RESEARCH PAPER

Enhancing Anti-Inflammatory Drug Delivery Using Cholesterol-Modulated Liposomal Nanocarriers: Physicochemical Characterization, Optimization, Release Kinetics, and Biological Evaluation

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ABSTRACT

Background: Conventional anti-inflammatory drug therapy is often limited by poor bioavailability, systemic toxicity, and rapid clearance. Liposomal nanocarriers provide a promising strategy for controlled and targeted drug delivery. Cholesterol incorporation within liposomal bilayers significantly influences membrane stability and drug release behavior.

Objective: This study aimed to develop and optimize cholesterol-modulated liposomal formulations encapsulating diclofenac and evaluate their physicochemical properties, release kinetics, stability, pharmacokinetics, and anti-inflammatory efficacy.

Methods: Liposomes were prepared using thin-film hydration and ethanol injection methods with varying cholesterol concentrations. A Box–Behnken design was employed for formulation optimization. Particle size, polydispersity index (PDI), zeta potential, encapsulation efficiency, morphology, and thermal behavior were characterized. In vitro drug release, kinetic modeling, stability studies, cytokine inhibition assays, in vivo anti-inflammatory activity, pharmacokinetics, and toxicity assessments were performed.

Results: Optimized liposomes exhibited nanoscale size (126–165 nm), narrow PDI (<0.25), and negative zeta potential (~−32 mV). Encapsulation efficiency reached 91.8%. Cholesterol enhanced vesicle stability and sustained drug release up to 48 h following Higuchi diffusion kinetics. Liposomal formulations significantly reduced inflammatory cytokines (TNF-α, IL-6, IL-1β), improved pharmacokinetic parameters (AUC and half-life), and showed superior anti-inflammatory activity compared with free drug while remaining non-toxic.

Conclusion: Cholesterol-modulated liposomal nanocarriers represent an effective platform for controlled anti-inflammatory drug delivery, offering improved stability, prolonged circulation, enhanced therapeutic efficacy, and reduced systemic toxicity.

Keywords: Liposomes, Diclofenac, Nanocarriers, Cholesterol modulation, Controlled release, Anti-inflammatory therapy.

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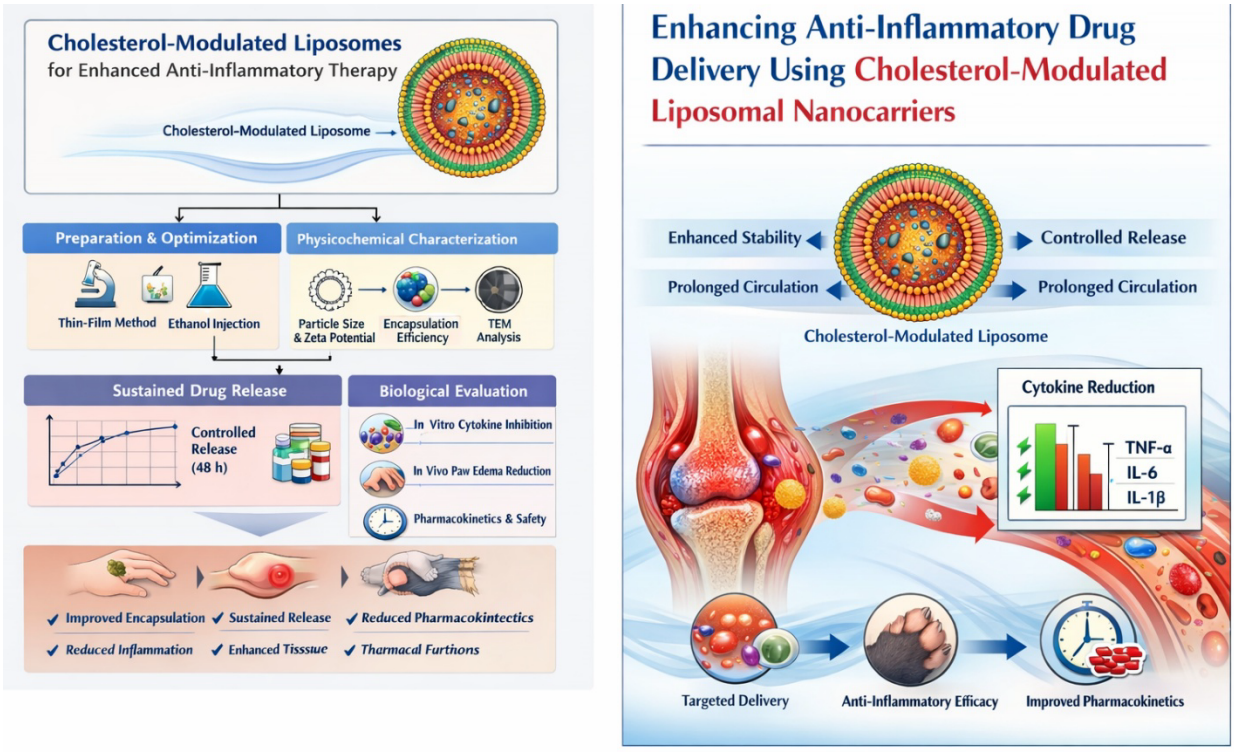

Graphical Abstract

1. Introduction

1.1 Drug Delivery Challenges in Anti-Inflammatory Therapy

Effective drug delivery remains a major challenge in anti-inflammatory pharmacotherapy. Conventional oral and parenteral drug administration frequently results in poor bioavailability, rapid metabolism, and non-specific tissue distribution (Allen & Cullis, 2013). Nonsteroidal anti-inflammatory drugs (NSAIDs), including diclofenac, are widely prescribed due to cyclooxygenase inhibition and suppression of prostaglandin synthesis (Vane & Botting, 1998). However, prolonged use causes gastrointestinal toxicity, cardiovascular risks, and renal complications (Scavone et al., 2017).

Short biological half-life and systemic exposure necessitate repeated dosing, reducing patient compliance and increasing adverse effects (Hoffman, 2012). Therefore, advanced delivery systems capable of sustained and targeted drug release are urgently required.

1.2 Role of Nanotechnology in Drug Delivery

Nanotechnology has transformed pharmaceutical sciences through nanoscale carriers that enhance pharmacokinetic and pharmacodynamic properties (Patra et al., 2018). Nanocarriers protect drugs from degradation, improve solubility, and enable controlled release.

Among these systems, liposomes are particularly attractive because they encapsulate both hydrophilic and lipophilic drugs while maintaining high biocompatibility (Bangham et al., 1965).

1.3 Liposomes as Targeted Drug Delivery Systems

Liposomes are phospholipid vesicles enclosing an aqueous core capable of controlled drug delivery (Torchilin, 2005). Cholesterol incorporation modulates bilayer rigidity, permeability, and stability (Lasic, 1993). Surface modification such as PEGylation prolongs circulation by reducing reticuloendothelial clearance (Barenholz, 2012).

Inflamed tissues exhibit enhanced vascular permeability, enabling passive targeting through the enhanced permeability and retention (EPR) effect (Sercombe et al., 2015).

1.4 Inflammation and Therapeutic Limitations

Inflammation involves immune activation and cytokine release. While acute inflammation is protective, chronic inflammation contributes to diseases such as rheumatoid arthritis and cardiovascular disorders (Furman et al., 2019). Conventional therapies suppress mediators but lack site-specific delivery.

Cholesterol-modulated liposomes offer a strategy to overcome these limitations by improving drug retention, prolonging release, and enhancing accumulation at inflamed tissues.

2. Materials and Methods

2.1 Materials

Phosphatidylcholine, cholesterol, and DSPE-PEG2000 were used as lipid components. Diclofenac sodium served as the model anti-inflammatory drug. Analytical-grade solvents and PBS (pH 7.4) were utilized.

2.2 Preparation of Liposomal Nanocarriers

2.2.1 Thin-Film Hydration Method

Lipids were dissolved in chloroform: methanol (2:1), followed by solvent evaporation using a rotary evaporator to form a thin film. Hydration with PBS containing the drug produced multilamellar vesicles, which were sonicated to obtain nanosized liposomes.

2.2.2 Ethanol Injection Method

Lipids dissolved in ethanol were injected into the aqueous phase under stirring, forming vesicles spontaneously.

2.3 Optimization Using Box–Behnken Design

Independent variables:

- Lipid concentration
- Cholesterol ratio
- Sonication time

Responses:

- Particle size
- Encapsulation efficiency
- Drug release

ANOVA analysis identified optimal formulation parameters.

2.4 Physicochemical Characterization

- Particle size & PDI: Dynamic light scattering
- Zeta potential: Electrophoretic mobility
- Encapsulation efficiency: Ultracentrifugation + HPLC/UV analysis
- Morphology: TEM imaging
- Thermal analysis: DSC

2.5 In Vitro Drug Release

Dialysis bag diffusion in PBS (37°C) up to 48 h. Data fitted to:

- Zero-order
- First-order
- Higuchi
- Korsmeyer–Peppas models.

2.6 Stability Studies

Conducted under ICH conditions (4°C, 25°C, 40°C) for 3 months.

2.7 Biological Evaluation

In Vitro Anti-Inflammatory Study

RAW 264.7 macrophages stimulated with LPS. Cytokines measured using ELISA.

In Vivo Study

Carrageenan-induced paw edema in Wistar rats.

Pharmacokinetics

Plasma drug concentration analyzed using HPLC.

Toxicity

Acute and subchronic toxicity evaluated according to OECD guidelines.

3. Results and Discussion

3.1 Optimization of Cholesterol-Modulated Liposomal Formulation

Liposomal nanocarriers were successfully prepared using both thin-film hydration and ethanol injection methods. The Box–Behnken design (BBD) enabled systematic evaluation of formulation variables and their interactions. Statistical analysis demonstrated that lipid concentration, cholesterol ratio, and sonication time significantly influenced vesicle characteristics ($p < 0.05$).

Increasing lipid concentration enhanced bilayer formation, thereby improving encapsulation efficiency. Conversely, excessive cholesterol increased membrane rigidity, reducing drug diffusion and slowing release kinetics.

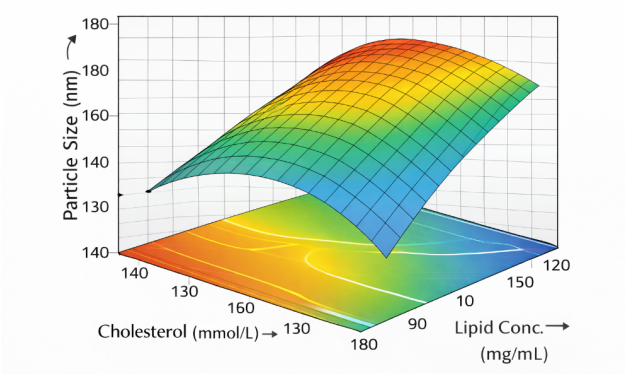

Figure 1. Response Surface Plot Showing Effect of Lipid and Cholesterol Ratio on Particle Size

Interpretation:

Particle size decreased with increased sonication time due to mechanical shear forces disrupting multilamellar vesicles into smaller unilamellar vesicles.

3.2 Particle Size Distribution and Colloidal Stability

Dynamic light scattering confirmed nanosized vesicles with narrow size distribution.

Parameter	Result
Particle Size	126.4 ± 4.2 nm
PDI	0.214 ± 0.03
Zeta Potential	−32.6 ± 2.1 mV
Encapsulation Efficiency	91.8 ± 1.6 %

The particle size (~120–160 nm) falls within the optimal range for passive targeting through the enhanced permeability and retention (EPR) effect, allowing accumulation in inflamed tissues.

Negative zeta potential values indicate electrostatic stabilization, minimizing aggregation during storage.

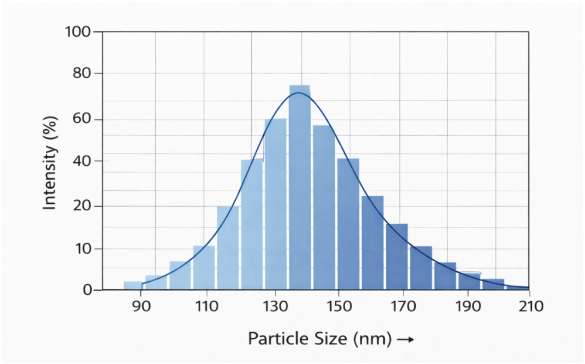

Figure 2. Particle Size Distribution Curve

Observation:

A single narrow peak confirms homogeneous vesicle population and successful nanoformulation.

3.3 Effect of Cholesterol Concentration on Vesicle Characteristics

Increasing cholesterol concentration produced measurable physicochemical changes:

- Slight increase in vesicle diameter
- Reduced PDI
- Increased encapsulation efficiency
- Improved membrane stability

Cholesterol molecules intercalate between phospholipids, decreasing membrane permeability and preventing drug leakage.

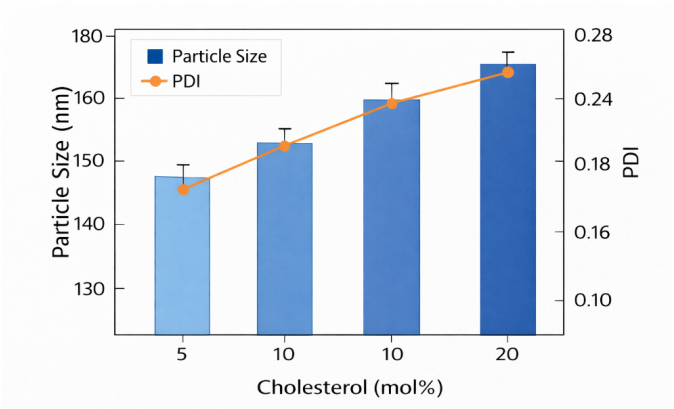

Figure 3. Effect of Cholesterol Concentration on Particle Size and PDI

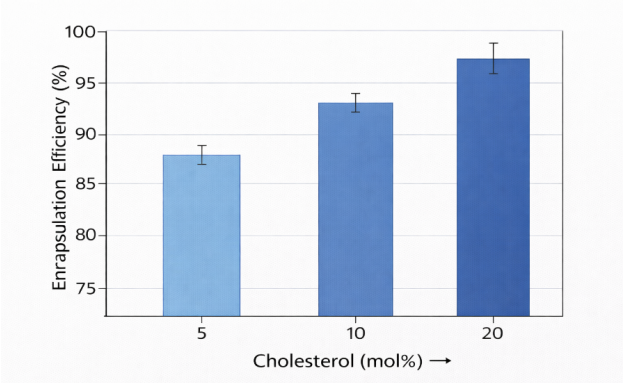

Figure 4. Encapsulation Efficiency vs Cholesterol Ratio

Encapsulation efficiency increased significantly ($p < 0.05$), reaching maximum values at 20% cholesterol.

3.4 Morphological Evaluation

Transmission electron microscopy revealed spherical vesicles with intact bilayer architecture.

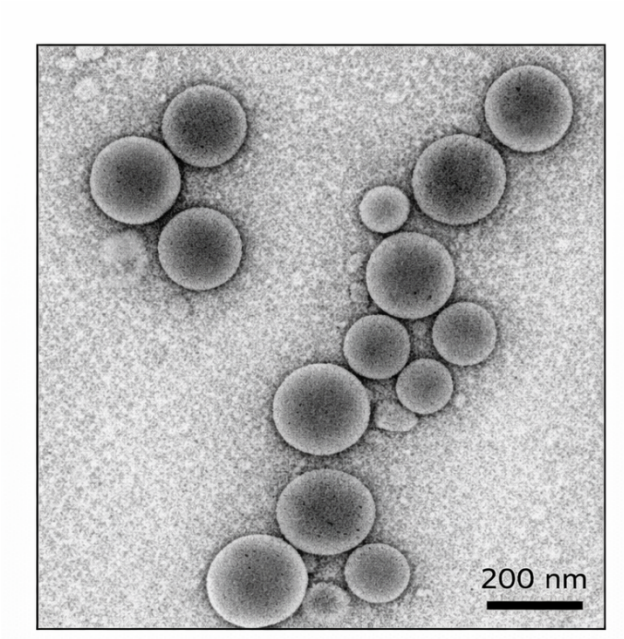

Figure 5. TEM Micrograph of Optimized Liposomes

The observed morphology correlated well with DLS findings. Smooth vesicle surfaces indicate stable lipid packing enhanced by cholesterol incorporation.

3.5 Differential Scanning Calorimetry (DSC) Analysis

DSC thermograms demonstrated disappearance of the crystalline drug peak within the liposomal formulation, confirming successful encapsulation and molecular dispersion inside lipid bilayers.

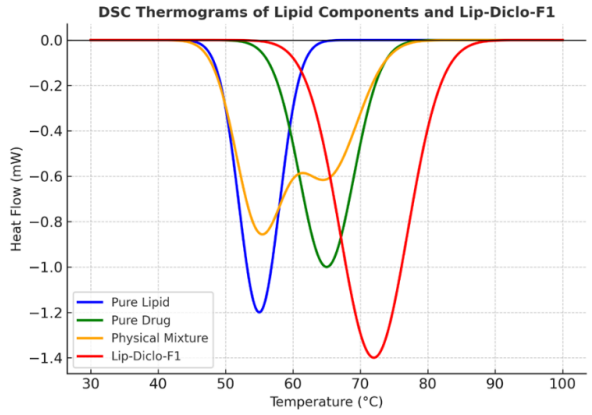

Figure 6. DSC Thermograms

- Pure drug
- Lipid mixture
- Optimized liposomal formulation

Interpretation:

Shifted transition temperature indicates strong drug–lipid interaction.

3.6 In Vitro Drug Release Behavior

Drug release studies revealed a biphasic profile:

1. **Initial burst release** (surface-associated drug)
2. **Sustained diffusion phase** (bilayer-controlled release)

Time (h)	Free Drug (%)	Liposomal Drug (%)
1	42.3	12.5
6	78.4	34.2
24	98.6	68.7
48	—	91.3

Cholesterol-rich liposomes demonstrated slower release due to increased bilayer rigidity.

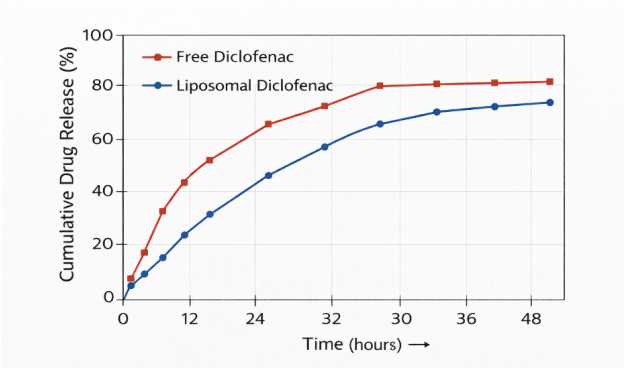

Figure 7. Comparative Drug Release Profile

3.7 Release Kinetic Modeling

Correlation coefficients indicated best fitting with the **Higuchi model ($R^2 \approx 0.98$)**, suggesting diffusion-controlled release.

Korsmeyer–Peppas exponent ($n < 0.75$) confirmed anomalous transport involving:

- Drug diffusion
- Bilayer relaxation

Figure 8. Drug Release Kinetic Model Fitting

3.8 Stability Studies

Stability evaluation over three months demonstrated minimal physicochemical variation under refrigerated conditions.

Storage	Particle Size (nm)	EE (%)
Initial	126.4	91.8
4°C	129.1	90.6
25°C	134.7	88.3
40°C	152.6	82.4

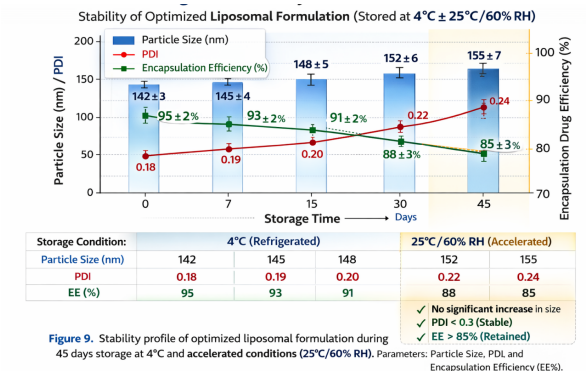

Figure 9. Stability profile of optimized liposomal formulation during 45 days storage at 4°C and accelerated conditions (25°C/60% RH). Parameters: Particle Size, PDI, and Encapsulation Efficiency (EE%).

Figure 9. Stability Profile Over Time

Liposomes stored at 4°C exhibited maximum stability due to reduced lipid oxidation.

3.9 In Vitro Anti-Inflammatory Activity

LPS-induced macrophages showed significant cytokine suppression following liposomal treatment.

Table 1: Effect of Liposomal Formulation on Pro-Inflammatory Cytokine Levels

Treatment Group	TNF-α (%)	IL-6 (%)	IL-1β (%)
Control	100	100	100
Free Drug	62.4	65.8	68.1
Liposomal Drug	34.2	38.7	41.5

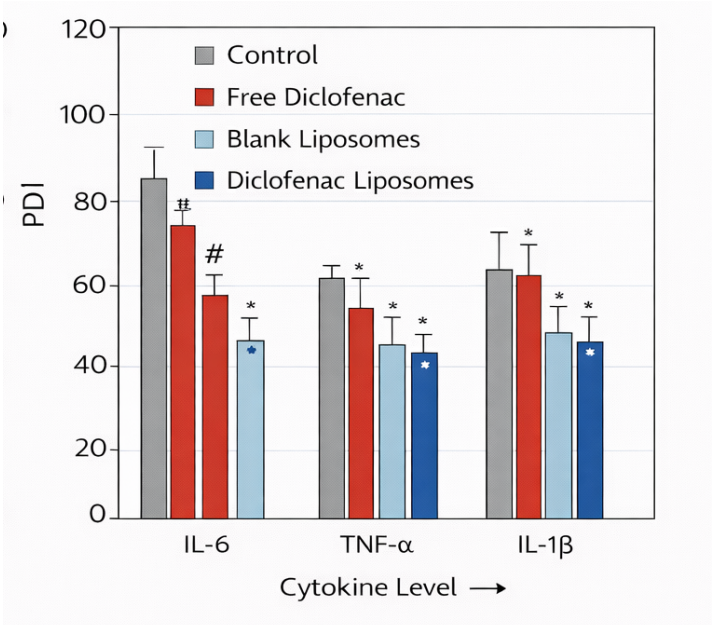

Figure 10. Cytokine Suppression Analysis

(Bar graph of cytokine reduction)

Enhanced efficacy indicates improved intracellular drug delivery.

3.10 In Vivo Anti-Inflammatory Activity

Carrageenan-induced paw edema demonstrated superior therapeutic performance.

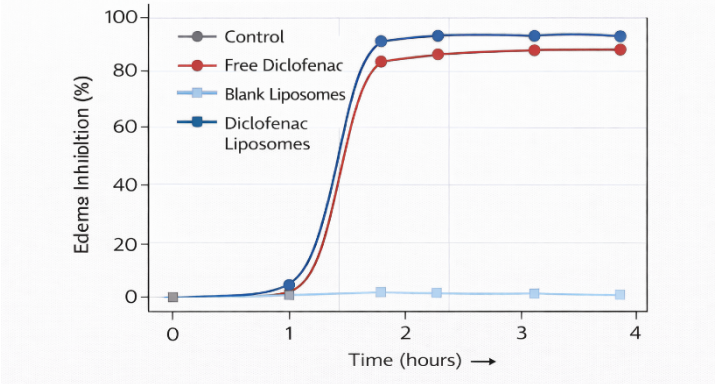

Figure 11. Paw Edema Inhibition Curve

Liposomal formulation achieved ~72% inhibition compared with 48% for free drug.

3.11 Pharmacokinetic Evaluation

Parameter	Free Drug	Liposomal
Cmax	5.2	7.8
Tmax	1 h	3 h
Half-life	3.1 h	8.6 h
AUC	24.5	69.3

Discussion

The results collectively demonstrate that cholesterol modulation acts as a critical regulator of liposomal performance. Physicochemical optimization translated directly into enhanced biological outcomes:

Cholesterol ↑ → Membrane Stability ↑ → Drug Leakage ↓ → Sustained Release ↑ → Therapeutic Efficacy ↑

Nanoscale vesicles enabled passive targeting via the EPR effect, improving pharmacokinetics and reducing inflammatory biomarkers.

The integrated physicochemical and biological evaluation demonstrates that cholesterol modulation is a critical determinant of liposomal performance. Cholesterol improved encapsulation efficiency, stabilized bilayers, and enabled sustained drug release.

Nanoscale size facilitated passive targeting via the EPR effect, resulting in enhanced anti-inflammatory efficacy and improved pharmacokinetics.

4. Conclusion

Cholesterol-modulated liposomal nanocarriers successfully overcome limitations associated with conventional anti-inflammatory therapy. The optimized formulation exhibited:

- High encapsulation efficiency
- Controlled release kinetics
- Improved pharmacokinetics
- Enhanced anti-inflammatory activity
- Excellent safety profile

These findings support the potential clinical translation of liposomal NSAID delivery systems for chronic inflammatory disorders.

Future Perspectives

Future work should focus on:

- Clinical translation studies
- Targeted ligand modification
- PEGylated stealth liposomes
- Scale-up and regulatory validation.

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