpm) produced a uniform non-sticky film at the sides, while at high speed (60 rpm and 90 rpm), lipid phase aggregated at the centre, possibly due to a high central force. The effect of hydrating buffer pH on entrapment efficiency was studied as the pH of hydrating buffer effect on entrapment of the drug into the lipid phase. Entrapment efficiency was varied at different pH values (5.0, 6.8, and 7.4). High entrapment efficiency was observed at a pH of 7.4 as the drug (BCNU) is unionized in aqueous fluid at that pH and more soluble in the lipidic phase while more ionized form at less pH and decreases entrapment into the lipid phase.[18] The results are depicted in Table 2.

3. 3. Full Factorial Design

When compared to unsaturated phospholipids, hydrogenated SPC is more stable and biocompatible. Based on earlier research, SPC and CH concentrations were chosen to produce stable liposomes devoid of any aggregation or fusion, with small vesicles and higher drug entrapment efficiencies. This reveals that the amount of SPC and CH is the more important element in liposome production. Optimized concentrations of SPC (60–80 mg) and CH (20-60 mg) were adequate to synthesize liposomes with small vesicle sizes, excellent drug entrapment, and no aggregation or sedimentation. A full factorial design was employed to investigate the factors systematically. Using DESIGN EXPERT® (version 8.0) software, the impact of different independent variables such as SPC (A) and CH (B) was examined by response surface plots. Figure 1 displays the response to the impacts of independent factors for liposomal vesicle size (Y_1) and PDE (Y_2). The following equations were produced, via regression and graphical analysis of data obtained from the experimental runs, where F ratios were statistically significant ($p < 0.05$), and Adj-R^2 values ranged from 0.8 to 1. The data was well-fit by these model equations.

The effect on vesicle size (Y_1) and PDE (Y_2) was observed to be significant by ANOVA and the linear equation was found as follows:

$$Y1 = +153.13 + 8.47A + 6.83B \quad (1)$$

$$Y2 = +63.68 + 3.88A - 10.84B \quad (2)$$

The response surface plots and regression equations mentioned above make it clear that the SPC and CH, at varying concentrations, produce a positive association concerning the vesicle size of BCNU-encapsulated liposomes. An increase in lipid concentration within the bilayer led to an increase in size. The level of CH was found to be closely correlated with a slight but substantial ($p < 0.05$) decline in entrapment efficiency. Similar outcomes for several lipophilic medications, such as alpha-tocopherol,[28] ciprofloxacin,[29] and triamcinolone acetonide,[30] have previously been observed. In the liposomal bilayer, CH molecules are positioned between the nearby phospholipid molecules. As a result, they take up some area and compete with BCNU for inclusion in the bilayer. Moreover, CH makes the bilayer stiffer, making it more challenging to incorporate drug molecules.

The adjusted determination coefficient (R^2= 0.8948 and 0.8873 for Y_1 and Y_2, respectively) and predicted determination coefficient (R^2 = 0.8217 and 0.8227 for Y_1 and Y_2, respectively) values were comparable and showed the high significance of the model. By rejecting the null hypothesis, these "p" values of 0.05 (Prob > F) show that the model terms are significant. The "p" values for vesicle size and PDE were 0.0005 and 0.0006, respectively. For 3^2 factorial design model, the sum of the "p" values and the "adjusted R^2" values reveals a substantial synergistic association between both independent variables at $P < 0.05$.

3. 4. Characterization of BCNU Loaded Liposome

3. 4. 1. Particle Size

The mean vesicle size of the various drug-loaded liposomal formulations, which had 20–60 mg CH and 60–80 mg SPC, was found to be between 141.0 and 170.9 nm. For drug-loaded liposomes, the polydispersity index ranged from 0.31 to 0.53, indicating narrow vesicle size dispersion shown in Table 4. A slightly small range of size distribution was present in every liposomal formulation. The amount of SPC and CH present was significantly related to the size of the drug-loaded liposomes. Rather than the lipid content in the liposomal dispersion, the CH enhances the stiffness of the membrane. Figure 2 shows a typical particle size distribution profile obtained for the optimized formulation (F4).

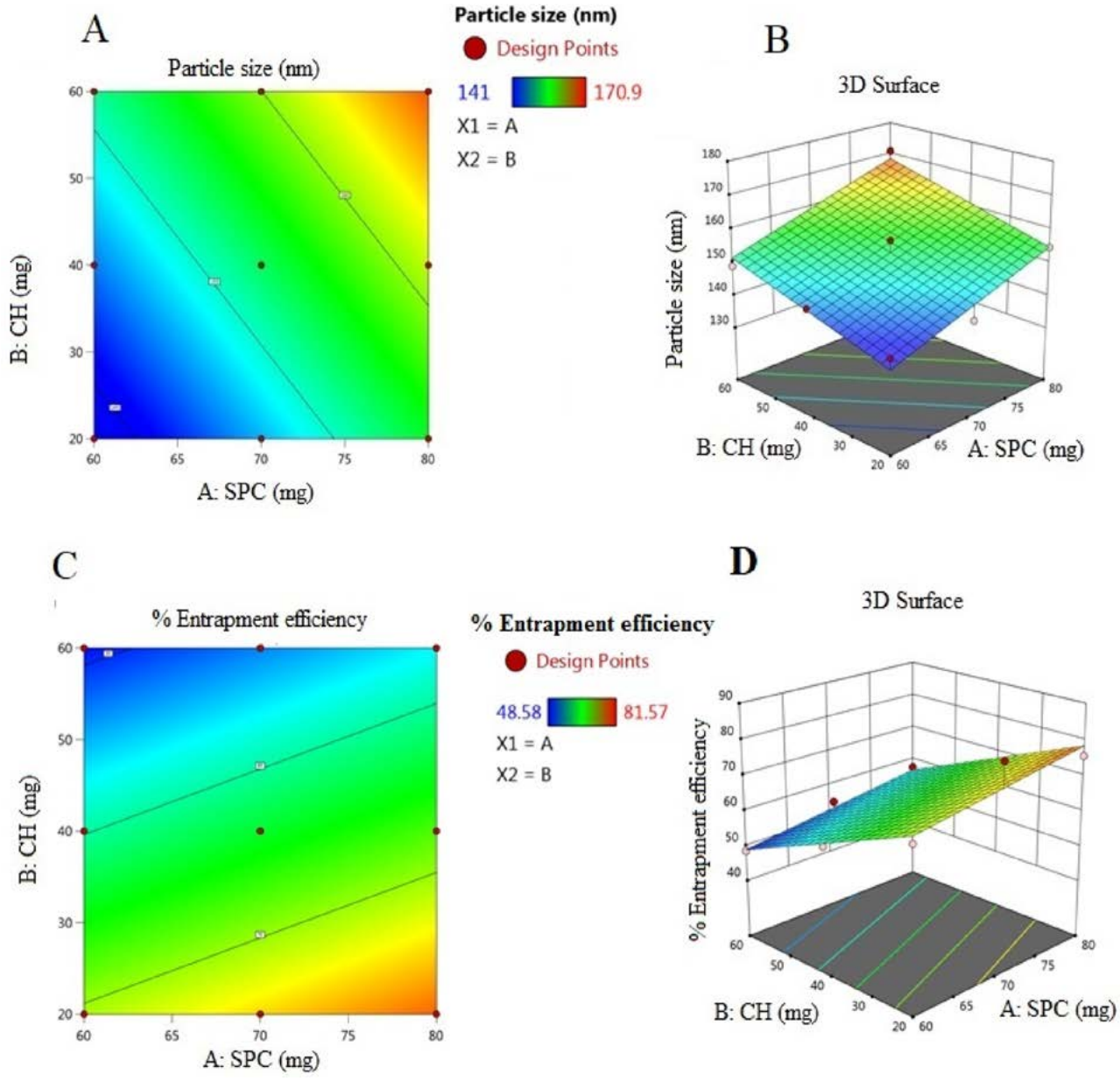

Figure 1. Linear plots (A, C) and Surface response plots (B, D) for particle size and % entrapment efficiency respectively

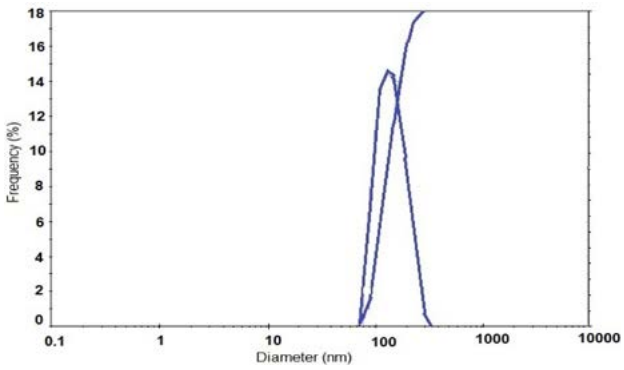

Figure 2. A typical particle size distribution curve of optimized formulation (F4)

3. 4. 2. Zeta Potential

Zeta potential measurements provide information on particle charge and the stability of the dispersion. Zeta potential shows the degree of repulsion between the charged particles in the dispersion. High zeta potential indicates highly charged particles, which avoids particle aggregation owing to electrostatic repulsion. If the zeta potential is low, attraction overcomes repulsion and the dispersion forms aggregates. A zeta potential value of +30 mV to −30 mV is thought to be optimal for good stabilization.[31] High zeta potential values, between ±20 and ±40 mV, offer system stability and are less prone to agglomeration formation or particle size growth. However, it should be noted that zeta potential

values are not an absolute measure of nanoparticle stability.[31]

The zeta potential of freshly prepared liposomes ranged from −18.9 mV to −32.7 mV revealing that they had enough charge and mobility to prevent vesicle aggregation (Table 4). The Zeta potential of the optimized formulation (F4) was depicted in Figure 3.

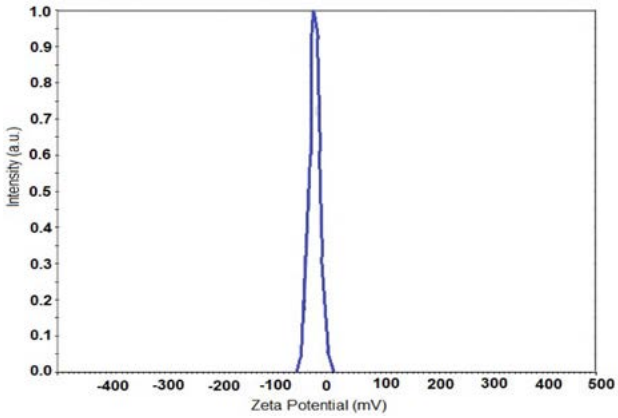

Figure 3. Zeta potential of optimized formulation (F4)

3. 4. 3. Percentage Drug Entrapment

PDE is measured as the drug retention in liposomes as a percentage of the total drug. Percent entrapment efficiency for all formulations was found to be between 48.58% – 81.57 % depicted in Table 4. The amount of SPC and CH optimized for liposomal formulation by considering the small vesicle size and maximum entrapment efficiency because these characteristics predominantly affect the encapsulation of the drug. Furthermore, smaller vesicle size offers better uptake by the cells and augmented drug deposition. Entrapment of the drug may be directly related to the overall surface area, as there are a higher number of vesicles more quantity of the drug will be entrapped. As the particle size decreases, the surface area increases that subsequently results in an increase in drug encapsulation. PDE in liposomes demonstrates that drug entrapment efficiency in the liposomes decreases with decreasing SPC concentrations. This is because the lipid bilayer is saturated with respect to the drug and has a restricted capacity for entrapment due to its low SPC content.

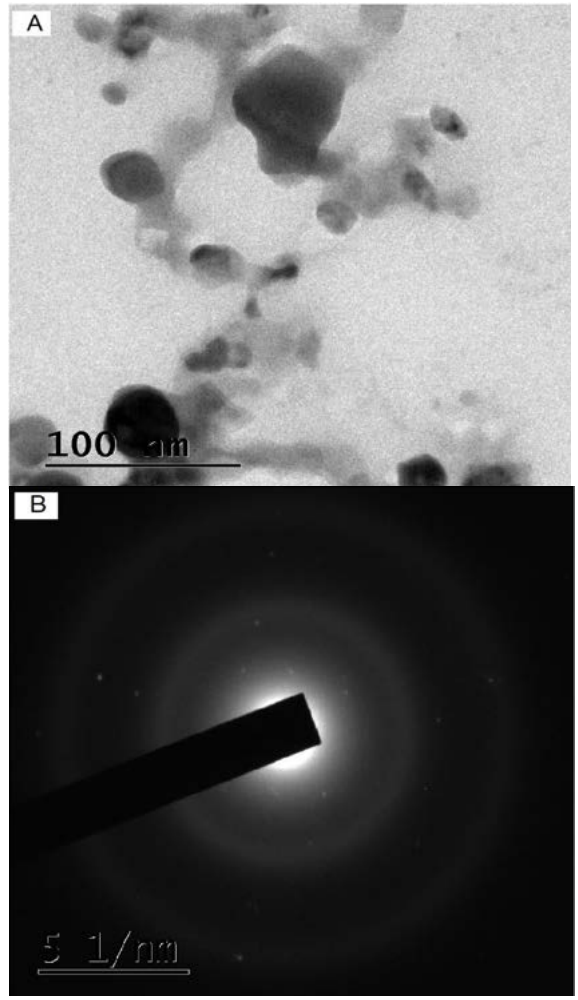

Figure 4. HR-TEM (A) and SAED (B) images of optimized formulation (F4)

Table 4. Vesicle size, PDI, Zeta potential, and PDE of different batches of liposomal formulations

Formulation codes–	Before HPH		After HPH		Zeta Potential (mV)	PDE
	Vesicle size (nm)	PDI	Vesicle size (nm)	PDI		
Blank	131.2±0.34	0.453±0.04	95.1±0.42	0.207±0.06	−21.4±0.32	–
F1	215.9±0.42	0.703±0.06	141.0±0.38	0.331±0.08	−11.5±0.42	64.64±0.43
F2	213.2±0.16	0.474±0.02	145.2±0.23	0.472±0.04	−23.2±0.12	58.94±0.74
F3	256.2±0.26	0.416±0.07	149.0±0.52	0.422±0.02	−36.1±0.26	48.58±0.63
F4	**219.8**±0.46	**0.487**±0.04	**141.7**±0.24	**0.251**±0.03	**−22.6**±0.36	**81.57±0.92**
F5	223.2±0.24	0.400±0.06	156.8±0.68	0.382±0.09	−32.6±0.35	61.36±0.34
F6	248.2±0.57	0.290±0.07	158.5±0.44	0.385±0.06	−25.8±0.48	54.60±0.64
F7	254.4±0.46	0.494±0.09	154.7±0.26	0.531±0.04	−18.4±0.16	75.61±0.83
F8	262.5±0.63	0.396±0.09	160.4±0.44	0.315±0.06	−28.9±0.23	62.22±0.93
F9	275.0±0.28	0.951±0.11	170.9±0.34	0.381±0.07	−30.2±0.28	57.60±0.46

Each value represents Mean ± SD, $n = 3$.

Based on the PDE data, it was revealed that when CH concentration increased, it provided rigidity to the bilayer and decreased PDE. Due to the high drug entrapment efficiency and small vesicle size of the F4 formulation, it was determined to be pertinent.

3. 4. 4. TEM Analysis

The TEM image of the optimized formulation (F4) showed spherical liposomes with a small vesicle size with an average particle size of 141.7nm (Figure 4A). Figure 4B showed the selected area electron diffraction (SAED) pattern of liposomes that confirms the formation of liposomes. This supports the results of particle size.

3. 4. 5. *In-Vitro* Drug Release Studies

The *in-vitro* drug release from the liposomal formulations and the pure BCNU was assessed using a PBS solution with a pH of 7.4. All formulations showed drug release up to 36 h, except the pure BCNU solution, which was released in less than 2 h. All formulations showed more than 90 % drug release within the 36 h (Figure 5). Formulation F4 showed a 96.64 % drug release over 36 h. which indicate controlled release of drug over a prolonged period of time.

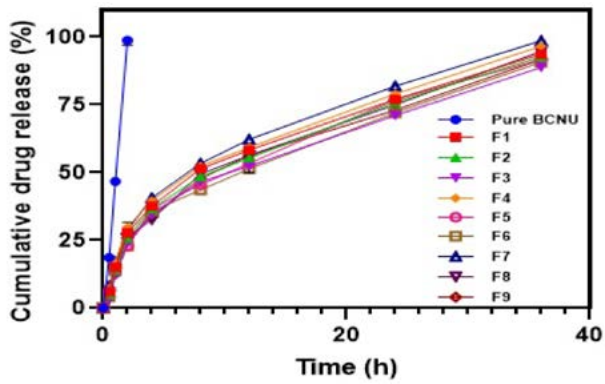

Figure 5. Cumulative % drug release from BCNU liposomes, and pure BCNU

3. 4. 6. Release Kinetic

The data obtained from the *in-vitro* drug release investigation of developed liposomes was fitted into kinetic models to identify the drug release mechanism. For the optimal fitting, the correlation coefficient value (R^2) was used. The values of R^2 for formulations ranged from 0.887 to 0.989. The correlation data for various models for all formulations are displayed in Table 5. According to the measured R^2 values, the Higuchi matrix kinetic model best describes the *in vitro* drug releases from BCNU liposomes. It demonstrates that a diffusion process was adopted to release the drug from the liposomes.

Table 5. Mathematical models in drug release kinetics of liposomal formulations

Formulation codes	Zero order (R^2)	First order (R^2)	Higuchi Matrix (R^2)	Hixon Crowell (R^2)	Korsmeyer-Peppas (R^2)
F1	0.887	0.974	**0.980**	0.977	0.939
F2	0.900	0.977	**0.984**	0.981	0.933
F3	0.893	0.978	**0.981**	0.972	0.899
F4	0.893	0.949	0.981	0.977	0.941
F5	0.913	0.987	**0.989**	0.986	0.963
F6	0.917	0.973	**0.986**	0.980	0.920
F7	0.893	0.924	**0.984**	0.978	0.959
F8	0.905	0.959	**0.987**	0.978	0.954
F9	0.901	0.972	**0.983**	0.976	0.901

3. 4. 7. Physical Stability of Liposomal Formulation

The stability of the liposomal formulation is a further essential factor in the development of an effective drug delivery system. As a result, we tested the durability of the improved liposomal formulation in various settings, including room temperature (25 °C / 60 %RH) and the refrigerator (4 °C). At initial, 30, 60, and 90-day intervals, all liposomal formulations were assessed and determined to be stable. At various storage conditions, caking and discoloration were not seen.

As a function of temperature, the mean particle size and formulation entrapment percentage were assessed. The results were depicted in Table 6 and a graphical representation of the change in particle size and entrapment efficiency is shown in Figure 6. Liposomes stored at 4 °C and 25 °C do not differ significantly in mean particle size. The entrapment efficiency showed a little decline, indicating a considerable loss of BCNU from the formulation over time when held at 25 °C. Therefore, based on the findings of the stability study, it is advised that the liposomal formulation be kept in a refrigerator for better stability.

3. 5. Optimization of Cryoprotectant and Freeze-Drying Process

Optimization of the cryoprotectant concentration used in the formulation is essential, along with careful consideration of the process parameter, to enable efficient stability of the liposomes with retaining formulation properties. We need to maintain the product's primary drying temperature below either the glass transition temperature (Tg′) or the somewhat higher collapse temperature (Tc) per guidelines for pharmaceutical freeze-drying. Typically, Tc and Tg′ can be used interchangeably because they are 1 to 2 °C apart. According to earlier research, the liposomal formulation with mannitol has a Tg′ of between –30 and –32 °C. Considering these values, the shelf temperature during primary drying was kept at –50 °C.[26]

Table 6. The average particle size and PDE of the formulation (F4) stored at various temperatures

Storage temperature	4±2 °C			25 ±2 °C (60±5 %RH)		
Parameter	**Vesicle size (nm)**	**PDE**	**Zeta Potential (mV)**	**Vesicle size (nm)**	**PDE**	**Zeta Potential (mV)**
Initial	141.7±0.24	81.57±0.92	−22.6±0.36	141.7±0.24	81.57±0.92	−22.6±0.36
30 days	150.4±0.32	80.16±0.42	−22.0±0.18	151.4±0.38	78.74±0.23	−24.6±0.12
60 days	156.9±0.25	79.68±0.12	−25.1±0.27	170.3±0.54	74.46±0.32	−25.3±0.24
90 days	167.1±0.37	78.72±0.14	−26.6±0.14	189.4±0.24	72.39±0.42	−25.5±0.18

Each value represents Mean ± SD, $n = 3$.

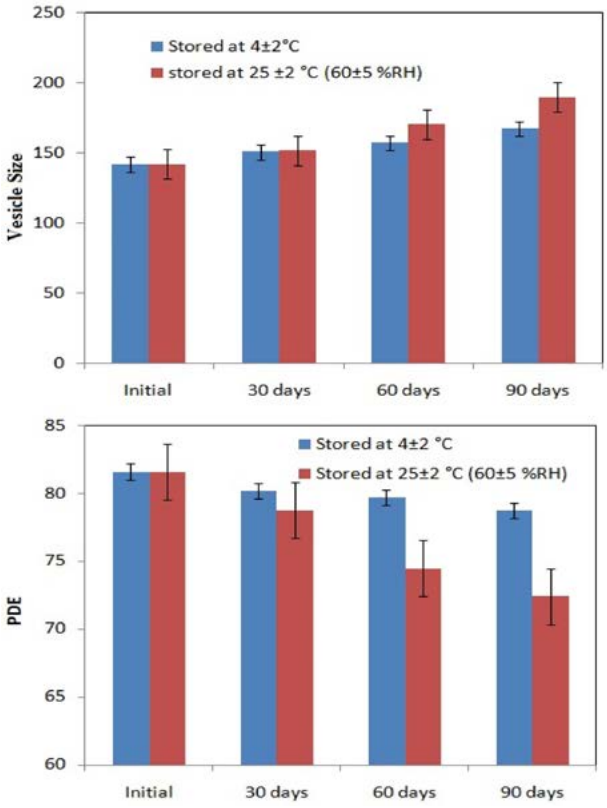

Figure 6. Physical stability of liposomes (F4) stored at different storage conditions; particle size (A) and % drug entrapment (B)

In the first section of the investigation, we explored how freeze-dried liposome stability was affected by mannitol content. This was accomplished by lyophilizing liposomal suspension in the presence of mannitol while varying the weight ratio of lipids to carbohydrates from 1:0 to 1:15. The stability of liposomes during freeze-drying was evaluated by measuring the proportion of the drug that was retained in the liposomes and comparing the size and PDI before and after freeze-drying. Since the drug retained after freeze-drying is closely correlated to the lipid phase transition and the aggregation of particles, it is considered the most sensitive measure that reflects all the harm caused by freeze-drying.

The physicochemical properties of the liposomes were examined before freeze-drying. The liposomes were 141.7 nm in size with a 0.251 PDI, indicating a narrow size distribution displayed in Table 4. In the case of non-cryoprotected liposomes, vesicle aggregation/fusion occurred during freeze-drying was evidenced by the size and PDI of the liposomes obtained after rehydration being significantly higher when freeze-dried without a cryoprotectant (control). It reveals that the freeze-drying process without cryoprotectant affects the integrity of the liposomes. Most of the drug that was encapsulated leaked during the process. In contrast, lyophilized formulations with cryoprotectant content demonstrated increased stability as evidenced by narrow size distribution with controlled vesicle size, and less amount of drug leakage shown in Table 7. However, the stability of the liposomes was significantly impacted by the cryoprotectant concentration. A Lipid: mannitol weight ratio of 1:15 during freeze-drying of liposomes produced vesicles that were two times larger than those of the fresh liposomes.

The distribution of population sizes within a given sample is essentially represented by PDI. The PDI's numerical value range is 0.0 (uniform or monodisperse) to 1.0. (Polydisperse). A PDI of 0.3 and below is thought to be acceptable in drug delivery applications using lipid-based carriers, such as liposome and nanoliposome formulations, and it denotes a homogenous (narrow) distribution of phospholipid vesicles.[32] Table 7 findings show that the freeze-drying procedure did not affect the PDI of rehydrated liposomes that included cryoprotectant in a different weight ratio, with the liposomes having a similar PDI to liposomes before the freeze-drying process (below 0.3). The size distribution of the liposomes was relatively wide, having a value of 0.661 at high lipid-to-mannitol ratios, 1:15, indicating that aggregation/fusion occurs during the processing. Over a limited range, the weight ratio of carbohydrate to lipid increased while the percentage of drug entrapment was reduced when more carbohydrate was added. The liposome membrane integrity was found to be best preserved at an intermediate ratio of 1:10 (lipid-to-mannitol). Previous literature has reported similar outcomes.[33]

Uniform cakes have been seen for all samples with an RM ≤ 5%. For all samples, the secondary drying process eliminated unfrozen water rather slowly, especially when it was done at a temperature of 20 °C. Table 7 displays the RM of cakes and the rehydrated liposomes' characteristics.

Table 7. The effects of mannitol concentration on vesicle size, PDI, PDE, Zeta potential, and RM of a freeze-dried liposomal formulation (F4).

Parameters	Lipid: mannitol weight ratio			
	1:0 (Control)	1:5	1:10	1:15
Vesicle size (nm)	416.6±0.46	237.3±0.62	191.4±0.46	218.5±0.54
PDI	0.933±0.07	0.299±0.08	0.226±0.06	0.661±0.04
PDE	56.40±0.57	72.60±0.53	80.48±0.68	74.67±0.56
Zeta potential (mV)	–26.7±0.38	–27.1±0.64	–28.5±0.49	–23.8±0.32
RM (%)	2.72±0.34	2.20±0.23	2.42±0.32	2.90±0.15

Each value represents Mean ± SD, $n = 3$.

3. 6. Compatibility Studies

Utilizing FTIR tests, it was assessed whether the drug was compatible with other excipients and formulations. Using an ATR-FTIR spectrometer, the infrared spectrum of a pure drug (BCNU) and a physical mixture of a BCNU with excipients and formulation were recorded (Figure 7). The distinctive peaks of the BCNU FTIR spectrum may be seen to correspond to COO^- groups at 1278 and 1456 cm^{-1} and to the double bond C=H at 1134 cm^{-1}. Additionally, peaks at 626, 1318, 1354, and 1432cm^{-1} were observed, which, respectively, corresponded to aromatic CH bending, C-N stretch, aliphatic CH bending, and CH2 bending. Typical characteristic peaks of the BCNU were also seen in the FTIR spectrum of the physical mixture and formulation with no obvious change from the spectra of the individual drug and excipients. This demonstrated that mannitol, the drug, lipids, and cholesterol did not interact chemically. These findings are consistent with previous research.[21]

3. 7. DSC study

DSC studies were used to analyse the thermal behaviour of pure BCNU, physical mixture and its formulation. The results are displayed in Figure 8. The DSC of pure BCNU shows a prominent endothermic peak at 31 °C and –141.4 J/g, indicating the melting of pure BCNU. The SPC endothermic peak was observed at 48.0 °C (the temperature of the phase transition) and had an enthalpy of –0.551 J/g, whereas the mannitol endothermic peak was observed at 161.0 °C and had an enthalpy of –157.4 J/g. At 148 °C, the typical cholesterol peak was discovered. It might be accounted for by the lipids' nanocrystalline structure in liposomes. The absence of an endothermic peak for BCNU in the formulation indicated that the lipid matrix had completely dissolved BCNU. The retention of the characteristic endothermic peak of mannitol in the formulation suggested its crystallinity and did not interact chemically. The conclusions of the DSC analysis were further supported by the PXRD data.

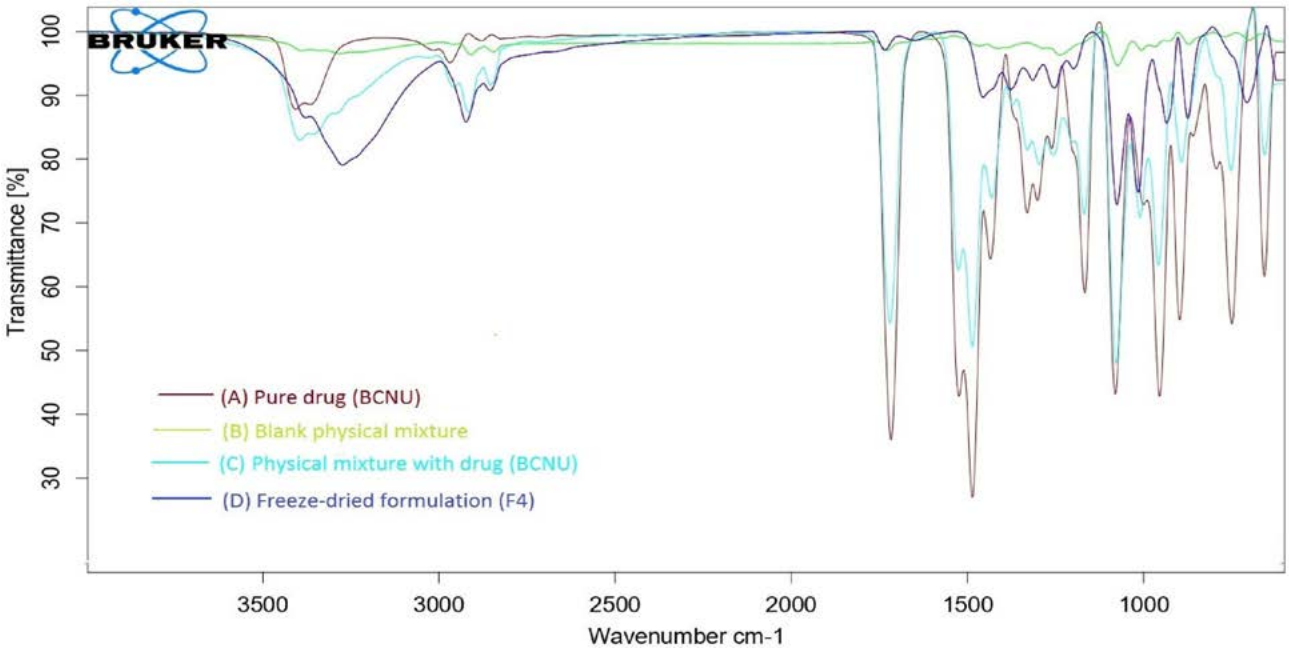

Figure 7. FTIR spectra of the pure drug (BCNU), physical mixture, and optimized formulation (F4)

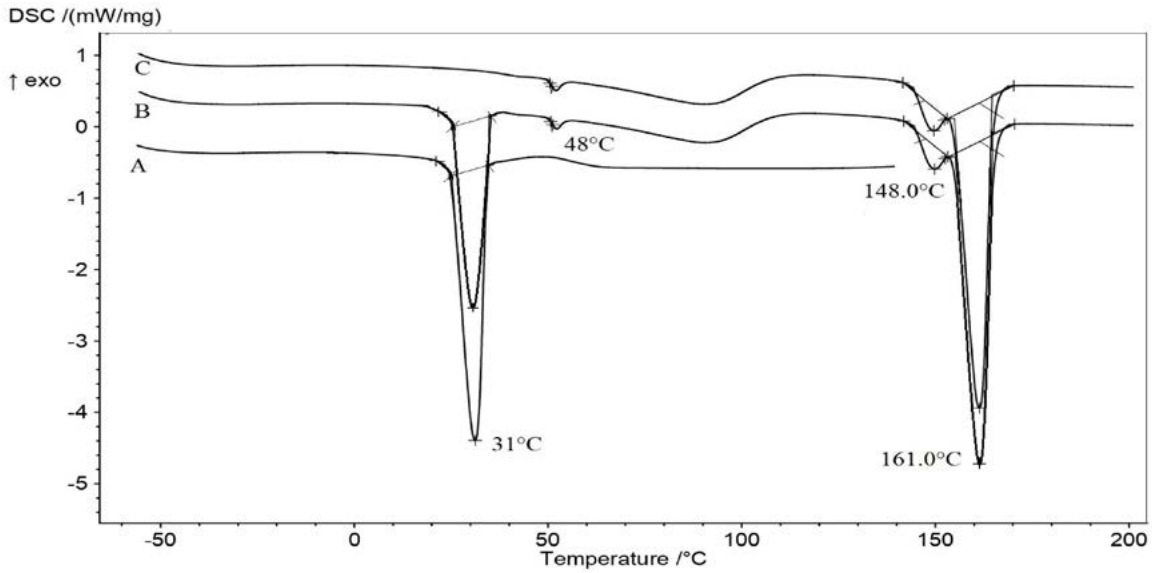

Figure 8. DSC thermogram of the pure drug (BCNU) (A), physical mixture (B), and freeze-dried formulation (F4) (C)

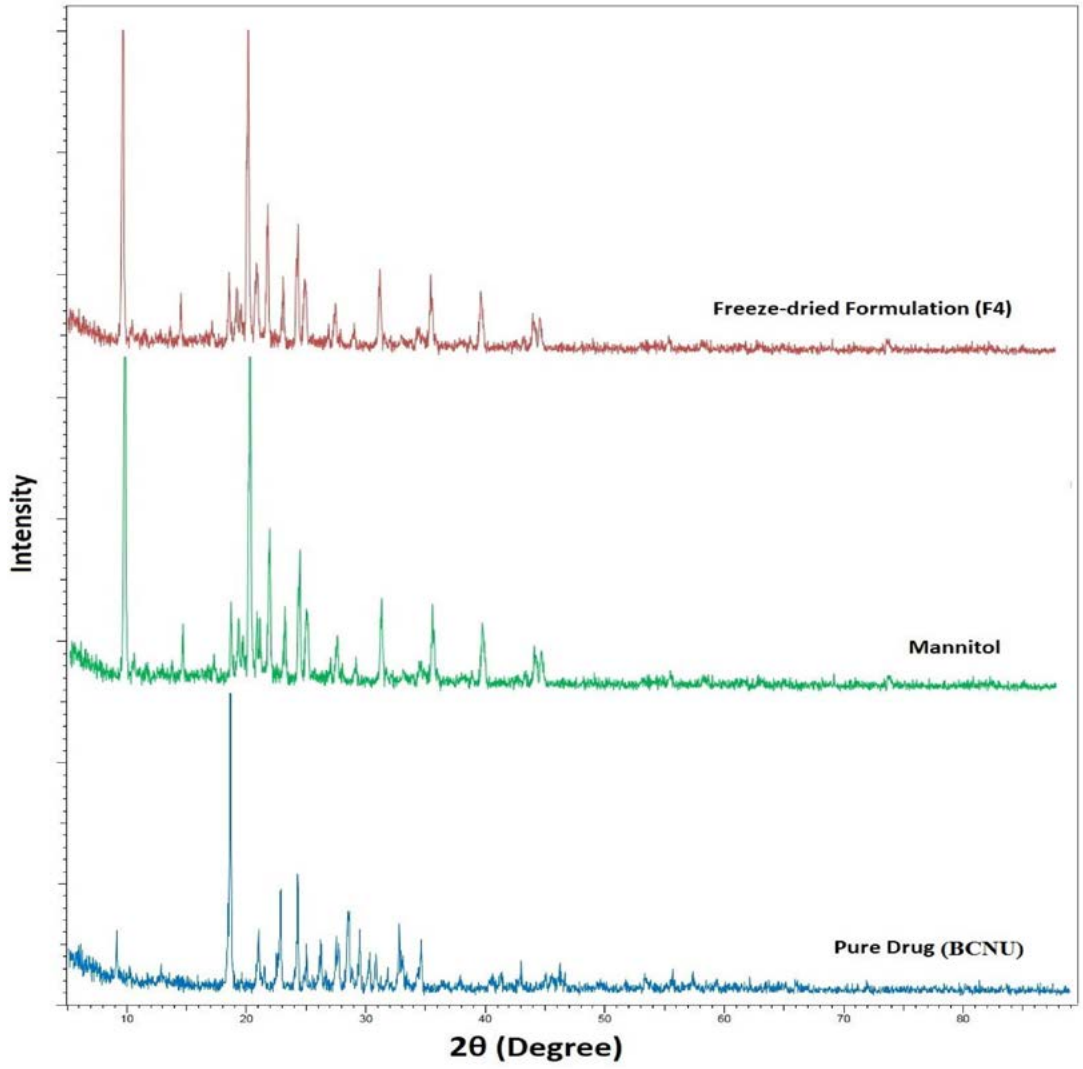

Figure 9. PXRD of the pure drug (BCNU), mannitol, and freeze-dried formulation (F4)

3. 8. PXRD

Figure 9 displays the PXRD of a lyophilized formulation (F4) and pure BCNU. Typical diffraction peaks remarkably at 2θ diffraction angles of 8.58°, 18.66°, 21.18°, 23.88°, 28.60°, 29.52°, 33.11°, and 34.90° were used to identify the crystalline nature of BCNU. The pure BCNU exhibits an intense crystalline peak between 5° and 50°. However, the peak of the pure drug (BCNU) in the lyophilized liposomal formulation (F4) was reduced; indicating a decrease in crystallinity. It was anticipated that BCNU was dispersed as a molecule in the thin lipid film layer. While the intense peak in the formulation might be due to the crystalline nature of mannitol.

3. 9. SEM

The microstructure of the product can be directly observed and the impact of the freeze-drying procedure on cake morphology can be determined by performing a microscopic examination of the freeze-dried cake. Figure 10 depicts a crystalline, porous matrix at a 200-fold magnification. Previous literature has reported similar outcomes.[33] The conclusions of the SEM analysis were further supported by the PXRD data.

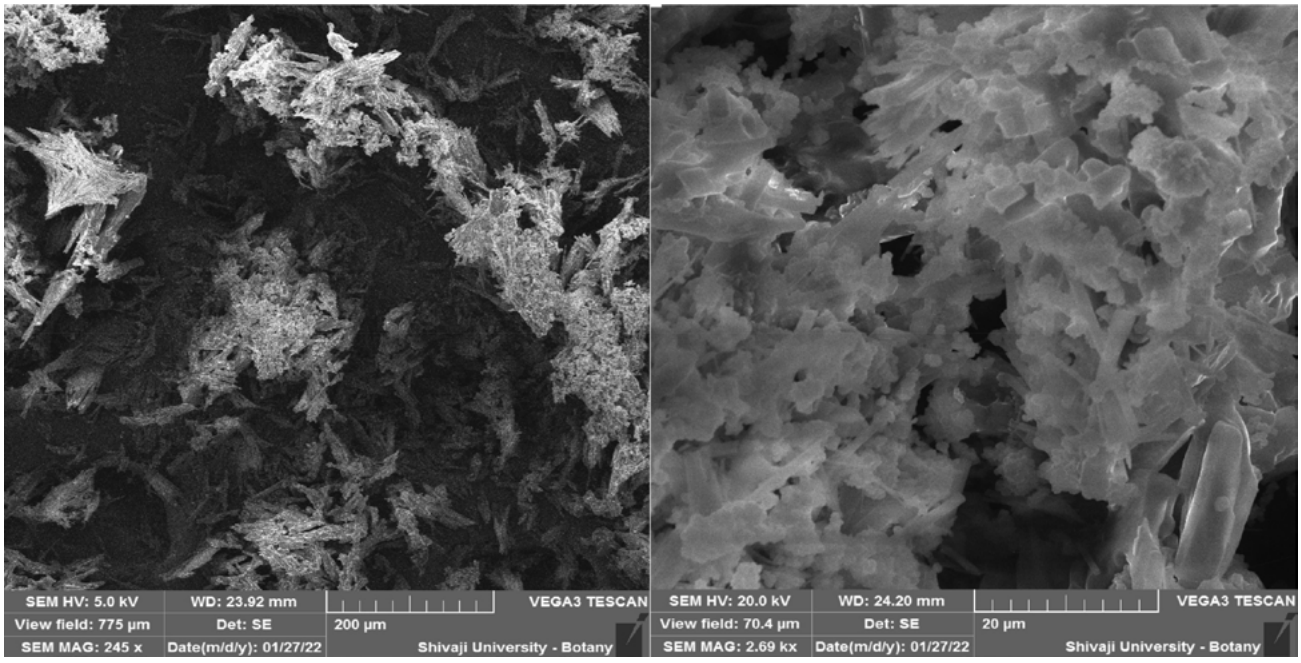

Figure 10. SEM images of freeze-dried formulation (F4)

4. Conclusions

For improving the characteristics and performance of nanoliposomal formulation of the anticancer drug (BCNU), we have assessed and examined the impact of various process parameters on formulation properties such as vesicle size, entrapment efficiency, zeta potential, and drug leakage after freeze-drying, to list a few. SPC and CH were investigated in various compositions using a 3^2-factorial design to fabricate nanoliposomes for targeted drug delivery. Surface response plots and regression equations showed a positive association between the vesicle size of BCNU-loaded liposomes and the SPC and CH at various ratios. A higher lipid content led to an increase in the size and stiffness of the liposomal bilayer. *In vitro* drug release and release kinetics investigations of BCNU-loaded liposomes revealed that the drug is released through a diffusion mechanism and the Higuchi matrix model is followed over a prolonged period. Stability studies showed that lipid compositions are stable under refrigerated storage (4 °C) conditions. FTIR and DSC analysis data demonstrated that mannitol as a cryoprotectant protects the liposomal structure at an optimum concentration during freeze-drying. In contrast, SEM microscopy revealed that the mannitol leads to the porous microstructure of the final product at an optimum concentration with some extent of crystallinity.

The crystalline nature of mannitol in the final lyophile provided mechanical strength to the final cake. In order to maintain mannitol in a crystalline state in the final product, it is necessary to ensure that mannitol does not crystallize when the system is in the glassy state (T < Tg'). As a result, it is evident from the outcomes of testing the parameters for the BCNU nanoliposomal formulation that it may minimize the dosing frequency and effectively be targeted at the site of action. Moreover, it will reduce the adverse effects brought on by the anticancer agent BCNU's high dose and non-targeted distribution. The pharmacokinetics and

pharmacodynamics properties of these formulations can be explored through *in vivo* bioavailability studies to develop an efficient drug delivery system for augmented anticancer therapy.

5. Future Prospects

The utilization of organic solvents in liposome-based pharmaceuticals has certain limitations. These solvents must be eliminated during the drug production process, which requires adhering to strict safety and regulatory standards. As a result, there is a rise in production expenses due to the need for further purification and waste management procedures. There are various techniques available to decrease the size and distribution of the initial hetero-dispersed liposome suspensions. Among these techniques, homogenization is widely employed as it is applicable for large-scale production and yields a desirable size reduction and distribution. This involves pumping the hetero-dispersed liposome preparation through a small reaction tank under high pressure in a cyclic manner until the desired average liposome size is attained. To decrease the size of liposomes, another technique is to subject them to sonication or ultrasonic irradiation, which generates shear forces during the process. Another effective size reduction method involves extruding the liposomes through membranes with uniform pore sizes to achieve uniform liposome preparation.

While the thin film hydration technique is a useful method for synthesizing liposomes, there are certain drawbacks that must be addressed. These include the need to use and completely remove organic solvents, the formation of multilamellar vesicles, and a broad distribution of particle sizes. Further investigation into the process design and preparation of liposomes through thin film hydration with homogenization techniques on an industrial scale is crucial. This is due to the current lack of continuous production at high levels and the drawbacks linked with the utilization of organic solvents. Nonetheless, before proceeding with large-scale liposome production, it is necessary to thoroughly examine the impact of each parameter of the thin film hydration-assisted process.

Author contribution statement

All authors listed have significantly contributed to the development and the writing of this article.

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Declarations

Conflict of Interest

The authors declare that they have no conflict of interest.

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Povzetek

Cilj raziskave je bil razviti in optimizirati novo liofilizirano liposomsko formulacijo protirakave učinkovine karmustin, ali bis-kloretil nitrosourea (BCNU), za podaljšano sproščanje, s čimer bi lahko odpravili od odmerka odvisne stranske učinke in izboljšali biološko uporabnost na mestu delovanja. Optimizacija je bila izvedena z uporabo 3^2-faktorskega pristopa, pri čemer sta bila sojin fosfatidilholin (SPC) in holesterol (CH) neodvisni spremenljivki. Optimizirana formulacija (F4) je pokazala visoko učinkovitost vključevanja (81,57 %) s povprečno velikostjo veziklov 141,7 nm in zeta potencialom -22,6 mV. In vitro študije sproščanja učinkovine iz vseh formulacij so pokazale, da se BCNU sprošča do 36 ur po Higuchijevem modelu matričnega sproščanja. Analize TEM, FTIR, DSC, PXRD in SEM potrjujejo nastanek liposomov. Nanoliposomska formulacija z BCNU je izkazovala podaljšano sproščanje, kar kaže, da bi jo lahko učinkovito uporabili za dopolnilno zdravljenja raka z zmanjšanjem od odmerka odvisnih stranskih učinkov.

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