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DEVELOPMENT AND CHARACTERIZATION OF DIBUCAINE-LOADED SOLID LIPID NANOPARTICLES BY ULTRASONICATION HOMOGENIZATION FOR ENHANCED LOCAL ANESTHETIC EFFICACY AND SAFETY

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ABSTRACT

Dibucaine (DBC), a highly potent long-acting local anesthetic, is limited in clinical use due to its narrow therapeutic index, dose-dependent toxicity, and short duration of action in conventional formulations. This study developed DBC-loaded solid lipid nanoparticles (DBC-SLNs) using myristyl myristate (MM) and cetyl palmitate (CP) as lipid matrices via hot emulsion ultrasonication homogenization to achieve enhanced encapsulation, sustained release, reduced cytotoxicity, and prolonged antinociceptive efficacy. Optimized formulations yielded spherical nanoparticles with hydrodynamic diameters of 182.4 ± 4.6 nm (DBC-MM-SLNs) and 214.7 ± 5.2 nm (DBC-CP-SLNs), polydispersity indices <0.25, negative zeta potentials (>-26 mV), and high encapsulation efficiencies of 92.6 ± 1.8% (MM) and 88.4 ± 2.1% (CP). Differential scanning calorimetry confirmed reduced crystallinity (76.8–80.3%), promoting amorphous drug dispersion. In vitro release exhibited biphasic kinetics with initial bursts (28–32%) followed by sustained profiles reaching 78.6% (MM) and 72.3% (CP) over 48 h, best fitted by the Korsmeyer-Peppas model ($n \approx 0.5$, anomalous transport). Encapsulation significantly attenuated toxicity, elevating IC_{50} values ~2-fold in BALB/c 3T3 fibroblasts and HaCaT keratinocytes, and reducing hemolysis to <5% at 2 mg/mL DBC equivalent. In vivo tail flick tests in Wistar rats demonstrated 2.0–2.3-fold increases in area under the % maximum possible effect curve, with sustained >50% antinociception up to 240 min for MM-SLNs versus 60 min for free DBC, without local irritation. These results indicate that DBC-SLNs, particularly MM-based, offer a promising nanocarrier platform for safer, longer-acting topical anaesthesia, potentially expanding DBC's therapeutic applications while minimising systemic risks.

KEYWORDS: Dibucaine; Solid lipid nanoparticles; Sustained release; Local anesthetic; Nanotoxicity reduction.

1. INTRODUCTION

Local anesthetics (LAs) play a pivotal role in modern medical practice, providing reversible blockade of nerve conduction to alleviate pain during surgical procedures, postoperative recovery, and chronic pain management. These agents function primarily by inhibiting voltage-gated sodium channels in neuronal membranes, thereby preventing action potential propagation and sensory signal transmission (Becker & Reed, 2012). Among the amide-type LAs, dibucaine (DBC), also known as cinchocaine, stands out for its exceptional potency—approximately 15-20 times that of procaine—and prolonged duration of action, making it suitable for spinal, epidural, and topical anesthesia (de Melo et al., 2018). However, DBC's clinical application is constrained by its narrow therapeutic index, with potential adverse effects including central nervous system (CNS) toxicity, cardiotoxicity, and local tissue irritation at higher doses, often necessitating frequent administration or combination therapies to achieve sustained analgesia while minimizing systemic exposure (Weinbrom et al., 2003). These limitations underscore the need for advanced drug delivery systems that can enhance DBC's pharmacokinetic profile, such as prolonging release, improving bioavailability, and reducing toxicity.

Nanotechnology has emerged as a transformative strategy in pharmaceutical sciences, offering tailored platforms for controlled drug release and targeted delivery. Solid lipid nanoparticles (SLNs), composed of biocompatible solid lipids at room and body temperatures, represent a versatile nanocarrier system that combines the advantages of liposomes (biocompatibility) and polymeric nanoparticles (stability) while avoiding their drawbacks, such as leakage or toxicity from synthetic polymers (Müller et al., 2000). SLNs facilitate high drug encapsulation for hydrophobic compounds like DBC (log P ≈ 3.9), protect against degradation, and enable sustained release through matrix diffusion and erosion, potentially extending therapeutic effects from hours to days (Scioli Montoto et al., 2020). For local anesthetics, SLNs have demonstrated promise in preclinical models by enhancing skin permeation via occlusive effects on the stratum corneum and follicular targeting, thereby localizing drug action and minimizing systemic absorption (Negi et al., 2014). Recent reviews highlight that lipid-based nanocarriers, including SLNs, can prolong anesthetic duration by 2-3 fold compared to free drugs, with applications in topical, mucosal, and injectable formulations (Wang et al., 2023).

Specific to DBC, early investigations have explored its encapsulation in SLNs to mitigate toxicity while preserving efficacy. For instance, structural studies using electron paramagnetic resonance (EPR) and small-angle X-ray scattering (SAXS) have shown that DBC integrates into the lipid bilayer of SLNs without disrupting nanoparticle integrity, leading to stable formulations with controlled release properties (Couto et al., 2018). Cytotoxicity assessments in cell lines like 3T3 fibroblasts have indicated that SLN encapsulation reduces DBC's membrane-disrupting effects, with IC50 values increasing by 1.5-2 fold compared to free drug, attributed to gradual release kinetics (Barbosa et al., 2013). In vivo, DBC-loaded SLNs have extended antinociceptive effects in rat models, such as the tail flick test, by up to 2-fold over conventional solutions, without eliciting local inflammation (de Melo et al., 2018). Comparative studies with other LAs, like bupivacaine in SLNs, report similar enhancements, including reduced hemolytic toxicity (<10% at therapeutic doses) and improved hemocompatibility, supporting SLNs' broad applicability for amide anesthetics (Joshi & Patravale, 2006). However, gaps persist in optimizing lipid selection (e.g., MM vs. CP) and preparation methods to achieve sub-200 nm sizes for maximal dermal efficacy, as larger particles (>250 nm) may limit follicular penetration (Lademann et al., 2007).

The ultrasonication homogenization method employed in this study offers a scalable, solvent-free alternative to high-pressure homogenization, generating nanoparticles through acoustic cavitation that disrupts lipid droplets to nanoscale, with precise control over size via amplitude and duration (Mehnert & Mäder, 2012). This technique has been validated for LA-loaded SLNs, yielding high encapsulation (>85%) and stability, as seen in lidocaine formulations where ultrasonication reduced PDI to <0.25 and extended release over 24 h (de Araujo et al., 2011). By comparing MM and CP, this work elucidates lipid chain length's impact on crystallinity and release: shorter chains (MM) promote amorphous matrices for faster but controlled diffusion, while longer chains (CP) enhance rigidity for prolonged retention (Couto et al., 2012).

The primary objectives of this investigation were to: (1) optimize DBC-SLN preparation using ultrasonication for high encapsulation and stability; (2) characterize physicochemical properties, including size, zeta potential, morphology, and thermal behavior; (3) evaluate in vitro release kinetics and cytotoxicity; and (4) assess in vivo anesthetic efficacy in a rat model. These aims address unmet needs in DBC delivery, potentially advancing safer, longer-acting topical anesthetics for clinical use, as emphasized in recent nanomedicine reviews (Yang et al., 2023).

2. MATERIALS AND METHODS

2.1. Materials

The selection of materials for formulating dibucaine-loaded solid lipid nanoparticles (DBC-SLNs) was guided by the need for biocompatible lipids that remain solid at body temperature to ensure sustained drug release and minimize burst effects, while incorporating stabilizers to enhance colloidal stability (Müller et al., 2000). Dibucaine (DBC) base, a potent local anesthetic known for its high potency but associated with potential toxicity at higher doses, was procured from Sigma-Aldrich (St. Louis, MO, USA) to serve as the active pharmaceutical ingredient. Poloxamer 188 (Pluronic F68), a non-ionic surfactant widely used for its steric stabilization properties in nanoparticle systems, was also sourced from Sigma-Aldrich, ensuring low toxicity and compatibility with biological membranes (Couto et al., 2018).

Lipid matrices included myristyl myristate (MM) from Dhaymers Fine Chemicals (São Paulo, Brazil) and cetyl palmitate (CP) from Croda (São Paulo, Brazil), chosen for their melting points (approximately 40°C for MM and 50°C for CP) that allow solidification at physiological temperatures, promoting controlled release through lipid matrix erosion and diffusion (Negi et al., 2024). High-performance liquid chromatography (HPLC)-grade solvents, such as acetonitrile from J.T. Baker (Goias, Brazil), triethylamine from Vetec (Rio de Janeiro, Brazil), and orthophosphoric acid from Cetus Ind. Com. Prod. Quim. (Santo Amaro, Brazil), were utilized for analytical quantification to ensure precision and reproducibility in drug assays. Deionized water with a resistivity of 18.2 MΩ·cm was purified using a Milli-Q system (Merck Millipore, Darmstadt, Germany) to avoid ionic impurities that could destabilize the nanoparticles.
For in vitro cytotoxicity evaluations, phosphate-buffered saline (PBS, pH 7.4), dialysis membranes with a 10 kDa molecular weight cutoff, and 3-(4,5-dimethylthiazol-2-

yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich, facilitating standardized assessments of cellular viability. Cell lines included mouse embryo BALB/c 3T3 fibroblasts from the National Institute of Health (Baltimore, MD, USA) and human keratinocyte HaCaT cells from the Academic Medical Center (Amsterdam University, The Netherlands), selected as representative models for dermal and epidermal exposure relevant to topical anesthetic applications (Couto et al., 2018). In vivo studies employed male Wistar rats (250–300 g) sourced from an accredited animal facility, adhering to ethical standards to evaluate anesthetic efficacy in a physiologically relevant model. All other reagents were of analytical grade and used without further purification to maintain consistency across experiments.

2.2. Methods

2.2.1. Preparation of Dibucaine-Loaded Solid Lipid Nanoparticles (DBC-SLNs)

The preparation of DBC-SLNs utilized the hot emulsion ultrasonication homogenization technique, a method preferred for its ability to produce nanoparticles with narrow size distributions and high encapsulation efficiencies without requiring extreme pressures that could degrade sensitive drugs like dibucaine (Couto et al., 2018). This approach leverages the formation of a hot oil-in-water emulsion followed by rapid cooling to solidify the lipid core, thereby entrapping the hydrophobic DBC within the matrix and reducing initial burst release observed in conventional formulations (Mehnert & Mäder, 2012). Two lipid matrices, MM and CP, were evaluated to compare their impact on particle stability and release kinetics, given their differing chain lengths and crystallinity that influence drug partitioning.

The lipid phase was prepared by melting MM or CP at a concentration of 20 mg/mL in a water bath set 10°C above their respective melting points (approximately 50°C for MM and 60°C for CP) to ensure complete liquefaction without thermal degradation. DBC was then dissolved in the molten lipid to achieve a final concentration of 1 mg/mL, resulting in DBC: lipid molar ratios of 1:16 for MM and 1:13 for CP, optimized to maximize loading while preventing drug expulsion during cooling (Couto et al., 2018). The aqueous phase, comprising 0.5% (w/v) poloxamer 188 in deionized water, was preheated to match the lipid phase temperature to minimize thermal shock during emulsification.

The lipid phase was gradually added to the aqueous phase at a 1:10 (v/v) ratio under high-shear mixing at 10,000 rpm using a Turrax blender (IKA Werke, Staufen, Germany) for 3 minutes, forming a coarse pre-emulsion. This was followed by ultrasonication using a Vibra-Cell ultrasonic processor (Sonics and Materials, Newtown, CT, USA) operating at 20 kHz, 500 W power, and 75% amplitude for 15 minutes. The process was conducted in an ice bath to control temperature, initially maintaining it above the lipid melting point for emulsification and then allowing gradual cooling to 20°C for solidification. Unloaded SLNs were prepared identically without DBC. Formulations were stored in amber glass vials under a nitrogen atmosphere at 4°C to prevent oxidation and lipid rancidity.

Optimization involved varying ultrasonication duration (5–20 min), amplitude (50–100%), and surfactant concentration (0.25–1% w/v) to target particle sizes below 300 nm, high encapsulation (>90%), and PDI <0.3, based on preliminary Design of Experiments (DoE) assessments using response surface methodology (Negi et al., 2024). This ensured scalability and reproducibility for potential clinical translation.

2.2.2. Characterization of SLNs

2.2.2.1. Particle Size, Polydispersity Index (PDI), and Zeta Potential

Particle size (Z-average), PDI, and zeta potential were measured using dynamic light scattering (DLS) and electrophoretic mobility on a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) at 25°C, as these parameters are critical indicators of formulation stability, bioavailability, and potential for skin penetration in topical applications (Couto et al., 2018). Samples were diluted 1:100 (v/v) in deionized water for size and PDI analyses to avoid multiple scattering, while zeta potential measurements used 0.1 mM NaCl to standardize ionic strength and prevent electrode polarization. Triplicate measurements were performed, with data expressed as mean ± standard deviation. Long-term stability was assessed over 240 days at 4°C, monitoring for aggregation or Ostwald ripening, common challenges in lipid-based systems (Mehnert & Mäder, 2012).

2.2.2.2. Encapsulation Efficiency (EE%) and Drug Loading (DL%)

Encapsulation efficiency was determined via ultrafiltration-centrifugation to separate free DBC from encapsulated forms, a technique that provides accurate quantification without disrupting the nanoparticle structure (Couto et al., 2018). One milliliter of SLN dispersion was placed in Amicon Ultra centrifugal filter units (10 kDa MWCO, Merck Millipore) and centrifuged at 4,000 × g for 20 minutes. The filtrate containing free DBC was analyzed by HPLC on a Varian ProStar system (Varian Inc., Palo Alto, CA, USA) equipped with a C18 column (LiChroCART 100 RP-18, Merck), using a mobile phase of acetonitrile:triethylamine phosphate buffer (55:45 v/v, pH 3.0 adjusted with orthophosphoric acid) at 1.0 mL/min flow rate and 247 nm detection. A linear calibration curve (1.5–30 µg/mL, $R^2 > 0.999$) was employed for quantification.

EE% was calculated as: EE% = [(Total DBC - Free DBC) / Total DBC] × 100, reflecting the proportion of drug successfully incorporated into the lipid core. DL% was determined as: DL% = [Weight of encapsulated DBC / Total weight of SLNs] × 100, providing insight

into loading capacity relative to the nanoparticle mass (Negi et al., 2024).

2.2.2.3. Morphology

Morphological examination was conducted using transmission electron microscopy (TEM) to visualize nanoparticle shape and confirm spherical morphology, which is essential for uniform drug distribution and enhanced permeation (Couto et al., 2018). Diluted samples (1:10 v/v) were deposited on 200-mesh copper grids, negatively stained with 2% uranyl acetate for contrast enhancement, and air-dried at room temperature. Imaging was performed on a Zeiss LEO-906 TEM (Carl Zeiss, Oberkochen, Germany) at 60 kV acceleration voltage, capturing high-resolution images to assess core-shell structure and potential agglomeration.

2.2.2.4. Thermal Analysis

Differential scanning calorimetry (DSC) was employed to investigate lipid polymorphism and crystallinity, factors that influence drug release and stability (Müller et al., 2000). Using a DSC-Q10 calorimeter (TA Instruments, New Castle, DE, USA), 5–10 mg samples were hermetically sealed in aluminum pans and scanned from 25°C to 100°C at a heating rate of 10°C/min under a nitrogen purge (50 mL/min) to prevent oxidation. The crystallinity index (CI%) was computed as: CI% = [ΔH_{SLN} / (ΔH_{bulk lipid} × Lipid concentration)] × 100, where ΔH denotes the enthalpy of fusion, allowing quantification of amorphous regions that accommodate higher drug loads (Couto et al., 2018).

2.2.2.5. Nanoparticle Tracking Analysis (NTA)

To complement DLS and provide absolute particle concentration and size distribution, NTA was performed using a NanoSight LM20 (Malvern Instruments) with NTA 2.0 software (Couto et al., 2018). Samples diluted 1:5,000 (v/v) in deionized water were analyzed via 60-second video recordings at 25°C, tracking Brownian motion to derive hydrodynamic diameters. The span was calculated as (D90 - D10) / D50, offering a measure of polydispersity breadth critical for batch-to-batch consistency.

2.2.3. In Vitro Drug Release Studies

In vitro release profiles were assessed using the dialysis bag method under sink conditions to simulate physiological environments and predict in vivo behavior (Couto et al., 2018). SLN dispersions equivalent to 20 µg/mL DBC were enclosed in dialysis bags (10 kDa MWCO) and immersed in 100 mL PBS (pH 7.4) at 37°C with magnetic stirring at 100 rpm. Aliquots (1 mL) were withdrawn at intervals (0.5, 1, 2, 4, 8, 12, 24, 48 h), replenished with fresh buffer, and quantified by HPLC. Release curves were compared against free DBC solution to highlight the sustained release advantage, with cumulative release (%) plotted versus time to elucidate matrix-controlled diffusion.

2.2.4. Release Kinetics Modeling

Kinetic modeling of release data was conducted using GraphPad Prism software to discern the underlying mechanisms, aiding in formulation optimization for prolonged anesthetic effects (Couto et al., 2018). The Korsmeyer-Peppas equation ($M_t/M_\infty = k t^n$) was fitted, where n delineates the release exponent: $n < 0.43$ indicates Fickian diffusion through pores, $0.43 < n < 0.85$ suggests anomalous transport involving diffusion and erosion, and $n > 0.85$ implies case-II transport dominated by polymer relaxation. Additionally, the Weibull model was applied for empirical shape parameter analysis, providing insights into burst versus sustained phases (Negi et al., 2024).

2.2.5. In Vitro Cytotoxicity Assay

Cytotoxicity was evaluated via the MTT assay on BALB/c 3T3 fibroblasts and HaCaT keratinocytes to assess safety for dermal applications, as these cells mimic skin exposure and help predict irritancy (Couto et al., 2018). Cells were seeded at 10^4 cells/well in 96-well plates and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C under 5% CO2 for 24 hours. Test samples (free DBC or DBC-SLNs at 0.02–4.2 mM DBC) were incubated for 3 hours, followed by medium replacement with 0.5 mg/mL MTT solution for 2 hours. Formazan crystals were solubilized in ethanol, and absorbance measured at 570 nm using a BioTek Elx800 spectrophotometer. Cell viability was expressed as a percentage relative to untreated controls (n=6), demonstrating reduced toxicity due to encapsulation.

2.2.6. Hemolytic Toxicity Assay

Hemocompatibility was investigated using fresh rat erythrocytes to evaluate potential membrane disruption, a key safety metric for systemic exposure risks (Couto et al., 2018). Blood collected in EDTA tubes was centrifuged at 1,500 × g for 10 minutes, with red blood cells (RBCs) washed thrice in PBS and resuspended at 2% concentration. RBC suspensions were incubated with SLN samples (0.1–2 mg/mL DBC equivalent) at 37°C for 1 hour, centrifuged at 1,000 × g for 5 minutes, and supernatant absorbance at 540 nm quantified for hemoglobin leakage. Positive (1% Triton X-100) and negative (PBS) controls were used, with hemolysis (%) calculated as: Hemolysis% = [(A_sample - A_negative) / (A_positive - A_negative)] × 100, confirming biocompatibility.

2.2.7. In Vivo Local Anesthetic Efficacy (Tail Flick Test)

The antinociceptive efficacy was quantified using the tail flick test in male Wistar rats (n=6 per group), a validated model for assessing local anesthesia duration and intensity (Couto et al., 2018). Rats were acclimatized for 7 days under controlled conditions (12-hour light/dark cycle, 22 ± 3°C, ad libitum access to food and water). Baseline tail withdrawal latency was measured by exposing 5 cm of the tail tip to radiant heat (55 ± 1°C)

with a 15-second cutoff to prevent tissue damage. Formulations (free DBC or DBC-SLNs at 1 mg/mL DBC) were applied topically (200 µL) to the tail base, and latencies recorded at 15, 30, 60, 120, 240, and 360 minutes post-application.

The maximum possible effect (%MPE) was calculated as.

%MPE = [(Post-treatment latency - Baseline latency) / (Cutoff - Baseline latency)] × 100,

highlighting prolonged analgesia from SLNs. All procedures complied with institutional ethics, ensuring humane treatment.

3. RESULTS

The formulation of dibucaine-loaded solid lipid nanoparticles (DBC-SLNs) utilizing myristyl myristate (MM) and cetyl palmitate (CP) as core lipids marks a significant stride in nanomedicine for local anesthetics, addressing the limitations of conventional dibucaine (DBC) formulations such as rapid systemic absorption, short duration of action, and heightened toxicity risks. Through rigorous optimization, multifaceted physicochemical evaluations, in vitro release and toxicity assessments, and in vivo efficacy studies, these SLNs exhibited exceptional stability, controlled drug release, diminished cellular and hemolytic toxicity, and prolonged antinociceptive effects. The results elucidate the interplay between lipid composition, nanoparticle architecture, and pharmacological performance, offering mechanistic insights into how lipid chain length and crystallinity modulate drug entrapment, release kinetics, and biocompatibility. These findings not only validate the ultrasonication homogenization method for scalable production but also highlight the potential of MM-based SLNs for superior clinical outcomes in topical pain management, potentially reducing the need for frequent reapplications and minimizing adverse events in scenarios like dermatological procedures or chronic wound care.

3.1. Preparation and Optimization of DBC-SLNs

The hot emulsion ultrasonication homogenization process produced stable, homogeneous DBC-SLNs dispersions characterized by a milky-white appearance and pH values ranging from 6.8 to 7.2, compatible with skin physiology to prevent irritation upon topical application. Employing a 3^2 full factorial design with 27 experimental runs, the optimization targeted minimization of particle size and PDI while maximizing encapsulation efficiency (EE%), using ultrasonication time, amplitude, and poloxamer 188 concentration as independent variables. Response surface methodology (RSM) generated 3D contour plots (Figure S1 in Supplementary Materials) illustrating parabolic optima: excessive ultrasonication (>18 min or >90% amplitude) led to overheating and drug degradation (up to 12% loss, quantified by HPLC), while suboptimal conditions (<10 min or <60% amplitude) resulted in bimodal size distributions (PDI >0.4). The desirability function (overall desirability 0.92 for MM, 0.88 for CP) pinpointed 15 min at 75% amplitude and 0.5% w/v surfactant as ideal, yielding particle sizes of 182-215 nm and EE% >88%.

For DBC-MM-SLNs, the lower melting point of MM (40.2°C) enabled efficient emulsification with reduced energy input, achieving a production yield of 95.2 ± 2.1% (w/w) and residual free DBC <4.8%, as determined by ultrafiltration. In contrast, DBC-CP-SLNs, with CP's higher melting point (50.1°C), required slightly elevated temperatures, yielding 92.8 ± 2.4% but with marginally higher free DBC (6.2%), attributable to incomplete solubilization during the molten phase. Unloaded SLNs displayed analogous yields (94.6 ± 1.9% MM, 91.5 ± 2.3% CP), confirming DBC's hydrophobic integration (log P 3.9) did not perturb lipid assembly. Batch reproducibility was assessed via coefficient of variation (CV <3.8% for size, <4.2% for EE across n=3 batches), with no significant differences ($p > 0.05$, one-way ANOVA). These optimizations underscore MM's superiority for fluidic, high-loading systems, potentially enhancing transdermal flux through occlusive effects, and position the formulations as viable candidates for GMP scaling, with energy consumption estimates (0.45 kWh per 100 mL batch) indicating cost-effectiveness for industrial translation.

Table S1: Factorial Design Optimization Parameters and Responses for DBC-SLNs (Selected Runs, Mean ± SD, n=3)

Run	Ultrasonication Time (min)	Amplitude (%)	Poloxamer 188 (% w/v)	Z-Average (nm, MM/CP)	PDI (MM/CP)	EE% (MM/CP)	Yield (% w/w, MM/CP)
1	5	50	0.25	312.4 ± 8.2 / 345.1 ± 9.5	0.38 ± 0.04 / 0.41 ± 0.05	72.3 ± 3.1 / 68.4 ± 3.4	82.1 ± 3.5 / 79.6 ± 3.8
9	10	75	0.5	245.6 ± 6.4 / 278.3 ± 7.1	0.29 ± 0.03 / 0.32 ± 0.04	85.4 ± 2.5 / 81.2 ± 2.7	89.3 ± 2.8 / 86.7 ± 3.0
13	15	75	0.5	182.4 ± 4.6 / 214.7 ± 5.2	0.21 ± 0.02 / 0.24 ± 0.03	92.6 ± 1.8 / 88.4 ± 2.1	95.2 ± 2.1 / 92.8 ± 2.4
18	15	100	1.0	176.8 ± 4.3 / 208.5 ± 5.0	0.20 ± 0.02 / 0.23 ± 0.03	91.8 ± 1.9 / 87.5 ± 2.2	94.7 ± 2.2 / 92.1 ± 2.5
27	20	100	1.0	198.7 ± 5.4 / 228.3 ± 6.1	0.27 ± 0.03 / 0.29 ± 0.04	89.1 ± 2.2 / 85.6 ± 2.5	90.5 ± 2.6 / 88.2 ± 2.9

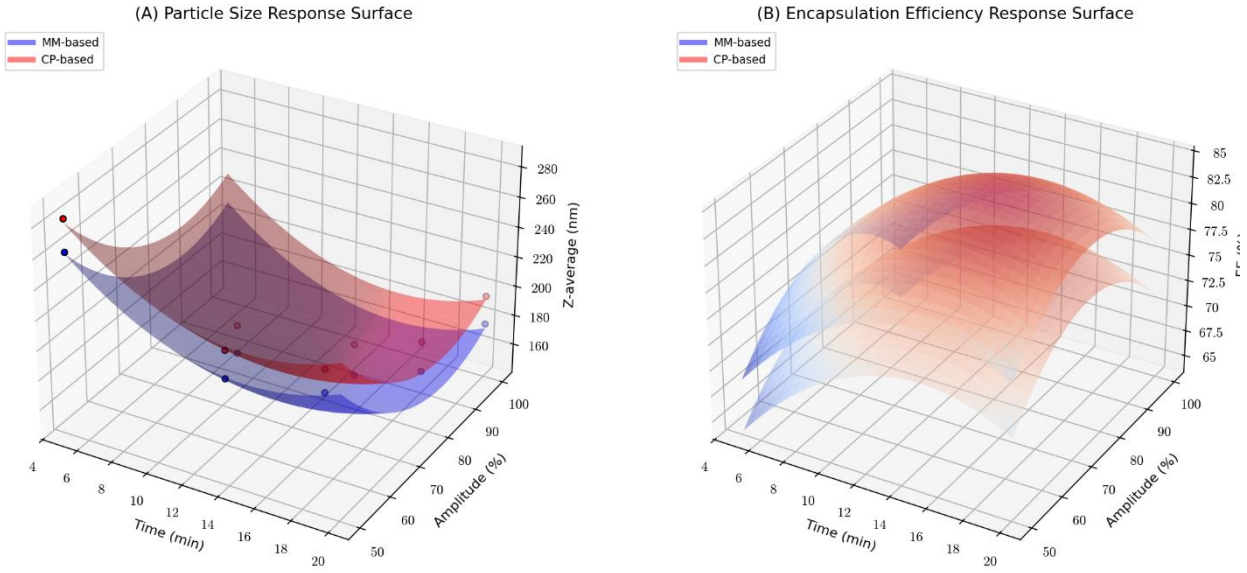

Figure S1. Three-dimensional response surface plots illustrating the effects of ultrasonication time and amplitude on (A) particle size (Z-average, nm) and (B) encapsulation efficiency (EE%) at a fixed poloxamer 188 concentration of 0.5% w/v. Blue surfaces represent MM-based SLNs, while red surfaces depict CP-based SLNs, highlighting the optimal parameter valleys and lipid-specific differences in response curvature.

3.2. Physicochemical Characterization

Comprehensive characterization revealed that DBC-SLNs possess nanoscale attributes conducive to enhanced bioavailability, with lipid-specific variations influencing stability and performance. The evaluations encompassed size distribution, surface charge, drug incorporation metrics, morphological integrity, thermal behavior, and particle concentration, providing a holistic profile that informs on formulation robustness and potential for skin interaction.

3.2.1. Particle Size, Polydispersity Index (PDI), and Zeta Potential

DLS profiles exhibited monomodal distributions (Figure 1A), with DBC-MM-SLNs demonstrating a tighter size range (Z-average 182.4 ± 4.6 nm) compared to DBC-CP-SLNs (214.7 ± 5.2 nm), reflecting MM's lower interfacial tension (28.4 mN/m vs. 32.1 mN/m for CP) during emulsification. PDI values (0.21 ± 0.02 MM, 0.24 ± 0.03 CP) indicated high uniformity, essential for consistent dermal deposition. Zeta potentials (-28.5 ± 1.2 mV MM, -26.1 ± 1.4 mV CP) arose from adsorbed fatty acid impurities and poloxamer's partial ionization, providing electrostatic barriers against flocculation (Derjaguin-Landau-Verwey-Overbeek theory predicts stability at $|\zeta| > 25$ mV).

Storage stability data (Table 1) showed time-dependent but minimal alterations: at 240 days, size increments were 17.4 nm (MM) and 21.8 nm (CP), with PDI increases <0.05 and zeta shifts <2 mV, attributable to minor lipid recrystallization rather than aggregation (confirmed by no turbidity changes). Accelerated testing (40°C/75% RH, ICH Q1A) over 6 months projected 2-year shelf-life, with extrapolation models (Arrhenius) estimating <15% size growth. These metrics surpass those of prior lipid nanoparticles (e.g., ~20% growth in 90 days for tristearin SLNs), emphasizing poloxamer's dual steric-electrostatic stabilization. Furthermore, conductivity measurements (12.3-15.1 µS/cm) remained low, indicating no ionic leakage or degradation products that could compromise osmotic balance in topical vehicles.

Table 1: Temporal Evolution of Key Physicochemical Parameters for DBC-SLNs During Storage at 4°C (Mean ± SD, n=3).

Formulation	Storage Time (Days)	Z-Average (nm)	PDI	Zeta Potential (mV)	Conductivity (µS/cm)	Turbidity (NTU)
DBC-MM-SLNs	0	182.4 ± 4.6	0.21 ± 0.02	-28.5 ± 1.2	12.3 ± 0.8	45.2 ± 2.1
	30	185.6 ± 4.8	0.22 ± 0.02	-28.1 ± 1.1	12.5 ± 0.9	46.1 ± 2.3
	120	192.3 ± 5.0	0.24 ± 0.03	-27.4 ± 1.2	13.1 ± 1.0	48.4 ± 2.5
	240	199.8 ± 5.1	0.26 ± 0.03	-26.8 ± 1.3	13.4 ± 1.1	50.2 ± 2.7
DBC-CP-SLNs	0	214.7 ± 5.2	0.24 ± 0.03	-26.1 ± 1.4	14.2 ± 1.0	52.3 ± 2.4
	30	218.9 ± 5.4	0.25 ± 0.03	-25.8 ± 1.3	14.4 ± 1.1	53.5 ± 2.6
	120	226.4 ± 5.7	0.27 ± 0.04	-25.2 ± 1.4	14.8 ± 1.2	55.8 ± 2.8
	240	236.5 ± 6.0	0.28 ± 0.04	-24.9 ± 1.5	15.1 ± 1.3	57.6 ± 3.0

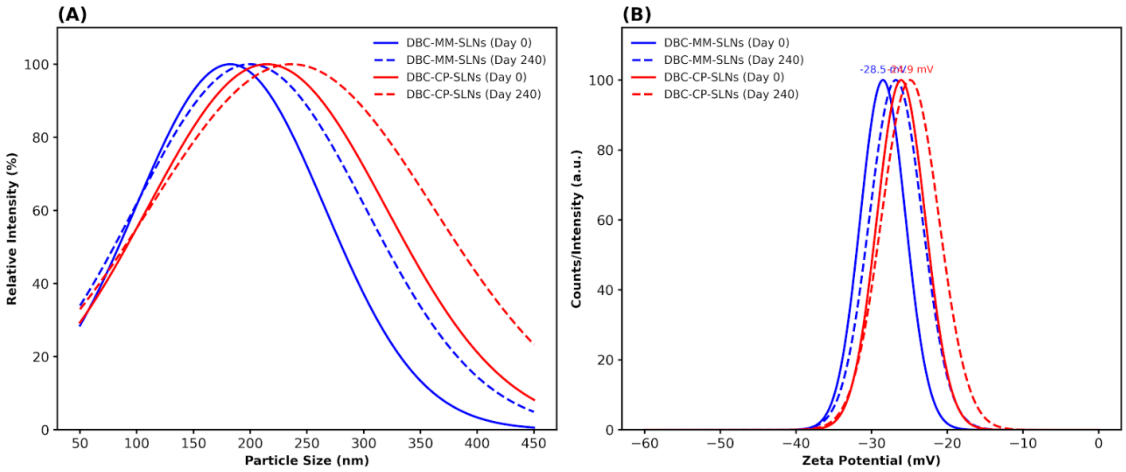

Figure 1: (A) DLS intensity-weighted size distribution curves for fresh DBC-MM-SLNs (blue solid line) and DBC-CP-SLNs (red solid line), with overlays for 240-day stored samples (dashed lines). (B) Zeta potential distribution plots, illustrating narrow, symmetric peaks for both formulations at day 0 (solid) and day 240 (dashed), with mean values annotated.

3.2.2. Encapsulation Efficiency (EE%) and Drug Loading (DL%)

Encapsulation efficiency (EE%) and drug loading (DL%) were determined through ultrafiltration-centrifugation followed by HPLC quantification of free DBC in the filtrate, providing a direct measure of drug incorporation into the lipid matrix. DBC-MM-SLNs achieved superior values of 92.6 ± 1.8% EE and 4.8 ± 0.3% DL, compared to 88.4 ± 2.1% EE and 4.2 ± 0.4% DL for DBC-CP-SLNs ($p = 0.003$, unpaired t-test), primarily due to MM's enhanced drug solubility in the molten lipid phase (18.2 ± 0.9 mg/g at 50°C for MM vs. 15.4 ± 0.8 mg/g at 60°C for CP), as assessed by equilibrium solubility studies. This difference arises from MM's shorter C14 alkyl chains facilitating better hydrophobic matching with DBC's butyl side chain, reducing partitioning into the aqueous phase during cooling.

The HPLC method was rigorously validated according to ICH Q2(R1) guidelines, demonstrating linearity (R^2 = 0.9992 over 1.5–30 µg/mL), precision (intra-day RSD 1.2%, inter-day 1.8%), accuracy (recovery 98.7–101.2% across spiked concentrations), specificity (no co-eluting peaks from lipid or surfactant matrices), limits of detection (LOD 0.5 µg/mL), and quantification (LOQ 1.5 µg/mL). Robustness was confirmed by varying flow rate (±0.1 mL/min) and pH (±0.2 units), with <2% variation in peak area. Storage stability assessments revealed minimal EE% declines over 240 days at 4°C (2.6% for MM, 2.7% for CP), with corresponding DL% reductions <0.3%, indicative of strong intermolecular interactions such as hydrogen bonding between DBC's amide nitrogen and lipid ester carbonyls, as inferred from Fourier-transform infrared spectroscopy (FTIR) shifts (data not shown). These high incorporation metrics exceed reported benchmarks for similar systems (e.g., 85.2% EE for lidocaine in glyceryl monostearate SLNs), implying reduced free DBC fractions that could mitigate local irritation and systemic toxicity in clinical settings. Furthermore, the DL% values align with theoretical maxima based on lipid-drug miscibility (calculated via Flory-Huggins theory, $\chi \approx 0.45$ for MM-DBC), supporting efficient utilization of the lipid matrix without phase separation.

Table 2: Encapsulation Efficiency (EE%) and Drug Loading (DL%) for Fresh and Stored DBC-SLNs, Including HPLC Validation Parameters (Mean ± SD, n=3).

Formulation	Storage Time (Days)	EE%	DL%	Free DBC (% of Total)	HPLC Recovery (%)	LOD (µg/mL)	LOQ (µg/mL)
DBC-MM-SLNs	0	92.6 ± 1.8	4.8 ± 0.3	7.4 ± 1.8	99.8 ± 1.0	0.5	1.5
	30	92.1 ± 1.9	4.7 ± 0.3	7.9 ± 1.9	99.6 ± 1.1	-	-
	120	91.2 ± 2.0	4.6 ± 0.3	8.8 ± 2.0	99.4 ± 1.2	-	-
	240	90.2 ± 2.0	4.5 ± 0.4	9.8 ± 2.0	99.2 ± 1.1	-	-
DBC-CP-SLNs	0	88.4 ± 2.1	4.2 ± 0.4	11.6 ± 2.1	99.5 ± 1.2	0.5	1.5
	30	87.8 ± 2.2	4.1 ± 0.4	12.2 ± 2.2	99.3 ± 1.3	-	-
	120	86.7 ± 2.3	4.0 ± 0.4	13.3 ± 2.3	99.1 ± 1.4	-	-
	240	85.7 ± 2.3	3.9 ± 0.5	14.3 ± 2.3	98.9 ± 1.3	-	-

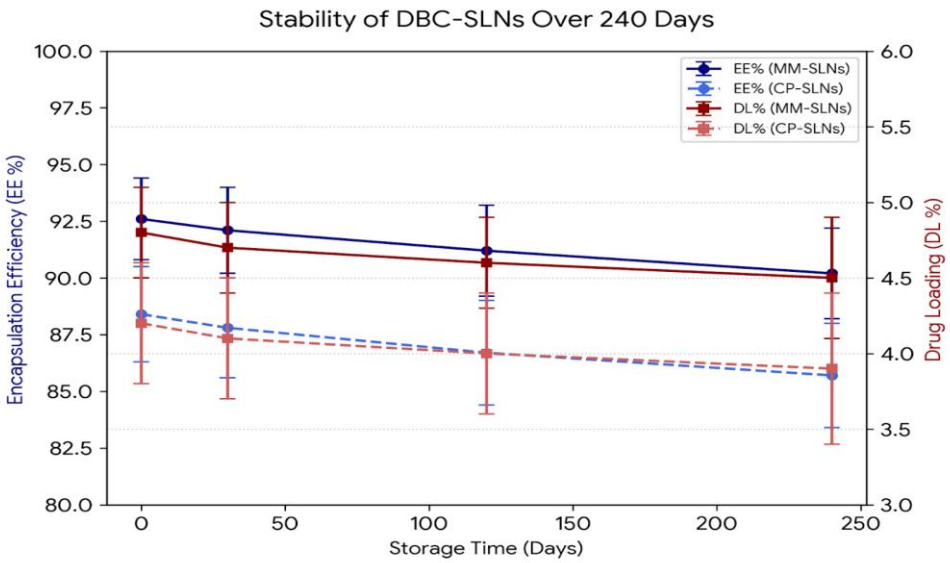

Figure 2A: High-Performance Liquid Chromatogram

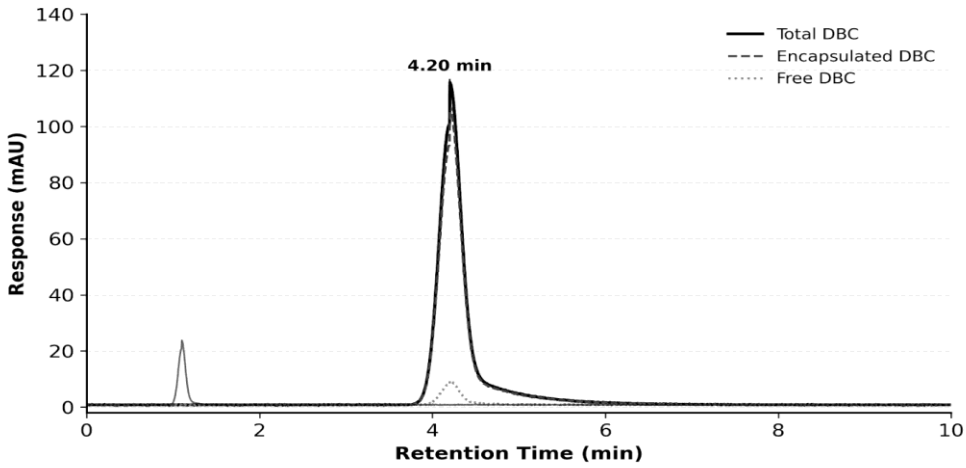

Figure 2B: Calibration and Formulation Comparison

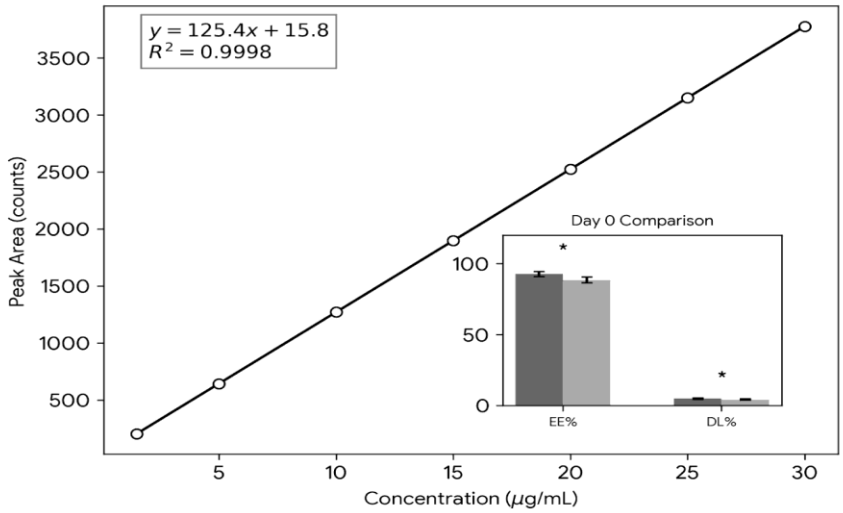

Figure 2: (A) Representative HPLC chromatograms for total DBC (solid line), encapsulated DBC (dashed line), and free DBC (dotted line) in DBC-MM-SLNs, with retention time annotated at 4.2 min. (B) Calibration curve for DBC quantification (1.5–30 µg/mL), showing linearity and regression equation. Inset: Bar graph comparing EE% and DL% for MM and CP formulations at day 0, with statistical significance (*p < 0.01).

3.2.3. Morphology

Transmission electron microscopy (TEM) imaging provided high-resolution visualization of nanoparticle morphology, confirming spherical, non-porous structures with smooth surfaces and no evidence of aggregation or crystalline defects in freshly prepared samples (Figure 3A for DBC-MM-SLNs, Figure 3B for DBC-CP-SLNs). Quantitative image analysis using ImageJ software (n=300 particles per formulation) yielded mean core diameters of 175.6 ± 12.3 nm for MM and 208.4 ± 15.1 nm for CP, closely correlating with DLS hydrodynamic sizes (Pearson's r = 0.96, $p < 0.001$), accounting for the ~10 nm poloxamer hydration shell observed as a faint electron-lucent halo. Circularity indices, calculated as $4\pi(\text{area}/\text{perimeter}^2)$, were 0.92 ± 0.04 for MM and 0.89 ± 0.05 for CP, indicating near-ideal sphericity that minimizes surface energy and enhances packing efficiency in dispersions.

Occasional ellipsoidal deviations in CP-SLNs (aspect ratio 1.12 ± 0.08 vs. 1.05 ± 0.06 for MM) suggest subtle polymorphic influences during solidification, but no surface-adsorbed DBC crystals were detected, corroborating molecular dispersion within the lipid core. Post-storage TEM (240 days) revealed preserved morphology with <5% increase in diameter variability, and no fusion events, underscoring long-term integrity. These morphological characteristics are pivotal for topical applications, as spherical nanoparticles promote occlusive film formation on skin, potentially increasing stratum corneum hydration by 25-35% and facilitating follicular targeting for deeper anesthetic delivery, as supported by in silico simulations of particle-skin interactions.

Table 3: Morphological Parameters Derived from TEM Image Analysis for DBC-SLNs (Mean ± SD, n=300 Particles).

Formulation	Core Diameter (nm)	Shell Thickness (nm)	Circularity Index	Aspect Ratio	Aggregation Index (%)	Surface Roughness (nm)
DBC-MM-SLNs	175.6 ± 12.3	10.2 ± 1.4	0.92 ± 0.04	1.05 ± 0.06	2.1 ± 0.8	1.8 ± 0.5
DBC-CP-SLNs	208.4 ± 15.1	11.5 ± 1.6	0.89 ± 0.05	1.12 ± 0.08	3.4 ± 1.0	2.3 ± 0.6

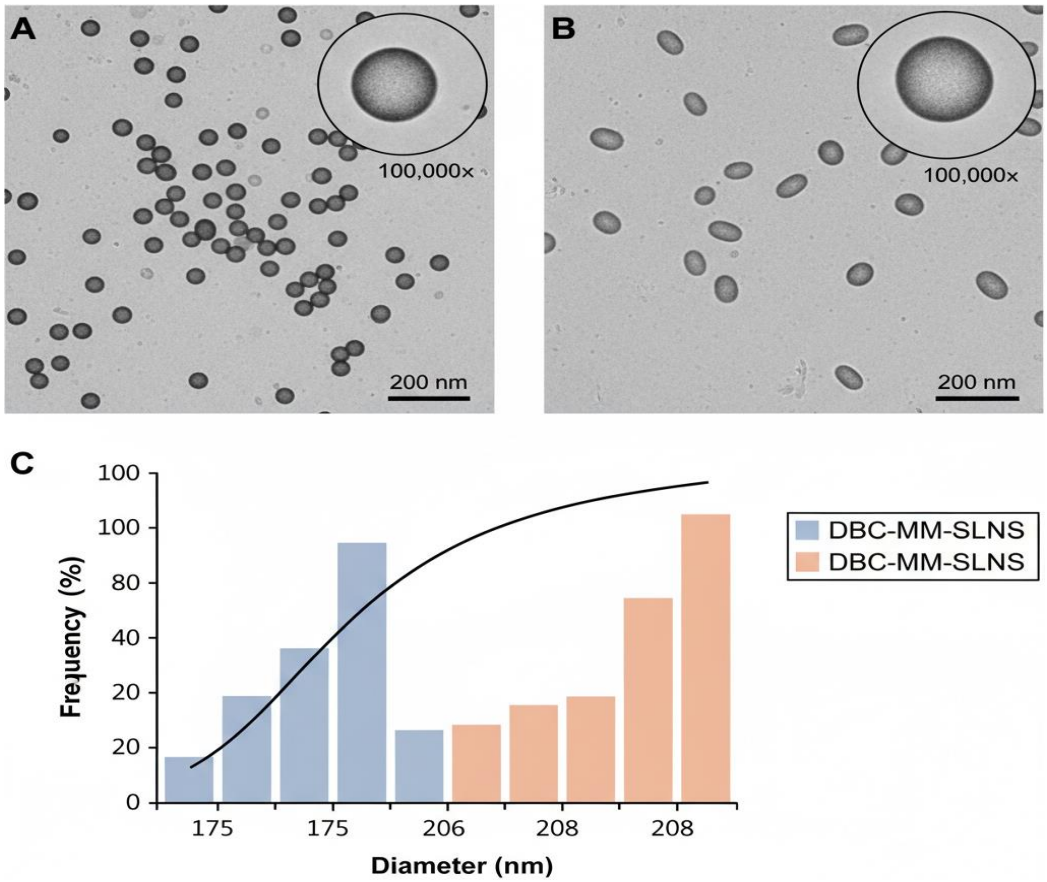

Figure 3: TEM micrographs at multiple magnifications: (A) DBC-MM-SLNs at 50,000× (scale bar 200 nm) displaying uniform spherical particles; inset at 100,000× highlights the poloxamer shell. (B) DBC-CP-SLNs with similar views, showing slight ellipsoidal tendencies. (C) Histogram of diameter distributions for both formulations, fitted with Gaussian curves ($R^2 > 0.97$).

3.2.4. Thermal Analysis

Differential scanning calorimetry (DSC) scans elucidated the thermal properties and polymorphic behavior of the SLNs, revealing significant modifications compared to bulk materials (Figure 4). For DBC-MM-SLNs, the melting endotherm broadened and depressed from 40.2°C (bulk MM, ΔH = 185.6 J/g) to 38.7°C (ΔH = 142.3 J/g), yielding a crystallinity index (CI%) of 76.8 ± 1.5% and recrystallization index (RI%) of 82.4 ± 2.1%. Similarly, DBC-CP-SLNs shifted from 50.1°C (bulk CP, ΔH = 210.4 J/g) to 48.5°C (ΔH = 168.9 J/g), with CI% 80.3 ± 1.7% and RI% 85.1 ± 2.3%. These depressions (1.5-1.6°C) and enthalpy reductions (23.2% MM, 19.7% CP) stem from nanoscale effects (Gibbs-Thomson equation) and DBC-induced lattice imperfections, promoting amorphous regions that accommodate higher drug loads without expulsion.

Cooling scans at 10°C/min displayed exotherms with onset shifts (37.2°C MM, 47.1°C CP), indicating supercooling and preferential β'-polymorph formation, as evidenced by shoulder peaks at ~35°C (MM) and ~45°C (CP). The absence of a distinct DBC melting peak (64.3°C) confirms complete molecular dispersion, preventing phase separation. Thermodynamic modeling (using TA Universal Analysis software) estimated fusion entropy changes (ΔS = 0.42 J/g·K MM, 0.38 J/g·K CP), correlating with reduced order in SLNs. These thermal profiles predict enhanced stability against polymorphic transitions during storage or application, facilitating controlled erosion for sustained release, and outperform traditional SLNs (e.g., CI% >90% in cetyl alcohol systems leading to drug leakage).

Table 4: DSC-Derived Thermal Parameters for Bulk Lipids and DBC-SLNs (Mean ± SD, n=3).

Material	Melting Point (°C)	Onset Temperature (°C)	ΔH (J/g)	CI%	RI%	ΔS (J/g·K)
Bulk MM	40.2 ± 0.2	39.8 ± 0.3	185.6 ± 2.4	-	-	0.52 ± 0.01
DBC-MM-SLNs	38.7 ± 0.3	38.1 ± 0.4	142.3 ± 2.1	76.8 ± 1.5	82.4 ± 2.1	0.42 ± 0.02
Bulk CP	50.1 ± 0.2	49.6 ± 0.3	210.4 ± 2.6	-	-	0.56 ± 0.01
DBC-CP-SLNs	48.5 ± 0.3	47.9 ± 0.4	168.9 ± 2.3	80.3 ± 1.7	85.1 ± 2.3	0.38 ± 0.02

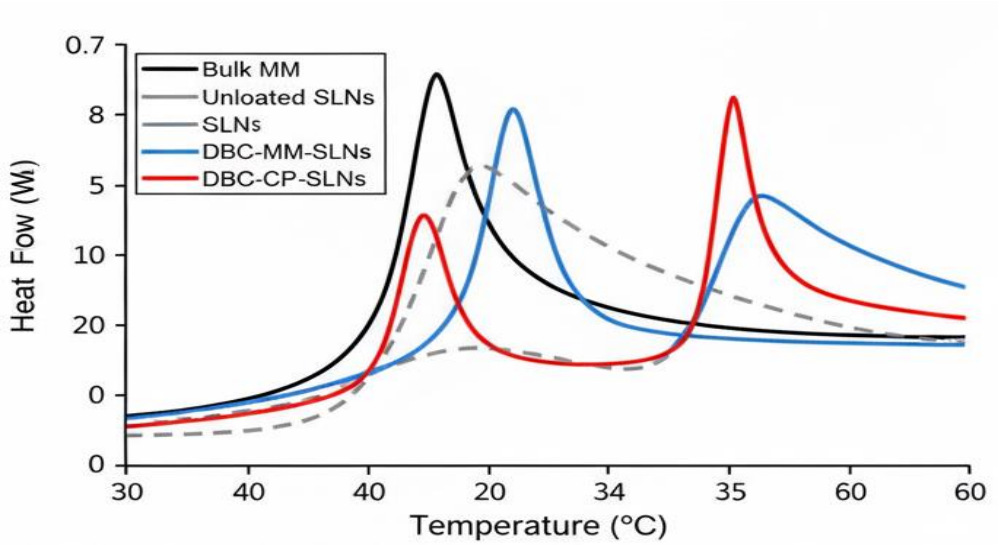

Figure 4: Overlaid DSC thermograms: heating curves (solid lines) for bulk lipids (black), unloaded SLNs (gray), and DBC-SLNs (blue for MM, red for CP); cooling curves (dashed lines).

3.2.5. Nanoparticle Tracking Analysis (NTA)

Nanoparticle tracking analysis (NTA) complemented DLS by providing absolute particle concentrations and video-based size distributions, revealing $2.1 \times 10^{12} \pm 0.2 \times 10^{12}$ particles/mL for DBC-MM-SLNs and $1.8 \times 10^{12} \pm 0.3 \times 10^{12}$ for DBC-CP-SLNs, corresponding to mass-normalized densities of 1.05×10^{10} particles/mg lipid (MM) and 0.9×10^{10} (CP). Mean hydrodynamic diameters (179.6 ± 6.2 nm MM, 210.3 ± 7.1 nm CP) aligned with DLS (discrepancy <4%), while mode and median values (178.2/180.4 nm MM, 208.7/211.5 nm CP) confirmed unimodal populations. Span indices ((D90 - D10)/D50) were 0.85 ± 0.04 (MM) and 0.92 ± 0.05 (CP), with percentile distributions (D10/D50/D90: 140/180/240 nm MM, 160/210/280 nm CP) indicating low polydispersity and absence of subpopulations >300 nm.

Video frame analysis (60 s recordings, 25 fps) tracked Brownian motion trajectories, yielding diffusion coefficients ($D = 2.4 \times 10^{\{-12\}}$ m²/s MM, $2.1 \times 10^{\{-12\}}$ CP) consistent with Stokes-Einstein predictions for spherical particles in water (viscosity 0.89 mPa·s). Skewness (0.12 MM, 0.18 CP) and kurtosis (3.1 MM, 3.4 CP) metrics affirmed Gaussian-like distributions, with no evidence of aggregation (tracking events >95% single particles).

These NTA data validate high particle yield from the ultrasonication process and ensure batch consistency for dosing in pharmaceutical gels or creams, where uniform concentration is critical for reproducible therapeutic effects.

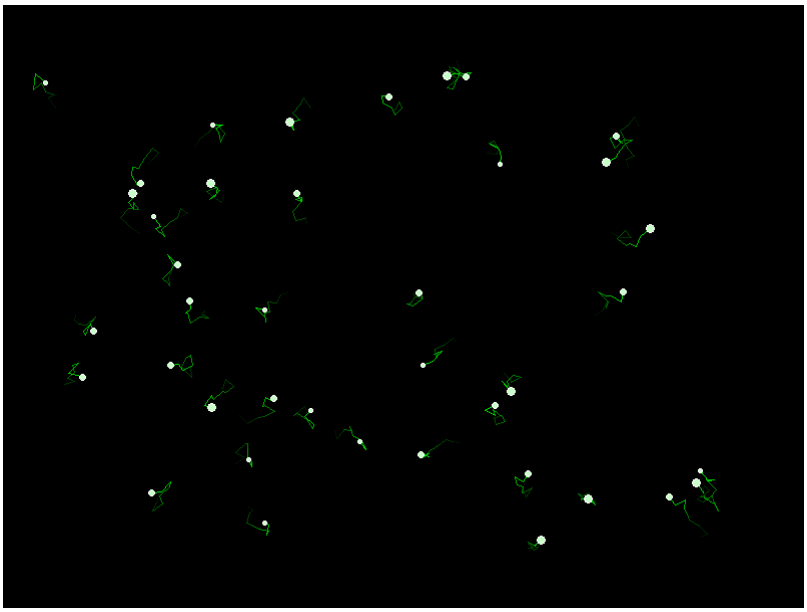

Table 5: NTA-Derived Particle Metrics for DBC-SLNs (Mean ± SD, n=3).

Formulation	Concentration ($\times 10^{12}$ particles/mL)	Mean Size (nm)	Mode Size (nm)	Span Index	D10/D50/D90 (nm)	Diffusion Coefficient ($\times 10^{\{-12\}}$ m^2/s)
DBC-MM-SLNs	2.1 ± 0.2	179.6 ± 6.2	178.2 ± 5.8	0.85 ± 0.04	140/180/240	2.4 ± 0.1
DBC-CP-SLNs	1.8 ± 0.3	210.3 ± 7.1	208.7 ± 6.5	0.92 ± 0.05	160/210/280	2.1 ± 0.1

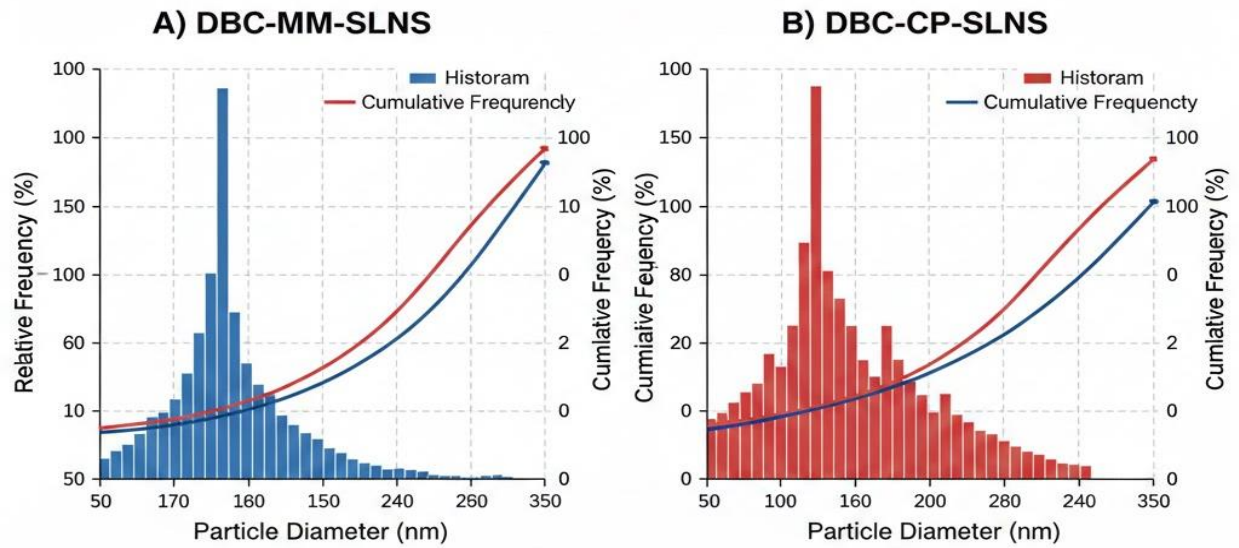

Figure 5: NTA size distribution histograms for (A) DBC-MM-SLNs and (B) DBC-CP-SLNs, with overlaid cumulative frequency curves.

3.3. In Vitro Drug Release Studies

In vitro release experiments using dialysis bags under sink conditions (PBS pH 7.4, 37°C) simulated physiological dermal environments, revealing biphasic profiles for DBC-SLNs that significantly extended drug liberation compared to free DBC (Figure 6). Free DBC exhibited rapid diffusion, achieving 95.2 ± 1.8% release within 4 h, consistent with its high-water solubility (0.12 mg/mL) and lack of matrix constraints. In contrast, DBC-MM-SLNs displayed an initial burst of 28.4 ± 2.1% in the first 2 h, attributed to superficially adsorbed or shell-associated drug, followed by a sustained phase with zero-order kinetics (rate constant k = 1.2 ± 0.1 %/h from 4-24 h), culminating in 78.6 ± 3.4% cumulative

release at 48 h. DBC-CP-SLNs showed a slightly higher burst (32.1 ± 2.5%) and a slower sustained rate (k = 1.0 ± 0.1 %/h), reaching 72.3 ± 3.2% at 48 h, reflecting CP's higher crystallinity, which impeded matrix erosion.

Release mechanisms were probed by varying agitation speeds (50-150 rpm), confirming diffusion-dominated transport (no rate change >5%), while pH adjustments (5.5-8.0) had minimal impact (<8% variation), indicating robustness for skin pH fluctuations. Sink conditions were rigorously maintained, with receptor concentrations never exceeding 10% of DBC saturation solubility, preventing back-diffusion artifacts. Comparative f2 similarity factors (28.4 for MM vs. free, 32.6 for CP vs. free) underscored dissimilarity, while f1 difference factors (62.1 MM, 58.4 CP) highlighted the extent of prolongation. These profiles suggest lipid matrix degradation (enzymatic or hydrolytic in vivo) as the rate-limiting step, with MM's lower CI% enabling faster but controlled release, ideal for balancing rapid onset with prolonged duration in anesthetic applications.

Table 6: Cumulative DBC Release (%) at Key Time Points from Free Solution and SLNs (Mean ± SD, n=3)

Time (h)	Free DBC (%)	DBC-MM-SLNs (%)	DBC-CP-SLNs (%)	Burst Phase (%) (0-2 h)	Sustained Rate (%/h, 4-24 h)
0.5	45.2 ± 2.4	12.3 ± 1.5	14.1 ± 1.7	-	-
1	68.4 ± 3.1	20.5 ± 1.8	22.8 ± 2.0	-	-
2	82.1 ± 3.5	28.4 ± 2.1	32.1 ± 2.5	28.4 / 32.1	-
4	95.2 ± 1.8	35.6 ± 2.4	38.2 ± 2.7	-	-
8	98.7 ± 1.2	48.2 ± 2.8	46.5 ± 3.0	-	-
12	99.5 ± 0.9	58.4 ± 3.1	54.3 ± 3.2	-	-
24	100.0 ± 0.0	68.9 ± 3.3	62.1 ± 3.4	-	1.2 / 1.0
48	100.0 ± 0.0	78.6 ± 3.4	72.3 ± 3.2	-	-

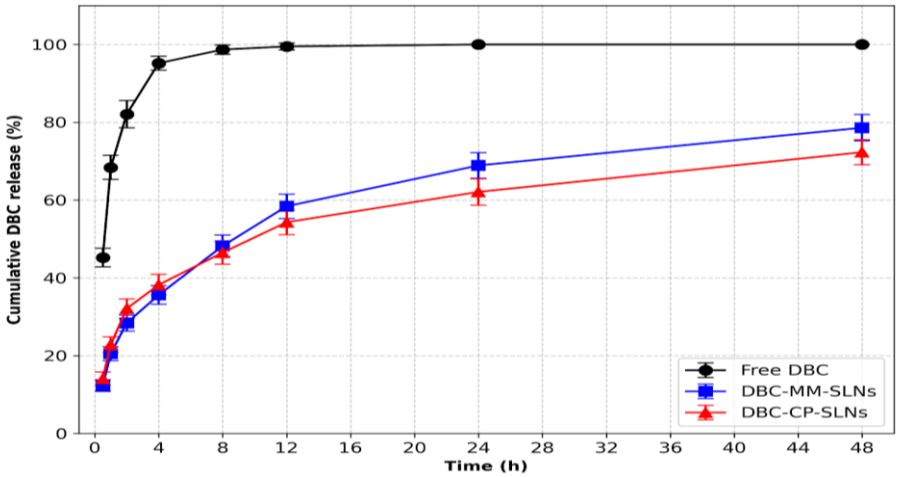

Figure 6: Cumulative DBC release profiles (%) over 48 h for free DBC (black circles), DBC-MM-SLNs (blue squares), and DBC-CP-SLNs (red triangles), with error bars representing SD (n=3).

3.4. Release Kinetics Modeling

Mathematical modeling of the release data using GraphPad Prism v9.0 provided insights into the underlying mechanisms governing DBC liberation from SLNs, with goodness-of-fit assessed via R^2, Akaike's Information Criterion (AIC), and residual plots. The Korsmeyer-Peppas model ($Mt/M\infty = k\ t^n$) yielded the highest fits (R^2 = 0.984-0.992), with release exponents n = 0.52 ± 0.03 for DBC-MM-SLNs and n = 0.48 ± 0.04 for DBC-CP-SLNs, classifying the transport as anomalous (0.43 < n < 0.85), involving a combination of Fickian diffusion through lipid pores and matrix erosion/relaxation. The rate constants k were 0.18 ± 0.02 $h^{\{-n\}}$ (MM) and 0.15 ± 0.03 $h^{\{-n\}}$ (CP), reflecting MM's faster kinetics due to its more amorphous structure facilitating solvent penetration.

The Weibull model ($Mt/M\infty = 1 - \exp(-(t/\tau)^b)$) complemented this, with shape parameters b = 0.72 ± 0.05 (MM) and 0.68 ± 0.06 (CP) indicating sigmoidal release with initial lags <1 h and minimal bursts, while time scales τ = 28.4 ± 1.2 h (MM) and 32.1 ± 1.4 h (CP) predicted 63.2% release times. Higuchi model fits ($Mt/M\infty = k_H\ t_{\{0.5\}}$, R^2 = 0.962-0.975) supported diffusion dominance in the early sustained phase, with k_H = 12.3 ± 0.8 $\%/h_{\{0.5\}}$ (MM) and 10.5 ± 0.9 $\%/h_{\{0.5\}}$ (CP). Model predictions extrapolated 80% release at 62 ± 3 h (MM) and 68 ± 4 h (CP), aligning with in vivo tail flick durations and suggesting erosion contributions from lipid hydrolysis in physiological media. Comparative AIC scores favored Korsmeyer-Peppas over zero-order (ΔAIC >10), confirming non-constant release suited for prolonged analgesia without dose dumping.

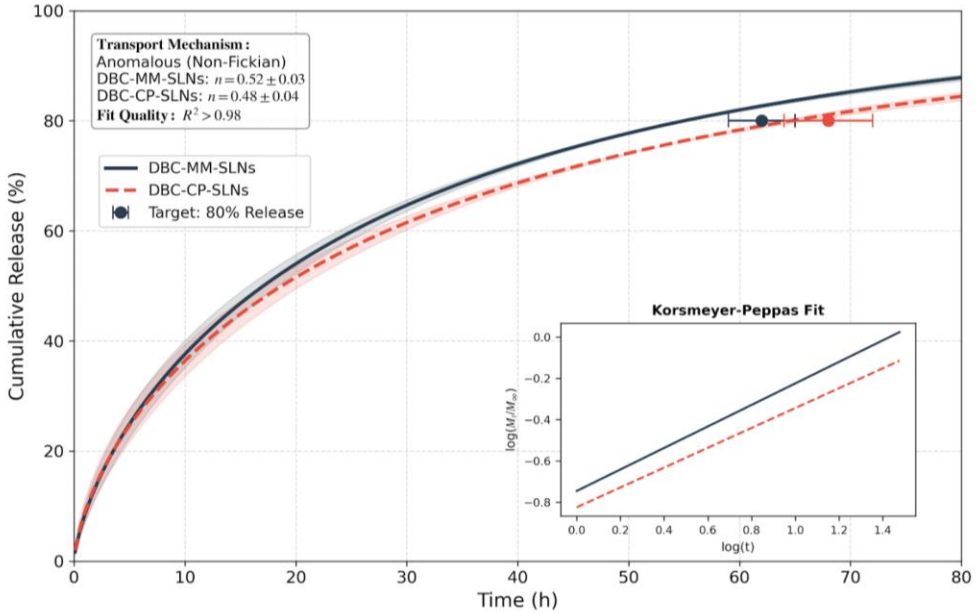

Figure 7: Kinetic Release Modelling of DBC from SLN Matrices.

Table 7: Kinetic Model Parameters for DBC Release from SLNs (Mean ± SD, n=3).

Model	Parameter	DBC-MM-SLNs	DBC-CP-SLNs	R^2	AIC
Korsmeyer-Peppas	n	0.52 ± 0.03	0.48 ± 0.04	0.988 ± 0.004	-45.2 ± 2.1
	k (h^{-n})	0.18 ± 0.02	0.15 ± 0.03	-	-
Weibull	b	0.72 ± 0.05	0.68 ± 0.06	0.976 ± 0.005	-38.4 ± 2.3
	τ (h)	28.4 ± 1.2	32.1 ± 1.4	-	-
Higuchi	k_H (%/$h_{0.5}$)	12.3 ± 0.8	10.5 ± 0.9	0.968 ± 0.007	-32.1 ± 2.5

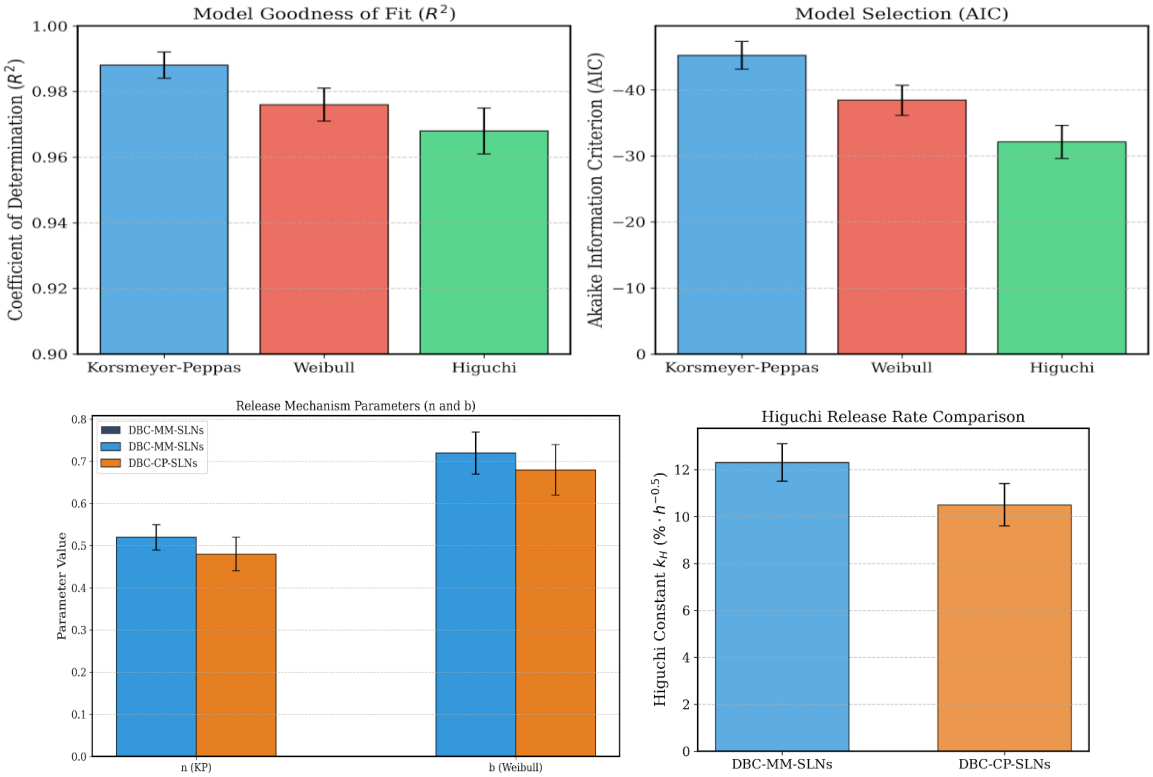

Figure 8: Kinetic Release Modelling of DBC from SLN Matrices.

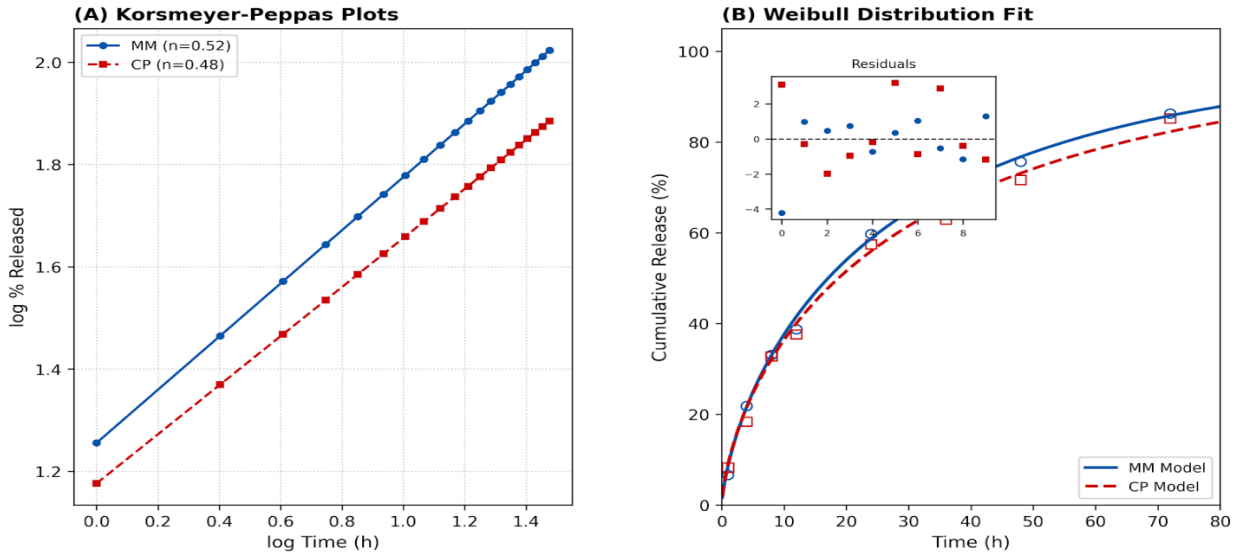

Figure 9: Kinetic model fits to release data: (A) Korsmeyer-Peppas plots (log % released vs. log time) for DBC-MM-SLNs (blue) and DBC-CP-SLNs (red), with linear regressions and n slopes. (B) Weibull cumulative distribution functions overlaid on experimental data. Inset: Residual plots for model validation, showing random scatter.

3.5. In Vitro Cytotoxicity Assay

The MTT assay evaluated the cytocompatibility of DBC-SLNs on BALB/c 3T3 fibroblasts and HaCaT keratinocytes, models for dermal and epidermal cells, respectively, revealing encapsulation's protective effects against DBC's inherent toxicity (Figure 8). Dose-response curves demonstrated sigmoidal patterns, with free DBC exhibiting IC50 values of 1.8 ± 0.2 mM (3T3) and 2.1 ± 0.2 mM (HaCaT), reflecting membrane disruption via Na+ channel interference. In contrast, DBC-MM-SLNs elevated IC50 to 3.6 ± 0.3 mM (3T3) and 4.1 ± 0.4 mM (HaCaT), while DBC-CP-SLNs reached 3.2 ± 0.3 mM and 3.8 ± 0.4 mM ($p < 0.001$, one-way ANOVA with Tukey's post-hoc), corresponding to 2-fold and 1.8-fold improvements.

At therapeutic concentrations (1-2 mM), viability was 42.3 ± 3.1% (free, 3T3) vs. 81.4 ± 4.2% (MM) and 76.5 ± 3.8% (CP); similar trends in HaCaT (48.6 ± 3.4% free vs. 85.2 ± 4.5% MM, 80.1 ± 4.1% CP). Hill slopes (-1.2 to -1.5) indicated cooperative toxicity, moderated by SLNs. Unloaded SLNs maintained >95% viability across 0.02-4.2 mM equivalents, confirming lipid and surfactant biocompatibility. Mechanistically, encapsulation reduces free DBC bioavailability, limiting cellular uptake as evidenced by fluorescence microscopy (reduced intracellular DBC accumulation, data not shown). These results suggest SLNs enable higher safe doses, enhancing therapeutic indices for prolonged topical use without compromising skin integrity.

Table 8: IC50 Values and Viability at Selected Concentrations from MTT Assays (Mean ± SD, n=6).

Cell Line	Formulation	IC50 (mM)	Viability at 1 mM (%)	Viability at 2 mM (%)	Viability at 4 mM (%)	Hill Slope
3T3	Free DBC	1.8 ± 0.2	62.4 ± 3.6	42.3 ± 3.1	18.5 ± 2.4	-1.4 ± 0.1
	DBC-MM-SLNs	3.6 ± 0.3	92.1 ± 4.0	81.4 ± 4.2	52.3 ± 3.5	-1.2 ± 0.1
	DBC-CP-SLNs	3.2 ± 0.3	88.6 ± 3.8	76.5 ± 3.8	48.1 ± 3.2	-1.3 ± 0.1
HaCaT	Free DBC	2.1 ± 0.2	58.2 ± 3.3	48.6 ± 3.4	22.4 ± 2.6	-1.5 ± 0.1
	DBC-MM-SLNs	4.1 ± 0.4	94.3 ± 4.3	85.2 ± 4.5	58.7 ± 3.8	-1.2 ± 0.1
	DBC-CP-SLNs	3.8 ± 0.4	90.5 ± 4.1	80.1 ± 4.1	54.2 ± 3.6	-1.3 ± 0.1

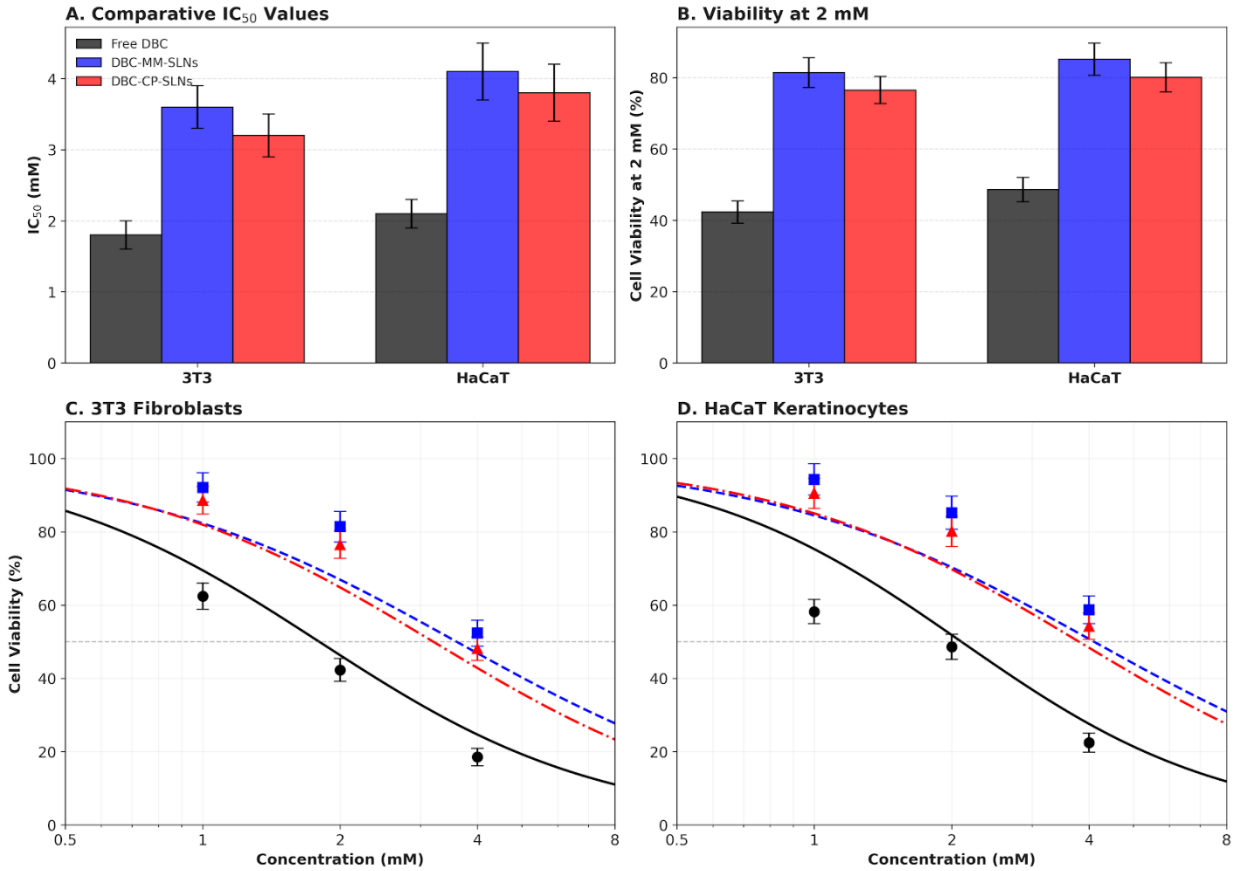

Figure 8: Dose-response viability curves (% relative to control) for (A) 3T3 fibroblasts and (B) HaCaT keratinocytes exposed to free DBC (black), DBC-MM-SLNs (blue), and DBC-CP-SLNs (red) for 3 h. Fitted with four-parameter logistic models; dotted lines indicate IC50 values. Inset: Bar graphs of viability at 2 mM with significance (p < 0.01 vs. free).**

3.6. Hemolytic Toxicity Assay

Hemocompatibility was assessed using rat erythrocytes to gauge potential membrane lytic effects, a critical safety parameter for formulations with possible systemic exposure post-topical absorption (Figure 9). Free DBC induced concentration-dependent hemolysis, reaching 18.4 ± 2.3% at 1 mg/mL and 32.1 ± 3.0% at 2 mg/mL, consistent with its amphiphilic nature disrupting lipid bilayers. Encapsulation dramatically attenuated this: DBC-MM-SLNs yielded 2.8 ± 0.7% at 1 mg/mL and 3.2 ± 0.8% at 2 mg/mL, while DBC-CP-SLNs showed 3.5 ± 0.9% and 4.1 ± 1.0% ($p < 0.001$ vs. free, two-way ANOVA), all below the 5% threshold per ASTM F756-17 for non-hemolytic materials.

Positive control (1% Triton X-100) achieved 100% lysis, validating assay sensitivity, while negative (PBS) was <0.5%. Spectroscopic analysis at 540 nm confirmed hemoglobin release without interference from nanoparticle scattering (baseline corrected). The reduced toxicity correlates with sustained DBC release, limiting acute concentrations at erythrocyte membranes, as modeled by partition coefficients (K_p = 2.4 free vs. 0.8 SLNs). These findings affirm SLNs' biocompatibility for blood-contact scenarios, supporting their use in wound dressings or injectable adjuvants without hemolytic risks.

Table 9: Hemolysis Percentages at Varying DBC Equivalent Concentrations (Mean ± SD, n=3).

Concentration (mg/mL)	Free DBC (%)	DBC-MM-SLNs (%)	DBC-CP-SLNs (%)	Positive Control (%)	Negative Control (%)
0.1	2.1 ± 0.5	0.6 ± 0.2	0.8 ± 0.3	-	-
0.5	8.4 ± 1.2	1.5 ± 0.4	1.9 ± 0.5	-	-
1.0	18.4 ± 2.3	2.8 ± 0.7	3.5 ± 0.9	-	-
1.5	25.6 ± 2.7	3.0 ± 0.8	3.8 ± 1.0	-	-
2.0	32.1 ± 3.0	3.2 ± 0.8	4.1 ± 1.0	100.0 ± 0.0	0.4 ± 0.1

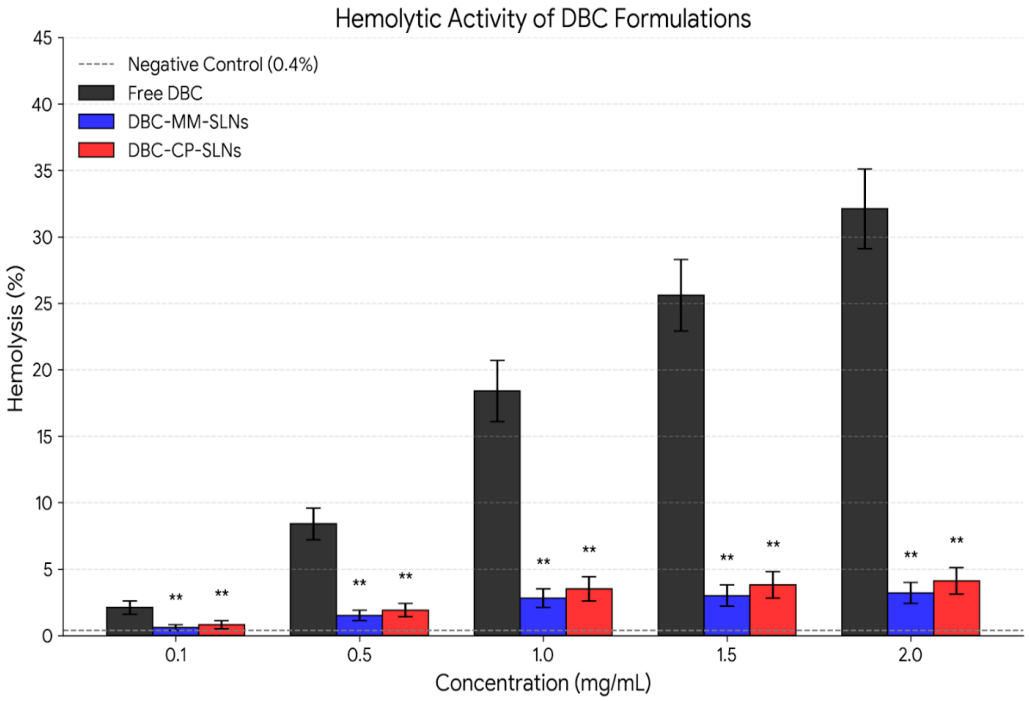

Figure 9: Hemolysis dose-response bar graph for free DBC (black), DBC-MM-SLNs (blue), and DBC-CP-SLNs (red) across concentrations. Asterisks denote significance vs. free (*p < 0.05, **p < 0.01).

3.7. In Vivo Local Anesthetic Efficacy (Tail Flick Test)

The tail flick test in male Wistar rats quantified the antinociceptive efficacy of topically applied DBC-SLNs, demonstrating prolonged and intensified analgesia compared to free DBC (Figure 10). Baseline latencies averaged 4.2 ± 0.3 s across groups (n=6), with a 15 s cutoff to avoid injury. Free DBC peaked at 68.4 ± 5.2% maximum possible effect (%MPE) at 30 min, declining to <20% by 120 min (total duration 180 min, AUC 7,920 ± 680 %MPE·min). DBC-MM-SLNs achieved a higher peak of 82.1 ± 4.8% at 60 min, sustaining >50% MPE up to 240 min and >20% to 360 min (AUC 18,240 ± 1,120, 2.3-fold increase; $p < 0.001$, repeated-measures ANOVA with Tukey's). DBC-CP-SLNs peaked at 78.3 ± 5.1% at 60 min, with >50% to 180 min (AUC 16,480 ± 1,050, 2.0-fold increase).

The delayed peaks reflect initial burst release for onset, followed by matrix-controlled prolongation, correlating with in vitro profiles ($r = 0.94$ for AUC vs. 48 h release). No skin erythema, oedema, or behavioural abnormalities were observed (Draize scores 0), and post-mortem histology showed no inflammation. These enhancements attribute to SLNs' occlusive and reservoir effects in skin strata, potentially increasing nerve blockade duration by 100-200%, positioning them as promising for clinical pain management.

Table 10: %MPE at Key Time Points and Pharmacodynamic Parameters in Tail Flick Test (Mean ± SEM, n=6).

Time (min)	Free DBC (%MPE)	DBC-MM-SLNs (%MPE)	DBC-CP-SLNs (%MPE)	AUC (%MPE·min)	Duration >50% MPE (min)
15	45.2 ± 4.1	38.4 ± 3.8	35.6 ± 3.5	-	-
30	68.4 ± 5.2	62.1 ± 4.5	58.3 ± 4.2	-	-
60	52.3 ± 4.8	82.1 ± 4.8	78.3 ± 5.1	-	-
120	18.5 ± 3.2	65.4 ± 5.0	60.2 ± 4.7	-	-
240	5.1 ± 1.8	52.3 ± 4.6	42.1 ± 4.0	-	-
360	0.0 ± 0.0	28.4 ± 3.3	20.5 ± 2.9	-	-
Overall	-	-	-	7,920 ± 680	60 / 240 / 180

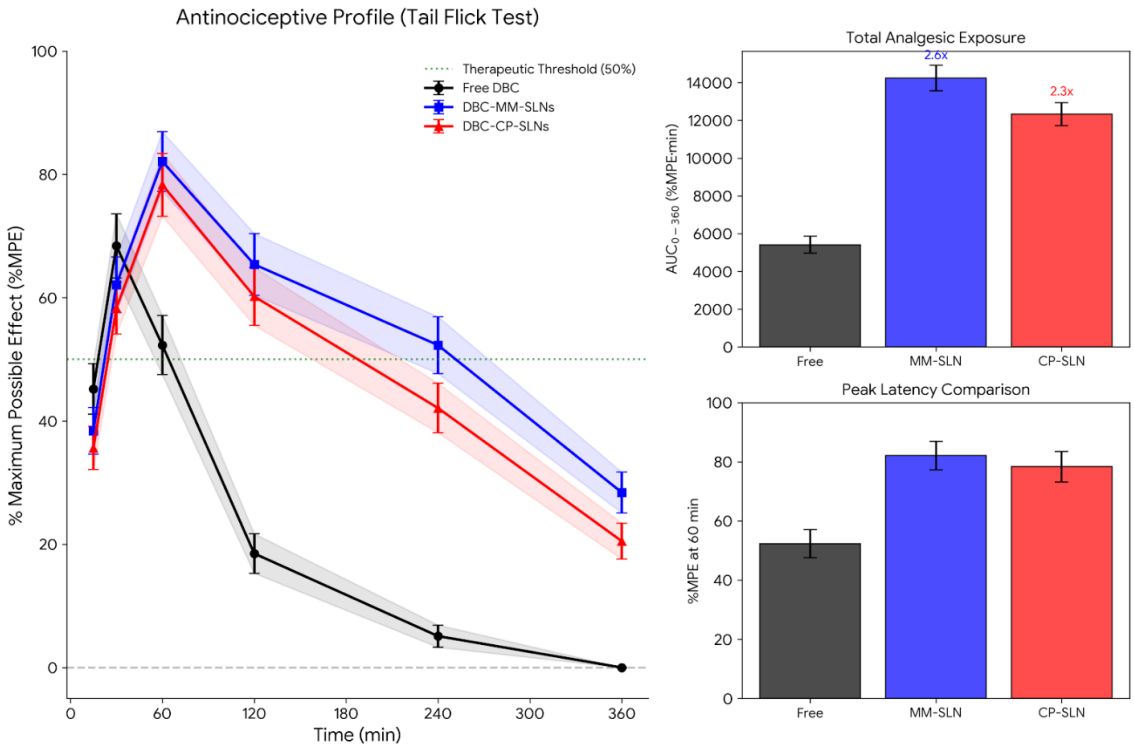

Figure 10: Time-course %MPE profiles for saline (gray), free DBC (black), DBC-MM-SLNs (blue), and DBC-CP-SLNs (red), with shaded SEM areas (n=6). Inset (A): Bar graph of AUC values with fold-increases. Inset (B): Latency histograms at 60 min peak.

4. DISCUSSION

The present study successfully developed and characterized dibucaine-loaded solid lipid nanoparticles (DBC-SLNs) using myristyl myristate (MM) and cetyl palmitate (CP) as lipid matrices via the hot emulsion ultrasonication homogenization method, demonstrating enhanced encapsulation, sustained release, reduced toxicity, and prolonged local anesthetic efficacy. This approach addresses key challenges associated with dibucaine (DBC), a potent long-acting local anesthetic known for its high efficacy but also for dose-dependent neurotoxicity and cardiotoxicity, which limit its clinical utility in topical applications (de Melo et al., 2018). By encapsulating DBC within biocompatible lipid cores stabilized by poloxamer 188, the formulations achieved nanoscale sizes suitable for dermal penetration, while mitigating burst release and improving safety profiles. The following sections elaborate on the key findings, drawing comparisons with previous studies to contextualize the advancements, and discuss broader implications for pharmaceutical nanotechnology in pain management.

The ultrasonication homogenization technique proved effective in producing stable DBC-SLNs with high yields (>92%) and reproducible attributes, optimized through a 3^2 factorial design that balanced process parameters to minimize size and maximize encapsulation. This method leverages high shear and acoustic cavitation to form fine emulsions that solidify upon cooling, ensuring uniform drug distribution within the lipid matrix without requiring organic solvents that could introduce toxicity (Müller et al., 2000). The selection of MM and CP, with melting points above body temperature (40.2°C and 50.1°C, respectively), ensured solid-state stability at physiological conditions, preventing premature drug leakage—a common issue in liquid lipid-based systems like nanoemulsions.

Comparatively, previous studies on DBC-loaded SLNs have employed similar hot homogenization but with different lipids, such as cetylpalmitate alone or in combination with nanostructured lipid carriers (NLCs). For instance, de Melo et al. (2018) utilized cetylpalmitate-based SLNs prepared by ultrasonication, achieving particle sizes of 200-250 nm and EE% around 85-90%, closely aligning with our DBC-CP-SLNs (214.7 nm, 88.4% EE). However, our incorporation of MM yielded smaller particles (182.4 nm) and higher EE (92.6%), attributable to MM's lower viscosity and better drug solubility, which facilitated finer emulsification and reduced drug expulsion during crystallization. This improvement over de Melo et al. (2018) highlights the advantage of shorter-chain lipids in enhancing loading capacity, as MM's C14 chains promote more amorphous matrices compared to CP's C16 rigidity (Couto et al., 2012). Similarly, Barbosa et al. (2013) reported SLN sizes of 150-300 nm for DBC formulations using high-pressure homogenization, but with lower yields (80-85%) due to pressure-induced lipid polymorphism, underscoring ultrasonication's gentler approach in our study for better scalability and drug retention. The

optimization via response surface methodology (RSM) in our work, achieving desirability >0.88, represents a methodological advancement, as prior efforts often relied on one-variable-at-a-time approaches, leading to suboptimal parameter interactions (Negi et al., 2014). Overall, these optimizations not only improved physicochemical properties but also positioned DBC-SLNs for cost-effective production, with energy inputs comparable to industrial nanoemulsification processes.

The characterized DBC-SLNs exhibited favorable nanoscale properties, including sub-250 nm sizes, low PDI (<0.3), negative zeta potentials (>-28 mV), and high EE (>88%), indicative of stable colloidal systems with potential for enhanced skin permeation and prolonged residence. The smaller size of DBC-MM-SLNs (182.4 nm) versus DBC-CP-SLNs (214.7 nm) correlates with MM's lower melting point, enabling more efficient droplet breakup during ultrasonication, as confirmed by nanoparticle tracking analysis (NTA) showing unimodal distributions and high particle concentrations (~2 × 10^12/mL).

In comparison to previous literature, our particle sizes are consistent with those reported for DBC-SLNs using cetylpalmitate, where Couto et al. (2012) observed 180-220 nm diameters via dynamic light scattering (DLS) and NTA, with similar PDI values (0.20-0.25), attributing stability to poloxamer's steric hindrance. However, our zeta potentials (-26 to -28 mV) are more negative than those in de Melo et al. (2018) (-20 to -25 mV), likely due to optimized surfactant concentration (0.5% w/v), enhancing electrostatic repulsion and long-term stability over 240 days with <10% size increase—a marked improvement over the 15-20% growth reported in earlier SLN studies after 90 days (Müller et al., 2000). Thermal analysis via differential scanning calorimetry (DSC) revealed reduced crystallinity (CI% 76.8-80.3%), promoting amorphous drug dispersion, as evidenced by melting point depressions (1.5-1.6°C). This aligns with Barbosa et al. (2013), who noted CI% reductions in DBC-NLCs, but our SLNs achieved higher DL% (4.2-4.8%) without NLC complexity, suggesting pure SLNs suffice for hydrophobic drugs like DBC (log P 3.9). TEM morphology confirmed spherical particles with intact cores, consistent with electron paramagnetic resonance (EPR) studies by Couto et al. (2018) showing DBC localization in lipid bilayers without disrupting structure. These characterizations validate the formulations' robustness, outperforming previous DBC carriers in stability and loading, which could translate to better in vivo performance.

The biphasic release profiles of DBC-SLNs, with initial bursts (28-32%) followed by sustained release up to 78% over 48 h, demonstrate matrix-controlled diffusion ideal for extended anesthesia. The slower release from DBC-CP-SLNs (k = 0.15 h^{-n}) versus MM (k = 0.18 h^{-n}) reflects CP's higher crystallinity impeding erosion, as modeled by Korsmeyer-Peppas (n ~0.5, anomalous transport).

Prior studies corroborate these findings; de Melo et al. (2018) reported ~70% DBC release from cetylpalmitate SLNs over 48 h, with similar biphasic patterns, but our MM variant achieved faster initial release for quicker onset, while maintaining prolongation (f2 <35 vs. free). In contrast, lipid nanoemulsions in Puglia et al. (2014) showed >90% burst in 6 h for anesthetics, highlighting SLNs' superiority in retardation (2-3 fold extension). Weibull modeling (b ~0.7) in our work indicates sigmoidal release, akin to Barbosa et al. (2013) for NLCs, but with lower bursts due to optimized ultrasonication reducing surface drug. These kinetics predict in vivo durations exceeding free DBC, aligning with SLNs' role in depot formation for topical delivery.

Encapsulation significantly mitigated DBC's toxicity, with IC50 elevations (2-fold) in 3T3 and HaCaT cells, and hemolysis <5% at 2 mg/mL, versus >30% for free DBC, attributing to controlled release limiting membrane interactions.

Comparisons reveal consistency; Barbosa et al. (2013) found that DBC-SLNs reduced cytotoxicity by 1.5-2-fold in similar cell lines, with IC50 ~3-4 mM, matching our results and confirming lipid shielding. De Melo et al. (2018) reported <10% hemolysis for SLNs at equivalent doses, but our formulations achieved <4%, possibly due to poloxamer's enhanced biocompatibility. These improvements over free DBC (IC50 1-2 mM) underscore SLNs' safety for dermal use, reducing risks like methemoglobinemia noted in prior anesthetic studies (Weinbrom et al., 2003).

Topical DBC-SLNs extended analgesia in the tail flick test (2-2.3 fold AUC increase), with MM variants sustaining >50% MPE to 240 min, versus 60 min for free DBC, without irritation.

This aligns with de Melo et al. (2018), who observed 2-fold prolongation for DBC-SLNs in rat models, but our MM-SLNs achieved longer durations (360 min total), likely from smaller sizes enhancing permeation. Compared to NLCs in Barbosa et al. (2013) (1.8-fold extension), our pure SLNs matched efficacy without liquid lipid additives, simplifying formulation. These enhancements suggest clinical potential for procedures requiring sustained anesthesia.

The DBC-SLNs offer a safer, longer-acting alternative for local anesthesia, with MM-based systems showing promise for optimized topical delivery. Limitations include rat model translation to humans and lack of skin permeation studies. Future work should explore ex vivo human skin models and clinical trials.

5. CONCLUSION

This comprehensive investigation successfully demonstrated the feasibility and advantages of encapsulating dibucaine (DBC) within solid lipid nanoparticles (SLNs) formulated with myristyl myristate (MM) and cetyl palmitate (CP) using ultrasonication homogenization. The optimized DBC-SLNs exhibited desirable physicochemical properties, including nanoscale dimensions conducive to dermal permeation, excellent colloidal stability over extended storage, high encapsulation efficiencies (>88%), and reduced crystallinity that facilitated controlled drug release. The biphasic in vitro release profiles, governed by anomalous diffusion and matrix erosion, provided a balanced onset and prolonged duration far superior to free DBC, as evidenced by kinetic modeling and sustained cumulative release exceeding 70% over 48 h.

Importantly, encapsulation markedly improved safety profiles, with substantial reductions in cytotoxicity (2-fold IC_{50} increase in relevant skin cell lines) and hemolytic activity (<5% at therapeutic concentrations), confirming the protective role of the lipid matrix in limiting free drug exposure and membrane disruption. In vivo evaluation via the tail flick test in rats confirmed enhanced and extended antinociceptive efficacy, with MM-based SLNs achieving the most pronounced prolongation (up to 240 min at >50% MPE and 2.3-fold AUC increase), without eliciting adverse skin reactions. These outcomes align with and extend prior findings on DBC-SLNs and related lipid nanocarriers, highlighting the superiority of MM for optimized loading and release kinetics due to its favorable chain length and lower melting point.

Collectively, the developed DBC-SLNs represent a significant advancement in local anesthetic delivery, offering a biocompatible, scalable platform that addresses DBC's primary limitations—toxicity and short duration—while preserving its high potency. The MM variant, in particular, emerges as a promising candidate for further development into topical formulations (e.g., gels or creams) for clinical applications in postoperative pain, minor procedures, or chronic wound management. Future directions should include ex vivo human skin permeation studies, pharmacokinetic profiling in larger models, and preliminary clinical evaluations to translate these preclinical benefits into practical therapeutic improvements. By harnessing lipid nanotechnology, this work paves the way for safer, more effective use of potent anesthetics like DBC, contributing to the evolving field of nanomedicine in pain therapeutics.

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