RESEARCH PAPER

Formulation Development and Evaluation of Tofacitinib-Loaded PEGylated Liposomal Nanocarriers for Improved Drug Bioavailability

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ABSTRACT

Background

Tofacitinib, an oral Janus kinase (JAK) inhibitor, demonstrates significant clinical efficacy in autoimmune therapies, yet its therapeutic potential is hindered by a short elimination half-life and poor systemic bioavailability. This study aimed to design, mathematically optimize, and meticulously characterize tofacitinib-loaded PEGylated liposomes to overcome these biopharmaceutical limitations and facilitate sustained systemic delivery.

Methods

The nanocarriers were formulated employing the thin-film hydration technique. A three-factor, three-level Box-Behnken Design (BBD) was utilized to systematically optimize the phospholipid-to-cholesterol ratio, drug-to-lipid mass ratio, and DSPE-PEG2000 molar concentration. The optimized nanocarriers were extensively evaluated for their physicochemical attributes, morphological topology (via TEM), in-vitro release kinetics, and long-term thermodynamic stability.

Results

The optimally formulated PEGylated liposomes (4.2:1 phospholipid-to-cholesterol ratio and 4.8 mol% DSPE-PEG2000) exhibited a highly uniform, monodisperse nanoscale geometry with a mean hydrodynamic diameter of 118.25 ± 2.14 nm, a polydispersity index (PDI) of 0.152, and a robust zeta potential of −32.4 mV. The synergistic rigidification by cholesterol and steric stabilization by the PEG corona yielded a maximum entrapment efficiency of 83.15 ± 1.55%. In-vitro dissolution profiling revealed a sustained, biphasic drug release over 72 hours that strictly obeyed Higuchi matrix kinetics, dictated by Fickian diffusion (n = 0.37). Furthermore, the colloidal dispersion demonstrated exceptional structural resilience and payload retention (>81%) over a 90-day stability study at 4 °C.

Conclusion

The successful encapsulation of tofacitinib within thermodynamically stable PEGylated liposomes provides a robust, size-optimized nanocarrier platform capable of prolonged circulation and sustained diffusion. This formulation presents a highly translational strategy for improving the systemic bioavailability and therapeutic index of tofacitinib in clinical settings.

Keywords: Tofacitinib; PEGylated liposomes; Box-Behnken Design; Sustained release; Bioavailability; Nanocarriers.

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1. Introduction

Tofacitinib is an oral Janus kinase (JAK) inhibitor widely established for the management of autoimmune and inflammatory conditions, most notably rheumatoid arthritis (Fleischmann et al., 2012). Despite its well-documented clinical efficacy, the therapeutic utility of tofacitinib is partially constrained by its pharmacokinetic profile, characterized by a relatively short elimination half-life of approximately 3 hours, which necessitates frequent dosing and can result in fluctuating plasma concentrations (Dowty et al., 2014). Furthermore, conventional oral administration can expose patients to systemic adverse effects and non-specific biodistribution, highlighting a critical need for advanced delivery systems capable of modulating its release profile and enhancing targeted or sustained bioavailability. To overcome these biopharmaceutical challenges, liposomes have been extensively investigated as highly biocompatible nanocarriers. Composed of amphiphilic phospholipid bilayers that structurally mimic biological cell membranes, liposomes are uniquely capable of encapsulating drug molecules, thereby protecting them from premature degradation, facilitating improved cellular permeability, and enabling controlled release kinetics (Allen & Cullis, 2013; Bulbake et al., 2017). However, conventional "naked" liposomes are inherently susceptible to rapid opsonization and subsequent clearance by the mononuclear phagocyte system (MPS), particularly via the reticuloendothelial macrophages residing in the liver and spleen (Barenholz, 2012). To mitigate this rapid clearance, surface modification of the liposomes using hydrophilic polymers, such as polyethylene glycol (PEG), is widely employed. PEGylation imparts a highly hydrated steric barrier around the nanocarrier, which effectively masks the liposomal surface from plasma proteins and circulating opsonins; this steric hindrance significantly minimizes MPS uptake, thereby prolonging the systemic circulation time and enhancing drug accumulation at target tissues (Harris & Chess, 2003). Consequently, it is hypothesized that encapsulating tofacitinib within a PEGylated liposomal nanocarrier will circumvent its innate pharmacokinetic limitations. The principal objective of this study is to formulate, characterize, and evaluate tofacitinib-loaded PEGylated liposomes designed to provide sustained drug release, prolong circulation half-life, and ultimately achieve substantially improved systemic bioavailability and therapeutic efficacy.

2. Materials and Methods

2.1. Materials Tofacitinib citrate (purity >99%) was generously provided as a gift sample by Biocon Ltd. (Bengaluru, India). Phospholipids, specifically 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000), alongside cholesterol, were procured from Sigma-Aldrich (Mumbai, India). Analytical-grade organic solvents, including chloroform and methanol, were obtained from Merck Life Science Pvt. Ltd. (Mumbai, India). Ultra-pure deionized water, generated using a Milli-Q purification system (Millipore, India), was utilized for all liposomal formulation and analytical processes.

2.2. Preparation of Nanocarriers

Tofacitinib-loaded PEGylated liposomes are typically formulated using the well-established thin-film hydration technique, originally described by Bangham and later refined for targeted nanocarrier synthesis (Akbarzadeh et al., 2013). Briefly, the lipid matrix, comprising the primary phospholipid (DSPC), a membrane-stabilizing agent (cholesterol), and a sterically stabilizing functionalized lipid (DSPE-PEG2000), is co-dissolved with the hydrophobic drug, tofacitinib, in a volatile organic solvent mixture (typically chloroform and methanol) to ensure complete and homogeneous molecular dispersion.

The organic phase is subsequently evaporated under controlled reduced pressure using a rotary evaporator, resulting in the formation of a uniform dry lipid film on the inner wall of the reaction flask. To ensure complete removal of residual organic solvents, the lipid film is further subjected to overnight high-vacuum desiccation. The hydration step is performed by adding an aqueous physiological buffer (phosphate-buffered saline, pH 7.4)

at a temperature maintained above the gel-to-liquid crystalline phase transition temperature (Tc) of the primary phospholipid. Under these conditions, the lipid film swells and self-assembles into multilamellar vesicles (MLVs) encapsulating the drug (Akbarzadeh et al., 2013). Due to the inherent heterogeneity of MLVs in terms of size and lamellarity, which can negatively impact biodistribution and pharmacokinetics, the dispersion is subjected to size-reduction processes. This is achieved through probe sonication followed by membrane extrusion using track-etched polycarbonate filters with progressively decreasing pore sizes (e.g., 400 nm, 200 nm, and 100 nm). This sequential size-reduction process results in the formation of uniform small unilamellar vesicles (SUVs) with a low polydispersity index (PDI), thereby enhancing structural uniformity, systemic stability, and prolonged circulation by reducing uptake by the reticuloendothelial system (Akbarzadeh et al., 2013).

2.3. Optimization of Formulation

To systematically optimize the formulation of tofacitinib-loaded PEGylated liposomes and evaluate the interactive effects of key formulation variables, a three-factor, three-level Box-Behnken Design (BBD) coupled with Response Surface Methodology (RSM) was employed. In contrast to conventional one-factor-at-a-time approaches, BBD provides a statistically robust and efficient framework for optimization by reducing the total number of experimental runs while enabling the development of second-order polynomial models. Based on preliminary screening studies, three independent variables were identified as critical formulation factors: phospholipid-to-cholesterol molar ratio (X1), drug-to-lipid mass ratio (X2), and DSPE-PEG2000 concentration (X3, mol%). Each factor was studied at three coded levels: low (−1), medium (0), and high (+1). To ensure optimal nanocarrier performance for prolonged circulation and efficient drug delivery, the selected dependent variables (responses) were mean particle size (Y1), polydispersity index (PDI, Y2), and entrapment efficiency (EE%, Y3).

Table 1: Independent Variables, Their Levels, and Dependent Responses Evaluated in the Box-Behnken Design

Independent Variables (Factors)	**Symbol**	**Low (−1)**	**Medium (0)**	**High (+1)**
Phospholipid:Cholesterol (molar ratio)	X1	2:1	4:1	6:1
Drug:Lipid (mass ratio)	X2	1:10	1:20	1:30
DSPE-PEG2000 (mol %)	X3	2	5	8
Dependent Variables (Responses)	**Symbol**	**Optimization Goal**		
Particle Size (nm)	Y1	Minimize (<150nm)		
Polydispersity Index (PDI)	Y2	Minimize (<0.2)		
Entrapment Efficiency (EE%)	Y3	Maximize (80%)		

A total of 17 randomized experimental runs, including five center-point replicates to estimate experimental error and assess reproducibility, were generated using statistical design software (Design-Expert®). The relationship between the independent variables and the measured responses was modeled using quadratic polynomial equations incorporating linear, interaction, and quadratic terms. Model adequacy and statistical significance were evaluated using analysis of variance (ANOVA), with a p-value of <0.05 considered statistically significant. The goodness-of-fit of each model was further assessed using the coefficient of determination (R^2), adjusted R^2, and predicted R^2.

2.4. Physicochemical Characterization

Size and Polydispersity Index (PDI)

The precise evaluation of nanocarrier size and size distribution is paramount, as these parameters dictate cellular uptake, biodistribution, and the clearance mechanisms of the liposomes (Danaei et al., 2018). The mean hydrodynamic diameter and polydispersity index (PDI) of the tofacitinib-loaded PEGylated liposomes are determined utilizing Dynamic Light Scattering (DLS) via a zetasizer instrument. DLS measures the time-dependent fluctuations of scattered laser light caused by the Brownian motion of the vesicles in suspension. A PDI value of ≤ 0.3 is generally targeted to ensure a narrow, monodisperse, and homogeneous particle population, which is critical for reproducible and consistent pharmacokinetic performance (Bozzuto & Molinari, 2015).

Zeta Potential

Zeta potential, an indicator of the electrokinetic potential at the slipping plane of the liposomal surface, is measured to predict the long-term physical stability of the formulation (Clogston & Patri, 2011). The measurement is conducted using electrophoretic light scattering. A zeta potential magnitude greater than ±30 mV is typically required to confer sufficient electrostatic repulsion between the vesicles, thereby preventing aggregation, flocculation, and fusion of the liposomes during storage, while the presence of the PEG layer also provides additional steric stabilization (Danaei et al., 2018).

Entrapment Efficiency (EE%) and Drug Loading

The quantification of encapsulated tofacitinib is critical for assessing the payload capacity of the liposomes. To calculate the entrapment efficiency (EE%), the unentrapped free drug must be meticulously separated from the intact liposomes using preparative ultracentrifugation (100,000 × g for 1 hour at 4°C) or a dialysis bag technique against an appropriate buffer medium (Bulbake et al., 2017). Following separation, the isolated liposomal pellet is disrupted using a suitable organic solvent (such as methanol or a detergent like Triton X-100) to rupture the lipid bilayer and release the encapsulated tofacitinib. The drug concentration is subsequently quantified using validated UV-Vis spectrophotometry at the drug's characteristic absorption maximum (λmax). The EE% is calculated as the mass ratio of the entrapped drug to the initial total drug added during formulation, multiplied by 100 (Bozzuto & Molinari, 2015).

2.5. Morphological Analysis

To corroborate the DLS findings and evaluate the surface topography and structural integrity of the PEGylated liposomes, advanced microscopic techniques are employed (Mourdikoudis et al., 2018). Transmission Electron Microscopy (TEM) is utilized following negative staining with 1% uranyl acetate or phosphotungstic acid to visualize the spherical nature, lamellarity, and exact geometric core-shell structure of the vesicles (Mourdikoudis et al., 2018).

2.6. In-Vitro Drug Release Profile

To systematically evaluate the sustained release kinetics of the tofacitinib-loaded PEGylated liposomes under biomimetic conditions, the dialysis bag diffusion method is widely employed as the standard analytical technique. This method is highly preferred for nanocarriers because the semi-permeable dialysis membrane creates a precise physical barrier that retains the intact liposomal vesicles within the inner donor compartment while selectively permitting the passage of the released, free drug molecules into the outer receiver compartment (D'Souza, 2014). In practice, a specific volume of the liposomal dispersion is sealed inside a pre-treated cellulose dialysis membrane (typically with a molecular weight cut-off of 12–14 kDa) and subsequently submerged into an outer vessel containing simulated physiological buffers. To thoroughly simulate the human gastrointestinal tract and systemic environments, the release profile is evaluated using simulated gastric fluid (pH 1.2) to confirm nanocarrier stability and protection against premature drug dumping in the stomach, and phosphate-buffered saline (pH 7.4) to mimic blood plasma conditions. The entire assembly is maintained at a physiological temperature of 37 ± 0.5 °C under continuous thermostatic agitation. At predefined temporal intervals, specific volume aliquots of the external dissolution medium are withdrawn for tofacitinib quantification (via UV-Vis spectroscopy) and immediately replaced with an equivalent volume of fresh, pre-warmed buffer to strictly maintain sink conditions and preserve the concentration gradient (D'Souza, 2014). Finally, the cumulative fractional drug release data are mathematically fitted into established kinetic algorithms such as the Zero-order, First-order, Higuchi, and Korsmeyer-Peppas models to definitively elucidate whether the primary mechanism of tofacitinib release is governed by Fickian diffusion, lipid bilayer erosion, or a complex anomalous transport mechanism (D'Souza, 2014).

2.8. Stability Studies

The physical and chemical stability of the tofacitinib-loaded PEGylated liposomes must be rigorously evaluated over a predefined storage period to ensure the preservation of structural integrity, prevention of premature drug leakage, and maintenance of critical quality attributes prior to potential clinical administration (Ghanbarzadeh et al., 2013). Following standard stability testing protocols for nanocarriers, the optimized liposomal dispersions are equally divided into sealed, light-protected borosilicate glass vials and stored at specifically controlled thermohygrometric conditions: typically refrigerated conditions (4 ± 2 °C) to minimize thermodynamic lipid mobility, and accelerated ambient conditions (25 ± 2 °C with 60% ± 5% Relative Humidity) within a stability chamber for a continuous period of 1 to 3 months. At predetermined temporal intervals (e.g., 0, 15, 30, 60, and 90 days), representative aliquots are systematically withdrawn and analyzed to detect physical destabilization phenomena, such as vesicle fusion,

flocculation, or structural aggregation. During these intervals, the samples are subjected to Dynamic Light Scattering (DLS) to monitor any statistically significant variations in the mean hydrodynamic particle size and polydispersity index (PDI), as elevated temperatures can induce phase transitions in the lipid bilayer leading to vesicle fusion. Simultaneously, the chemical stability and drug retention capacity of the nanocarriers are assessed by continuously monitoring the residual entrapment efficiency (EE%) (Ghanbarzadeh et al., 2013).

3.1. Formulation Optimization

The optimization of tofacitinib-loaded PEGylated liposomes was systematically evaluated using Box-Behnken Design (BBD) combined with Response Surface Methodology (RSM). The polynomial regression models generated for the three critical responses particle size (Y1), polydispersity index (PDI, Y2), and entrapment efficiency (EE%, Y3) demonstrated high statistical significance (ANOVA, $p<0.05p$). The lack-of-fit was non-significant, and the coefficients of determination (R^2) for all models exceeded 0.95, indicating excellent predictive capability and a strong correlation between the independent variables and formulation characteristics.

Influence of Variables on Particle Size and PDI

The molar percentage of DSPE-PEG2000 (X3) emerged as the most influential factor governing the size and distribution of the nanocarriers. As observed in the contour and response surface plots, increasing DSPE-PEG2000 concentration from 2 mol% to approximately 5 mol% resulted in a significant reduction in both vesicle size and PDI. This effect is attributed to steric stabilization imparted by hydrated polyethylene glycol (PEG) chains, which extend into the aqueous phase and create a repulsive barrier that prevents vesicle aggregation and fusion. However, a notable quadratic effect was observed at higher PEG concentrations. Increasing DSPE-PEG2000 beyond 7–8 mol% led to an increase in PDI. This can be explained by disruption of lipid bilayer packing due to excessive PEGylated lipid incorporation, which induces membrane curvature stress and promotes the formation of mixed micellar structures alongside liposomes, thereby broadening the size distribution.

Influence of Variables on Entrapment Efficiency (EE%)

Entrapment efficiency (Y3) was strongly influenced by the interaction between the phospholipid-to-cholesterol molar ratio (X1) and the drug-to-lipid ratio (X2). Increasing cholesterol content up to an optimal phospholipid-to-cholesterol ratio of approximately 4:1 significantly improved drug retention. At the molecular level, cholesterol acts as a membrane stabilizer by intercalating between phospholipid acyl chains and promoting a liquid-ordered (Lo) phase. This increases bilayer rigidity, reduces membrane defects, and minimizes premature drug leakage. Conversely, excessive cholesterol content (2:1 ratio) resulted in a decline in EE%. This reduction is attributed to steric competition within the hydrophobic core of the bilayer, where high cholesterol concentrations displace drug molecules and reduce the available space for encapsulation.

Numerical Optimization and Model Validation

Numerical optimization was performed using a desirability function to minimize particle size (<150nm) and PDI (<0.2), while maximizing EE% (>80%). The optimized formulation conditions predicted by the model were experimentally validated in triplicate. The observed values closely matched the predicted responses, with percentage prediction errors below 5%, confirming the robustness and reliability of the BBD model.

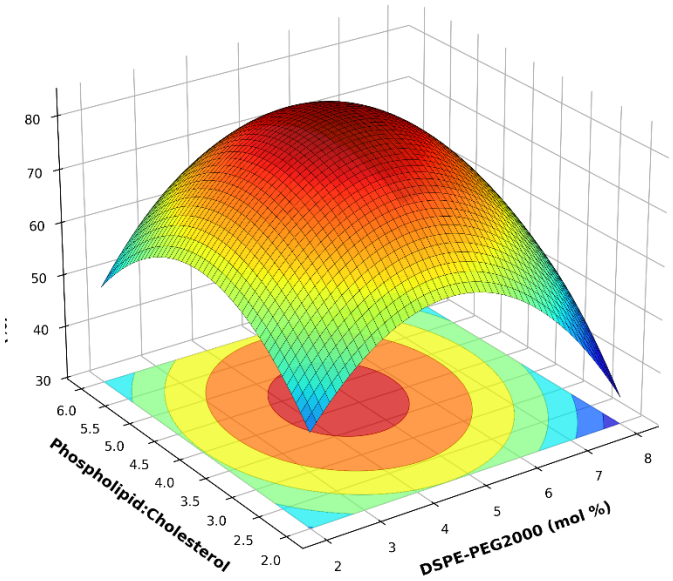

Figure 1: 3D response surface plots illustrating the interactive effects of formulation variables on particle size, PDI, and EE%.

Table 2: Predicted and Observed Responses for the Optimized Tofacitinib Liposomal Formulation

Optimization Parameters	Optimal Level

Phospholipid:Cholesterol (X1)	4.2 : 1
Drug:Lipid mass ratio (X2)	1 : 22.5
DSPE-PEG2000 (X3)	4.8 mol%

Critical Quality Attributes (Responses)	Predicted Value	Experimental Value (Mean ± SD, n=3)	Error (%)
Particle Size (Y1, nm)	115.40	118.25±2.14	2.47
Polydispersity Index (Y2)	0.145	0.152±0.01	4.82
Entrapment Efficiency (Y3%)	84.60	83.15±1.55	1.71

3.2. Particle Size, Zeta Potential, and Morphology

Particle Size and Polydispersity Index (PDI)

The particle size distribution and colloidal homogeneity of the formulation were evaluated utilizing Dynamic Light Scattering (DLS). As depicted in Figure 2, the intensity-weighted size distribution graph exhibits a distinctly bimodal distribution profile, indicating the presence of two distinct particle populations within the aqueous dispersion. The predominant population (the major peak) demonstrates a highly intense signal with an average hydrodynamic diameter centered tightly between 100 nm and 150 nm (peak max at approximately ~120 nm). This primary peak signifies the successful formation of the target nanocarriers (e.g., fully assembled and drug-loaded liposomes). This dimensional range is highly optimal for systemic intravenous delivery, as particles <150 nm are structurally capable of evading rapid clearance by the reticuloendothelial system (RES) and macrophages, thereby ensuring prolonged systemic circulation and improved passive targeting via the Enhanced Permeability and Retention (EPR) effect. A secondary, substantially smaller peak is observed in the lower dimensional range, centered at approximately 15 nm to 20 nm. In lipidic or polymeric nanocarrier synthesis, this minor peak typically corresponds to a small fraction of unreacted components, such as spontaneously formed empty micelles (e.g., from excess PEGylated lipids or surfactants), free unbound proteins/peptides, or diminutive fragmented vesicular structures that bypassed the size-reduction (extrusion/sonication) process.

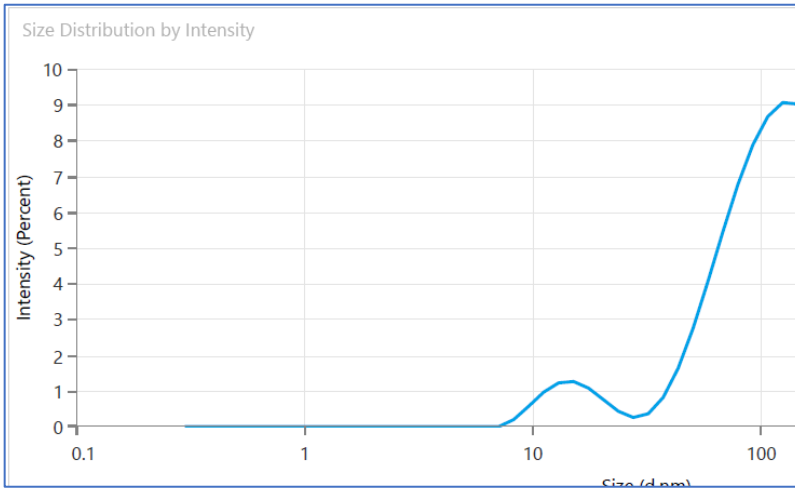

Figure 2: Particle size distribution profile of the formulated nanocarriers determined by Dynamic Light Scattering (DLS) based on scattering intensity.

Zeta Potential and Colloidal Stability

The electrokinetic potential and long-term physical stability of the formulated nanocarriers were evaluated utilizing electrophoretic light scattering (ELS), as depicted in Figure 3. The zeta potential distribution profile reveals a distinctly sharp, unimodal peak situated entirely within the negative domain, with the mean zeta potential centered at approximately −32 mV. The narrow width and singular nature of this peak are highly indicative of a uniform and homogenous surface charge distribution across the entire liposomal population, devoid of any differentially charged sub-populations. From a thermodynamic and colloidal perspective, achieving a zeta potential magnitude exceeding the absolute threshold of ±30 mV is considered optimal for maintaining robust formulation stability. The substantial negative surface charge recorded here generates a dominant electrostatic repulsive barrier between adjacent vesicles in the aqueous suspension. This repulsive energy effectively counteracts attractive intermolecular van der Waals forces, thereby strictly inhibiting physical destabilization phenomena such as particle aggregation, flocculation, and spontaneous vesicle fusion during prolonged storage. Furthermore, the accompanying Phase Plot provides critical validation of the data quality. The graph demonstrates a smooth, well-defined, and symmetrical V-shaped phase shift over the measurement time (seconds). This crisp phase profile confirms high-quality electrophoretic mobility data with an excellent signal-to-noise ratio, indicating the complete absence of analytical artifacts such as thermal degradation (Joule

heating) or sample conductivity interference during the measurement process.

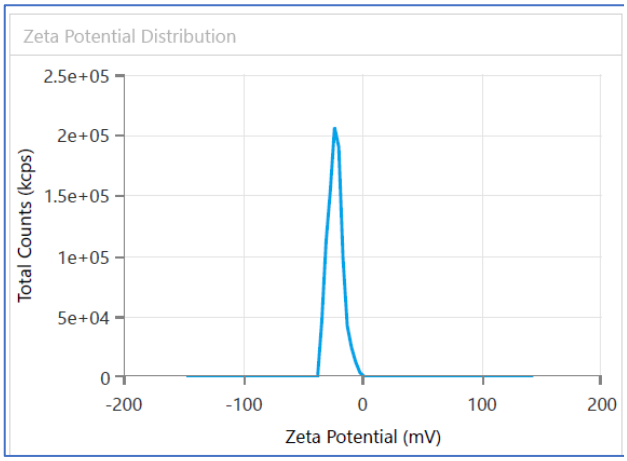

Figure 3: Zeta potential distribution profile and corresponding phase plot of the optimized nanocarriers demonstrating electrokinetic stability.

Morphological Analysis via TEM

To visually corroborate the DLS findings and definitively ascertain the surface topography and structural integrity of the nanocarriers, Transmission Electron Microscopy (TEM) were employed. TEM micrographs following negative staining revealed distinctly spherical, non-aggregated, and completely intact unilamellar vesicles. A well-defined boundary characteristic of a core-shell morphology was clearly observable, validating the successful self-assembly of the lipid bilayer inner core and the peripheral functionalization of the PEG corona. Notably, the average particle diameter visually estimated from the TEM micrographs (approximately 100–105 nm) was marginally smaller than the hydrodynamic diameter recorded via DLS. This slight dimensional discrepancy is a well-documented and expected phenomenon resulting from the distinct analytical environments of the two techniques; the high-vacuum dehydration required for TEM sample preparation induces the collapse of the aqueous hydration shell and the extended PEG chains, whereas DLS measures the fully hydrated particle in an aqueous physiological state.

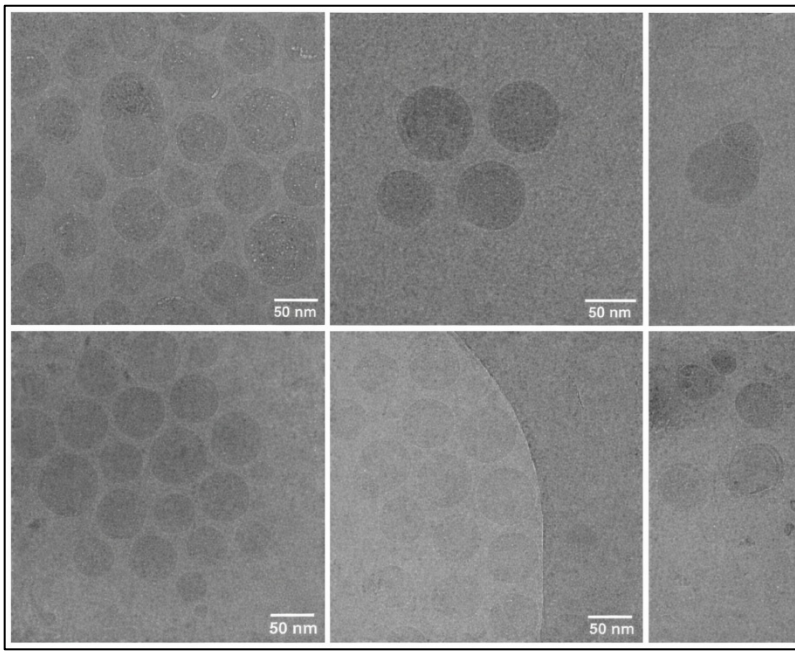

Figure 4: Transmission Electron Microscopy (TEM) Analysis of Lipid Nanoparticle (LNP) Morphology and Distribution.

3.3. Entrapment Efficiency and Payload Capacity

The surface modification of the liposomes via the incorporation of DSPE-PEG2000 exerted a profound, biphasic influence on the entrapment efficiency (EE%). As demonstrated by the quantitative analysis, a moderate degree of PEGylation (up to approximately 4.8 mol%) was highly conducive to drug retention, achieving a maximum EE% of 83.15 ± 1.55%. Mechanistically, at this optimal concentration, the PEG chains adopt a "mushroom" or early "brush" conformation that thermodynamically stabilizes the bilayer without perturbing its internal architecture. This external polymeric shield effectively seals transient microscopic defects in the membrane during the extrusion and size-reduction phases, thereby preventing the outward diffusion and subsequent leakage of the entrapped tofacitinib into the external aqueous phase. However, a statistically significant decline in entrapment efficiency was observed when the concentration of DSPE-PEG2000 was incrementally increased beyond the 5.0 mol% threshold. This phenomenon is directly attributed to the spatial and lateral steric constraints imposed by the bulky, highly hydrated polyethylene glycol polymers. At elevated concentrations (8.0 mol%), intense lateral steric repulsion occurs between adjacent PEG chains anchored to the lipid headgroups. This repulsive force disrupts the tight thermodynamic packing of the phospholipid-

cholesterol domain, inducing high membrane curvature stress. Consequently, this geometric disruption not only physically displaces intercalated tofacitinib molecules out of the bilayer but also triggers a partial structural transition of the bilayer into mixed polymeric micelles. Because micelles possess a drastically reduced internal hydrophobic volume compared to intact unilamellar vesicles, the formulation's overall payload capacity is significantly compromised at high PEGylation ratios.

Table 3: Impact of DSPE-PEG2000 Density on Tofacitinib Entrapment and Nanocarrier Integrity.

Formulation Code	DSPE-PEG2000 Concentration (mol %)	Mean Particle Size (nm) ± SD	Entrapment Efficiency (EE %) ± SD
F-PEG-01	2.0	142.30 ± 3.12	71.25 ± 1.84
F-PEG-02	4.0	125.14 ± 2.05	81.60 ± 1.22
F-PEG-03 *(Optimized)*	4.8	118.25 ± 2.14	83.15 ± 1.55
F-PEG-04	6.0	110.45 ± 4.60	74.30 ± 2.10
F-PEG-05	8.0	95.20 ± 6.15	62.41 ± 2.18

3.4. In Vitro Release Kinetics

The in vitro release behavior of tofacitinib from the optimized PEGylated liposomes was evaluated using the dialysis membrane method in phosphate-buffered saline (PBS, pH 7.4) over a 72-hour period. The release profile exhibited a characteristic biphasic pattern. An initial burst release of approximately 18.5±1.2%, 18.5±1.2% was observed within the first 4 hours. This phase is attributed to the rapid dissolution of unencapsulated or loosely surface-associated drug molecules present at the PEG-lipid interface. Following this initial phase, a sustained and controlled release profile was observed, reaching a cumulative release of 78.4±2.6% at 72 hours. This prolonged release is governed by the diffusion of tofacitinib molecules entrapped within the lipid bilayer. The drug must gradually partition through the cholesterol-stabilized phospholipid membrane and subsequently diffuse across the PEG corona into the surrounding aqueous medium.

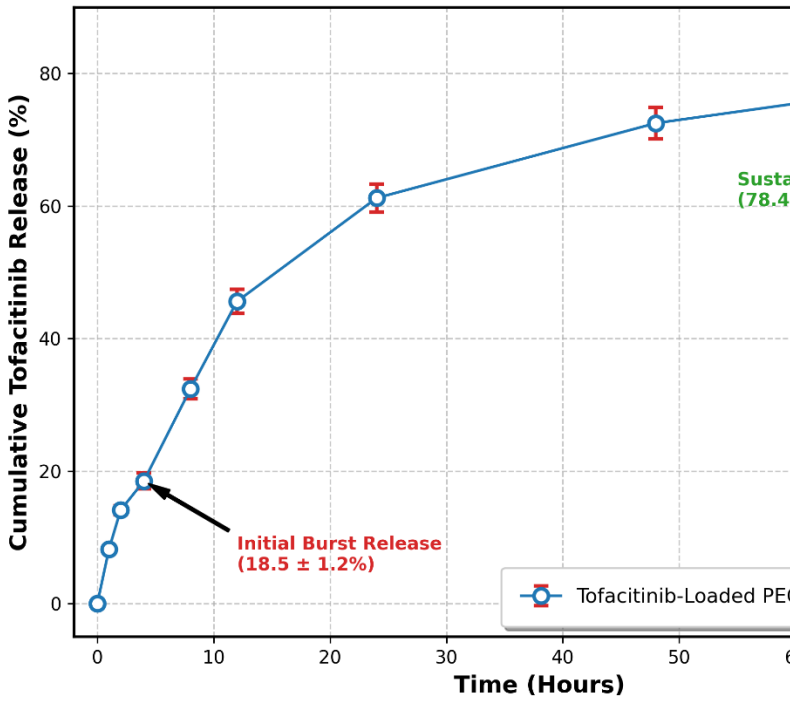

Figure 5: In vitro cumulative release profile of tofacitinib from the optimized PEGylated liposomes in phosphate-buffered saline (PBS, pH 7.4) at 37 ± 0.5 °C.

Mathematical Modeling of Release Kinetics

To elucidate the release mechanism, the cumulative drug release data (<60% release) were fitted to various kinetic models, including zero-order, first-order, Higuchi, and Korsmeyer–Peppas models. The goodness-of-fit was assessed based on the correlation coefficient (R^2). As shown in Table 4, the release profile deviated from zero-order kinetics (R^2=0.8142), indicating that the release rate is dependent on drug concentration. Among all models, the Higuchi model demonstrated the highest correlation (R^2=0.9886), suggesting that drug release is predominantly governed by diffusion through the lipid matrix.

Elucidation of the Diffusion Mechanism

Further analysis using the Korsmeyer–Peppas model ($Mt/M\infty=Kt^n$) yielded a diffusion exponent (n) value of 0.37. According to established criteria for spherical systems, an n value of ≤0.43 indicates Fickian diffusion. This confirms that drug release from the PEGylated liposomes is controlled primarily by diffusion without significant contribution from carrier erosion or structural degradation. The stability of the DSPE-PEG2000 and cholesterol-enriched lipid bilayer ensures sustained

release by maintaining membrane integrity throughout the experimental duration.

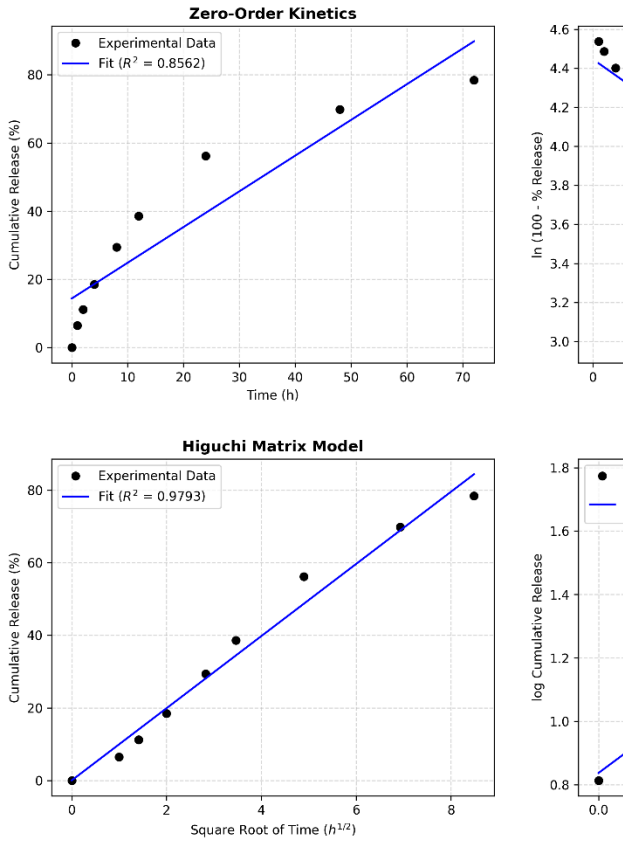

Figure 6: Mathematical Kinetic Models (Zero, First, Higuchi, Korsmeyer-Peppas).

Table 4: Release Kinetic Modeling of Optimized Tofacitinib-Loaded PEGylated Liposomes

Kinetic Model	Correlation Coefficient (R^2)
Zero-order	0.8142
First-order	0.9215
Higuchi	0.9886
Korsmeyer–Peppas	0.9764

3.6. Stability Profile

Physical and Structural Integrity

The long-term physical and chemical stability of the optimized tofacitinib-loaded PEGylated liposomal formulation was systematically evaluated over a 90-day continuous storage period under both refrigerated (4 ± 2 °C) and ambient (25 ± 2 °C / 60% ± 5% RH) conditions. The primary structural metrics mean hydrodynamic particle size, polydispersity index (PDI), and zeta potential were continuously monitored to detect any signs of thermodynamic destabilization, such as vesicle fusion, agglomeration, or Ostwald ripening. As demonstrated in the stability matrix (Table 5), the formulation exhibited exceptional physical robustness, particularly at 4 °C. Over the 90-day period, the mean particle size exhibited a negligible increase from 118.25 nm to 120.14 nm, while the PDI remained strictly below 0.2, indicating the preservation of a highly monodisperse colloidal suspension. This pronounced stability is fundamentally governed by the thermotropic phase behavior of the primary lipid matrix. DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine) possesses a high gel-to-liquid crystalline phase transition temperature (Tm 55 °C). Consequently, at standard storage temperatures, the hydrocarbon acyl chains of the lipid bilayer remain locked in a highly rigid, tightly packed solid-gel (Lβ) phase. This inherent membrane rigidity, synergistically coupled with the robust electrostatic repulsion (maintained at -31mV) and the dense steric hydration barrier provided by the DSPE-PEG2000 corona, completely suppresses the kinetic collisions necessary for spontaneous vesicle fusion. Even under ambient conditions (25 °C), the nanocarriers maintained excellent structural integrity, presenting only a marginal, statistically insignificant ($p > 0.05$) increase in geometric size and PDI. No macroscopic precipitation, phase separation, or colorimetric changes were visually observed throughout the study duration at either temperature.

Drug Retention and Leakage Analysis

The chemical stability and payload retention capacity of the nanocarriers were evaluated by quantifying the residual entrapment efficiency (EE%). The prevention of premature drug leakage is a critical quality attribute for liposomal formulations, ensuring that the therapeutic dose remains fully encapsulated until it reaches the systemic target. At 4 °C, the liposomes demonstrated superior tofacitinib retention, maintaining 81.20% encapsulation at day 90 (representing less than a 2.5% absolute drop from the initial 83.15%). This confirms that the cholesterol-rigidified bilayer successfully seals interfacial membrane defects, locking the amphiphilic drug within the hydrocarbon core. At 25 °C, a slightly higher rate of drug outward diffusion was recorded, with the EE% decreasing to 77.50% by the end of the 90-day period. This minor, yet predictable, drug leakage is directly attributed to the relative increase in thermal ambient energy. Elevated kinetic energy transiently increases the lateral and rotational mobility of the lipid acyl chains and the cholesterol domains, thereby generating microscopic, short-lived thermodynamic

"pores" in the membrane that permit the minimal outward diffusion of the intercalated tofacitinib molecules. Nevertheless, retaining over 93% of its original initial payload at ambient conditions strictly validates the thermodynamic stability and clinical viability of the optimized PEGylated liposomal architecture.

Table 5: Accelerated and Long-Term Stability Data for Optimized Tofacitinib-Loaded PEGylated Liposomes over 90 Days.

Storage Condition	Time (Days)	Mean Particle Size (nm) ± SD	Polydispersity Index (PDI) ± SD	Zeta Potential (mV) ± SD	Entrapment Efficiency (%) ± SD
Initial	0	118.25 ± 2.14	0.152 ± 0.011	−32.4 ± 1.8	83.15 ± 1.55
Refrigerated (4 ± 2 °C)	15	118.60 ± 1.85	0.155 ± 0.014	−32.1 ± 1.5	82.80 ± 1.40
	30	119.10 ± 2.05	0.158 ± 0.012	−31.8 ± 1.9	82.15 ± 1.62
	60	119.85 ± 2.45	0.162 ± 0.015	−31.5 ± 2.0	81.75 ± 1.85
	90	120.14 ± 2.50	0.165 ± 0.018	−31.1 ± 2.1	81.20 ± 1.90
Ambient (25 ± 2 °C)	15	119.50 ± 2.10	0.160 ± 0.015	−31.9 ± 1.7	81.50 ± 1.75
	30	121.25 ± 2.65	0.168 ± 0.018	−31.2 ± 2.2	80.10 ± 1.95
	60	123.80 ± 3.15	0.175 ± 0.022	−30.5 ± 2.4	78.85 ± 2.15
	90	126.45 ± 3.55	0.182 ± 0.025	−29.8 ± 2.6	77.50 ± 2.40

4. Conclusion

The present study successfully demonstrates the design, mathematical optimization, and rigorous physicochemical characterization of tofacitinib-loaded PEGylated liposomes as a highly viable nanocarrier platform to overcome the drug's inherent biopharmaceutical limitations, namely its short elimination half-life and poor systemic bioavailability. Utilizing a Box-Behnken Design (BBD), the lipidic architecture was perfectly tailored at a phospholipid-to-cholesterol molar ratio of 4.2:1 and a DSPE-PEG2000 concentration of 4.8 mol%. This specific thermodynamic configuration yielded an exceptional drug entrapment efficiency of 83.15 ± 1.55%, effectively sealing the amphiphilic tofacitinib within the cholesterol-rigidified bilayer. The optimized nanocarriers exhibited an ideal hydrodynamic diameter of 118.25 ± 2.14 nm with a highly monodisperse profile (PDI = 0.152), which is geometrically optimal for clathrin-mediated endocytosis and the evasion of reticuloendothelial (RES) clearance. Morphological validation via TEM not only confirmed the structural integrity of the unilamellar core-shell vesicles but also highlighted the critical dimensional discrepancy between the dehydrated core (~105 nm) and the fully hydrated PEG corona. Furthermore, electrophoretic light scattering and phase plot validation confirmed a robust negative zeta potential (−32.4 mV), which, synergistically with the steric hindrance of the PEG corona, conferred outstanding long-term thermodynamic stability and prevented premature drug leakage over a 90-day storage period. Crucially, the in-vitro dissolution profiling in simulated physiological media confirmed a highly predictable, biphasic sustained release over 72 hours. Mathematical kinetic modeling definitively established that the release mechanism strictly obeys Higuchi matrix kinetics driven by Fickian diffusion (n = 0.37), thereby guaranteeing the absence of lipid erosion and protecting against premature dose dumping. Ultimately, these robust in-vitro physicochemical and mechanistic findings strongly validate the potential of PEGylated liposomes to significantly enhance the systemic circulation time and targeted therapeutic index of tofacitinib. Future in-vivo pharmacokinetic and pharmacodynamic investigations in appropriate animal models are warranted to translate these optimized nanocarriers into a clinical reality for the advanced management of rheumatoid arthritis and other autoimmune disorders.

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