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ORIGINAL ARTICLE

Development, characterization and evaluation of doxorubicin nanostructured lipid carriers for prostate cancer

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Summary

Purpose: *The purpose of this study was to develop an optimised formulation for a nanostructured lipid carrier (NLC) loaded with doxorubicin.*

Methods: *A doxorubicin-loaded NLC was prepared using an emulsification solidification method. The Box-Behnken design response surface methodology was used to optimise formulations of the doxorubicin-loaded NLC.*

Results: *The drug entrapment efficiency, drug loading efficiency, particle size, and zeta potential of the doxorubicin-loaded NLC were 74.18%, 13.28%, 170 nm, and –14.8 mV, respectively. Transmission electron microscopy of the optimised NLC showed spherical particles. Furthermore, the doxorubicin-loaded NLC was found to exhibit good therapeutic efficacy with remarkably improved oral bioavailability of doxorubicin.*

Conclusion: *The NLC system demonstrated potential for the targeted delivery of doxorubicin in prostate cancer.*

Key words: *Box-Behnken design, doxorubicin, nanostructured lipid carrier, prostate cancer, target delivery*

Introduction

While most prostate cancer cases progress slowly, some of them grow relatively quickly and become metastatic. Once the disease becomes metastatic, treatment with chemotherapy drugs (e.g., doxorubicin) must be considered. Normally, these chemotherapeutic agents act by inducing apoptosis. With respect to prostate cancer specifically, doxorubicin has been proved successful in treating the disease [1]. However, doxorubicin is linked with certain adverse events such as alopecia [2,3] and cardiac disorders the heart muscle [4,5]. Similar to other clinical chemotherapy regimens, doxorubicin requires specificity to effectively kill the most rapidly dividing cancer cells. Hence, a delivery system is required to deliver doxorubicin to its target cells enhancing its efficacy while minimising toxicity.

Drug delivery and toxicity are two main issues to consider when treating cancer with chemotherapy drugs, for which nanomedicine has recently been demonstrated to be a reasonable solution [6-9]. In fact, targeted delivery is possible with nanomedicine as it can be used to deliver drugs to a specific site. Currently, the focus of nanomedicine is to enhance chemotherapeutic efficacy and solve the issues of biocompatibility.

NLCs are a new generation of lipid nanoparticles. They are designed to exhibit the benefits of increased loading efficiency and sustained drug release [10], and possess high biocompatibility and bioavailability. NLCs are generally comprised of a mixture of both liquid and solid lipids, with drug materials entrapped in the latter. It is important that these solid lipids be both biocompat-

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ible and biologically useful. The particle size of NLCs is typically less than 1000 nm. NLCs can be delivered through oral, intravenous, pulmonary and transdermal routes [11-16].

The objectives of this study were 1) to prepare an NLC system by an emulsification solidification method [17-19] to deliver doxorubicin; 2) to optimise the formulation using the Box-Behnken design response surface methodology; 3) to characterise the morphology, particle size, zeta potential, entrapment, and drug loading efficiency of the prepared NLC system; 4) to investigate the oral bioavailability of the doxorubicin-loaded NLCs; and finally 5) to study the enhanced anticancer potential of the doxorubicin-loaded NLCs.

Methods

Doxorubicin was purchased from JinKang Fine Chemicals Co (LianYun-Gang, China). Glyceryl monostearate (GMS) was procured from Shanghai Chemical Reagent Co. Ltd. (Shanghai, China). Soya lecithin was purchased from Shanghai Taiwei Pharmaceutical Co. Ltd. (Shanghai, China). Distearoyl phosphatidylethanolamine (DSPE-PEG, PEG molecular weight: 2000) was procured from NOF Corporation (Tokyo, Japan). Polyethylene glycol 40 stearate (PEG-40-St) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Soybean and PEG 400 were purchased from Shanghai Chemical Reagent Co. Ltd. (Shanghai, China). All other chemicals and solvents were of analytical reagent grade.

Preparation of doxorubicin NLCs

The emulsion evaporation–solidification method was used to prepare the doxorubicin-loaded NLCs. The formulation was optimised by the Box-Behnken design response surface methodology, as follows: organic and aqueous phases were prepared separately and mixed together. Two organic phases were prepared, the first by dissolving doxorubicin, monostearin, and soybean oil in 10 mL of methanol, and the second by dissolving soya lecithin in 10 mL of alcohol. The two organic phases were then mixed together and left in a water bath at 75°C for 30 min. An aqueous phase was prepared by dissolving PEG-40-St and DSPE-PEG in 30 mL of double-distilled water at 75°C. Next, the organic phase was rapidly introduced into the aqueous phase using an injection needle and stirred at a speed of 1000 rpm. The resultant suspension was stirred continually at 75°C for 2 hrs with an electric stirrer. Once the organic solvents had been removed and an emulsion had formed, the suspension was quickly added to 10 mL of water and stirred continually at 1000 rpm at 0°C for 2 hrs. The doxorubicin-loaded NLCs were separated and stored at 4°C.

Experimental design

The variables of stirring speed, time, and lipid concentration were the main factors that impacted the particle size, zeta potential, and entrapment efficiency of NLCs in our preliminary experiments. To investigate the impact of stirring speed, time, and lipid concentration on particle size, zeta potential, and entrapment efficiency, the Box-Behnken design response surface methodology was used. Details of this design are shown in Table 1. We could not simply estimate the experimental range for each factor. As such, we conducted preliminary experiments with various experimental ranges. From these results, we adjusted the values of the range for each factor in our study.

Table 1. Independent variables and their levels of doxorubicin-loaded NLCs prepared for Box-Behnken design response surface methodology

Variables	*Levels*		
	-1	0	+1
Stirring speed (rpm)	500	750	1000
Time (min)	1	2	3
Lipid concentration (%)	1	1.5	2

Characterisation of doxorubicin-loaded NLCs

Transmission electron microscopy was used to study the morphology of doxorubicin-loaded NLCs. Dynamic light scattering with a Malvern Zetasizer 3000 HSA (Malvern Instruments, Malvern, UK) was used to analyse both the particle size and the zeta potential.

Chromatographic conditions

The doxorubicin levels were analysed using assays as per a high-performance liquid chromatography (HPLC) method [20]. A 50-µL aliquot of daunorubicin (1 µg/mL) was used as an internal standard. To precipitate proteins and extract doxorubicin, a 1-mL aliquot of acetonitrile was first added to each 200-µl sample of daunorubicin. This mixture was then stirred for 2 min and centrifuged at 13,000 rpm for 10 min. The upper layer was separated and an aliquot of 0.8 mL was transferred to a microtube and evaporated under nitrogen gas at 38°C. The resultant residue was reconstituted in a 200-µL mobile phase prior to injection into a C18 reverse-phase column. The mobile phase consisted of 20 mM phosphate buffer (pH 3.8), acetonitrile, and methanol at a ratio of 45:20:35 v/v/v, respectively. The flow rate of the mobile phase was 1.0 mL/min and a fluorescence detector was used to monitor the eluent at an excitation wavelength of 460 nm with an emission cut-off filter of 580 nm. Doxorubicin and daunorubicin had approximate retention times of 3.5 and 5.8 min, respectively. The detection limit of doxorubicin in the plasma of rats was 2 ng/mL.

Determination of drug entrapment efficiency and drug loading percentage

Both drug loading and entrapment efficiency were determined by measuring the amount of drug encapsulated within the nanoparticles. The nanoparticle dispersion was filtered through a 3-µm nitrocellulose membrane filter to remove any drug that had not been entrapped. To extract the drug from the lipid, 9.5 mL of methanol was added to 0.5 mL of filtrate and mixed well with a cyclomixer. The mixture was centrifuged for 15 min at 5,000 rpm, followed by collection of the resultant supernatant. The supernatant was then assayed by an HPLC method for determining the drug concentration. Briefly, the nanoparticle dispersion was centrifuged for 30 min at 5,000 rpm and the aqueous phase was assayed by an HPLC method to determine the amount of drug.

Drug encapsulation and loading efficiency were calculated using equations 1 and 2, respectively:

$$EE\ (\%) = \frac{W_{total} - W_{free}}{W_{total}} \times 100 \ldots\ldots\ldots Equation\ 1$$

$$DL\ (\%) = \frac{W_{total} - W_{free}}{W_{lipid}} \times 100 \ldots\ldots\ldots Equation\ 2$$

where W_{total} is the total amount of drug, W_{free} is the amount of drug not entrapped, and W_{lipid} is the weight of the lipid.

Pharmacokinetic study in rats

Male Sprague–Dawley rats were used to perform an in vivo pharmacokinetic study. The animals were fasted overnight and divided into two groups of 6 animals each. One group was administered an oral dose of 50 mg/kg doxorubicin suspension, while the other was given an oral dose of 50 mg/kg doxorubicin-loaded NLCs. Blood samples of 0.45 mL were collected in heparinised tubes from the femoral artery at 0, 0.1, 0.25, 0.5, 1, 2, 3, 5, 6, 8, 12 and 24 hrs post-treatment. The blood samples were centrifuged at 13,000 rpm for 5 min. Approximately 200 µL aliquots of plasma samples were stored at –40°C until the HPLC analysis. The plasma drug concentration versus time profile curve shows both C_{max} and T_{max}. The trapezoidal method was used to calculate the area under the curve.

Cell culture

The PC3 cell line of human bone metastatic prostate cancer was procured from American Type Culture collection (ATCC, Manassas, VA, USA). Both 10% foetal FBS and 1% penicillin/streptomycin/amphotericin were added to the medium as supplements. The cell line was cultured in T75 flasks. The PC3 cells were allowed to proliferate in 48-well plates for 24 hrs prior to the experiment.

Cytotoxicity assay of doxorubicin-loaded NLCs in PC3 cells

The doxorubicin NLC system was evaluated for efficacy by treating PC3 cells. Cells were exposed to doxorubicin suspension and doxorubicin-loaded NLCs for 32 hrs in serum-free medium. The cell viability was quantified with a 3-(4,5-dimethylthiazol-2-yl)-5-(3-car-

Table 2. Experimental responses and Box-Behnken design arrangements

Design points	*Factor level*			*Responses*		
	Stirring speed (rpm)	*Time (min)*	*Lipid concentration (%)*	*Particle size (nm)*	*Zeta potential (-mV)*	*Entrapment efficiency (%)*
1	-1	-1	0	236	-0.17	67.17
2	+1	-1	0	182	-14.80	74.18
3	-1	+1	0	208	-11.34	76.12
4	+1	+1	0	180	-15.8	78.27
5	-1	0	-1	226	0.14	76.51
6	+1	0	-1	229	-17.21	79.67
7	-1	0	+1	231	0.583	73.37
8	+1	0	+1	186	-13.23	84.52
9	0	-1	-1	230	-12.21	45.19
10	0	+1	-1	180	-1.33	72.48
11	0	-1	+1	195	0.52	62.51
12	0	+1	+1	191	0.42	68.50
13	0	0	0	174	0.18	47.72
14	0	0	0	175	0.17	47.23
15	0	0	0	175	0.18	46.64
16	0	0	0	175	0.19	46.32
17	0	0	0	170	0.22	43.79

boxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium assay (MTT). Briefly, MTT assay reagent was added to the cells and the plates were incubated for 2 hrs. The absorbance at 490 nm was read with a microplate reader. Cell viability was presented as percentages in reference to the control.

Statistics

Statistical analysis was performed using GraphPad Prism software. Data are expressed as averages with standard deviation. Statistical significance was determined by Student's paired t-test. A value of $p<0.05$ was considered as statistically significant. Response models and statistical parameters for Box-Behnken design were analysed using Design Expert version 9 software and statistical significance was determined by one way ANOVA. A p value<0.05 was considered as statistically significant.

Results

The experiments performed and the responses obtained are detailed in Table 2. The quadratic mathematical model for three independent factors is provided in Eq. (3):

$Y=\beta 0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{23}X_2X_3$.... Equation 3

where Y is the response to be modelled, β is the regression coefficient, and X1, X_2, and X_3 represent factors A, B, and C, respectively. Table 3 represents the statistical parameters, such as adjusted R^2, model p value, adequate precision, and % CV obtained from ANOVA for the reduced models. Figure 1 illustrates the response surface plots for particle size, zeta potential, and entrapment efficiency. The analysis of perturbation plots and response plots of the optimisation models revealed that stirring speed and time influenced all three responses of particle size, zeta potential, and loading efficiency. The investigated procedure was well explained through the perturbation plots presented in Figure 2. Figure 2 shows that, in order of influence, particle size was highly influenced by stirring time followed by stirring speed, zeta potential was significantly influenced by stirring speed, and entrapment efficiency was influenced by stirring speed, followed by stirring time, and then lipid concentration. Table 4 demonstrates that the experimental values were very close to the predicted values for NLCs prepared under optimal assay conditions for the doxorubicin NLC formulation. The low percentage bias obtained indicates that the formulations being optimised were reliable and reasonable. The desirability D value was found to be 0.8302 and is presented in Figure 3.

Particle size and zeta potential measurement

The mean particle size and zeta potential of the optimised doxorubicin NLC formulation were 170 nm and −14.8 mV, respectively. This zeta potential is sufficient to produce stable NLCs [23]. The particle size distribution is presented in Figure 4. The percentages of drug loading and entrapment efficiency were found to be 13.28±0.16% and 74.18±0.32%, respectively.

Table 3. Response models and statistical parameters obtained from ANOVA for Box-Behnken design

Responses	*Regression model*	*Adjusted R^2*	*Model p values*	*Adequate precision*	*% CV*
Particle size	+175.95-15.50×A-10.50×B-7.75×C-12.00×A×C+11.50×B×C+23.37×A^2+20.87×C^2	0.8961	<0.0001	3.95	12.48
Zeta potential	+0.64-8.45×A-0.21×B-0.33×C-0.29×AB+0.035×AC+0.080×BC-8.43×A^2+0.64×B^2-0.59×C^2	0.9932	<0.0003	3.24	18.16
Entrapment efficiency	+46.04+2.88×A+5.75×B-5.25×B×C+21.98×A^2+5.73×B^2+9.98×C^2	0.9379	<0.0001	3.56	15.66
Acceptance criteria	≥0.80	<0.05	<4%	>10%	

Table 4. Experimental and predicted values under optimal assay conditions for doxorubicin-loaded NLC formulation

Stirring speed (rpm)	*Time (hr)*	*Lipid concentration (%)*	*Particle size (nm)*	*Zeta potential (-mV)*	*Entrapment efficiency (%)*
1000	*2*	1			
Desirability D value	0.810				
Predicted			168	-14.8	73.17
Experimental			170	-13.3	74.32
Bias (%)			1.17	-1.3	1.34

Acceptance criteria = 2 %

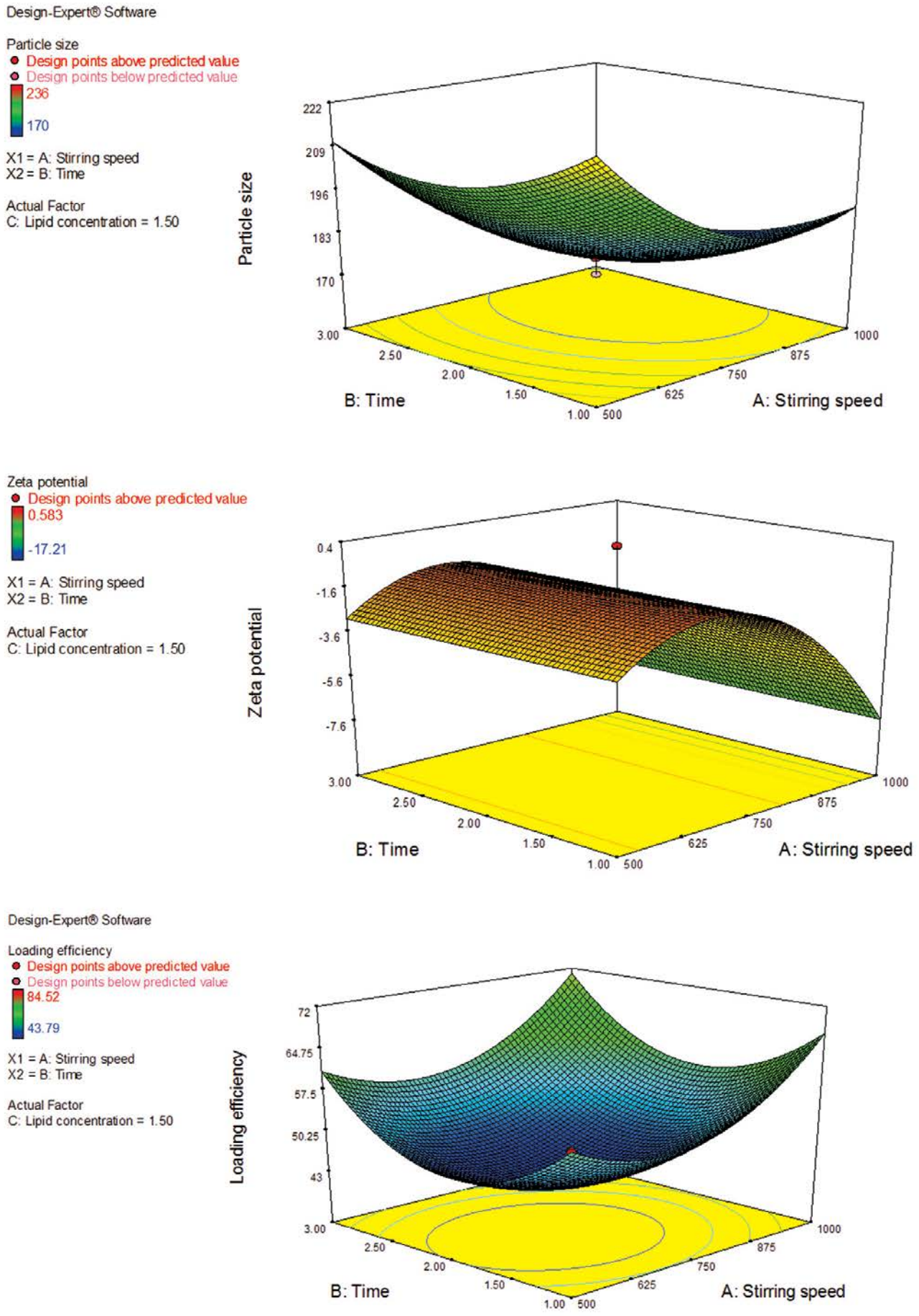

Figure 1. Three-dimensional (3D) response surface plots showing the impact of the variables on the response.

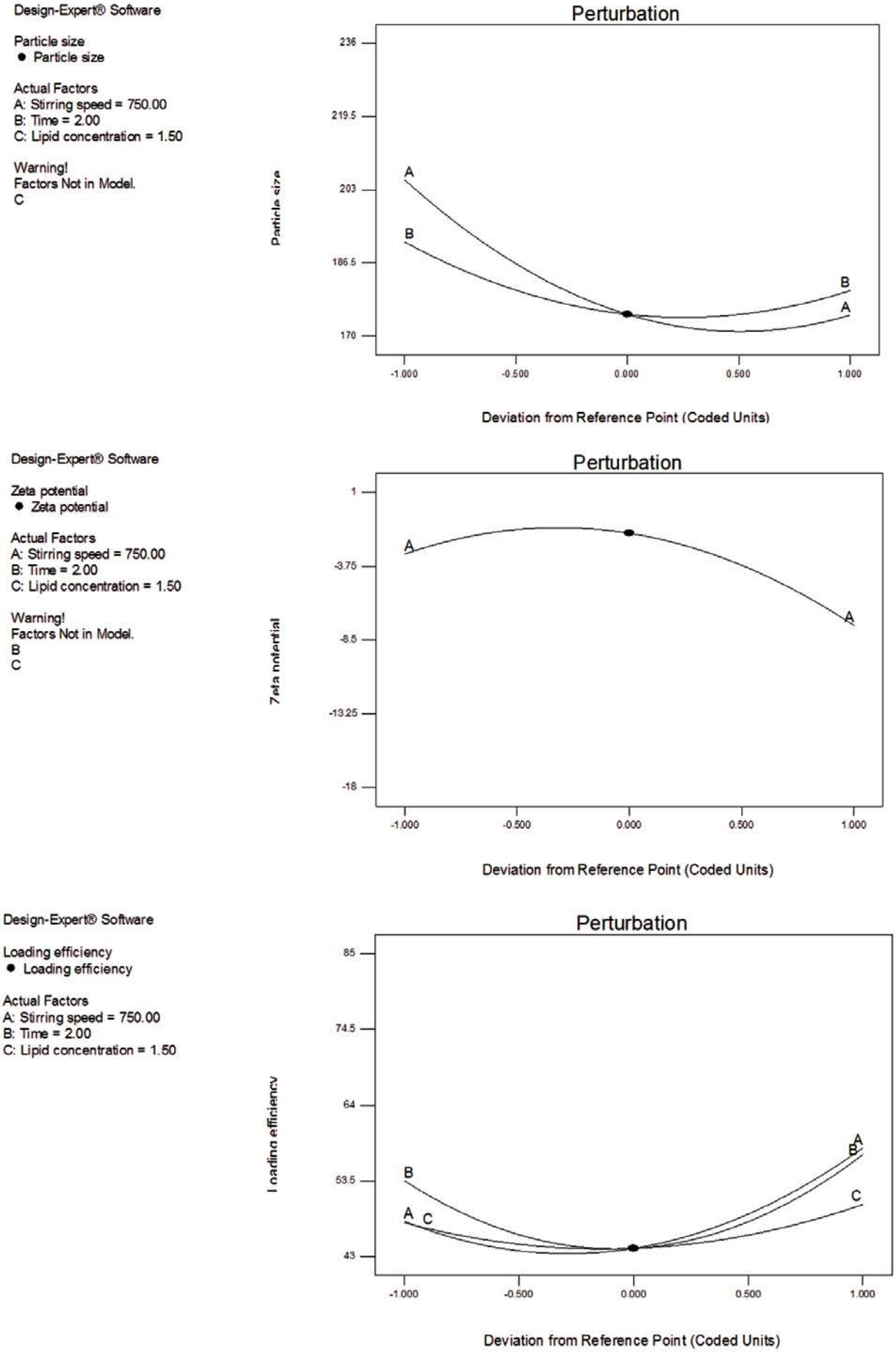

Figure 2. Perturbation plots showing the impact of each of the independent variables on particle size, zeta potential, and entrapment efficiency.

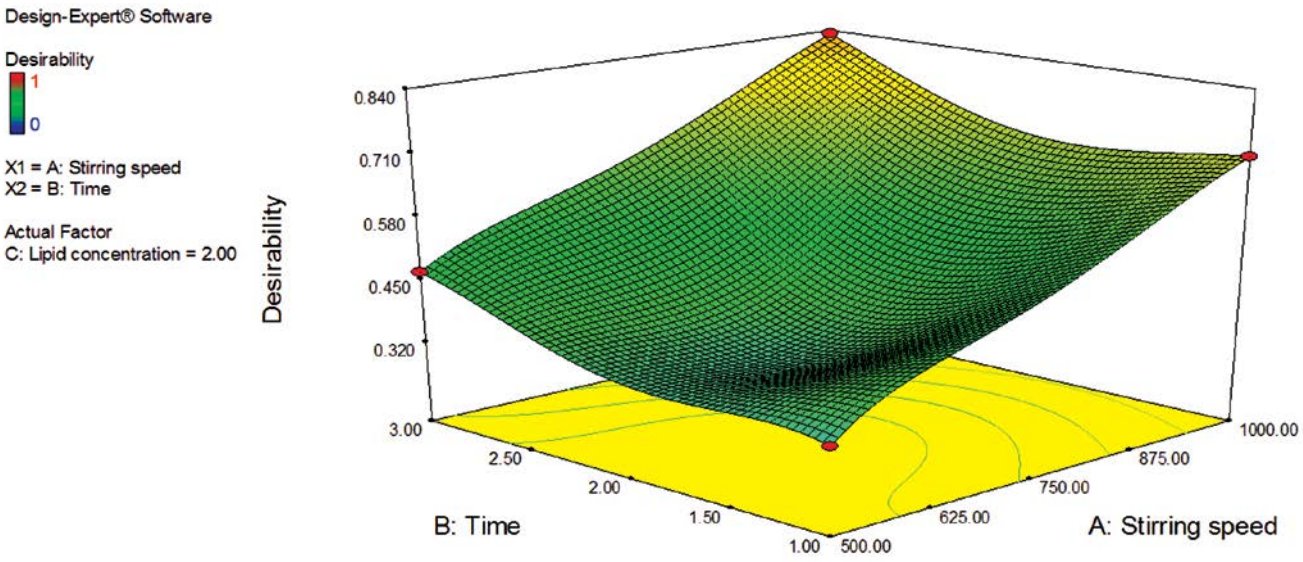

Figure 3. Three-dimensional response surface plot representing overall desirability function.

Table 5. Mean pharmacokinetic parameters of doxorubicin after oral administration at a dose of 50 mg/kg to rats (n=6, each)

Parameters	*Doxorubicin suspension*	*Doxorubicin NLC*
AUC (ng.h/mL)	204±42.0	463±88.2**
Cmax (ng/mL)	20±3.32	52±7.76*
Tmax (h)	0.32±0.12	0.28±0.12
$T_{1/2}$	13.7±2.21	14.5±2.98
Relative bioavailability	226.96	

*p<0.05, **p<0.01 (significant differences were calculated by comparing the doxorubicin NLC group with the doxorubicin suspension group)

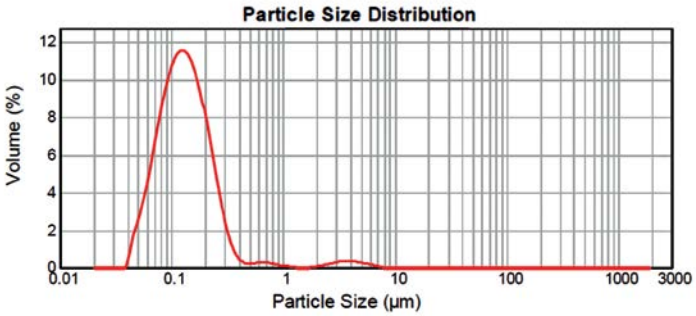

Figure 4. Particle size distribution of optimised doxorubicin NLC formulation.

Transmission electron microscopy

A TEM image of the optimised doxorubicin NLC formulation is shown in Figure 5. The formulation technique used in this study produced NLCs that were smooth and round in structure [24]. The NLC formulation was uniform in size, indicating that the formulation method was efficient.

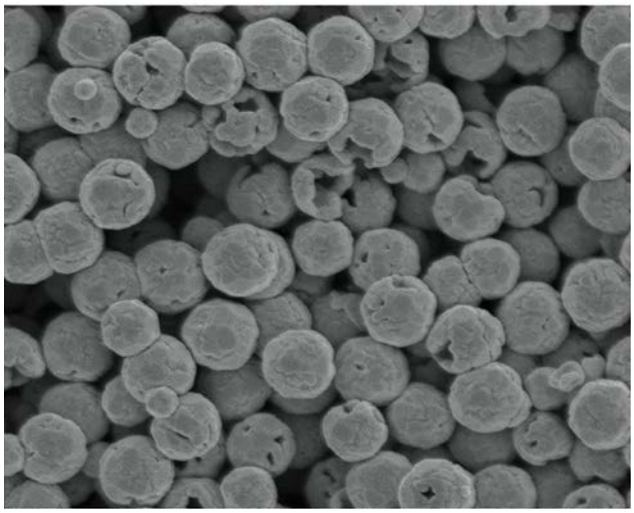

Figure 5. TEM image of optimised doxorubicin NLC formulation taken at 3.0 kV and 50,000 magnification.

Oral bioavailability

The mean plasma drug concentration versus time profile curve of orally administered doxorubicin is illustrated in Figure 6. The mean pharmacokinetic parameters of doxorubicin are listed in Table 5.

In vitro cytotoxicity

Doxorubicin-loaded NLCs and suspension were studied for their cytotoxicity *in vitro* with the aid of cell viability experiments. The results of these experiments are summarised in Table 6. Figure 7 shows the cytotoxicity of PC3 cells incubated with the doxorubicin-loaded NLCs and suspension, *in vitro*.

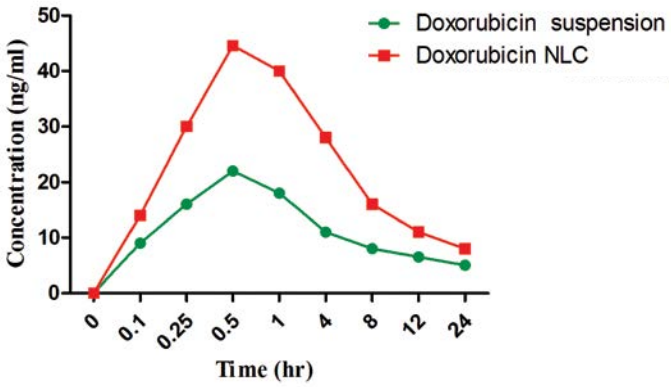

Figure 6. Mean plasma drug concentration versus time profile curve of doxorubicin after oral administration of doxorubicin as a suspension and NLC at a dose of 50 mg/kg. Doxorubicin NLC group showed a significant difference compared to doxorubicin suspension group ($p<0.01$). Values are means ± SEM (n=6).

Table 6. IC_{50} value of doxorubicin suspension and NLC formulation for PC3 cells

Group	*IC_{50} (nm) (mean ±SD)*
Doxorubicin suspension	209.87 ±9.68
Doxorubicin NLC	40.61±6.15**

**p<0.01 compared with doxorubicin suspension

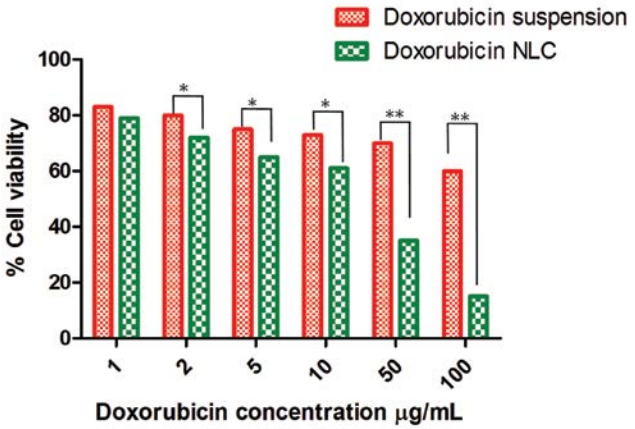

Figure 7. *In vitro* cytotoxicity determination using MTT assay on PC3 cells (*p < 0.05, **p < 0.01, compared with the doxorubicin suspension).

Discussion

Optimisation of doxorubicin-loaded NLCs by Box-Behnken design response surface methodology

Design-Expert® software (version 8.0.1) was used to design the experiment and perform both the data analysis and the desirability function calculations. The Box-Behnken design response surface methodology was used to investigate the curvature term before starting an optimisation procedure. The curvature was significant for all responses (X_1, X_2, and X_3) (p value < 0.05 by ANOVA), showing that a quadratic model should be considered. Stirring speed (X_1), time (X_2), and lipid concentration (X_3) were the factors selected for the optimisation process, while particle size, zeta potential, and entrapment efficiency were selected as the responses. These selections were based on the preliminary experiments. The experiments were randomly conducted to minimise the effects of uncontrolled variables that could bias the measurements.

To obtain a simple and realistic model, the insignificant terms ($p>0.05$) were removed using a backward elimination process. Notably, the R^2 value in a regression model always decreases when regression variables are removed. Hence, an adjusted R^2 value that accounts for many regression variables is selected in statistical modelling [21,22].

The experimental data demonstrated a good fit with the equations of second-order polynomials. The adjusted R^2 value was also within the acceptable limit, further confirming the fitness of the data. Notably, all of the reduced models were shown to be significant ($p<0.05$). Adequate precision is a measure of the signal (response) to noise (deviation) ratio and has reached a desirable level when the ratio is less than 4. In our analysis, the ratio was indeed calculated to be less than 4, which was a sign of the adequacy of this approach and indicates the significance of the model [21]. The coefficient of variation (CV) is a measure of the reproducibility of this model. CV values greater than 10% indicate that models can be reasonably reproduced. In our experiments, the CV values calculated for the models were greater than 10%.

Perturbation plots are used to explain the impact of an independent factor on a specific response, while all remaining factors are held constant at a reference point. The sensitivity of the response to a specific factor is indicated by a steep slope or curvature.

Oral bioavailability

The plasma concentration of doxorubicin significantly increased when administered as part of the NLC formulation, compared with that for doxorubicin alone. Specifically, there were significant increases in both the area under the plasma concentration time curve from zero to infinity (AUC) ($p<0.01$) and the peak concentration (C_{max}) ($p<0.05$). The relative bioavailability (RB) of doxorubicin in the NLC formulation was 2–3 times higher than for doxorubicin alone. There were no

significant differences observed in T_{max} and $t_{1/2}$.

There are a few factors that affect the bioavailability of NLCs. First, our results clearly demonstrated that the absorption of doxorubicin could be increased by orally administering doxorubicin as part of NLCs. In addition, it has been shown that NLCs are readily absorbed through the gastrointestinal tract, which is facilitated by their small particle size [25]. Surfactant also helped to increase the permeability of NLCs, while minimising both degradation and clearance. Indeed, a small particle size and the mechanism of gastrointestinal uptake both increase the bioavailability of NLCs in cells and tissues [26].

In vitro cytotoxicity

Our results clearly demonstrated that the inhibitory effect was significantly higher when doxorubicin was administered as part of NLCs compared with suspension alone. The IC_{50} values of cells incubated for 24 h in doxorubicin suspension and NLC formulation were 40.61 ± 6.15 and 209.87 ± 9.68 nM, respectively. When doxorubicin was administered as a suspension and as part of the NLC formulation, its IC_{50} value decreased to 210 and 40 nM, respectively. This potentially explains the cytotoxic effect of NLCs. Specifically, as NLCs are absorbed by cells, doxorubicin is released closer to its target sites and could ultimately lead to a higher drug concentration in these areas [27, 28].

Conclusion

An emulsification and solidification method was effectively applied to prepare doxorubicin-loaded NLCs. Nano drug delivery systems could be revolutionary for cancer therapeutics and drug delivery, particularly for bone-metastatic prostate malignancies. Transmission electron microscopy confirmed that doxorubicin-loaded NLCs had an appropriate hydrodynamic size and zeta potential. The PC3 cell line was used to confirm the adequacy of doxorubicin delivered by an NLC system. Pharmacokinetic studies showed delayed t_{max} and increased C_{max} values, with enhanced bioavailability of doxorubicin when administered as part of NLCs rather than a suspension. MTT assay demonstrated significantly higher cytotoxicity of doxorubicin-loaded NLCs in prostate cancer cells. As such, there are some barriers to be overcome before NLCs can be used as drug delivery systems for cancer therapeutics. Future studies should focus on how doxorubicin-loaded NLCs could enhance therapeutic efficacy by specifically targeting malignancies, while working to diminish the associated toxicities.

Conflict of interests

The authors declare no confict of interests.

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