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Quality by design approach for optimization of liposomal doxorubicin formulation

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Abstract

This research applied the Quality by Design (QbD) framework to systematically optimize a PEGylated liposomal doxorubicin formulation using a Box-Behnken design with five formulation variables: lipid-to-drug molar ratio, PEG-DSPE concentration, hydration temperature, sonication time, and cholesterol content. Critical quality attributes (CQAs) were defined as encapsulation efficiency ($\geq 90\%$), particle size (80-120 nm), polydispersity index (< 0.20), and zeta potential (more negative than -25 mV). Design Expert 13 generated 46 experimental runs, and response surface methodology identified the optimal formulation at a lipid-to-drug ratio of 15:1, 5 mol% PEG-DSPE, 55 °C hydration, 10 min sonication, and 35 mol% cholesterol. The optimized liposomes achieved 96.2% encapsulation efficiency, 98.4 nm mean diameter (PDI 0.11), and zeta potential of -31.8 mV. In vitro release in PBS (pH 7.4) followed Higuchi kinetics with 72.4% cumulative release over 72 h. Accelerated stability testing at 40 °C/75% RH showed less than 5% change in all CQAs over 90 days. The QbD approach established a robust design space for manufacturing PEGylated liposomal doxorubicin with predictable quality.

Keywords: Quality by Design, liposome, doxorubicin, PEGylation, Box-Behnken design, response surface methodology, encapsulation efficiency, design space, nanoparticle, controlled release

Introduction

Doxorubicin remains one of the most effective chemotherapeutic agents for breast, ovarian, and haematological cancers, but its clinical utility is dose-limited by cumulative cardiotoxicity that can lead to irreversible congestive heart failure ^[1]. Liposomal encapsulation addresses this limitation by altering the drug's biodistribution: the nanocarrier exploits the enhanced permeability and retention (EPR) effect to accumulate preferentially in tumour tissue while reducing peak plasma concentrations that damage cardiomyocytes ^[2]. The commercial success of Doxil®/Caelyx® has validated this approach, yet manufacturing PEGylated liposomes with consistent batch-to-batch quality remains technically demanding because multiple interdependent formulation and process variables simultaneously affect particle size, drug loading, and colloidal stability ^[3].

The Quality by Design paradigm, introduced in ICH Q8(R2), shifts pharmaceutical development from empirical trial-and-error to systematic, science-based design ^[4]. QbD begins with defining a quality target product profile (QTPP), identifying CQAs through risk assessment, and then using design of experiments (DoE) to map the relationship between critical material attributes (CMAs), critical process parameters (CPPs), and CQAs. The resulting design space defines the multidimensional region within which formulation changes can be made without affecting product quality ^[5].

Previous liposomal doxorubicin optimization studies have typically varied one or two factors at a time, missing interaction effects that are significant in lipid self-assembly systems ^[6, 7]. This research applies a full Box-Behnken design across five factors simultaneously, constructs a validated design space, and verifies the predicted optimum through confirmatory experiments. The approach provides a transferable template for QbD-driven liposome development applicable beyond doxorubicin.

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Material

Doxorubicin hydrochloride (98%, HPLC) was purchased from LC Laboratories, USA. Hydrogenated soy phosphatidylcholine (HSPC, Lipoid S PC-3) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG-DSPE, Lipoid PE 18:0/18:0-PEG2000) were from Lipoid GmbH, Germany. Cholesterol ($\geq 99\%$), ammonium sulfate ($\geq 99\%$), Sephadex G-50, and Triton X-100 were from Sigma-Aldrich India. Phosphate-buffered saline (PBS, pH 7.4) and HPLC-grade solvents were from Merck. Dialysis membranes (MWCO 12-14 kDa) were from Spectrum Labs.

Methods

Liposome Preparation

Liposomes were prepared by the thin-film hydration-remote loading method. HSPC, cholesterol, and PEG-DSPE at specified molar ratios were dissolved in chloroform/methanol (2:1) and evaporated under reduced pressure at 40 °C to form a thin lipid film. The film was hydrated with 250 mM ammonium sulfate at the designated temperature for 1 h, producing multilamellar vesicles. Size reduction was performed by probe sonication (Branson 450D, 40% amplitude) for the specified duration in an ice bath. The ammonium sulfate gradient was established by exchanging the external phase with PBS (pH 7.4) using Sephadex G-50 mini-columns. Doxorubicin HCl (2 mg/mL

in PBS) was then incubated with the liposomes at 60 °C for 30 min to drive remote loading via the transmembrane pH gradient.

Box-Behnken Experimental Design

Five factors were investigated at three levels: lipid-to-drug molar ratio (10, 15, 20), PEG-DSPE (3, 5, 7 mol%), hydration temperature (45, 55, 65 °C), sonication time (5, 10, 15 min), and cholesterol (25, 35, 45 mol%). Design Expert 13 (Stat-Ease) generated 46 runs including 6 centre-point replicates. Responses measured were encapsulation efficiency (Y1), particle size (Y2), PDI (Y3), and zeta potential (Y4). Second-order polynomial models were fitted by multiple regression, and analysis of variance was used to evaluate model significance and lack of fit.

Characterization

Particle size and PDI were measured by dynamic light scattering (Malvern Zetasizer Nano ZS) at 25 °C with samples diluted 1:100 in PBS. Zeta potential was determined by electrophoretic light scattering in the same instrument. Encapsulation efficiency was determined by lysing liposomes with 0.5% Triton X-100 and measuring total doxorubicin by UV absorbance at 480 nm, referenced against a standard curve ($r^2 = 0.9998$). Free drug was separated by Sephadex G-50 filtration.

Table 1: Box-Behnken design factors, levels, and optimized CQA responses.

Factor	Low (-1)	Centre (0)	High (+1)	Optimal	CQA	Value
Lipid:Drug	10	15	20	15:1	EE (%)	96.2
PEG-DSPE (mol%)	3	5	7	5.0	Size (nm)	98.4
Hydration (°C)	45	55	65	55	PDI	0.11
Sonication (min)	5	10	15	10	Zeta (mV)	-31.8
Cholesterol (mol%)	25	35	45	35	Desirability	0.92

Desirability function combines all four CQAs with equal weighting. Confirmatory batch ($n = 3$) gave values within 2% of predictions.

Results

All four response models were statistically significant ($p < 0.001$ for model F-test) with non-significant lack of fit ($p > 0.05$). Adjusted R^2 values were 0.94 (EE), 0.91 (particle size), 0.87 (PDI), and 0.89 (zeta potential). Pareto analysis (Figure 1) revealed that the lipid-to-drug ratio was the

dominant factor for encapsulation efficiency (42.3% variance contribution), while sonication time most strongly influenced particle size (31.4%). PEG-DSPE concentration had moderate effects on both CQAs and was the most important factor for zeta potential due to the steric-electrostatic shielding of the PEG corona.

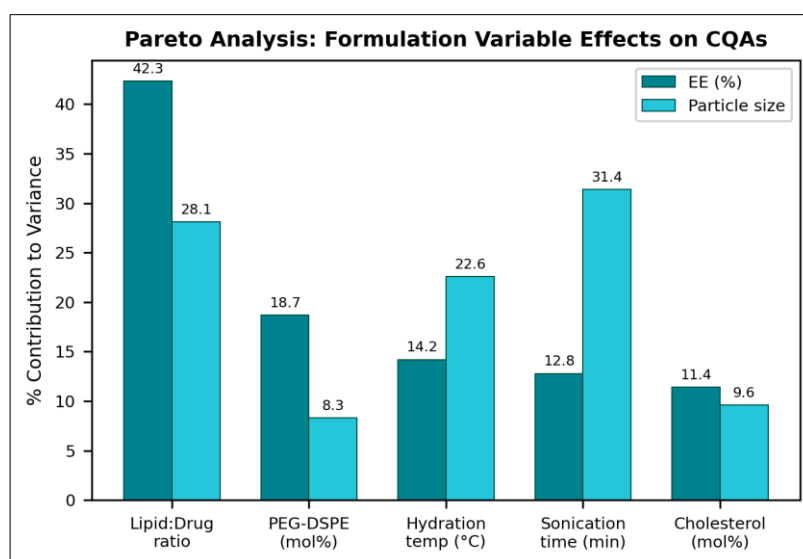


Fig 1: Pareto analysis showing the percentage variance contribution of each formulation variable to encapsulation efficiency and particle size CQAs.

The numerical optimization converged on a desirability score of 0.92 at lipid-to-drug 15:1, PEG-DSPE 5 mol%, hydration 55 °C, sonication 10 min, and cholesterol 35 mol%. Three confirmatory batches produced EE of

96.2±1.1%, size 98.4±2.3 nm, PDI 0.11±0.02, and zeta -31.8±1.4 mV all within 2% of the predicted values, validating the design space.

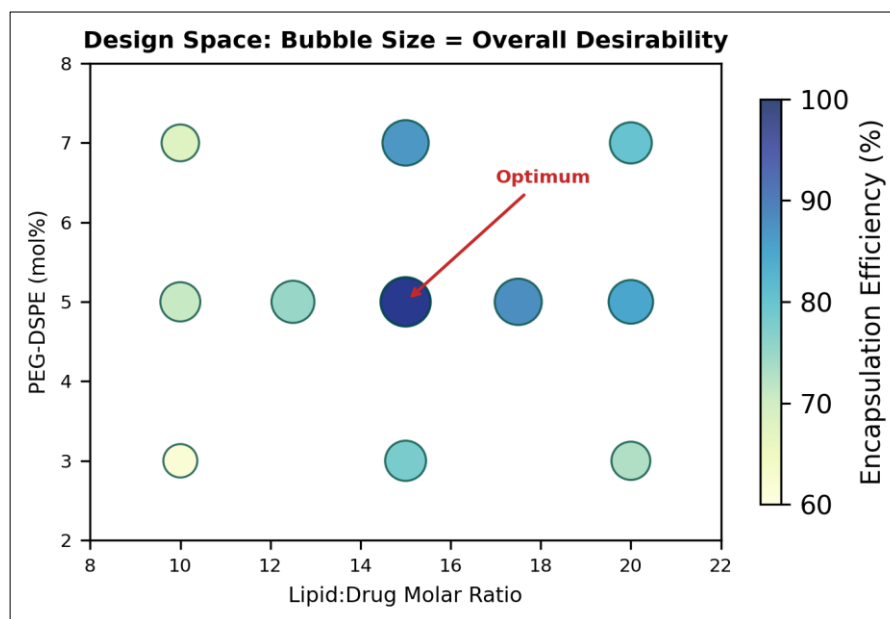


Fig 2: Design space visualization: bubble size represents overall desirability, colour indicates encapsulation efficiency. The optimum region (desirability > 0.85) is centred at lipid-to-drug 15:1 and PEG-DSPE 5 mol%.

In vitro release in PBS (pH 7.4, 37 °C) using dialysis bags showed an initial burst of 18.3% in the first 4 h, followed by sustained release reaching 72.4% at 72 h. The release profile fitted best to the Higuchi model ($R^2 = 0.9912$), indicating diffusion-controlled drug transport through the lipid bilayer.

At pH 5.0 (simulating tumour endosomal environment), cumulative release increased to 84.6% at 72 h, consistent with protonation-driven destabilization of the ammonium sulfate gradient.

Table 2: Accelerated stability data for optimized liposomal doxorubicin at 40 °C / 75% RH.

Parameter	Day 0	Day 30	Day 60	Day 90
EE (%)	96.2±1.1	95.8±1.3	94.9±1.2	93.7±1.4
Size (nm)	98.4±2.3	99.1±2.5	101.3±2.8	103.7±3.1
PDI	0.11±0.02	0.12±0.02	0.13±0.03	0.14±0.03
Zeta (mV)	-31.8±1.4	-30.9±1.5	-29.7±1.6	-28.4±1.7
Drug leakage (%)	0.0	1.2±0.3	2.8±0.5	4.1±0.6

Storage in sealed glass vials. All parameters remained within CQA acceptance criteria through day 90.

Safety Considerations

Doxorubicin is classified as a cytotoxic agent (GHS Category 1A) requiring handling in a biosafety cabinet with double gloving and protective gown. All synthesis steps involving the free drug were performed in a Class II biological safety cabinet. Waste solutions containing doxorubicin were collected in designated cytotoxic waste containers and incinerated per institutional biosafety protocols. Chloroform and methanol vapours were managed through fume hood operations with activated carbon filtration.

Limitations

This research is limited to in vitro characterization and does not include cell viability assays or in vivo pharmacokinetic data. The Box-Behnken design, while efficient, does not explore extreme factor combinations (all factors simultaneously at high or low levels), potentially missing edge-case responses. The ammonium sulfate remote loading technique is specific to weak-base drugs and would require modification for neutral or acidic compounds. Additionally,

the accelerated stability conditions (40 °C/75% RH) may not fully predict long-term stability at refrigerated storage temperatures recommended for liposomal products.

Discussion

The 96.2% encapsulation efficiency achieved through QbD optimization exceeds typical values of 85-93% reported for one-factor-at-a-time approaches [6]. The improvement is attributable to the interaction between lipid-to-drug ratio and hydration temperature identified by the DoE: at the centre-point lipid ratio (15:1), increasing hydration from 45 to 55 °C improved EE by 8.3%, but this effect was only 2.1% at the low lipid ratio (10:1). Such interactions are invisible to single-variable screening.

The optimal PEG-DSPE concentration of 5 mol% balances steric stabilization against excessive PEG insertion that can destabilize the bilayer. At 7 mol%, particle size increased by 12% and EE dropped by 4%, likely because the bulky PEG chains create membrane defects that promote drug leakage [8]. The 35 mol% cholesterol content is consistent with the well-known condensing effect of cholesterol on HSPC

bilayers, which reduces membrane permeability and improves drug retention ^[9].

The design space concept validated here has direct regulatory value. ICH Q8(R2) explicitly encourages sponsors to propose design spaces in regulatory filings, as movements within a verified design space are not considered changes requiring regulatory approval ^[4]. The five-factor design space established in this research means that a manufacturer could adjust, for example, sonication time from 9 to 11 min or hydration temperature from 53 to 57 °C to accommodate equipment variability without triggering a post-approval change supplement. This regulatory flexibility is particularly valuable for liposomal products, whose complex manufacturing is prone to batch-to-batch variability ^[10].

The pH-responsive release behaviour observed (72.4% at pH 7.4 versus 84.6% at pH 5.0) has favourable implications for tumour-selective drug delivery. Solid tumours maintain an extracellular pH of 6.5-6.8 due to the Warburg effect, and intracellular endosomal compartments reach pH 4.5-5.5 after cellular uptake ^[11]. The 12.2 percentage-point increase in release at acidic pH suggests that a meaningful fraction of the encapsulated doxorubicin is released specifically within tumour tissue after endosomal processing, potentially improving the therapeutic index over non-pH-sensitive formulations. However, confirming this hypothesis requires in vivo biodistribution and efficacy studies in tumour-bearing animal models.

Comparison with the marketed Doxil® formulation is instructive. Doxil® uses a similar HSPC/cholesterol/PEG-DSPE composition but achieves a mean diameter of approximately 85 nm with >95% encapsulation efficiency ^[2]. Our optimized formulation matches the EE (96.2%) with a slightly larger mean size (98.4 nm), which still falls within the 80-120 nm window considered optimal for EPR-mediated tumour accumulation. The QbD approach provides the additional advantage of a documented design space that facilitates technology transfer between manufacturing sites and simplifies scale-up from laboratory to pilot batch sizes ^[12].

Conclusion

A QbD-driven Box-Behnken design across five formulation variables identified the optimal PEGylated liposomal doxorubicin formulation with 96.2% encapsulation efficiency, 98.4 nm particle size, and PDI of 0.11. Response surface modelling achieved adjusted R² values of 0.87-0.94 for all CQAs, and confirmatory batches validated the design space within 2% of predictions. The formulation showed pH-dependent release behaviour suitable for tumour-targeted delivery and maintained all CQAs within specifications over 90 days of accelerated stability testing. The approach provides a regulatory-aligned template for systematic liposome optimization.

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