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Targeting the Colon with Nanostructured Lipid Carriers: A Promising pH-sensitive Delivery of Azathioprine

by

Mudha Tariq

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Abstract

Inflammatory bowel disease (IBD) is a chronic condition that relapses and there are still gaps in the therapy available. Azathioprine is a commonly used immunosuppressant drug for treating IBD, but unfortunately it has poor oral bioavailability, chemically unstable in a range of GI pH and has systemic toxicity due to non-targeted delivery. For this purpose, azathioprine loaded nanostructured lipid carriers (NLCs) were prepared and surface modified with pH-sensitive enteric polymer cellulose acetate phthalate (CAP) for colon-specific drug delivery. Thirteen formulations (NLC1-13) were prepared by hot high-speed homogenization, in which the solid lipid composition, the concentration of the liquid lipid, the surfactant system and the homogenization time were systematically varied. The parameters used to evaluate the formulations were entrapment efficiency (EE $\geq 80\%$), particle size (100-250 nm), polydispersity index (PDI < 0.30), zeta potential value ($> |30\text{ mV}|$), gastric release ($\leq 10\%$), and colonic release ($\geq 80\%$). Among all the formulations, NLC10 was uniquely identified as the only formulation satisfying all the six CQAs simultaneously with EE of $93.47 \pm 0.42\%$, particle size of $110.3 \pm 5.1\text{ nm}$, PDI of 0.285 ± 0.01 , zeta potential of $-40.4 \pm 0.8\text{ mV}$, gastric release of $8.10 \pm 0.4\%$, and colonic release of $91.84 \pm 1.8\%$. The release of the drug at pH 1.2 was followed by super Case-II transport ($n = 1.312$, $R^2 = 0.988$), which is indicative of the superior structural integrity of the intact CAP barrier. For pH 7.4, release occurred in a manner similar to Case-II transport ($n = 0.944$, $R^2 = 0.995$), with the release process being primarily matrix relaxation-controlled and a small diffusion contribution due to CAP dissolution. Drug-excipient compatibility was confirmed by solid-state characterization using FTIR and successful amorphous drug dispersion in the lipid matrix was confirmed using DSC and XRD. The SEM showed the formation of spherical nanoparticles, which were homogeneous and distributed. Stability studies showed that the product was excellently physically and chemically stable when stored at $4 \pm 2^\circ\text{C}$ for 90 days. PDI rose to 0.43 after 90 days at $40 \pm 2^\circ\text{C}/75 \pm 5\%\text{ RH}$ which shows that refrigerated storage is better for long term shelf life. Acute oral toxicity test conducted in BALB/c mice at 2 g/kg showed total biocompatibility without any

significant haematological, biochemical or histopathological changes. The results indicate that azathioprine loaded CAP coated NLCs would be a good carrier for pH responsive colon targeted therapy for IBD.

Keywords: Azathioprine; Nanostructured lipid carriers; Cellulose acetate phthalate; Colon-targeted delivery; Inflammatory bowel disease; pH-responsive drug release

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Abbreviations

6-MMP	6-Methylmercaptopurine
6-MP	6-Mercaptopurine
6-TGN	6-Thioguanine Nucleotides
6-TGTP	6-Thioguanine Triphosphate
ATG16L1	Autophagy-Related 16-Like 1
BCS	Biopharmaceutics Classification System
CAP	Cellulose Acetate Phthalate
CD	Crohn's Disease
COX-2	Cyclooxygenase-2
CQA	Critical Quality Attributes
DSC	Differential Scanning Calorimetry
DSS	Dextran Sulfate Sodium
EE	Entrapment Efficiency
EP	European Pharmacopoeia
FMT	Faecal Microbiota Transplantation
FTIR	Fourier Transform Infrared (Spectroscopy)
GI	Gastrointestinal
GPAT	Glutamine Phosphoribosylpyrophosphate Amido transferase (PRPP Amidotransferase)
GR	Glucocorticoid Receptor
GRAS	Generally Recognized As Safe
GWAS	Genome-Wide Association Studies
HGPRT	Hypoxanthine-Guanine Phosphoribosyltransferase
HLB	Hydrophilic–Lipophilic Balance

HPMCP	Hydroxypropyl Methylcellulose Phthalate
HRQoL	Health-Related Quality of Life
IBD	Inflammatory Bowel Disease
IBDU	Inflammatory Bowel Disease – Unclassified
ICH	International Council for Harmonisation
ICH Q1A(R2)	ICH Guideline on Stability Testing of New Drug Substances and Products (Revision 2)
IFN-γ	Interferon Gamma
IL	Interleukin e.g., IL-5, IL-6, IL-10, IL-12 IL-13, IL-17, IL-23
IL23R	Interleukin 23 Receptor
IRGM	Immunity-Related GTPase M
JAK	Janus Kinase
LMICs	Low- and Middle-Income Countries
LT	Leukotriene
MTX	Methotrexate
NF-κB	Nuclear Factor Kappa B
NLC	Nanostructured Lipid Carrier
NOD2	Nucleotide-Binding Oligomerization Domain-Containing Protein 2
NP	Nanoparticle
OA	Oleic Acid
OECD	Organisation for Economic Co-operation & Development
PDI	Polydispersity Index
PG	Prostaglandin
PLGA	Poly(Lactic-co-Glycolic Acid)
PRPP	Phosphoribosyl Pyrophosphate
PVAP	Polyvinyl Acetate Phthalate
RH	Relative Humidity
ROS	Reactive Oxygen Species
SCFAs	Short-Chain Fatty Acids
SEM	Scanning Electron Microscopy

SLN	Solid Lipid Nanoparticle
TGF-β	Transforming Growth Factor Beta
Th1 / Th2 / Th17	T Helper Cell Types 1, 2, and 17
TIMP	Thioinosine Monophosphate
TNBS	Trinitrobenzene Sulfonic Acid
TNF-α	Tumor Necrosis Factor Alpha
TPMT	Thiopurine S-Methyltransferase
Treg	Regulatory T Cell
UC	Ulcerative Colitis
USP	United States Pharmacopeia
XRD	X-Ray Diffraction
log P	Logarithm of the Partition Coefficient
mp	Melting Point
o/w	Oil-in-Water

Chapter 1

Introduction

1.1 Background: The Burden of Inflammatory Bowel Disease and the Case for Site-specific Therapy

Inflammatory bowel disease (IBD), comprising Crohn's disease and ulcerative colitis, is a chronic, relapsing immune-mediated disorder of the gastrointestinal tract that now affects an estimated 6.8 million people across 195 countries, with rising incidence not only in the West but increasingly across Asia, Africa, and South Asia, including Pakistan [1, 2]. Beyond this growing global burden, the more fundamental challenge for pharmaceutical scientists is one of drug delivery: IBD is, at its core, a disease of the colonic mucosa, yet the therapies used to treat it are overwhelmingly systemic in their distribution. This mismatch between where a drug acts and where it is actually delivered is the central problem this thesis addresses. Rather than pursuing a new chemical entity, the present work formulates and develops a novel colon-targeted nanoparticulate drug delivery system for an established immunosuppressant, azathioprine, demonstrating that re-engineering how a drug is delivered can be as therapeutically consequential as discovering a new one.

1.2 Current Therapeutic Landscape and the Gap in Site-specific Delivery

Pharmacological management of IBD follows a stepwise algorithm, aminosalicylates, corticosteroids, immunosuppressants, biologics, and small-molecule inhibitors, chosen according to disease location, severity, and prior response. Aminosalicylates such as mesalazine remain first-line for mild-to-moderate ulcerative colitis but are ineffective for maintenance in Crohn's disease, while corticosteroids induce remission quickly but cannot be sustained long-term without dose-dependent toxicity [3]. Biological agents targeting TNF- α , mucosal integrins, or the IL-12/23 axis have transformed outcomes in moderate-to-severe disease, yet remain expensive, require parenteral administration, and are largely inaccessible in resource-limited settings such as Pakistan [4]. What unites every option on this ladder is the same structural limitation: none of them delivers drug preferentially to the inflamed colon. Closing this gap does not require a new molecule; it requires a delivery platform engineered specifically to place an existing, well-characterised drug at the site where it is needed, which is precisely the formulation strategy developed in this thesis.

1.3 Azathioprine: Therapeutic Value and the Delivery Barrier

Azathioprine is one of the most established immunosuppressants used for maintenance therapy in IBD, but its clinical utility is constrained less by its pharmacology than by how it is delivered. Conventional oral azathioprine is absorbed non-selectively across the small intestine and undergoes extensive first-pass hepatic metabolism, so that only a fraction of the administered dose ever reaches the colonic mucosa, while the rest circulates systemically and accumulates in non-target tissues [5]. This is compounded by the drug's poor aqueous solubility (0.130

mg/mL at pH 1.2), its high-lattice-energy crystalline structure, and its susceptibility to acid-catalysed degradation, physicochemical properties that make oral bioavailability inconsistent even before the delivery problem is considered.

Azathioprine's pharmacological profile is well understood and clinically proven; what it lacks is a delivery vehicle capable of directing it to its intended site of action. It is precisely this gap that the drug delivery system formulated in this study is designed to close [6].

1.4 Nanostructured Lipid Carriers as an Enabling Delivery Platform

Nanoparticulate carriers have emerged as a practical way to correct exactly this kind of solubility–distribution mismatch, by reducing particle size to enhance dissolution, encapsulating labile drugs within protective matrices, and using surface and size characteristics to bias where in the body a drug ends up [7].

Nanostructured lipid carriers (NLCs) were selected as the delivery platform for this study because they are particularly well suited to azathioprine's specific limitations. Developed as a second generation of solid lipid nanoparticles, NLCs use a partially disordered binary mixture of solid and liquid lipids — in this study, Compritol[®] 888 ATO and Precirol[®] ATO 5 as solid lipids with oleic acid as the liquid lipid — to create a matrix with structural imperfections that overcome the low drug-loading capacity and storage-related drug expulsion that limited first-generation solid lipid nanoparticles [8, 9].

In formulating azathioprine within this matrix, the drug is converted from its thermodynamically stable crystalline state to an amorphous, more soluble form, while the lipid matrix simultaneously shields it from gastrointestinal degradation — directly resolving the physicochemical barrier identified in Section 1.3 through formulation design rather than chemical modification of the drug itself.

1.5 pH-Sensitive Cellulose Acetate Phthalate Coating: Achieving Colon-specific Release

An NLC core alone still releases its payload throughout the gastrointestinal tract; achieving true colon-specificity requires a second, independent control mechanism, engineered in this study through a post-applied coating of cellulose acetate phthalate (CAP). CAP is a pharmacopoeial enteric polymer that remains protonated and hydrophobic at gastric pH (1.0–3.0), forming a compact barrier that restricts drug release to below 10% over a 2-hour gastric phase [10].

As the coated NLC transits into the small intestine and reaches the proximal colon (pH 6–7.4), progressive ionisation of the polymer’s phthalate groups triggers its dissolution, exposing the lipid core and initiating sustained release into the colonic environment [11]. By engineering this two-stage, pH-responsive release profile — gastric protection followed by colonic release — onto an already optimised NLC core, this study assembles a single delivery system in which both stages of site-specific targeting are achieved through formulation design alone, without altering azathioprine’s molecular structure.

1.6 Problem Statement

Conventional immediate-release oral azathioprine formulations, such as Imuran[®] tablets, are fundamentally incompatible with the pharmacokinetic requirements of colon-targeted therapy. Following oral administration, azathioprine is rapidly absorbed across the proximal small intestine, a site anatomically and physiologically distant from the inflamed distal ileum and colon, and undergoes extensive first-pass hepatic biotransformation via thiopurine methyltransferase (TPMT) and xanthine oxidase, generating cytotoxic 6-thioguanine nucleotide and 6-methylmercaptopurine metabolites that enter systemic circulation before any drug reaches the target mucosa.

The consequence is a pharmacokinetic profile inverted relative to therapeutic need: peak metabolite exposure occurs in non-target bone marrow, liver, and lymphoid tissue, while the diseased colonic mucosa, lacking a dedicated absorptive or retentive mechanism for the drug, receives only a residual, sub-therapeutic fraction delivered indirectly through systemic redistribution rather than direct luminal contact.

This exposure pattern is not merely inefficient but causally linked to azathioprine's dose-limiting toxicity profile: myelosuppression in up to 15% of patients, clinically significant hepatotoxicity in 3–10%, idiosyncratic pancreatitis in 3–5%, and a four- to fivefold elevated risk of non-Hodgkin lymphoma with long-term use, toxicities that arise directly from systemic thiopurine metabolite burden rather than from any deficiency in the drug's intrinsic immunosuppressive mechanism. The problem is compounded at the formulation level: azathioprine's low aqueous solubility (0.130 mg/mL at pH 1.2), high crystal lattice energy, and susceptibility to acid-catalysed hydrolytic degradation in the stomach introduce dissolution-rate variability that further destabilizes an already unfavourable exposure profile, producing inter-patient variability in bioavailability that complicates dose titration and necessitates continuous hematological and biochemical monitoring.

While enteric-coated systems such as Eudragit S100-coated chitosan beads have been reported for azathioprine, these are micro-sized carriers that lack the nano-scale advantages of NLCs. No clinically translatable, nanoparticulate platform has been reported that simultaneously achieves solubility enhancement, gastric-phase protection, and pH-triggered colonic release within a single, pharmaceutically scalable system for azathioprine. Enteric-coated and delayed-release approaches address gastric protection but do not confer true colon-specificity, and no clinically translatable, nanoparticulate platform has been reported that simultaneously achieves solubility enhancement, gastric-phase protection, and pH-triggered colonic release within a single, pharmaceutically scalable system. This unresolved convergence of a pharmacokinetic delivery failure, a physicochemical stability limitation, and the absence of a mechanistically integrated solution constitutes the specific scientific problem addressed by the present study.

1.7 Rationale of the Study

In the present study, a novel colon-targeted azathioprine drug delivery system is formulated by combining two independently validated technologies — NLC-based solubilisation and pH-triggered enteric coating — into a single, integrated platform, an approach that has not previously been reported for azathioprine. The binary solid lipid system of Compritol[®] 888 ATO and Precirol[®] ATO 5 was selected for its established compatibility and its ability to produce imperfect-type NLCs with the structural disorder required for high azathioprine loading; Tween[®] 80 (HLB ~15) stabilises the oil-in-water interface, while Transcutol[®] P acts as a co-solvent and penetration enhancer to improve drug solubility in the lipid phase and support mucosal absorption.

Layering the CAP coating onto this optimised core converts a conventional nanocarrier into a genuinely site-specific delivery system, engineered so that gastric protection and colonic release occur as sequential, independently controlled formulation events rather than as a single uncontrolled release process. The resulting system is expected to achieve entrapment efficiency above 80%, particle size within 100–250 nm, gastric release below 10%, and colonic release above 80% over 24 hours — quality attributes that would represent a clinically meaningful advance over conventional oral azathioprine therapy, achieved entirely through formulation innovation.

1.8 Research Aim and Objectives

1.8.1 Aim

The aim of this research is to formulate and optimise azathioprine-loaded nanostructured lipid carriers (NLCs) with a pH-sensitive cellulose acetate phthalate (CAP) polymer coating to achieve colon-specific drug delivery, ensuring site-specific release and improved stability with minimised systemic exposure.

1.8.2 Objectives

The specific objectives of this study are:

- i. To formulate and optimise azathioprine-loaded NLCs with high encapsulation efficiency, nanoscale particle size (100–250 nm), and amorphous drug incorporation using the hot high-speed homogenisation technique, employing a binary solid lipid system (Compritol[®] 888 ATO and Precirol[®] ATO 5) with oleic acid and a binary surfactant system (Tween[®] 80 and Transcutol[®] P).
- ii. To apply a pH-sensitive CAP coating to the NLC surface using a solvent evaporation–adsorption technique to achieve colon-specific drug delivery with effective gastric protection ($\leq 10\%$ release at pH 1.2).
- iii. To characterise the optimised coated NLC formulation comprehensively using particle size analysis, PDI, zeta potential, entrapment efficiency, FTIR spectroscopy, DSC, XRD, and SEM.
- iv. To evaluate in vitro drug release under physiological pH-switching conditions (pH 1.2 $\rightarrow$ pH 7.4) and characterise release kinetics using zero-order, first-order, Higuchi, and Korsmeyer–Peppas mathematical models.
- v. To assess the physical and chemical stability of the optimised formulation under ICH Q1A(R2) storage conditions at 4°C and 25°C/60% RH over 90 days.
- vi. To conduct an acute oral toxicity study in BALB/c mice to evaluate the safety and biocompatibility of the optimised CAP-coated NLC formulation per OECD Guideline 423.

1.9 Research Hypothesis

Azathioprine-loaded NLCs, prepared using a binary solid lipid matrix of Compritol[®] 888 ATO and Precirol[®] ATO 5 with oleic acid as the liquid lipid and an optimised Tween[®] 80:Transcutol[®] P binary surfactant system (75:25), and

subsequently post-coated with CAP, will demonstrate high entrapment efficiency ($\geq 80\%$), nanoscale particle size (100–250 nm), minimal gastric drug release ($\leq 10\%$ at pH 1.2), and sustained colonic drug release ($\geq 80\%$ at pH 7.4 over 24 h), governed by anomalous non-Fickian transport kinetics. The optimised formulation will maintain adequate physicochemical stability over 90 days under ICH storage conditions and will exhibit complete biocompatibility with no evidence of acute oral toxicity in the murine model, establishing the platform as a promising, safe strategy for site-specific IBD therapy.

Chapter 2

Literature Review

2.1 Inflammatory Bowel Disease: Global and Clinical Perspective

2.1.1 Definition and Classification of Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a chronic, relapsing, immune-mediated inflammatory disorder of the gastrointestinal tract resulting from a complex interaction among genetic susceptibility, immune dysregulation, intestinal microbiota, epithelial barrier dysfunction, and environmental factors [12]. IBD primarily comprises two major clinical entities: Crohn's disease (CD) and ulcerative colitis (UC). Although both disorders share several immunological mechanisms, they differ in disease distribution, histopathology, and clinical manifestations. A small proportion of patients (approximately 5-10%) cannot be clearly classified as either CD or UC and are therefore diagnosed with IBD-unclassified (IBDU) based on inconclusive clinical, endoscopic, radiological, and histopathological findings [13].

2.1.2 Crohn's Disease

Crohn's disease is characterized by chronic transmural inflammation that can involve any part of the gastrointestinal tract, although the terminal ileum and colon are most commonly affected. The disease typically presents with discontinuous "skip lesions" and may lead to complications such as intestinal strictures, fistulae, abscess formation, and bowel obstruction. Patients frequently experience abdominal pain, chronic diarrhoea, weight loss, and fatigue. Despite advances in medical therapy, many patients require surgical intervention because of progressive disease or treatment failure, and postoperative recurrence remains common [14].

2.1.3 Ulcerative Colitis

Ulcerative colitis is a chronic inflammatory disease confined to the colon and rectum, characterized by continuous inflammation limited primarily to the mucosal and submucosal layers. The disease invariably begins in the rectum and extends proximally without skip lesions. Common clinical manifestations include bloody diarrhoea, rectal bleeding, urgency, tenesmus, and abdominal discomfort. Disease severity ranges from mild proctitis to extensive pancolitis, with long-standing extensive disease associated with an increased risk of colorectal cancer and severe complications such as toxic megacolon and colonic perforation [15].

2.1.4 Epidemiology and Global Burden of Inflammatory Bowel Disease

A systematic review analyzing population-based data from 195 countries estimated that the global prevalence of IBD exceeded 6.8 million cases in 2017, representing a dramatic increase from prior decades and establishing IBD as a genuinely global public health priority [16]. The highest incidence and prevalence rates have historically been reported in North America and Northern Europe, where IBD affects 0.3-0.5% of the general population, but a striking epidemiological transition

is underway as newly industrializing nations in Asia, Latin America, and the Middle East report rapidly increasing IBD incidence attributed to westernization of diet, lifestyle, and gut microbial ecology. The parallel trends of stable or plateauing incidence in high-prevalence Western countries and rapidly rising incidence in previously low-prevalence developing countries suggest that environmental exposures including dietary patterns, antibiotic use, reduced enteric pathogen exposure, and changes in the gut microbiome interact with background genetic susceptibility to drive the global IBD epidemic [15].

2.1.5 Prevalence of Inflammatory Bowel Disease in Developing Countries and Pakistan

Although previously considered uncommon in South Asia, the incidence of IBD is steadily increasing in Pakistan and neighboring developing countries. Hospital-based studies from major Pakistani cities have reported a growing number of diagnosed cases, with ulcerative colitis occurring more frequently than Crohn's disease. The absence of a national IBD registry limits accurate estimation of disease prevalence; however, the increasing clinical burden highlights the need for improved diagnostic services and affordable long-term treatment strategies suitable for resource-limited healthcare settings [17].

2.2 Pathophysiology of Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) develops through a complex interaction between genetic susceptibility, environmental factors, intestinal microbiota, epithelial barrier dysfunction, and an abnormal immune response. In genetically predisposed individuals, disruption of immune homeostasis results in an exaggerated inflammatory response against the normal intestinal microbiota, leading to chronic intestinal inflammation and progressive tissue damage [18].

Under physiological conditions, the intestinal mucosal immune system maintains tolerance toward commensal microorganisms while providing protection against pathogens. In IBD, this balance is disrupted, leading to activation of dendritic cells and macrophages that present microbial antigens to naive T lymphocytes. Consequently, Crohn's disease is predominantly associated with a Th1/Th17-mediated immune response characterized by increased production of tumor necrosis factor-alpha (TNF- α), interferon-gamma (IFN- γ), and interleukin-17 (IL-17), whereas ulcerative colitis exhibits a relatively Th2-skewed response involving elevated IL-5 and IL-13 levels. These cytokines amplify inflammation, recruit additional immune cells, and contribute to continuous mucosal injury [19].

Genetic predisposition plays a significant role in disease susceptibility. Genome-wide association studies have identified more than 240 susceptibility loci associated with IBD. Among these, NOD2, ATG16L1, IRGM, and IL23R are the most extensively studied genes. Mutations in NOD2 impair bacterial recognition and innate immune responses, whereas alterations in ATG16L1 and IRGM disrupt autophagy and intracellular bacterial clearance. Variants in IL23R influence Th17-cell differentiation and cytokine signaling, contributing to persistent intestinal inflammation. However, genetic factors alone do not fully explain disease development, highlighting the importance of environmental and microbial influences [20].

Alterations in the intestinal microbiota, commonly referred to as dysbiosis, are another hallmark of IBD. Patients typically exhibit reduced microbial diversity, depletion of beneficial bacteria such as *Faecalibacterium prausnitzii* and *Roseburia intestinalis*, and increased abundance of potentially pathogenic microorganisms, including members of the Enterobacteriaceae family. Dysbiosis reduces the production of beneficial short-chain fatty acids, particularly butyrate, weakens epithelial barrier function, and promotes continuous activation of mucosal immune responses, thereby sustaining chronic inflammation [21].

The integrity of the intestinal epithelial barrier is essential for preventing the translocation of luminal microorganisms and their products into the underlying tissue. This barrier is maintained by epithelial cells connected through tight-junction proteins, including occludin, claudins, and zonula occludens proteins. In

IBD, inflammatory cytokines such as $\text{TNF-}\alpha$, $\text{IFN-}\gamma$, and $\text{IL-1}\beta$ disrupt these tight junctions, increasing intestinal permeability. Concurrent goblet cell dysfunction and depletion of the protective mucus layer further compromise barrier integrity, allowing bacterial antigens to penetrate the lamina propria and perpetuate immune activation.

The inflammatory process is sustained by an extensive network of cytokines and inflammatory mediators. Among these, $\text{TNF-}\alpha$ is considered a key regulator because it activates the $\text{NF-}\kappa\text{B}$ signaling pathway, promotes leukocyte recruitment, induces epithelial apoptosis, disrupts barrier function, and stimulates tissue remodeling. The therapeutic success of anti-TNF monoclonal antibodies, including infliximab and adalimumab, has confirmed the central role of $\text{TNF-}\alpha$ in IBD pathogenesis [22].

Oxidative stress further contributes to disease progression through excessive production of reactive oxygen species (ROS) by activated neutrophils and macrophages. Elevated ROS levels damage lipids, proteins, and

DNA, resulting in epithelial injury, mitochondrial dysfunction, and increased intestinal permeability. Persistent oxidative stress not only aggravates inflammation but also contributes to fibrosis in Crohn's disease and increases the long-term risk of colorectal cancer in ulcerative colitis [23].

Chronic inflammation in IBD is ultimately maintained by defective immune regulation. Impaired regulatory T-cell function, reduced secretion of anti-inflammatory cytokines such as IL-10 and transforming growth factor-beta ($\text{TGF-}\beta$), and prolonged activation of memory T and B lymphocytes prevent resolution of the inflammatory response.

Consequently, continuous cycles of inflammation, tissue injury, and repair lead to progressive structural damage of the intestinal wall. These pathogenic mechanisms emphasize the importance of therapeutic strategies capable of delivering anti-inflammatory agents directly to the diseased colonic mucosa while minimizing systemic exposure and associated adverse effects [24]. Simplified scheme is shown in figure 2.1.

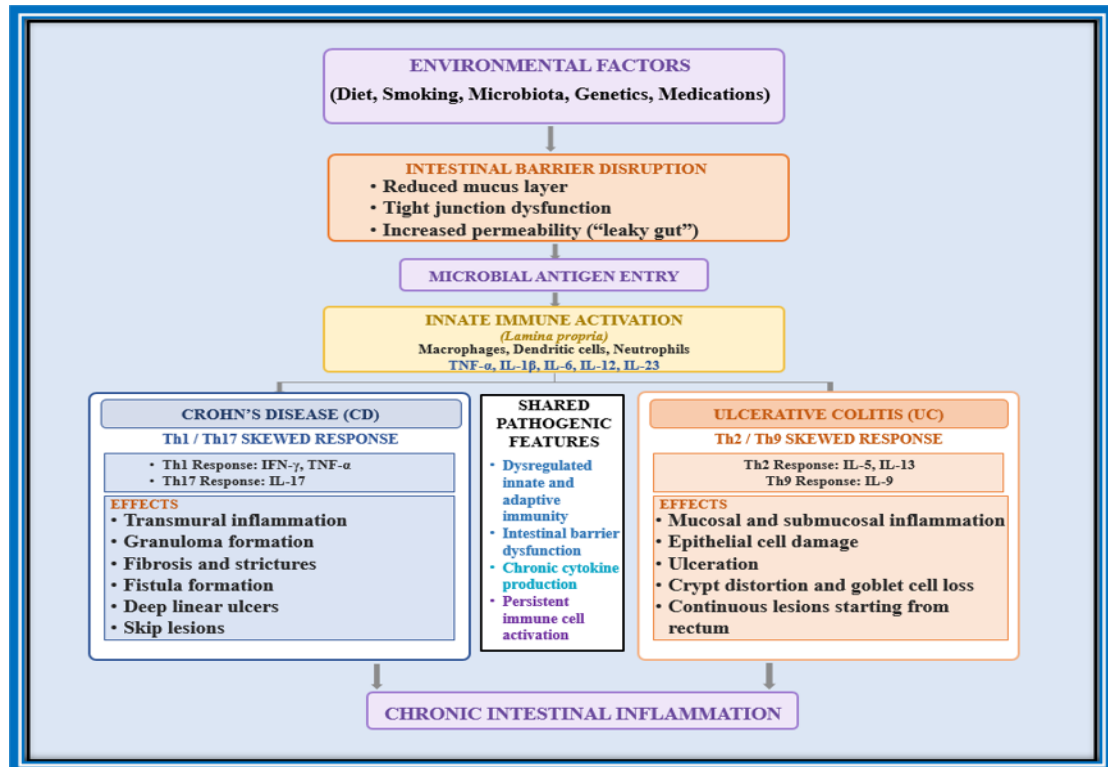


FIGURE 2.1: Simplified schematic of the immunopathogenesis of inflammatory bowel disease. Crohn's disease is predominantly mediated by Th1/Th17 immune responses with increased TNF- α , IFN- γ , and IL-17, whereas ulcerative colitis is characterized by a Th2-skewed response involving IL-5 and IL-13. Both conditions share epithelial barrier dysfunction, dysbiosis, and dysregulated innate immunity as central pathogenic mechanisms.

2.3 Current Therapeutic Approaches for IBD and Their Limitations

The management of inflammatory bowel disease (IBD) aims to induce and maintain clinical remission, promote mucosal healing, reduce disease complications, and improve patients' quality of life. Treatment selection depends on disease type, severity, location, and individual patient characteristics.

Current therapeutic strategies include aminosalicylates, corticosteroids, immuno modulators, biologics, and small-molecule targeted therapies, each with distinct mechanisms of action and limitations. The major therapeutic agents used in IBD management are summarized in Table 2.1.

TABLE 2.1: Summary of current therapeutic agents used in the management of IBD, with mechanisms of action and key limitations.

Drug Class	Agents	Mechanism of Action	Key Limitations
Aminosalicylates	Mesalazine, Sulfasalazine	Local inhibition of prostaglandin and leukotriene synthesis; anti-inflammatory	Limited efficacy in Crohn's disease; gastrointestinal intolerance
Corticosteroids	Prednisolone, Budesonide	Broad suppression of inflammatory cytokine production	Unsuitable for long-term maintenance due to systemic adverse effects
Immunomodulators	Azathioprine, 6-MP, Methotrexate	Inhibition of purine synthesis and lymphocyte proliferation	Slow onset, myelosuppression, hepatotoxicity
Anti-TNF Biologics	Infliximab, Adalimumab	Neutralization of TNF- α and suppression of inflammation	High cost, immunogenicity, infection risk
Gut-Selective Biologics	Vedolizumab, Ustekinumab	Inhibition of leukocyte trafficking or IL-12/23 signaling	Expensive; limited availability in developing countries
JAK Inhibitors	Tofacitinib, Upadacitinib	Inhibition of Janus kinase signaling pathways	Risk of infections, thrombosis, and uncertain long-term safety

CD = Crohn's disease; UC = Ulcerative colitis; 6-MP = 6-mercaptopurine; JAK = Janus kinase.

2.3.1 Aminosalicylates

Aminosalicylates, including mesalazine and sulfasalazine, remain the first-line therapy for patients with mild-to-moderate ulcerative colitis. These agents exert their therapeutic effects through local inhibition of inflammatory mediators, suppression of cytokine production, and antioxidant activity within the colonic mucosa. Various oral and rectal formulations have been developed to optimize colonic drug delivery. However, their clinical benefit in Crohn's disease is limited,

and they are generally ineffective in moderate-to-severe IBD, where escalation to immunomodulatory or biological therapy is often required [25].

2.3.2 Corticosteroids

Systemic corticosteroids such as prednisolone and intravenous methylprednisolone, as well as locally acting budesonide, are highly effective for inducing remission during acute IBD exacerbations. Their potent anti-inflammatory effects result from suppression of multiple inflammatory signaling pathways. Nevertheless, prolonged corticosteroid therapy is associated with serious adverse effects, including osteoporosis, hyperglycemia, hypertension, adrenal suppression, and increased susceptibility to infection. Consequently, corticosteroids are recommended only for short-term induction therapy and not for long-term disease maintenance [26].

2.3.3 Immunosuppressive Agents

Thiopurine immunomodulators, particularly azathioprine and 6-mercaptopurine, are widely used for maintaining long-term remission in both Crohn's disease and ulcerative colitis, especially in steroid-dependent patients. Methotrexate serves as an alternative in selected cases of Crohn's disease. Despite their clinical effectiveness, these agents exhibit a delayed onset of action and may produce significant adverse effects, including myelosuppression, hepatotoxicity, and pancreatitis. Variability in thiopurine metabolism further necessitates individualized dosing and careful patient monitoring [27].

2.3.4 Biologics and Targeted Therapies

Biological agents have significantly improved the management of moderate-to-severe IBD by targeting specific inflammatory pathways. Anti-TNF agents, including infliximab and adalimumab, effectively induce mucosal healing and reduce disease progression. More recently, gut-selective biologics such as vedolizumab and

IL-12/23 inhibitors such as ustekinumab have expanded available treatment options with improved safety profiles. Small-molecule Janus kinase (JAK) inhibitors offer the advantage of oral administration and rapid clinical response but require careful monitoring because of potential risks of serious infections and thromboembolic events [28].

2.3.5 Limitations of Conventional Therapies and Need for Site-specific Drug Delivery

Despite considerable advances in IBD therapy, conventional oral and systemic drug delivery systems remain limited by non-specific drug distribution, resulting in inadequate drug concentrations at the inflamed colonic mucosa and unnecessary systemic exposure. This limitation is particularly important for immunosuppressive agents such as azathioprine, whose therapeutic use is frequently restricted by dose-dependent toxicities, including bone marrow suppression and hepatotoxicity. Consequently, site-specific colon-targeted drug delivery systems have emerged as a promising strategy to maximize local drug concentrations while minimizing systemic adverse effects. Such targeted delivery approaches have the potential to improve therapeutic efficacy, enhance patient safety, and reduce the overall treatment burden in IBD management [29, 30].

2.4 Azathioprine

2.4.1 Introduction to Azathioprine

Azathioprine is a thiopurine prodrug chemically designated as 6-[(1-methyl-4-nitroimidazol-5-yl)thio]purine that has been used for more than five decades as an immunosuppressive agent in organ transplantation and autoimmune diseases, including inflammatory bowel disease (IBD). Following oral administration, azathioprine undergoes rapid non-enzymatic conversion to 6-mercaptopurine (6-MP), which is subsequently metabolized into active and inactive metabolites responsible

for its therapeutic effects and toxicity [31]. It belongs to BCS class 4 meaning low bioavailability and permeability [32].

2.4.2 Physicochemical Properties

Azathioprine is a yellow crystalline powder with a molecular formula of $C_9H_7N_7S$ and a molecular weight of 277.26 g/mol. It exhibits poor aqueous solubility, with solubility increasing under neutral to slightly alkaline conditions, low lipophilicity ($\log P \approx 0.10$), and a high melting point (243-244°C) [33–35].

In addition, it is susceptible to hydrolytic degradation under acidic and alkaline conditions. These physicochemical limitations contribute to poor oral performance and justify the development of lipid-based nanoformulations, such as nanostructured lipid carriers (NLCs), to improve drug stability, solubility, and colon-targeted delivery [36].

2.4.3 Pharmacological Basis for Colon Targeting

Azathioprine is recommended for maintaining remission in patients with Crohn's disease and ulcerative colitis, particularly those with steroid-dependent or frequently relapsing disease. The SONIC trial demonstrated that combination therapy with azathioprine and infliximab achieved superior corticosteroid-free remission and mucosal healing compared with either therapy alone. Furthermore, azathioprine reduces corticosteroid dependence, decreases postoperative recurrence, and improves long-term disease control [36].

Despite its clinical efficacy, conventional oral azathioprine is absorbed mainly in the proximal small intestine, whereas its therapeutic target in IBD is the inflamed colonic mucosa. This pharmacokinetic mismatch results in unnecessary systemic exposure and dose-related toxicity while limiting local drug availability. Therefore, colon-targeted drug delivery systems are considered a promising approach to increase drug concentration at the site of inflammation while minimizing systemic

adverse effects. This concept provides the pharmacological basis for the development of CAP-coated azathioprine-loaded nanostructured lipid carriers (NLCs) [33].

2.4.4 Mechanism of Action

The immunosuppressive activity of azathioprine is mediated through its active metabolites following conversion to 6-mercaptopurine (6-MP). Subsequently, thioinosine monophosphate (TIMP) and methyl-TIMP inhibit PRPP amidotransferase (amidophosphoribosyltransferase or GPAT), the rate-limiting enzyme of de novo purine synthesis, thereby reducing the availability of purine nucleotides required for DNA and RNA synthesis in rapidly proliferating lymphocytes. In parallel, 6-thioguanine nucleotides (6-TGNs), particularly 6-thioguanine triphosphate (6-TGTP), are incorporated into DNA in place of guanine, resulting in DNA damage, inhibition of lymphocyte proliferation, and cell cycle arrest. Additionally, 6-TGTP inhibits the small GTPase Rac1, disrupting Vav1-dependent T-cell activation and inducing mitochondrial apoptosis through the intrinsic pathway [37].

The balance between active and inactive metabolites is regulated by competing metabolic enzymes. Thiopurine S-methyltransferase (TPMT) converts 6-MP to inactive 6-methylmercaptopurine (6-MMP), whereas xanthine oxidase metabolizes it to inactive 6-thiouric acid. Genetic polymorphisms in TPMT contribute to considerable interpatient variability in therapeutic response and toxicity, making TPMT genotyping or phenotyping an important consideration before initiating thiopurine therapy [38].

2.4.5 Limitations of Conventional Azathioprine Therapy

Despite its proven efficacy in maintaining remission in IBD, the clinical use of azathioprine is limited by its pharmacokinetic characteristics and systemic toxicity. Following oral administration, azathioprine is rapidly absorbed from the proximal

small intestine, reaching peak plasma concentrations within 1-2 h. However, its oral bioavailability is reduced to less than 47% because of extensive intestinal and hepatic first-pass metabolism.

Intracellular 6-thioguanine nucleotide (6-TGN) concentrations determine therapeutic efficacy and hematological toxicity, while interpatient variability in metabolism further complicates dose individualization.

The major dose-limiting adverse effect is myelosuppression, which manifests as leucopenia, thrombocytopenia, and anemia due to suppression of hematopoietic precursor cells by 6-TGN. The risk is markedly increased in patients with reduced TPMT activity. Other clinically significant adverse effects include hepatotoxicity, reported in 3-10% of patients, idiosyncratic pancreatitis in 3-5%, gastrointestinal intolerance, and an increased risk of infections and malignancies, including a four- to five-fold higher risk of non-Hodgkin lymphoma during long-term immunosuppressive therapy [39, 40].

Conventional immediate-release azathioprine tablets release the drug throughout the gastrointestinal tract, with absorption occurring predominantly in the proximal small intestine rather than at the inflamed colonic mucosa. In addition, its poor aqueous solubility, crystalline nature, and extensive first-pass metabolism contribute to variable bioavailability and systemic drug exposure. These limitations provide a strong rationale for developing colon-targeted CAP-coated azathioprine-loaded nanostructured lipid carriers (NLCs) capable of improving local drug delivery while minimizing systemic toxicity [41].

2.4.6 Previous Azathioprine Delivery Systems

Compared with other anti-inflammatory drugs used in IBD, relatively few advanced drug delivery systems have been developed specifically for azathioprine. One notable study reported a pH-sensitive chitosan bead formulation coated with Eudragit[®] S100, which achieved colon-specific drug release and improved anti-inflammatory activity in an experimental colitis model.

Although this study demonstrated the feasibility of colon-targeted azathioprine delivery, the formulation employed microsized beads rather than nanoparticles. Unlike nanoparticulate systems, bead-based carriers provide limited mucus penetration, reduced interaction with inflamed colonic tissue, and less efficient controlled drug release. Furthermore, studies investigating azathioprine-loaded lipid nanoparticles remain scarce, highlighting a significant gap in the literature.

To date, no published study has reported a cellulose acetate phthalate (CAP)-coated azathioprine-loaded nanostructured lipid carrier (NLC) that combines pH-responsive colon targeting with the advantages of NLCs, including enhanced drug loading, controlled release, and improved stability. This research gap provides the scientific rationale for the present study and is discussed further in the literature gap section.

2.5 Colon-targeted Drug Delivery: Rationale and Strategies

2.5.1 Physiological Basis for Colon Targeting

Inflammatory bowel disease (IBD), comprising Crohn's disease (CD) and ulcerative colitis (UC), is characterized by chronic inflammation primarily affecting the intestinal mucosa, with the colon representing the principal site of disease involvement in ulcerative colitis and a major site of pathology in colonic Crohn's disease.

As discussed in Chapter 1, conventional oral drug therapy exposes the entire gastrointestinal tract and systemic circulation to therapeutic agents despite the pathological target being localized predominantly within the inflamed colonic mucosa. Consequently, colon-targeted drug delivery has emerged as a rational pharmaceutical strategy aimed at maximizing local drug concentration at the disease site while simultaneously minimizing systemic drug exposure and associated adverse effects [42].

The colon possesses several unique physiological characteristics that distinguish it from the upper gastrointestinal tract and make it particularly suitable for targeted drug delivery. Compared with the stomach and small intestine, the colon exhibits a prolonged transit time ranging from approximately 20 to 48 hours, allowing sufficient residence time for sustained drug release.

Furthermore, the colonic environment is characterized by a near-neutral pH, generally ranging between 6.4 and 7.4 under physiological conditions, although minor variations may occur depending on disease activity and individual patient characteristics [43].

In addition, the colon contains an exceptionally dense microbial population exceeding 10^{11} - 10^{12} microorganisms per gram of luminal content, together with relatively low digestive enzyme activity compared with the upper gastrointestinal tract. These physiological features provide multiple opportunities for designing drug delivery systems capable of resisting gastric and intestinal conditions before releasing their payload selectively within the colon [44].

For colon-specific diseases such as IBD, the objective of drug delivery extends beyond improving conventional oral bioavailability. Instead, the principal aim is to redirect drug exposure toward the inflamed colonic mucosa while minimizing unnecessary systemic absorption.

Such site-specific delivery has the potential to improve therapeutic efficacy by maintaining higher local drug concentrations while simultaneously reducing dose-dependent systemic toxicity [45].

This principle is particularly important for immunosuppressive agents such as azathioprine, whose therapeutic action is required primarily within inflamed intestinal tissues rather than throughout the systemic circulation.

Therefore, successful colon-targeted formulations must achieve two fundamental objectives simultaneously. First, the delivery system must effectively protect the encapsulated drug from premature release during gastric and small intestinal transit.

Second, drug release should occur selectively after the carrier reaches the colonic environment, thereby ensuring maximum therapeutic exposure at the disease site. These requirements have driven the development of several colon-targeting strategies exploiting the unique physiological characteristics of the distal gastrointestinal tract [46].

2.5.2 Strategies for Colon-targeted Drug Delivery

Several pharmaceutical approaches have been developed to achieve site-specific drug delivery to the colon. These strategies exploit different physiological triggers encountered during gastrointestinal transit and may be broadly classified into pH-dependent, time-dependent, microflora-activated, and pressure-controlled delivery systems.

Among these approaches, pH-dependent drug delivery is the most extensively investigated and clinically established strategy. It utilizes enteric polymers that remain insoluble under acidic gastric conditions but dissolve once the surrounding pH exceeds a predetermined threshold. Because gastrointestinal pH gradually increases from approximately 1-3 in the stomach to about 6-7 in the distal small intestine and colon, appropriately selected enteric polymers can delay drug release until the formulation reaches the lower gastrointestinal tract [47]. Several commercially available colon-targeted products successfully employ this principle, demonstrating its pharmaceutical reliability and clinical applicability.

Time-dependent delivery systems depend on the relatively predictable gastrointestinal transit time to delay drug release following gastric emptying. These systems usually employ polymer coatings or capsule designs that provide a predetermined lag time before drug release begins. Although conceptually simple, their clinical performance is often compromised by substantial inter-patient variability in gastric emptying and intestinal transit times. Such variability becomes even more pronounced in patients with inflammatory bowel disease due to diarrhea, altered intestinal motility, and active inflammation, thereby limiting the reliability of time-controlled formulations [48].

Microflora-activated systems exploit the dense bacterial population residing within the colon. Polysaccharides such as pectin, guar gum, dextran, chitosan, and inulin remain largely resistant to digestion in the upper gastrointestinal tract but undergo enzymatic degradation by colonic bacterial enzymes, resulting in localized drug release. While this approach offers excellent colon specificity, its performance may be influenced by alterations in gut microbiota composition. Since IBD is frequently associated with significant microbial dysbiosis, degradation rates of these polysaccharides may become unpredictable, reducing formulation consistency among patients [49].

A fourth strategy involves pressure-controlled drug delivery, in which specially engineered coatings rupture under the increased luminal pressure generated by colonic peristalsis. Although this approach theoretically provides colon-selective release, abnormal intestinal motility commonly observed in IBD patients may reduce its reproducibility, limiting its widespread application [50].

Among these various approaches, pH-responsive delivery systems remain the preferred strategy because they combine relatively simple manufacturing processes, reproducible drug release characteristics, regulatory acceptance, and extensive clinical validation. Consequently, pH-triggered colon targeting forms the fundamental basis of the present formulation.

2.5.3 pH-Responsive Polymers for Colon-Targeted Drug Delivery

The success of pH-dependent colon targeting depends largely upon selecting an enteric polymer with an appropriate dissolution threshold. Several polymers have been investigated for this purpose, including Eudragit[®] L100, Eudragit[®] S100, hydroxypropyl methylcellulose phthalate (HPMCP), polyvinyl acetate phthalate (PVAP), and cellulose acetate phthalate (CAP). Although each polymer exhibits pH-sensitive solubility, differences in their dissolution characteristics determine the region of the gastrointestinal tract where drug release occurs [51].

Eudragit[®] L100 dissolves at approximately pH 6.0 and therefore begins releasing drug within the distal ileum. While suitable for certain applications, premature dissolution may reduce the amount of drug reaching the colon.

In contrast, Eudragit[®] S100 dissolves only at pH values above 7.0, delaying drug release until the distal colon. Although beneficial for diseases involving the distal colon, this higher dissolution threshold may postpone drug release beyond regions of active inflammation in many IBD patients [52].

TABLE 2.2: pH-responsive enteric polymers used for colon-targeted delivery.

Polymer		Dissolution pH	Approximate release site	Notes
Eudragit L100	[®]	≥ 6.0	Distal ileum	Releases before the colon proper; risk of premature release
Eudragit S100	[®]	≥ 7.0	Distal colon	Higher threshold delays release into distal colon, where inflammation may already be resolving
HPMCP 55/HP-50)	(HP-	5.5-6.0	Terminal ileum/proximal colon	Similar range to CAP; considerably less pharmacopoeial precedent in colon-targeted NLC coating specifically
PVAP		~ 5.0	Terminal ileum	Lower threshold; least colon-specific of the group
Cellulose etate phthalate (CAP)	ac-	≥ 6.0	Proximal colon	>60-year pharmacopoeial history (USP/BP/EP); threshold aligns closely with proximal colonic pH

Hydroxypropyl methylcellulose phthalate exhibits dissolution behavior similar to CAP, generally dissolving between pH 5.5 and 6.0. However, compared with CAP, its application in colon-targeted lipid nanoparticle formulations remains relatively limited. Polyvinyl acetate phthalate possesses an even lower dissolution threshold of approximately pH 5.0, increasing the likelihood of premature drug release before the formulation reaches the colon [53].

Among these enteric polymers, cellulose acetate phthalate (CAP) represents a particularly suitable choice for colon-targeted lipid nanocarrier systems. CAP remains

stable under gastric conditions but undergoes rapid ionization and dissolution once exposed to the near-neutral pH of the proximal colon.

This dissolution threshold closely corresponds to the physiological pH encountered at the beginning of the colon, enabling earlier and more localized drug release than polymers requiring higher pH values. Furthermore, CAP possesses an extensive pharmaceutical history spanning more than six decades and is officially recognized by major pharmacopoeias, including the United States Pharmacopeia (USP), British Pharmacopoeia (BP), and European Pharmacopoeia (EP). Its well-established safety profile, regulatory acceptance, and compatibility with pharmaceutical manufacturing further support its selection as the enteric coating polymer for the present study [54].

Therefore, among the available pH-sensitive polymers, CAP provides an optimal balance between gastric protection and timely drug release within the proximal colon. This characteristic makes it particularly appropriate for delivering azathioprine directly to inflamed colonic tissues while minimizing premature drug release in the upper gastrointestinal tract [55].

Beyond its long-standing role in conventional tablet and pellet enteric coatings, a growing body of more recent literature has explored CAP specifically at the nanoparticulate and microparticulate scale, lending further support to its selection for the present nanostructured lipid carrier (NLC) formulation. Mandal *et al.* fabricated dolutegravir-loaded CAP nanoparticles (~ 200 nm, polydispersity index < 0.2) by a modified water-in-oil-in-water emulsification technique and demonstrated that the particles remained intact at acidic pH while releasing over 80% of the entrapped drug within one hour upon exposure to near-neutral pH, directly confirming the pH-triggered disintegration behaviour of nanoscale CAP carriers [56].

Similarly, Singh *et al.* developed CAP-coated pectin microparticles co-loaded with mesalamine and the probiotic *Saccharomyces boulardii* for site-specific colonic delivery, and reported that the CAP coating minimised premature release in simulated gastric and intestinal fluid while permitting a synergistic anti-inflammatory

effect in a 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis model, with a subsequent in vivo evaluation confirming reduced colonic inflammation and preserved macroscopic architecture of the colon [57, 58]. In the same vein, Vidal-Romero *et al.* prepared CAP-based nanospheres and nanocapsules (approximately 250-300 nm) loaded with chlorhexidine for periodontal application, and reported pH-dependent, diffusion- and erosion-controlled drug release governed by the same ionizable phthalate carboxylic acid groups ($pK_a \sim 5.28$) that underlie CAP's colon-targeting mechanism in particulate and coated systems [59].

Collectively, these nanoscale and microparticulate studies, published after 2015, reinforce that the pH-responsive dissolution behaviour of CAP established for conventional dosage forms is preserved and, in several cases, accelerated when the polymer is applied as a thin coating on sub-micron drug carriers, supporting its rational extension to NLC-based platforms.

The combination of pH-sensitive enteric polymers with lipid-based nanocarriers for colon targeting has also gained momentum in the recent literature, even where CAP itself has not yet been the coating polymer of choice. Kim *et al.* developed a ternary nanocomposite carrier consisting of a liposomal core, an aminoclay intermediate layer, and an outer coating of the structurally related enteric polymer Eudragit[®] S100 for the colon-targeted delivery of budesonide; the dual-coated liposomes released less than 10% of the entrapped drug after two hours in simulated gastric fluid (pH 1.2) but underwent rapid size reduction and drug release at pH 7.4, together with enhanced colonic residence time and reduced systemic exposure in a rodent model [60].

In a closely related and directly comparable example, Dubey *et al.* developed Eudragit S-100-coated nanostructured lipid carriers for enhanced colonic delivery of rebamipide in ulcerative colitis, demonstrating that an enteric-polymer-coated NLC platform very similar in design to the present azathioprine system can achieve pH-triggered, colon-restricted release from a lipid nanoparticle core [61]. In a related direction, nanostructured lipid carriers loaded with trimebutine maleate have been evaluated for their protective effect in acute experimental colitis, and reviews of NLC-based colon delivery platforms have specifically identified

pH-sensitive polymer coating as one of the principal surface-modification strategies for achieving colon-restricted release from lipid nanocarriers [62, 63]. Notably, however, the great majority of pH-sensitive polymer-coated lipid nanocarriers reported to date rely on Eudragit-type methacrylate copolymers rather than CAP, and CAP-coated NLC systems for colon-targeted, small-molecule immunomodulatory drugs such as azathioprine remain scarcely reported in the literature. This relative gap, together with CAP's lower dissolution pH threshold relative to Eudragit S100 and its long clinical track record, further justifies the selection of CAP as the coating polymer for the azathioprine-loaded NLC system developed in the present work.

A more detailed comparison of CAP with alternative enteric polymers in the context of lipid nanoparticle coating is presented later in Section 2.4 after discussing the nanostructured lipid carrier platform.

The application of pH-sensitive CAP coating to NLCs for colon-targeted delivery leverages the specific interaction between the polymer's ionizable phthalate carboxylic acid groups ($pK_a \sim 5.28$) and the gastrointestinal pH gradient. At gastric pH (1.0-3.0), protonated CAP remains hydrophobic and intact, forming a compact barrier that prevents water ingress and drug diffusion from the NLC core. As the formulation transits through the small intestine and enters the proximal colon at pH 6.0-7.4, progressive deprotonation of CAP carboxylate groups generates negatively charged, hydrophilic moieties whose electrostatic repulsion drives polymer chain hydration, swelling, and dissolution, exposing the NLC lipid matrix to colonic fluid and initiating sustained drug release.

This pH-dependent ionization behaviour has been further corroborated in the recent pharmaceutical technology literature discussing CAP within the broader family of cellulose-ester and cellulose-ether-ester enteric polymers used for oral colon-targeted delivery, including comparative reviews that continue to cite CAP alongside cellulose acetate trimellitate and hydroxypropyl methylcellulose phthalate/acetate succinate as functionally equivalent, phthalate- or succinate-bearing alternatives distinguished mainly by their differing dissolution pH thresholds and degrees of substitution [64]. Reviews of polysaccharide- and cellulose-based oral

colon-targeted delivery systems have also continued to reference CAP-coated formulations of azathioprine and other colon-directed drugs as established precedents for combining CAP with polysaccharide or particulate cores, underscoring the continuity between earlier CAP-coated tablet/pellet systems and the CAP-coated lipid nanocarrier strategy adopted in the present work [65].

2.6 Nanostructured Lipid Carriers: An Enabling Platform

2.6.1 Evolution from Solid Lipid Nanoparticles to Nanostructured Lipid Carriers

Nanostructured lipid carriers (NLCs) are second-generation lipid nanoparticles developed to overcome the limitations of solid lipid nanoparticles (SLNs). Although SLNs provide good biocompatibility and protect drugs from degradation, their highly ordered crystalline matrix often results in limited drug loading and progressive drug expulsion during storage due to lipid recrystallization [66]. To address these limitations, NLCs were developed by incorporating a liquid lipid into the solid lipid matrix, creating structural imperfections that increase drug accommodation and improve formulation stability.

TABLE 2.3: Comparison of principal nanocarrier systems for pharmaceutical drug delivery

Property	Liposomes	Polymeric NPs	Dendrimers	SLNs	NLCs
Drug loading	Moderate	High	Moderate	Limited	★ High
Stability	Poor	Good	Good	Moderate	★ Excellent
Biocompatibility	Good	Moderate	Cytotoxic	Good	★ Excellent
Scale-up	Difficult	Moderate	Difficult	Feasible	★ Feasible
Organic solvents	Required	Required	Required	Not needed	★ Not needed
Drug expulsion	Low	Low	Low	High risk	★ Minimized
Controlled release	Yes	Yes	Yes	Yes	★ Superior

★ = best relative performance

Compared with other nanocarriers such as liposomes, polymeric nanoparticles, and dendrimers, NLCs offer several pharmaceutical advantages, including higher drug loading, improved physical stability, solvent-free preparation, excellent biocompatibility, and easier scale-up. These advantages make NLCs particularly suitable for oral drug delivery systems intended for long-term treatment of chronic diseases such as inflammatory bowel disease (IBD). For azathioprine, the selection of NLCs is based not only on improved drug encapsulation but also on their ability to enhance drug stability, provide sustained release, and serve as a platform for pH-responsive colon targeting

2.6.2 Structural Classification of Nanostructured Lipid Carriers

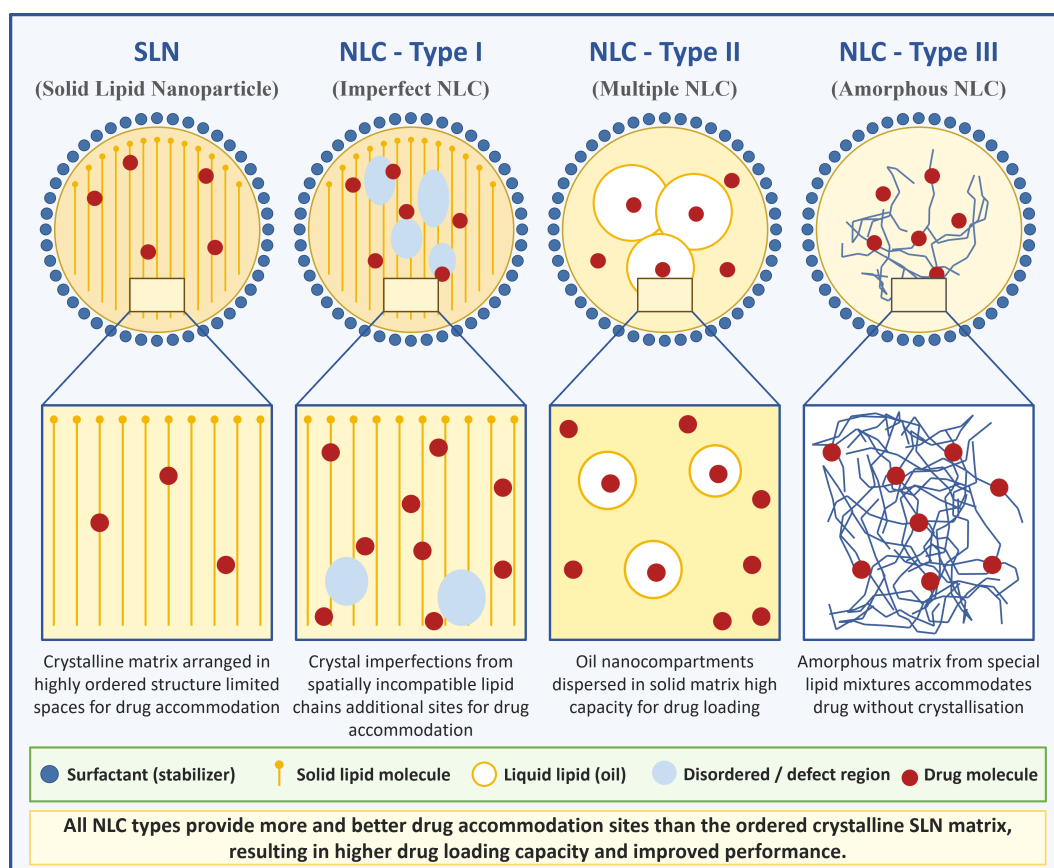


FIGURE 2.2: Schematic illustration of NLC classification created using BioRender.com. Type I (imperfect NLCs): spatial incompatibility between solid and liquid lipid chains creates crystal imperfections and disordered domains that accommodate drug molecules. Type II (multiple NLCs): liquid lipid oil nanocompartments dispersed within the solid lipid matrix provide high drug loading capacity. Type III (amorphous NLCs): special lipid combinations yield an amorphous matrix that accommodates drug without crystallization. All types offer advantages over the ordered crystalline SLN matrix

The extent of this structural heterogeneity depends on the solid-lipid: liquid-lipid ratio, the chemical compatibility of the lipid pair selected, and the processing conditions employed [67]. Based on the nature of the solid-liquid lipid interaction, NLCs are illustrated in figure 2.2.

2.6.3 Composition and Functional Role of Nanostructured Lipid Carrier Components

An NLC formulation consists of a solid lipid, a liquid lipid, surfactants, and the therapeutic drug, with each component performing a specific function that contributes to overall formulation performance.

TABLE 2.4: Physicochemical properties and formulation roles of excipients in the proposed azathioprine-loaded CAP-coated NLC.

Excipient	Category	Key properties	Role in this formulation
Compritol [®] 888 ATO	Solid lipid	Glyceryl behenate; mp $\sim 70^{\circ}\text{C}$; waxy solid	Primary structural matrix; controls release rate
Precirol [®] 5 ATO	Solid lipid	Glyceryl palmitostearate; mp $\sim 57^{\circ}\text{C}$	Binary blend partner; introduces crystal imperfections; increases loading capacity
Oleic acid	Liquid lipid	C18:1 unsaturated; mp $\sim 16^{\circ}\text{C}$; GRAS	Creates the disordered NLC matrix; enhances drug accommodation
Tween [®] 80	Surfactant	HLB ~ 15 ; non-ionic; polyoxyethylene sorbitan monooleate	Primary emulsifier; stabilises o/w interface; prevents particle aggregation
Transcutol [®] P	Co-surfactant/co-solvent	High solubilizing capacity for lipophilic and hydrophilic drugs; low viscosity; miscible with water, oils, and surfactants	Improves drug solubility in the lipid phase; aids self-Nano emulsification; enhances mucosal permeation
CAP	pH-sensitive polymer	Dissolves above pH ~ 6 ; Ionizable phthalate carboxylic acid groups	pH-triggered colon-targeting coat; prevents gastric drug release

The solid lipid forms the structural matrix responsible for controlling drug release and maintaining particle integrity. In the present study, Compritol[®] 888 ATO and Precirol[®] ATO 5 were selected as binary solid lipids because their different fatty acid chain lengths create structural imperfections that increase drug loading while improving storage stability [68].

Oleic acid functions as the liquid lipid. Its incorporation disrupts the crystalline packing of the solid lipid matrix, generating additional space for drug entrapment while improving matrix flexibility and sustained drug release [69].

The surfactant system, consisting of Tween[®] 80 and Transcutol[®] P, stabilizes the nanoparticles by reducing interfacial tension and preventing particle aggregation. Transcutol[®] P additionally enhances drug solubility within the lipid matrix and facilitates formulation stability [70].

To achieve colon-specific delivery, the optimized NLCs are coated with cellulose acetate phthalate (CAP), a pH-responsive polymer that remains intact under gastric conditions but dissolves in the near-neutral pH of the colon. This coating protects azathioprine from premature release and ensures site-specific drug delivery.

2.6.4 Advantages of Nanostructured Lipid Carriers in Oral and Colon-targeted Drug Delivery

NLCs offer a compelling set of advantages for oral drug delivery applications that directly address the physicochemical and pharmacokinetic challenges of azathioprine. The conversion of crystalline drug to an amorphous or molecularly dispersed state within the NLC matrix substantially enhances apparent aqueous solubility and dissolution rate. The solid lipid matrix protects encapsulated drugs from acid-catalyzed and enzymatic degradation during gastrointestinal transit, while lipid excipients stimulate bile secretion and promote the formation of mixed micelles capable of solubilizing and transporting drug to the enterocyte brush border membrane [71]. However, chylomicron-mediated intestinal lymphatic transport a mechanism well-documented for highly lipophilic drugs (typically $\log P > 5$) that

bypasses hepatic first-pass metabolism and can increase bioavailability 2-5-fold is not expected to contribute meaningfully to azathioprine absorption given its hydrophilic nature ($\log P \approx 0.10$). Instead, bioavailability enhancement for azathioprine arises primarily from improved solubilization, protection from presystemic degradation, and prolonged mucosal residence time. For colon-targeted applications such as azathioprine delivery in IBD, these mechanisms are complemented by the drug-release-controlling properties of the CAP coating, ensuring that enhanced dissolution occurs selectively within the colonic target compartment rather than in the upper gastrointestinal tract [72]. The application of pH-sensitive polymer-coated NLCs to colon-specific drug delivery in IBD has been reported in a growing body of literature that consistently demonstrates the feasibility and superiority of this hybrid approach over conventional formulations, with CAP- and Eudragit S100-coated NLCs loaded with budesonide, curcumin, tacrolimus, and celecoxib demonstrating gastric drug release below 10% and colonic release above 80% in representative published studies [73].

2.6.5 Drug Release Mechanisms and Physical Stability of Nanostructured Lipid Carriers

Drug release from NLC formulations occurs through a combination of interrelated mechanisms: diffusion-driven release, in which drug molecules migrate from the high-concentration NLC core through the disordered lipid matrix along a concentration gradient governed by Fick's first law, and lipid matrix erosion by pancreatic lipase and other gastrointestinal lipolytic enzymes, which progressively degrades the outer lipid layers and reduces the effective diffusion path length [74]. The Korsmeyer-Peppas release exponent (n) provides mechanistic discrimination between Fickian diffusion ($n \leq 0.45$), anomalous non-Fickian transport combining diffusion and matrix relaxation ($0.45 < n < 0.89$), and Case II transport dominated by matrix erosion ($n \geq 0.89$) [75]. The long-term physical stability of NLC dispersions is influenced primarily by particle aggregation driven by van der Waals attractive forces, Ostwald ripening, and lipid polymorphic transitions that alter matrix crystallinity over time; non-ionic surfactant stabilization provides

a steric energy barrier resisting aggregation, and the binary solid lipid system retards polymorphic transitions by introducing molecular heterogeneity that stabilizes metastable crystalline forms [76].

2.6.6 pH-Sensitive Polymer Coating for Colon - Targeted Nanostructured Lipid Carriers Delivery

The application of pH-sensitive CAP coating to NLCs for colon-targeted delivery leverages the specific interaction between the polymer's ionizable phthalate carboxylic acid groups ($pK_a \sim 5.28$) and the gastrointestinal pH gradient. At gastric pH (1.0-3.0), protonated CAP remains hydrophobic and intact, forming a compact barrier that prevents water ingress and drug diffusion from the NLC core. As the formulation transits through the small intestine and enters the proximal colon at pH 6.0-7.4, progressive deprotonation of CAP carboxylate groups generates negatively charged, hydrophilic moieties whose electrostatic repulsion drives polymer chain hydration, swelling, and dissolution, exposing the NLC lipid matrix to colonic fluid and initiating sustained drug release.

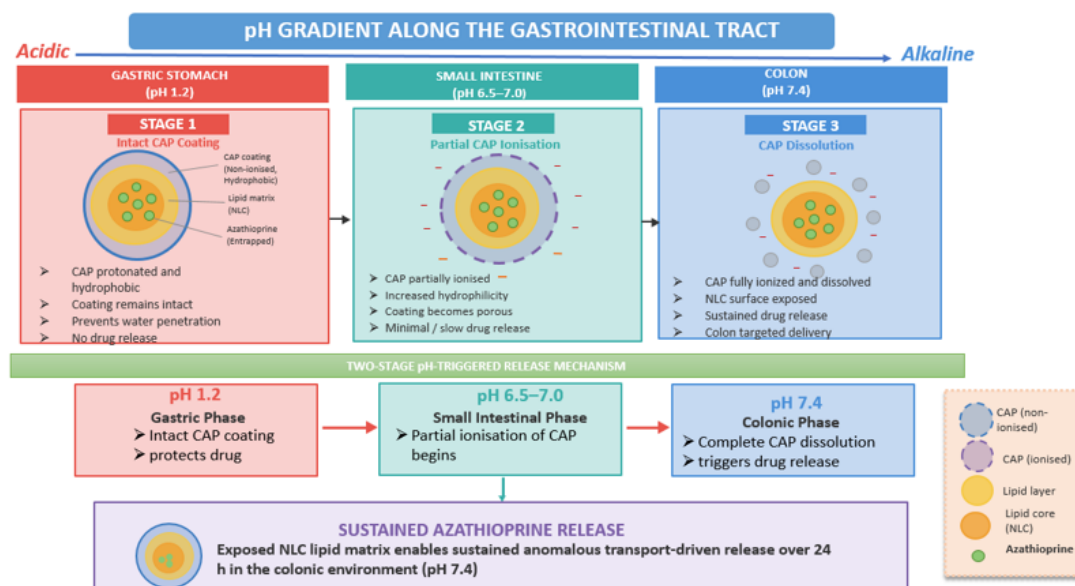


FIGURE 2.3: Schematic illustration of the two-stage pH-triggered release mechanism of CAP-coated azathioprine-loaded NLCs. At gastric pH (1.2), protonated CAP remains hydrophobic and intact, preventing drug release. In the small intestine (pH 6.5-7.0), partial CAP ionization begins. At colonic pH (7.4), complete CAP ionization and dissolution exposes the NLC surface, initiating sustained release over 24 h.

The combination of NLC drug delivery technology with pH-responsive CAP polymer coating creates a hybrid system with synergistic advantages that neither component provides independently. Compared with conventional pH-sensitive tablets or capsules, the nanoscale particle size of CAP-coated NLCs offers the potential for mucus penetration, intimate colonic mucosal contact, and macrophage-mediated uptake at the inflammatory lesion site, mechanisms that have been reported for lipid and polymeric nanoparticles in IBD models but require experimental validation in the specific azathioprine, CAP-NLC system [77–79].

Compared with uncoated NLCs, the CAP coating dramatically reduces gastric drug release to below the clinically important 10% threshold that defines effective gastric protection, while the controlled-release lipid matrix of the NLC provides sustained drug release over 24 h at colonic pH following CAP dissolution, avoiding the immediate burst release that would occur with simple pH-triggered systems lacking an intrinsic controlled-release matrix.

This sequential mechanism, pH-triggered CAP dissolution followed by sustained, anomalous-transport-governed drug release from the exposed NLC lipid matrix (a release pattern combining diffusion, matrix erosion, and swelling that has been directly demonstrated for orally administered NLCs transiting simulated gastric and intestinal fluids [80]), ensures that azathioprine is delivered preferentially to the inflamed colonic mucosa in a sustained, concentration-gradient-driven manner that maintains therapeutic drug levels throughout the 20–48 h colonic residence time of the dosage form [81], addressing the fundamental pharmacokinetic mismatch between systemic drug distribution and the colonic therapeutic target that limits the utility of conventional azathioprine therapy.

This pharmacokinetic rationale is directly supported by dedicated azathioprine colon-targeting studies published in the recent literature. Zhang *et al.* developed a colon-targeted, microbiota-modulating azathioprine nanoparticle built from pectin, zein, and the pH-sensitive polymer Eudragit® S100 (APZE) and demonstrated that, relative to a conventional azathioprine suspension, the coated nanoparticle achieved markedly higher oral bioavailability, prolonged colonic retention through

mucoadhesion, and greater colonic drug accumulation in rats, together with reduced formation of the inactive, toxicity-associated metabolite 6-thiouric acid and superior efficacy in a dextran-sulfate-sodium colitis model [82, 83].

These findings, obtained with an enteric-polymer-coated particulate carrier structurally analogous in concept to the CAP-coated NLC developed in the present work, provide direct, drug-specific evidence that colon-restricted, sustained release of azathioprine from a nanocarrier can simultaneously improve local drug exposure and reduce the systemic metabolite burden implicated in azathioprine-associated hepatotoxicity, a risk that has been mechanistically linked to elevated levels of methylated thiopurine metabolites generated via first-pass hepatic metabolism of systemically absorbed drug [84], reinforcing the pharmacokinetic argument advanced above for the CAP-NLC platform.

2.7 Previous Nanostructured Lipid Carriers - based Studies for Colon - targeted and Immunosuppressive Delivery

Published studies on the application of NLCs to colon-specific and immunosuppressive drug delivery are summarized in Table 2.5.

Numerous studies have demonstrated the potential of nanostructured lipid carriers for improving the oral delivery of anti-inflammatory and immunosuppressive drugs used in IBD. NLC formulations developed for drugs such as budesonide, curcumin, celecoxib, tacrolimus, and pioglitazone have consistently reported high encapsulation efficiency, sustained drug release, improved stability, and enhanced therapeutic efficacy compared with conventional formulations.

However, not all NLC systems provide colon-specific drug delivery. Many formulations improve oral bioavailability but release the drug immediately after administration due to the absence of a pH-responsive coating.

Consequently, although these studies confirm the advantages of NLC technology, they do not adequately address the requirement for selective drug release within the colon.

TABLE 2.5: Summary of key published studies on NLC formulations for colon-specific delivery and immunosuppressive or anti-inflammatory drug delivery relevant to the present study

Drug	Lipid system	Coating / Strategy	Key outcome	Research gap / Limitation	Ref
Azathioprine	Not applicable (polymeric chitosan bead system)	Eudragit [®] S100-coated chitosan beads	Achieved pH-dependent drug release and reduced systemic toxicity compared with conventional therapy.	The formulation employed a microparticulate chitosan bead system, which lacks the nanoscale advantages of NLCs, including enhanced mucus penetration, larger surface area, and controlled release from a lipid matrix. Furthermore, the study did not investigate CAP-mediated pH-responsive coating, formulation optimization, release kinetics, or long-term physicochemical stability, leaving the potential of a CAP-coated azathioprine-loaded NLC unexplored.	[85]
Budesonide	Compritrol [®] + Labrafac WL 1349	Eudragit [®] S100 coating	Demonstrated colon-specific drug release with sustained mucosal drug levels and improved local drug delivery.	Although Eudragit [®] S100-coated, the formulation was developed for budesonide rather than azathioprine. The study did not evaluate azathioprine encapsulation or compare different lipid compositions for formulation optimization.	[86]
Curcumin	Stearic acid + Tristearin (Solid Binary Lipid Nanoparticles)	Uncoated SBLNs prepared by emulsion solvent evaporation	Enhanced anti-inflammatory activity and improved therapeutic efficacy in an experimental IBD model.	Although the formulation improved the oral delivery and anti-inflammatory efficacy of curcumin, the nanoparticles were not designed for colon-specific drug release through a pH-responsive enteric coating.	[87]

Table 2.5 continued from previous page

Drug	Lipid system	Coating / Strategy	Key outcome	Research gap / Limitation	Ref
Celecoxib	Glycerol monostearate (GMS) + geraniol	Eudragit [®] S100-coated nanostructured lipid carriers (NLCs)	Demonstrated colon-specific sustained drug release, significantly reduced DSS-induced ulcerative colitis severity, and suppressed inflammatory biomarkers (COX-2 and iNOS).	Although the formulation successfully achieved pH-responsive colon targeting using Eudragit [®] S100-coated NLCs, the study was developed for celecoxib rather than azathioprine. Furthermore, formulation optimization for azathioprine encapsulation, comparative evaluation of alternative lipid compositions, and long-term physicochemical stability were not investigated.	[24]
Tacrolimus	Compritol [®] 888 ATO + Capmul MCM C8 [®]	Uncoated NLC	Significantly enhanced oral bioavailability (~7.2-fold) and lymphatic drug distribution (~19-fold) by improving drug solubilization and intestinal lymphatic transport.	The formulation was developed to enhance systemic oral bioavailability through lymphatic transport rather than achieve colon-specific drug delivery. Moreover, it did not incorporate a pH-responsive enteric coating or evaluate colon-targeted drug release for inflammatory bowel disease.	[71]
Pioglitazone	Compritol [®] + Labrasol [®]	Uncoated NLC	Developed optimized NLCs with high drug encapsulation efficiency, nanoscale particle size, improved physicochemical properties	The NLCs were not protected by an enteric coating, resulting in drug release before reaching the colon. The work focused on systemic delivery for diabetes rather than localized IBD therapy.	[73]
Curcumin (Bead-NLC hybrid)	NLC incorporated into pH-sensitive beads	Bead-NLC combination	Achieved >80% colonic drug release with minimal gastric release and superior performance compared with either beads or NLCs alone.	Although the hybrid system improved colon targeting, it employed curcumin instead of azathioprine and involved a more complex multiparticulate design that may complicate large-scale manufacturing. CAP coating was not investigated.	[76]

Table 2.5 continued from previous page

Drug	Lipid system	Coating / Strategy	Key outcome	Research gap / Limitation	Ref
Fenofibrate	Chitosan-coated NLC	Chitosan (mucoadhesive)	Produced approximately 2.7-fold higher bioavailability through enhanced intestinal absorption.	The formulation was designed for systemic delivery of a highly lipophilic drug (log P 5.2-6.3) rather than localized colon targeting. Its absorption mechanism is not applicable to azathioprine.	[88]
5 - Amino salicylic acid (5 - ASA)	Bile salt-containing liposomes	Eudragit [®] S100 coating	Optimized colon-targeted liposomes achieved pH-dependent release, enhanced cellular uptake, prolonged gastrointestinal residence, and improved pharmacokinetic performance.	The formulation employed liposomes rather than NLCs, which generally exhibit lower physical stability. The study focused on 5-ASA, did not use CAP coating, and did not investigate a binary lipid matrix, formulation optimization of NLCs, or long-term stability.	[89]
Prednisolone	Chitosan-coated liposomes	Eudragit [®] S100 encapsulation	Successfully protected prednisolone from gastric release and achieved colon-specific delivery with improved therapeutic performance in vitro, ex vivo, and in vivo.	The carrier system was liposomal rather than an NLC, and the drug investigated was prednisolone rather than azathioprine. CAP-coated NLCs, formulation optimization, release kinetics, and storage stability were not evaluated.	[90]
Azathioprine ★ (Present study)	Compritrol [®] 888 ATO + Precirol [®] ATO 5 + oleic acid	CAP coating	Designed to achieve high entrapment efficiency ($\geq 80\%$), minimal gastric drug release ($\leq 10\%$ at pH 1.2), sustained colonic release ($\geq 80\%$ at pH 7.4 over 24 h), comprehensive physicochemical characterization, release kinetics, and stability evaluation.	Addresses the limitations of previous studies by combining a binary lipid NLC system with CAP-mediated pH-responsive colon targeting for azathioprine, together with formulation optimization, detailed characterization, in vitro release kinetics, and stability studies.	Present study

The combination of a pH-responsive coating with an NLC core provides two independent levels of drug release control: the CAP coating determines the site of

release, whereas the lipid matrix controls the subsequent release rate. This dual-release mechanism offers greater targeting efficiency than either approach alone.

2.8 Literature Gap and Rationale of the Present Study

2.8.1 Literature Gap

Despite significant advances in colon-targeted drug delivery systems, several limitations remain in the current literature. Previous studies have demonstrated the potential of nanostructured lipid carriers (NLCs) to improve drug encapsulation, stability, and controlled release, while enteric polymers have been successfully employed to achieve site-specific delivery to the colon. However, these approaches have largely been investigated independently or with drugs other than azathioprine.

Although azathioprine remains a cornerstone therapy for the maintenance treatment of inflammatory bowel disease (IBD), relatively few studies have focused on developing advanced delivery systems for this drug. The available formulations are predominantly conventional oral dosage forms that release the drug in the upper gastrointestinal tract, resulting in extensive systemic exposure before reaching the site of inflammation. Consequently, the therapeutic benefits of azathioprine are often accompanied by dose-dependent adverse effects, including myelosuppression, hepatotoxicity, and gastrointestinal intolerance.

A limited number of studies have explored colon-targeted azathioprine delivery using polymer-based systems. However, these formulations were generally based on micro-sized carriers and did not incorporate lipid nanocarriers capable of providing enhanced drug protection, sustained release, and improved interaction with the inflamed colonic mucosa. Furthermore, no published study has reported the development of a cellulose acetate phthalate (CAP)-coated azathioprine-loaded nanostructured lipid carrier designed specifically for colon-targeted delivery.

In addition, the literature lacks comprehensive investigations evaluating the optimization, physicochemical characterization, drug release behaviour, release kinetics, and stability of CAP-coated azathioprine-loaded NLCs. Therefore, an important gap remains in the development of a delivery system capable of combining pH-responsive colon targeting with the advantages of lipid nanoparticle technology.

2.8.2 Rationale of the Present Study

The present study was designed to address the limitations identified in previous research by developing a CAP-coated azathioprine-loaded nanostructured lipid carrier for colon-targeted drug delivery. The proposed formulation integrates two complementary strategies within a single delivery system.

First, the NLC core provides a stable lipid matrix capable of encapsulating azathioprine, protecting the drug from degradation, improving drug loading, and providing sustained release characteristics. Second, the CAP coating acts as a pH-responsive barrier that prevents premature drug release in the stomach and small intestine while enabling drug release upon exposure to the higher pH environment of the colon.

This dual-functional approach is expected to increase drug availability at the site of inflammation while reducing unnecessary systemic exposure. By combining the advantages of NLC technology with the site-specific release properties of CAP, the formulation may offer a more effective strategy for the management of inflammatory bowel disease than conventional immediate-release azathioprine tablets.

Therefore, the present research focuses on the formulation, optimization, characterization, in vitro release evaluation, release kinetic analysis, and stability assessment of CAP-coated azathioprine-loaded NLCs as a potential colon-targeted delivery system.

Chapter 3

Material and Methods

3.1 Materials

Azathioprine (purity $\geq 99\%$) was selected as the model drug and procured from Mass Pharma (Pvt) Ltd. Compritol[®] 888 ATO (glyceryl dibehenate) and Precirol[®] ATO 5 (glyceryl palmitostearate) were obtained from Gattefossé (France) and employed as solid lipid matrices for NLC fabrication. Cellulose acetate phthalate (CAP) was procured from Evonik Industries (Germany) and used as the pH-sensitive enteric polymer for colon-targeted surface coating. Oleic acid (purity $\geq 99\%$, Sigma-Aldrich, USA) served as the liquid lipid component responsible for inducing controlled structural imperfection within the solid lipid matrix. Tween[®] 80 (polysorbate 80, Sigma-Aldrich, USA) was used as the primary surfactant and Transcutol[®] P (diethylene glycol monoethyl ether, Gattefossé, France) was used as the co-surfactant. Absolute ethanol (analytical grade, Merck, Germany) was used as the solvent for CAP coating. All other chemicals, solvents, and reagents used in this study were of analytical or spectroscopic grade and were used as received without further purification.

3.2 Preparation of Nanostructured Lipid Carriers

NLCs were prepared using a modified hot high-speed homogenization method, followed by post-coating with CAP for colon-specific delivery. The lipid phase was prepared by accurately weighing and melting solid lipids (Compritol® 888 ATO and/or Precirol® ATO 5) on a thermostatically controlled hot plate at 75°C. Oleic acid was incorporated into the molten lipid under gentle magnetic stirring to yield a homogeneous lipid melt. Azathioprine, equivalent to 5% w/w of the total lipid content, was accurately weighed and uniformly dispersed into the molten lipid phase under continuous magnetic stirring prior to addition of the aqueous phase, ensuring homogeneous drug distribution within the lipid melt.

The aqueous phase was prepared by dissolving surfactants (Tween® 80 and/or Transcutol® P) in 10 mL distilled water followed by sonication for 5 min and heating to 75°C. The heated aqueous phase was added dropwise to the molten lipid phase under gentle stirring to form a coarse pre-emulsion, which was then subjected to high-speed homogenization (Model T25, IKA, Germany) at 12,000 rpm for a predetermined time. The resulting nanoemulsion was cooled gradually to room temperature under continuous slow stirring, enabling lipid recrystallization and formation of uncoated drug-loaded NLCs [91, 92].

For colon-targeted delivery, uncoated NLCs were post-coated with CAP using a solvent displacement-based surface deposition technique. For colon-targeted delivery, CAP (40 mg, equivalent to 10% w/w of the total lipid content) was dissolved in sufficient absolute ethanol and added dropwise to the NLC dispersion under continuous magnetic stirring (400-600 rpm) over 10-15 min. Stirring was continued for an additional 30-45 min to allow uniform polymer deposition. Residual ethanol was removed by rotary evaporation. Stirring was continued for an additional 30-45 min to allow uniform polymer deposition on the nanoparticle surface and to permit equilibration of the coating layer. Residual ethanol was removed by rotary evaporation under reduced pressure at 30-35°C (Rotavapor R-100, Buchi,

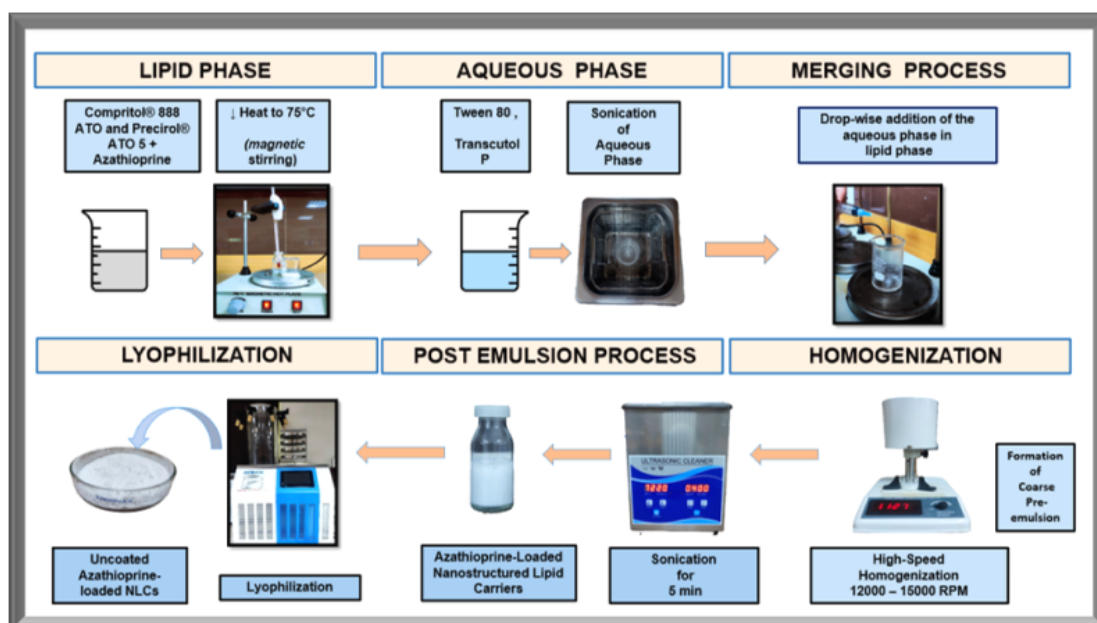


FIGURE 3.1: The complete schematic of NLC preparation and coating is presented

Switzerland). CAP-coated nanoparticles were then separated from the bulk dispersion by centrifugation at 2,000 rpm for 15 min at 4°C (Eppendorf Centrifuge 5430R, Germany), washed twice with distilled water to remove unbound polymer and free drug, and dried under vacuum at $25 \pm 2^\circ\text{C}$ for 24 h to obtain a free-flowing powder. The dried, CAP-coated azathioprine-loaded NLCs were stored in airtight, light-protected containers at 4°C until further characterization [7].

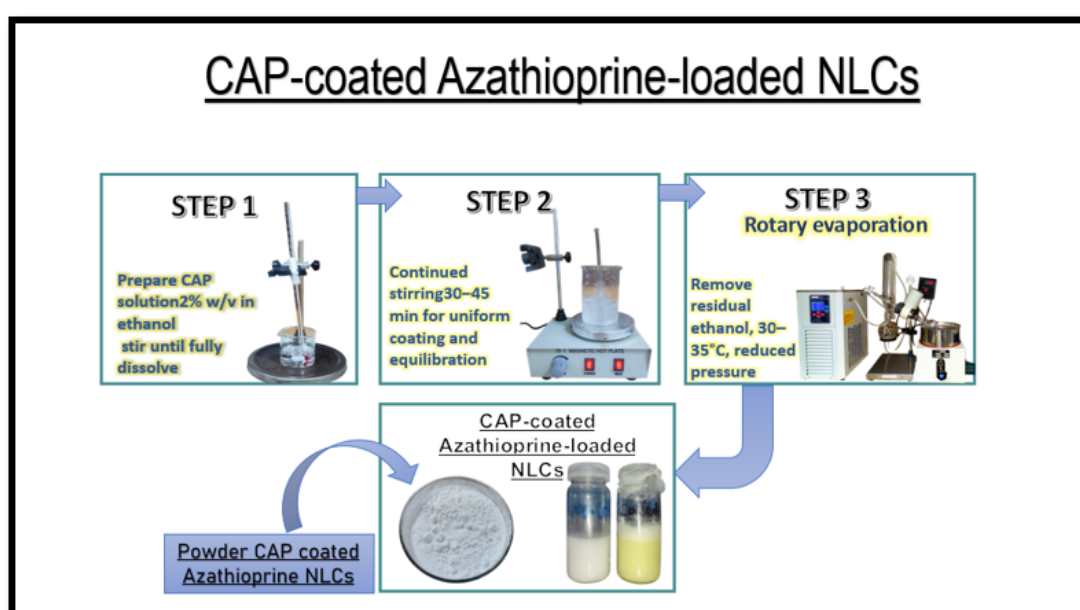


FIGURE 3.2: Steps showing the process of coating Azathioprine loaded NLCs

3.3 Experimental Design and Formulation Optimization

A one-factor-at-a-time (OFAT) design was employed to evaluate the influence of four formulation and process variables on the physicochemical and release performance of azathioprine-loaded NLCs: solid lipid composition (relative ratio of Compritol[®] 888 ATO to Precirol[®] ATO 5), liquid lipid concentration (oleic acid, as a percentage of total lipid content), surfactant system (relative ratio of Tween[®] 80 to Transcutol[®] P), and homogenization time [93]. Thirteen formulations (NLC1-NLC13) were prepared and characterized in parallel, with each formulation varying a single factor while the remaining three factors were held constant at fixed values (70:30 Compritol[®]: Precirol[®] blend, 30% oleic acid, 100:0 Tween[®] 80: Transcutol[®] P at 2% w/v total surfactant concentration, and 10 min homogenization, except where the factor under investigation required otherwise) [94]. The optimal formulation was subsequently identified from the complete data set against the critical quality attributes defined in Section 3.4.

The effect of solid lipid composition was assessed using a 70:30 Compritol[®]: Precirol[®] blend (NLC3, corresponding to 49% and 21% of total lipid content, respectively), Compritol[®] 888 ATO alone (NLC1), and Precirol[®] ATO 5 alone (NLC2), with oleic acid concentration, surfactant system, and homogenization time held constant. The effect of liquid lipid concentration was assessed by incorporating oleic acid at 10% (NLC4), 20% (NLC5), 30% (NLC3), and 50% (NLC6) of total lipid content, using a 70:30 Compritol[®]: Precirol[®] blend with surfactant system and homogenization time held constant [95]. The effect of surfactant system was assessed across five Tween[®] 80: Transcutol[®] P ratios 100:0 (NLC1), 0:100 (NLC7), 50:50 (NLC8), 85:15 (NLC9), and 75:25 (NLC10) at a constant total surfactant concentration of 2% w/v, with solid lipid composition and oleic acid concentration held constant. The effect of homogenization time was assessed at 5 min (NLC11), 10 min (NLC10), 15 min (NLC12), and 20 min (NLC13), using a 70:30 Compritol[®]: Precirol[®] blend, 30% oleic acid, and a 75:25 Tween[®] 80: Transcutol[®] P surfactant system. Across all thirteen formulations, total lipid

content (400 mg), drug loading (5% w/w of total lipid), CAP coating (10% w/w of total lipid), total surfactant concentration (2% w/v), homogenization speed (12,000 rpm), and processing temperature (75°C) were held constant to isolate the effect of each tested variable [96]. The complete composition of all thirteen formulations is presented in Table 3.1. The OFAT approach was selected as an exploratory screening strategy to independently assess the directional influence of each formulation variable prior to any multivariate optimization, given the limited number of variables and the absence of prior NLC formulation data for azathioprine

TABLE 3.1: Composition of azathioprine-loaded nanostructured lipid carrier formulations (NLC1-NLC13)

Code	Compritol ® (%)	Precirol ® (%)	Oleic Acid (%)	Tween ® 80 (%)	Transcutol ® P (%)	Homog. Time (min)	CAP (%) w/w
NLC1	70	0	30	2.0	0	10	10
NLC2	0	70	30	2.0	0	10	10
NLC3	49	21	30	2.0	0	10	10
NLC4	63	27	10	2.0	0	10	10
NLC5	56	24	20	2.0	0	10	10
NLC6	35	15	50	2.0	0	10	10
NLC7	49	21	30	0	2.0	10	10
NLC8	49	21	30	1.0	1.0	10	10
NLC9	49	21	30	1.7	0.3	10	10
NLC10	49	21	30	1.5	0.5	10	10
NLC11	49	21	30	1.5	0.5	5	10
NLC12	49	21	30	1.5	0.5	15	10
NLC13	49	21	30	1.5	0.5	20	10

Total lipid = 400 mg; Drug = 5% w/w of lipid; CAP = 40 mg (10% w/w of total lipid); Speed = 12,000 rpm; Temp = 75°C for all formulations

3.4 Formulation Selection Criteria

The optimal formulation was selected based on six critical quality attributes (CQAs). A formulation was considered optimal only if it satisfied all six criteria simultaneously (Table 3.2).

TABLE 3.2: Critical quality attributes (CQAs) and acceptance criteria for formulation selection

Quality Attribute		Acceptance Criterion	Scientific Rationale
Entrapment (EE %)	Efficiency	>80%	Ensures adequate drug loading for therapeutic efficacy at the colonic target site
Particle Size		100–250 nm	Facilitates mucosal penetration, GI transit stability, and colloidal integrity
Polydispersity (PDI)	Index	< 0.30	Indicates homogeneous particle size distribution and formulation reproducibility
Zeta Potential		> ± 30 mV	Predicts colloidal stability and electrostatic resistance to particle aggregation
Gastric Release (pH 1.2)		<10%	Prevents premature drug release in stomach; reduces systemic azathioprine absorption
Colonic Release (pH 7.4)		>80%	Ensures site-specific delivery of azathioprine to the inflamed colonic mucosa

3.5 Characterization of Nanostructured Lipid Carrier Formulations

3.5.1 Entrapment Efficiency

The entrapment efficiency (%EE) of azathioprine-loaded NLCs was determined using an ultrafiltration-centrifugation method to separate encapsulated drug from untrapped (free) drug. Briefly, a 0.5 mL aliquot of each NLC dispersion was centrifuged at $14,000 \times g$ for 20 min at 4°C (Eppendorf 5430R, Germany). The filtrate, containing untrapped azathioprine, was collected and passed through a 0.45 μm PVDF membrane filter to eliminate any residual lipid particulates. The amount of free azathioprine in the filtrate was measured using UV-Vis spectrophotometry at $\lambda_{\text{max}} = 280$ nm (UV-1900i, Shimadzu, Japan) using a standard

calibration curve ($R^2 \geq 0.999$). The percentage entrapment efficiency was calculated by using following equation 1:

$$\%EE = \frac{W_t - W_f}{W_t} \times 100 \quad (3.1)$$

W_t - total amount of azathioprine incorporated at the time of formulation, and W_f - amount of free (unentrapped) drug in the supernatant. Total drug content (W_t) was determined by dissolving a known quantity of the NLC dispersion into methanol (1:10, v/v) under sonication for 10 min, and filtering and spectrophotometrically analyzing it at 280 nm. All measurements were performed in triplicate, and results are expressed as mean $\pm$ standard deviation (SD) [9].

3.5.2 Particle Size, Polydispersity Index and Zeta Potential

Particle size (Z-average), polydispersity index (PDI), and zeta potential of the NLC formulations were determined using dynamic light scattering (DLS) and laser Doppler micro-electrophoresis, respectively, using a Zetasizer Nano ZS instrument (Malvern Instruments, Worcestershire, UK). Prior to analysis, each NLC dispersion was appropriately diluted with deionized water to achieve a suitable scattering intensity. Measurements were performed at 25°C with a detection angle of 173° (backscattering). Each sample was measured in triplicate, and results are expressed as mean $\pm$ standard deviation. A PDI value below 0.30 was considered indicative of a monodisperse and homogeneous particle population, while a zeta potential magnitude of $> \pm 30$ mV was used as the threshold for adequate colloidal stability [97].

3.5.3 In Vitro Drug Release Studies and Kinetics

In vitro drug release studies of azathioprine-loaded NLCs were performed using the dialysis bag diffusion method to evaluate the release behavior under simulated gastrointestinal conditions relevant to colon-targeted delivery. Dialysis

membranes (MWCO 10 kDa, Spectrum Laboratories, USA) were hydrated and pre-conditioned by soaking in phosphate-buffered saline (PBS, pH 7.4) for 30 min at room temperature prior to use. An accurately weighed quantity of each NLC dispersion equivalent to 5 mg azathioprine was transferred into the pre-hydrated dialysis bag, which was securely sealed with dialysis clips to prevent leakage [98].

The release study was conducted separately in two different dissolution media to evaluate the pH-dependent release behavior of the CAP-coated NLCs: (i) simulated gastric fluid (SGF, pH 1.2) and (ii) simulated colonic fluid (SCF, pH 7.4 phosphate buffer). For each medium, the dialysis bag was immersed in 100 mL of the respective dissolution medium containing 0.1% w/v Tween[®] 80 to maintain sink conditions. The dissolution study was carried out for 24 h in each medium independently, maintaining the temperature at $37 \pm 0.5^\circ\text{C}$ under continuous magnetic stirring at 100 rpm using a digitally controlled water bath to ensure uniform temperature distribution and sink conditions throughout the study [99].

At predetermined time intervals (0.5, 1, 2, 4, 6, 8, 10, 12, 16, 20, and 24 h), 1 mL aliquots of the release medium were withdrawn using a pre-calibrated pipette and replaced immediately with an equal volume of fresh pre-warmed (37°C) dissolution medium to maintain constant volume and sink conditions. Withdrawn samples were filtered by a $0.45\ \mu\text{m}$ PVDF syringe filter (Millipore, USA) to remove all particulate matter and analysed spectrophotometrically at $\lambda_{\text{max}} = 280\ \text{nm}$ by using a double-beam UV-Vis spectrophotometer (UV-1900i, Shimadzu, Japan). The concentration of drug in the samples was determined by using a pre-constructed standard calibration curve ($R^2 \geq 0.999$) of Azathioprine in respective dissolution media. Each formulation was repeated three times and the results are presented as the mean $\pm$ standard deviation (SD). The mechanism and the kinetics of azathioprine release from NLC formulations were explained by fitting the cumulative drug release data to four different models, namely, zero order, first order, Higuchi model (Square root of time) and Korsmeyer-Peppas model (Power law). Nonlinear regression analysis was performed on the experimental data to calculate the model parameters: release rate constants (k) and release exponent (n) using GraphPad

Prism (Version 9.0, GraphPad Software, USA). The model with the highest value of the correlation coefficient (R^2) was selected as the best fit model [85].

3.5.4 Fourier Transform Infrared Spectroscopy

Fourier transform infrared (FTIR) spectroscopy was used to assess the compatibility of azathioprine with the excipients used for the nanostructured lipid carrier (NLC) formulation and to check for any possible physicochemical interaction after drug incorporation. FTIR spectra of pure azathioprine, blank NLCs and optimized azathioprine loaded NLCs (NLC10) was taken by KBr pellet method. In brief, the samples were in turn triturated with spectroscopic grade KBr in 1:99 ratio and were compressed into translucent pellets by a hydraulic press. The prepared pellets were scanned in the spectral range 4000-400 cm^{-1} by using an FTIR spectrophotometer at room temperature. The absorption peaks were characteristic of the functional groups of azathioprine and formulation excipients and compared. The spectra of the optimized formulation were compared to check the shift in the peak, broadening, disappearance or reduction in intensity compared to pure drug spectrum. The minor variations in the peak positions and intensities were thought to be due to intermolecular interactions and hydrogen bonding between azathioprine and the lipid matrix. But no major changes or additional peak were observed thus showing the compatibility of azathioprine with the excipients used and successful incorporation of the drug into the selected system without chemical degradation or instability [100].

3.5.5 Differential Scanning Calorimetry

The thermal behavior, the crystalline state and the physicochemical compatibility of azathioprine with the lipid excipients was investigated by differential scanning calorimetry (DSC). The DSC analysis of pure azathioprine, Compritol® 888 ATO and optimized azathioprine-loaded NLC formulation were carried out. The

samples were accurately weighed in aluminium pans (5-10mg) and an empty aluminium pan was used as a reference. The PerkinElmer DSC 8000 (USA) was used for analysis at a nitrogen flow rate of 20 mL/min. The temperature was ramped from 30°C to 350°C at the heating rate of 10°C/min. The absence or marked decrease in the characteristic endothermic melting peak of the drug in the formulation thermogram was taken as a proof of drug amorphization or its molecular dispersion in the lipid matrix [101].

3.5.6 X-Ray Diffraction Analysis

To assess the solid-state crystallinity, X-ray powder diffraction (XRD) analysis was performed on azathioprine, the lipid excipients, blank NLC and optimized azathioprine loaded NLC formulation. Before being analyzed, each of the solid samples was finely ground and evenly placed on glass sample holders. Diffraction patterns were measured using Bruker D8 Advance diffractometer (Bruker, Germany) with Cu K α radiation ($\lambda = 1.5406 \text{ \AA}$) and at an accelerating voltage of 40 kV and a current of 30 mA. Samples were scanned between 5-60° 2 θ with a scanning rate of 2°/min and a step size of 0.02° 2. The characteristic diffraction peaks were used to evaluate the changes in the crystalline structure and consequently, the amorphization/molecular dispersion of azathioprine in the NLC matrix [102].

3.5.7 Scanning Electron Microscopy

External appearances of the optimized azathioprine loaded NLC formulation in terms of surface morphology, particle geometry and spatial distribution were analyzed by scanning electron microscopy (SEM). NLC samples were diluted appropriately, drop cast onto the carbon coated copper stubs and air dried at room temperature before imaging. The dried samples were then sputter-coated with thin layer of gold-palladium alloy under vacuum to increase conductivity and reduce charging artefacts. Scanning electron microscope (SEM) images were taken

by a scanning electron microscope (JEOL JSM-7610F, Japan) at various magnifications ranging from $1000\times$ to $100,000\times$, using an accelerating voltage of 5-15 kV. For the images, analysis was carried out regarding uniformity of particle shape, surface texture, appearance of drug crystal on the particle surface and homogeneity of the formulation [103].

3.6 Stability Studies

The physical stability of the optimized azathioprine - loaded NLC formulation (NLC10) was assessed in accordance with ICH Q1A(R2) guidelines for stability testing of new drug substances and products. Samples were stored in tightly sealed, light-resistant amber glass vials under three different storage conditions: refrigerated ($4 \pm 2^\circ\text{C}$), ambient ($25 \pm 2^\circ\text{C}$ / $60 \pm 5\%$ RH), and accelerated ($40 \pm 2^\circ\text{C}$ / $75 \pm 5\%$ RH). Stability evaluations were performed at predetermined time intervals (0, 1, 2, and 3 months). At each time point, formulations were evaluated for visual appearance (color, clarity, presence of precipitation, phase separation, or aggregation) and entrapment efficiency (EE %) [104].

The formulation was considered physically stable if no visible changes such as aggregation, phase separation, or precipitation were observed during storage. Stability was further assessed by monitoring changes in entrapment efficiency and particle size over the storage period. Formulations retaining high drug entrapment ($>85\%$) while maintaining particle size within the nanometer range and without statistically significant deterioration ($p > 0.05$) were considered stable under the tested storage condition. All stability samples were analyzed in triplicate, and results are expressed as mean $\pm$ standard deviation (SD) [105].

3.7 Acute Oral Toxicity Study

Acute oral toxicity of the optimized azathioprine-loaded NLC formulation was evaluated following a modified single-dose oral toxicity protocol adapted from

OECD Test Guidelines 423, using parallel control and treated groups. Body weight 30-36 g healthy adult female BALB/c mice were randomly divided into two groups: the control group (Group I, n=4) was administered 1 mL/kg body weight of normal saline orally and the treatment group (Group II, n=4) was administered a single dose of the optimized NLC formulation at an equal dose of 2,000 mg/kg body weight of mice by adjusting the volume to 1 mL/kg for equal dosing volume per group. All the animals were kept in standard laboratory conditions ($22 \pm 2^\circ\text{C}$, $55 \pm 5\%$ relative humidity, 12 hours light/dark cycle) and had free access to food and water for the whole duration of the experiment [106]. The Research Ethics Committee, Faculty of Pharmacy, Capital University of Science and Technology, Islamabad accepted the study protocol (Approval Ref: CUST/DERC/PH/ERC/PG/2026/012)

The animals were continuously monitored for 4 hours after administration, and then daily for 14 days. Mortality, general behavior, locomotor activity, grooming, salivation, piloerection, convulsions, and neurological and systemic toxicity were observed. Weight was taken on days 0, 7 and 14. At the end of the observation period, blood samples were collected by cardiac puncture under light anesthesia for hematological analysis (hemoglobin, WBC, platelets) and biochemical evaluation of liver function (ALT, AST, ALP) and kidney function (creatinine, urea, glucose). Following sacrifice, major organs (liver, kidneys, heart, spleen, stomach, and intestines) were excised, fixed in 10% neutral buffered formalin, processed for histopathology, and stained with hematoxylin and eosin (H&E) for microscopic examination. Statistical comparisons between groups were performed using Student's unpaired t-test, with $p < 0.05$ considered statistically significant [107].

3.8 Statistical Analysis

All experimental data are expressed as mean $\pm$ standard deviation (SD) of at least three independent replicates ($n = 3$). Statistical analysis was performed using SPSS Statistics (Version 26.0, IBM, USA). Comparisons among multiple formulation groups were performed using one-way analysis of variance (ANOVA)

followed by Tukey's post-hoc test to identify statistically significant pairwise differences. Comparisons between two groups (e.g., control vs. treated in the toxicity study) were performed using Student's unpaired two-tailed t-test. In all cases, a p-value of less than 0.05 was considered statistically significant [108].

Chapter 4

Results and Discussion

Thirteen formulations (NLC1-NLC13) were prepared by systematically varying solid lipid composition, liquid lipid concentration, surfactant system, and homogenization time. One of the most important quality parameter of the nanocarrier system is entrapment efficiency (EE%) which represents the quantity of the drug loaded in the nanocarrier and has a direct impact on the therapeutic efficacy at the target site. The EE% for azathioprine of all NLC formulations was determined by the ultrafiltration-centrifugation method and a zeta potential measurement was performed to predict the electrostatic resistance to particle aggregation and the colloidal stability.

4.1 Entrapment Efficiency

One of the most important parameters for the nanostructured lipid carriers (NLCs) is its entrapment efficiency (EE), which indicates the drug incorporation and its retention in the lipid matrix during the preparation of NLC. Table 4.1 shows that the EE of azathioprine loaded in NLC formulations varied between $68.23 \pm 0.94\%$ to $93.47 \pm 0.42\%$, indicating that the composition of the formulations and the processing parameters had a significant effect on drug entrapment.

NLC1, formulated with Compritol[®] 888 ATO as the sole solid lipid, exhibited an EE of $71.19 \pm 0.83\%$. Compritol[®] possesses a highly ordered crystalline structure that limits the availability of imperfections within the lipid matrix for drug accommodation. Consequently, drug molecules may be expelled during lipid recrystallization, resulting in lower entrapment efficiency. Substituting Compritol[®] with Precirol[®] ATO 5 in NLC2 increased EE to $74.36 \pm 0.72\%$, suggesting that the comparatively less ordered crystalline arrangement of Precirol[®] provided improved space for drug incorporation. Further enhancement was observed in NLC3, which contained a binary blend of Compritol[®] and Precirol[®] (49:21) with 30% oleic acid, yielding an EE of $82.54 \pm 0.59\%$. The combination of two structurally different solid lipids likely generated a less ordered crystalline network with increased lattice imperfections, thereby creating additional sites for drug entrapment. These findings indicate that binary lipid matrices with optimized solid lipid ratios are more favorable for azathioprine incorporation than single-lipid systems. NLC3 served as the foundational binary lipid formulation for subsequent evaluation of surfactant system (NLC7-NLC10) and homogenization time (NLC11-NLC13), with identical lipid composition maintained across these series to isolate the effects of surfactant ratio and processing parameters [109].

Liquid lipid concentration was studied using NLC4-NLC6 with a constant surfactant amount of 2.0% Tween[®] 80 to determine the effect of oleic acid. The EE of NLC4 with a 63:27 ratio of solid lipid (Compritol[®]: Precirol[®]) and 10% oleic acid was $77.42 \pm 0.65\%$. The further increase in EE of $79.50 \pm 0.71\%$ for NLC5 containing 20% oleic acid and 56:24 solid lipid ratio was seen whereas the NLC6 in which 50% oleic acid and 35:15 solid lipid ratio was used showed a significant decrease in EE value of $68.23 \pm 0.94\%$. Both formulations failed to meet CQA and the increase was significant in EE from NLC4 to NLC5 is a result of moderate disruption of ordered crystalline lattice by liquid lipid. The liquid lipid forms some amorphous space in the solid lipids with partial intercalation between the acyl chains of solid lipids at oleic acid content of 20%, which can accommodate the drug in more free volume pockets. This effect however is concentration dependent [110]. The phase inversion phenomenon is predominant: the solid lipids

are not continuous any longer but dispersed in the liquid lipid continuous phase when the oleic acid content is 50% (NLC6). The partitioning of azathioprine is preferentially in the continuous liquid lipid domain, and not trapped in the solid lipid crystallites. During cooling, the lack of a rigid crystalline scaffold prevents stable nanoparticle formation, and drug molecules readily diffuse across the fluid lipid-aqueous interface into the external aqueous phase.

Moreover, the high oleic acid content results in a low viscosity of the molten lipid phase, which leads to greater drug leakage from the newly formed nanoparticles due to enhanced Brownian motion of the drug before solidification. In the liquid lipids series, therefore, 30% oleic acid is the value for maximum EE while 50% causes excessive fluidity and drug expulsion. The observed trends of NLC4-NLC6 correspond to the combined effects of the oleic acid concentration and solid lipid ratio changes, since the solid lipid ratios have to be necessarily changed to keep the total lipid mass constant [111].

The influence of the surfactant composition was studied with NLC7-NLC10 with identical lipid composition: 49:21 Compritol[®]: Precirol[®] and processing conditions. NLC7, stabilized solely with Transcutol[®] P (0% Tween[®] 80, 2.0% Transcutol[®] P), exhibited an EE of $84.67 \pm 0.63\%$. Formulations containing mixed surfactant systems showed variable performance, with NLC8 (1.0% Tween[®] 80, 1.0% Transcutol[®] P) and NLC9 (1.7% Tween[®] 80, 0.3% Transcutol[®] P) achieving EE values of $81.34 \pm 0.68\%$ and $80.29 \pm 0.57\%$, respectively [112]. Notably, NLC10, prepared using a Tween[®] 80: Transcutol[®] P ratio of 75:25 (1.5%:0.5%), demonstrated the highest EE among all formulations ($93.47 \pm 0.42\%$). The progressive reduction in EE from NLC7 (84.67%) to NLC8 (81.34%) and NLC9 (80.29%) with increasing incorporation of Transcutol[®] P indicates competitive micellar partitioning at suboptimal surfactant ratio of the formulations. Tween[®] 80 will have micelle formation in the aqueous phase when the concentration is greater than the CMC ($\sim 0.01\%$ w/v). As Tween[®] 80 concentration increases from 0% (NLC7) to 1.0% (NLC8) and 1.7% (NLC9), the micellar concentration rises proportionally [10]. Azathioprine partitions into these micelles during homogenization, and this micellar solubilization competes with lipid matrix

entrapment. During ultrafiltration-centrifugation, micelle-associated drug passes through the filter membrane, artificially lowering apparent EE. The lowest EE in this subset (NLC9, 80.29%) corresponds to the highest Tween[®] 80 concentration, confirming the micellar competition mechanism [113].

However, this mechanism operates only when Tween[®] 80 dominates the interface without sufficient Transcutol[®] P to stabilize the lipid core. NLC7 (0% Tween[®] 80, 2.0% Transcutol[®] P) achieves 84.67% EE because Transcutol[®] P, as a lipophilic co-solvent, penetrates the lipid matrix and enhances drug solubilization within the core rather than partitioning drug into the aqueous phase. The limitation of NLC7 is poor colloidal stability (large size, low zeta potential), not low EE. The micellar competition trend is broken by NLC10 (75:25, 93.47%) which is a dual mechanism synergy, and works only within a narrow window of surfactant ratios. At 75:25 Tween[®] 80:Transcutol[®] P: (i) Transcutol[®] P penetrates the lipid matrix and increases azathioprine solubility within the core via hydrogen bonding with the thiopurine moiety, effectively “locking” the drug molecules in the lipid phase; (ii) Tween[®] 80 adsorbs at the interface and forms a densely packed mixed monolayer with Transcutol[®] P, with the polyoxyethylene chains of Tween[®] 80 providing a steric barrier and the glycol ether groups of Transcutol[®] P providing interfacial fluidity for optimized surfactant packing density. This mixed monolayer results in zero net drug permeability across the interface: the steric barrier blocks outward diffusion of drug, the increased core solubilisation removes the thermodynamic driving force for drug escape. The percentages of NLC8 and NLC9 were lower than 75:25, in which the Transcutol[®] P content was not enough to increase the core solubilization and Tween[®] 80 micelles extracted the drug. When the ratio is greater than 75:25 (NLC7), inadequate amount of Tween[®] 80 does not stabilize the interface, resulting in colloidal instability despite high EE. The 75:25 ratio therefore is the thermodynamic sweet spot of maximum core retention and maximum interfacial stabilization [114].

The NLC11-NLC13 were subjected to different homogenization times and the effect was compared with NLC10. The EE of NLC11 (prepared with 5 min homogenization) was $73.15 \pm 0.76\%$. The EE was significantly improved at 10 min

homogenization (NLC10; EE of $93.47 \pm 0.42\%$), likely due to better dispersion of the lipid phase and greater incorporation of azathioprine into the lipid matrix. However, further increases resulted in reduced EE values of $79.80 \pm 0.67\%$ for NLC12 (15 min) and $74.30 \pm 0.81\%$ for NLC13 (20 min). The lower EE at 5 min is due to the incomplete emulsification - the insufficient energy leads to the presence of a bimodal droplet population, including poorly stabilized large aggregates and a small number of nanoparticles [115].

Large droplets have low surface area to volume ratio, and therefore a decreased ability to adsorb surfactant, leaving drug in the bulk lipid phase. These aggregates release drug into the aqueous phase during the cooling process via the mechanism of Ostwald ripening and phase separation. The uniform sustained shear allows complete dispersion of the droplets and consistent surfactant coverage at 10 min for maximum EE. The decrease after 10 min is due to thermomechanical degradation.

These cumulative energy dissipations increase the local temperature above 75°C which results in: (i) increased drug diffusion from the lipid core to the aqueous phase, (ii) hydrolysis of the Tween[®] 80, affecting the interface stabilization, and (iii) partial melting of the solid lipid crystallites, affecting drug entrapment sites.

Free radical formation by cavitation also causes the chemical degradation of azathioprine. 10 min is the time point for optimal dispersion efficiency with good formulation integrity. Thus, 10 min of homogenization time was considered to be the most favourable for the efficient entrapment of the drug [116].

The EE of NLCs containing binary lipid matrices (NLC1-NLC3) was greater than that of the single-lipid systems, with the highest EE for NLC3 at $82.54 \pm 0.59\%$. NLC10 (surfactant composition: 75:25 Tween[®] 80: Transcutol[®] P) showed the highest EE ($93.47 \pm 0.42\%$) of all the thirteen formulations, which was about 11% higher than NLC3 only due to the change in the surfactant composition. This comparison validates that binary solid lipids serve as a basis of matrix for efficient drug entrapment, but surfactant composition is the most important factor that influences EE in the binary solid lipid formulation system.

4.2 Particle Size, Polydispersity Index and Zeta Potential

Nanostructured lipid carriers (NLCs) have important physicochemical characteristics, such as particle size, polydispersity index (PDI), and zeta potential that determine their performance, stability, and biological behaviour. As presented in Table 4.1, the particle size of the prepared formulations ranged from 110.3 ± 5.1 nm to 282.1 ± 6.9 nm, while PDI values ranged from 0.18 ± 0.01 to 0.391 ± 0.03 . The zeta potential values varied between -10.5 ± 1.3 mV and -40.4 ± 0.8 mV. Together, these results indicate that the lipid composition, surfactant ratio, and homogenization time have a significant effect on the properties of the nanoparticles [117].

NLC1-NLC3 were used to evaluate the effect of solid lipid composition. NLC1, prepared with Compritol[®] 888 ATO as the sole solid lipid, exhibited a particle size of 268 ± 9.0 nm, a PDI of 0.391 ± 0.03 , and a zeta potential of -24.2 ± 1.0 mV. Using Precirol[®] ATO 5 as a replacement for Compritol[®] in NLC2 led to a slightly smaller particle size (263 ± 7.1 nm), a reduced PDI (0.351 ± 0.02) and a slightly higher magnitude of the zeta potential (-27.2 ± 1.2 mV). The binary solid-lipid system used to form NLC3 yielded particles of similar size (264 ± 8.2 nm) and PDI (0.39 ± 0.02), and a zeta potential of -26.9 ± 0.9 mV. However, the PDI value of 0.39 is above the acceptance limit of < 0.30 , which suggests a broad particle size distribution, despite the favorable effect of the binary lipid blend on the entrapment efficiency [118]. This increased PDI is probably due to the crystallization of Compritol[®] and Precirol[®] during the cooling process, because the lipids have different melting points and crystallization speeds, leading to the formation of a bimodal distribution of small particles crystallizing fast and large aggregates crystallizing slowly. Thus, although NLC3 has the highest EE of the solid lipid series (82.54%), it does not fulfill the PDI CQA. These results show that the binary lipid blend greatly increased the entrapment efficiency but had a limited effect on the reduction of particle size. However, it is possible that mixing two structurally different lipids has resulted in a less ordered lipid matrix which

led to better accommodation of the drug without adversely affecting the colloidal properties [119].

The influence of the solid lipid composition on the zeta potential was not too pronounced, but still noticeable. NLC1 (100% Compritol[®]) exhibited a zeta potential of -24.2 ± 1.0 mV, while NLC2 (100% Precirol[®]) showed -27.2 ± 1.2 mV, and the binary blend NLC3 displayed -26.9 ± 0.9 mV. These values are below the more stringent criterion for colloidal stability of $|\pm 30|$ mV. The trend is attributed to differences in the surface chemistry of the solid lipids, with Compritol[®] (glyceryl dibehenate) having longer alkyl chains which can have different orientations at the surface, thus influencing the charge density. The binary lipid blend seems to form a more heterogeneous surface with slightly increased charge density, which enhanced the colloidal stability [120].

The effect of different concentrations of liquid lipid (NLC4, NLC5 and NLC6) was studied. Increasing the oleic acid concentration from 10% (NLC4) to 20% (NLC5) resulted in an increase in particle size from 240 ± 6.5 nm to 282.1 ± 6.9 nm and an increase in PDI from 0.28 ± 0.02 to 0.35 ± 0.02 . This non-monotonic behavior of the relationship between oleic acid concentration and particle size is due to the interplay of the two opposing phenomena: matrix fluidity and surfactant stabilization efficiency. The high amount of solid lipid (90% total) with a predominantly crystalline solid lipid matrix at 10% oleic acid (NLC4) imparts a firmness to the particle and prevents droplet coalescence during cooling, resulting in a compact particle size of 240 ± 6.5 nm. When the liquid lipid content is higher (NLC5, 20% oleic acid), the higher amount of fluidity in the intermediate melt state destabilizes the emulsion during the homogenization step, due to the low viscosity of the molten droplets, which is less able to withstand the shear forces present during the process, while the droplets are also not fluid enough to efficiently break up into smaller droplets. The droplet fusion in this transitional state is facilitated by the critical cooling window before the total lipid recrystallization and leads to the largest particle size observed so far (282.1 ± 6.9 nm). Therefore, 20% oleic acid is a critical instability zone where fluidity of the matrix is enough to break the crystal stabilization but not to allow effective fragmentation of the droplet [121].

Interestingly, further increase of oleic acid in the NLC to 50% (NLC6) decreased the particle size to 214 ± 7.3 nm and PDI to 0.29 ± 0.02 . The decrease in particle size at 50% oleic acid is due to a change from a solid lipid-dominated system to a liquid lipid-dominated system. The molten lipid phase at 50% oleic acid has nanoemulsion-like fluidity, which allows efficient break-up of droplets when subjected to high shear homogenization (12,000 rpm) and with minimal resistance from crystalline domains.

The lower viscosity of the NLC6 lipid melt helps to create smaller primary droplets which remain stable on cooling. This is in contrast to NLC5, which has an intermediate viscoelastic state due to the 20% oleic acid, which does not coalesce because it is too fluid, but also does not break up efficiently, thus giving rise to the peculiar size maximum. The non-monotonic trend ($240 \rightarrow 282 \rightarrow 214$ nm) thus is not a linear relationship between fluidity and particle size but reflects a phase-transition phenomenon in the lipid matrix [122].

The zeta potential values of NLC4, NLC5, and NLC6 were -21.8 ± 1.1 mV, -10.5 ± 1.3 mV, and -13.6 ± 1.2 mV, respectively. The zeta potential was shown to be strongly dependent on the concentration of the liquid lipid, with a non-monotonic dependence that was dependent on the interaction between the fluidity of the matrix and the charge density of the surface. NLC4 (10% oleic acid) exhibited a zeta potential of -21.8 ± 1.1 mV, while NLC5 (20% oleic acid) showed a pronounced decline to -10.5 ± 1.3 mV. This reduction is a consequence of the initial disturbance to the ordered crystalline lipid matrix, in which an increase in the amount of liquid lipid in the core results in a higher mobility of the molecules and a consequent decrease in the packing density and surface exposure of ionizable fatty acid groups [123]. As the concentration of oleic acid is increased to 20%, some disorder occurs in the lipid matrix, causing the carboxyl groups at the particle-aqueous interface to lose their orientation, resulting in a low surface charge density and the smallest magnitude of zeta potential in the liquid lipid series. NLC6 (50% oleic acid) exhibited a slight improvement from -13.6 ± 1.2 mV for NLC5, however still below the colloidal stability limit of $|\pm 30|$ mV. The near complete loss of ordered solid lipid matrix at this high liquid lipid loading creates a highly

fluid system, but the absolute amount of oleic acid (an unsaturated C18:1 fatty acid with only a single ionizable carboxyl group) provides a degree of offset to the disorder-induced decrease in charge density by increasing absolute number of potentially ionizable groups present at the interface. However, the high fluidity does not allow good packing and orientation of these groups, reducing the ability to express surface charge, and hence NLC6 is limited by its high oleic acid content. All together these data showed that zeta potential in liquid lipid series (NLC4, NLC5, NLC6) does not decrease progressively with increasing oleic acid content but rather showed a minimum at 20% and a partial recovery at 50% [124].

The differences in the effect of the surfactant composition were evaluated by using NLC7 and NLC10 which had the same lipid composition and processing parameters. NLC7, stabilized solely with Transcutol[®] P, exhibited a particle size of 264.9 ± 8.2 nm, a PDI of 0.38 ± 0.02 , and a zeta potential of -15.8 ± 0.9 mV. NLC7 had a high EE ($84.67 \pm 0.63\%$) and a large particle size (264.9 ± 8.2 nm), high PDI (0.38 ± 0.02), and low zeta potential magnitude (-15.8 ± 0.9 mV), which can be explained by the unique physicochemical properties of Transcutol[®] P (diethylene glycol monoethyl ether). Transcutol[®] P is a very lipophilic co-solvent that penetrates deeply into the lipid matrix and not at the particle-aqueous interface. This deep penetration contributes to the high EE because of the solubilisation of the drugs in the lipid core. Transcutol[®] P is however, not a classic surfactant with a defined hydrophilic head group, therefore it is very weak in providing interface stabilization at the surface of the nanoparticles. The poor surface adsorption leads to: (i) inadequate ζ -potential; (ii) inadequate steric hinderance against coalescence of the droplets during homogenization (large particle size); (iii) a wide size distribution (high PDI) because of uncontrolled aggregation of droplets. However, Transcutol[®] P is only able to dissolve azathioprine in the lipid matrix and it does not work as a single stabilizer for the formulation of nanoparticles, which requires the use of Tween[®] 80 to obtain a better colloidal behavior [68].

The use of Tween[®] 80 in combination with Transcutol[®] P progressively improved particles characteristics. NLC8 (50:50 Tween[®] 80: Transcutol[®] P) showed a particle size of 225 ± 6.2 nm and a PDI of 0.25 ± 0.02 , while NLC9 (85:15) further

reduced the particle size to 210 ± 5.8 nm with a PDI of 0.21 ± 0.01 . NLC10 (75:25 Tween[®] 80: Transcutol[®] P) demonstrated the smallest particle size (110.3 ± 5.1 nm), a PDI of 0.285 ± 0.01 , and the highest zeta potential magnitude (-40.4 ± 0.8 mV). The achieved PDI of 0.285 is within the limits, demonstrating a desirable outcome that reflects improved particle size uniformity while successfully harmonizing the objective of minimizing particle size [125].

This slight increase in PDI compared to NLC12 (0.18 ± 0.01) may be due to the wider spectrum of droplet break-up behaviors at the 75:25 surfactant ratio: some droplets break completely into the ~ 100 nm range, whereas others stabilize in the ~ 120 nm range, thus generating a slightly wider but still acceptable distribution. This marginal PDI is counterbalanced by the outstanding performance of NLC10 on all the other CQAs. The significant decrease in particle size for NLC10 shows that the 75:25 surfactant ratio helped in achieving a good level of interfacial stabilization during the homogenization process, thus creating smaller droplets that did not coalesce during cooling. Moreover, the estimated zeta potential was highly negative indicating the good electrostatic stability and mitigating the particle aggregation in the storage period [126].

The zeta potential was highly impacted by the surfactant system, mainly by the type of surfactant and the amount of surfactant adsorbed on the surface of the nanoparticles. NLC1 (Tween[®] 80 alone, 2.0%) produced a zeta potential of -24.2 ± 1.0 mV, whereas NLC7 (Transcutol[®] P alone, 2.0%) gave -15.8 ± 0.9 mV. The single-surfactant formulations did not provide sufficient zeta potential values (above $|\pm 30|$ mV) required for electrostatic stabilization. The non-ionic surfactant Tween[®] 80 is not directly involved in the surface charge, but can adsorb onto the surface of the nanoparticles and affect the orientation and ionization of other surface groups [127]. The glycol ether Transcutol[®] P is able to penetrate the lipid matrix and affect the hydration layer on the particle surface. This synergistic effect of the combination of these two surfactants may be due to increased surface coverage and/or orienting of the head groups of the lipids, which maximized the negative charge density, and hence the magnitude of the zeta potential. The 75:25 ratio of Tween[®] 80: Transcutol[®] P offers a favorable balance of hydrophilic and

lipophilic properties that leads to a densely packed layer of surfactant molecules at the interface with favorable orientation of ionizable groups, causing the greatest possible zeta potential magnitude (-40.4 mV for NLC10). This is important to guarantee the long-term stability: higher values of the zeta potential translate to greater electrostatic repulsion, which will inhibit the aggregation of particles and Ostwald ripening during storage [128].

The homogenization time was also an important factor affecting the nanoparticles. NLC11, prepared using 5 minutes of homogenization, exhibited a particle size of 218 ± 5.5 nm, a PDI of 0.22 ± 0.01 , and a zeta potential of -22.9 ± 0.8 mV. The particle size of NLC10 after 10 minutes of homogenization was much decreased to 110.3 ± 5.1 nm and the zeta-potential value was increased to -40.4 ± 0.8 mV, reflecting better dispersion and stability of the particles. When the homogenization time was further increased to 15 min (NLC12) and 20 min (NLC13), the particle size of 195 ± 5.0 nm and 208 ± 6.1 nm were obtained [129].

In spite of having the lowest PDI value (0.18 ± 0.01), which indicated a highly uniform particle population, the particle size and zeta potential of NLC12 were not as favourable as those of NLC10. The very low PDI value of NLC12 indicates that the particle population is very monodisperse and mechanically this can be attributed to the possibility of size-selective degradation during prolonged homogenization. After 15 min, the long shear stress serves as a kinetic filter, the smaller particles with higher surface energy being less prone to further fragmenting, and the larger particles still being broken down until a critical particle size is reached.

This size focusing effect results in a narrow size distribution of particles with mean size 195 ± 5.0 nm. In exchange for this monodispersity, however, the colloidal stability of these is compromised: the prolonged homogenisation has caused a reduction in the magnitude of the zeta-potential of the particles to -23.8 ± 0.7 mV and also increased their susceptibility to aggregation during storage. Furthermore, the particle size of 195nm is in the acceptable range, but much larger than the 110.3nm obtained with NLC10, which is important for mucosal penetration and for stable GI transit. In this way, NLC12 is a locally good formulation in

terms of size uniformity but not as well in other CQAs (particle size, zeta potential and colonic release), demonstrating that PDI alone is not sufficient to assess formulation, where other CQAs are compromised. The rise in particle size after 10 minutes may be due to the excessive mechanical energy input that can lead to excessive particle collision frequency, creating some particle recoalescence. Hence the homogenization time of 10 min resulted in the best compromise in particle size reduction, size distribution and colloidal stability [130].

Homogenization time also influenced zeta potential, with NLC11 (5 min) showing -22.9 ± 0.8 mV, NLC10 (10 min) showing -40.4 ± 0.8 mV, and NLC12 (15 min) showing -23.8 ± 0.7 mV. The slightly lower value of zeta potential at 5 min may be because surfactants did not cover the particle surface completely because of the insufficient energy input, which led to the lower efficiency of the surface charge expression. The maximum value of zeta potential (-40.4 mV) at 10 min suggests that the surface was fully covered and the ionizable groups were arranged properly. The slightly lower magnitude at 15 min (-23.8 mV) may be due to the loss of surfactant due to displacement or degradation under long high shear conditions, which could expose less charged lipid surfaces or change the hydration layer at the particle interface. NLC10 was above the stability criterion of $|\pm 30|$ mV and NLC12 was below at -23.8 mV. The zeta potential obtained at 20 min (NLC13, -22.1 ± 0.9 mV) was similar to that obtained at 5 min (NLC11, -22.9 mV); longer homogenization does not seem to sustain the high surface charge density achieved after 10 min. The decrease of the magnitude of the zeta potential with increase in homogenization time is progressive and it is directly related with the degradation of the surfactant and change in the structure of the interface, which affects the electrostatic stabilization capacity of the nanoparticle system [131].

The overall superiority of the NLC10 can be explained in terms of systems level mechanistic integration of the formulation components. The binary solid lipid matrix (49% Compritol[®] + 21% Precirol[®]) results in a crystalline structure that is heterogeneous with lattice defects that are favorable for the entrapment of the drug (EE = 93.47%) and, at the same time, exhibits a sufficient structural rigidity to prevent the expulsion of the drug. The 30% oleic acid concentration

compromises the drug accommodation and sustains the matrix integrity through moderate lattice disruption, while avoiding phase inversion. The rational combination of 75:25 Tween[®] 80: Transcutol[®] P (TS) results in a synergic mixed surfactant monolayer at the lipid-aqueous interface: Tween[®] 80 stabilizes the lipid-aqueous interface sterically through its polyoxyethylene chains, and TS is responsible for solubilizing the drug within the lipid core and fluidizing the interface, which favors good packing of surfactant molecules.

This is a mixed monolayer which reduces drug leakage during the nanoparticles preparation and achieves the highest electrostatic charge density (zeta potential = -40.4 mV). It has been found that a 10 min homogenization time is sufficient to disperse the droplets completely and for the surfactant to adsorb to them, without inducing thermomechanical degradation [132].

The resulted particle size 110.3 ± 5.1 nm helps in mucosal penetration and the targeting of the colon and the magnitude of the zeta potential is high, ensuring long term colloidal stability. The particle size, PDI, and zeta potential of optimized NLC10 formulation are presented in figure 4.1.

TABLE 4.1: Physicochemical characterization of NLC1-NLC13 (mean $\pm$ SD, n = 3)

CODE	EE %	Size (nm)	PDI	Zeta Potential (mV)
NLC1	71.19 ± 0.83	268.0 ± 9.0	0.391 ± 0.03	-24.2 ± 1.0
NLC2	74.36 ± 0.72	263 ± 7.1	0.351 ± 0.02	-27.2 ± 1.2
NLC3	82.54 ± 0.59	264 ± 8.2	0.39 ± 0.02	-26.9 ± 0.9
NLC4	77.42 ± 0.65	240 ± 6.5	0.28 ± 0.02	-21.8 ± 1.1
NLC5	79.50 ± 0.71	282.1 ± 6.9	0.35 ± 0.02	-10.5 ± 1.3
NLC6	68.23 ± 0.94	214 ± 7.3	0.29 ± 0.02	-13.6 ± 1.2
NLC7	84.67 ± 0.63	264.9 ± 8.2	0.38 ± 0.02	-15.8 ± 0.9
NLC8	81.34 ± 0.68	225 ± 6.2	0.25 ± 0.02	-21.1 ± 1.1
NLC9	80.29 ± 0.57	210 ± 5.8	0.21 ± 0.01	-22.4 ± 0.9
NLC10	93.47 ± 0.42	110.3 ± 5.1	0.285 ± 0.01	-40.4 ± 0.8
NLC11	73.15 ± 0.76	218 ± 5.5	0.22 ± 0.01	-22.9 ± 0.8
NLC12	79.8 ± 0.67	195 ± 5.0	0.18 ± 0.01	-23.8 ± 0.7
NLC13	74.3 ± 0.81	208 ± 6.1	0.21 ± 0.01	-22.1 ± 0.9

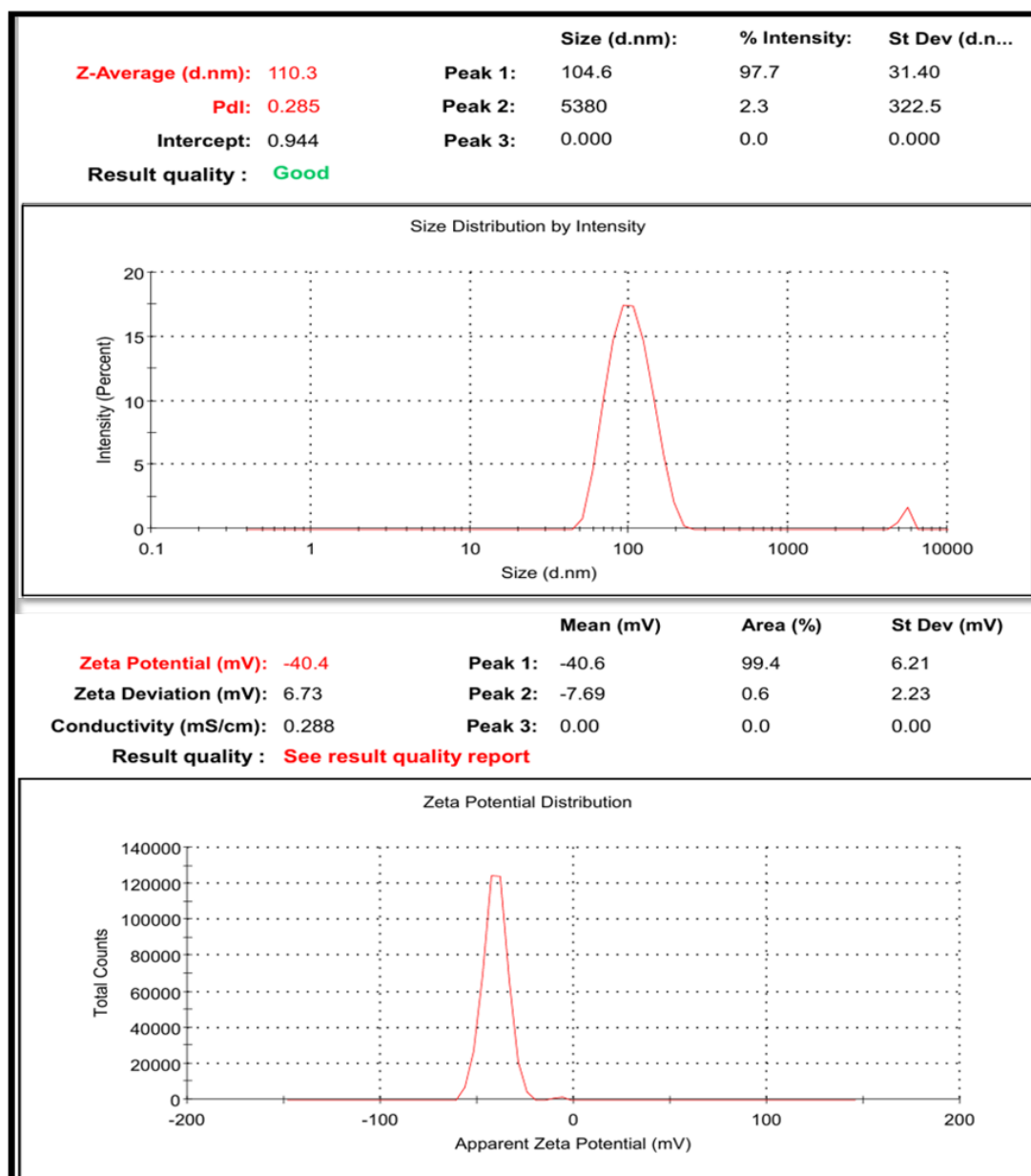


FIGURE 4.1

4.3 In Vitro Drug Release Profile and Kinetics

All the azathioprine (AZA)-loaded nanostructured lipid carrier (NLC) formulations (NLC1-NLC13) were assessed for colon-targeted drug delivery by simulating the in vitro release behavior in simulated gastric fluid (SGF, pH 1.2) and simulated colonic fluid (SCF, pH 7.4). The cumulative drug release profiles showed significant pH-dependent profiles with limited drug release in gastric pH and higher sustained

release at colonic pH. This pH-responsive release is important for colon-targeted drug delivery system as it ensures that minimal amount of drug is released in upper gastrointestinal tract and maximum drug is released at the site of intestinal inflammation [133].

TABLE 4.2: In vitro drug release data of NLC1-NLC13 formulations (Mean $\pm$ SD, n = 3)

Code	SGF (%) pH 1.2	SCF (%) pH 7.4
NLC1	18.33 $\pm$ 1.01	62.47 $\pm$ 3.44
NLC2	12.74 $\pm$ 0.70	50.37 $\pm$ 2.77
NLC3	17.21 $\pm$ 0.95	69.47 $\pm$ 3.82
NLC4	19.14 $\pm$ 1.05	57.38 $\pm$ 3.16
NLC5	18.67 $\pm$ 1.03	70.94 $\pm$ 3.90
NLC6	10.88 $\pm$ 0.60	31.28 $\pm$ 1.72
NLC7	19.42 $\pm$ 1.07	65.28 $\pm$ 3.59
NLC8	16.58 $\pm$ 0.91	68.73 $\pm$ 3.78
NLC9	18.94 $\pm$ 1.04	55.84 $\pm$ 3.07
NLC10	8.10 $\pm$ 0.4	91.84 $\pm$ 1.8
NLC11	14.93 $\pm$ 0.82	72.37 $\pm$ 3.98
NLC12	19.02 $\pm$ 1.05	32.84 $\pm$ 1.81
NLC13	17.86 $\pm$ 0.98	60.17 $\pm$ 3.31

The cumulative release of azathioprine at pH 1.2 after 24 h was NLC10 (8.10%) - NLC7 (19.42%). Overall, all formulations exhibited minimum drug release in the acidic medium, confirming the pH responsive protective function of cellulose acetate phthalate (CAP) coating, which effectively preventing release of encapsulated drug under simulated gastric conditions. CAP has phthalate groups with a pKa of $\sim$ 5.0 - 5.5, which means that below this pH, the polymer is largely unionized and insoluble, corresponding to a dense hydrophobic barrier around the surface of the nanoparticle. This barrier prevents gastric fluid to penetrate into the lipid matrix and therefore limits the diffusion of azathioprine from the core of NLC. NLC10 showed the least gastric drug release (8.10%) among all formulations which means that it has greatest acid resistance and it provides the most effective protection to the stomach [134]. Drug release from NLC10 increased gradually from 0.50% at 0.5 h to 0.69% at 1 h, 1.08% at 2 h, 2.11% at 4 h, 3.53% at 8 h,

6.84% at 16 h, 7.40% at 20 h, and ultimately 8.10% after 24 h. The very low release seen during the dissolution time suggests that most of the encapsulated azathioprine was protected from the acid exposure by the CAP coated lipid matrix. The decreased release from NLC10 can be explained by the favourable ratio between surfactant and lipid which facilitated the uniform generation of nanoparticles and successful deposition of CAP on the nanoparticles. This compact coating structure probably not only lowered surface defects but also diffusion pathways, which led to the minimization of drug leakage under an acidic condition [135].

In contrast, NLC7 exhibited the highest gastric release ($19.42 \pm 1.07\%$), followed by NLC4 ($19.14 \pm 1.05\%$), NLC12 ($19.02 \pm 1.05\%$), NLC9 ($18.94 \pm 1.04\%$), and NLC5 ($18.67 \pm 1.03\%$). The drug release from these formulations was comparatively higher in acidic media, indicating the less barrier efficiency and higher matrix permeability. This can be explained by less favorable lipid packing, higher matrix imperfections or incomplete CAP surface coverage which allows the diffusion of dissolved drug molecules through the coating layer [136].

The effect of the lipid composition was seen in the formulation series. Generally, formulations with more gastric release had less compact lipid matrices allowing more water penetration and drug diffusion. The greater liquid lipid content can result in disruption of the crystal form of the solid lipid matrix, creating structural defects that promote the migration of the drug molecules to the surface of the nanoparticles. On the other hand, formulations with less gastric release, probably had more homogeneous lipid matrices that were able to protect the encapsulated drug more during the exposure to the acidic condition [137].

Gastric release behavior was also greatly influenced by the surfactant system. When preparing the nanoparticles, using the right surfactant combination enhances the emulsion formation and leads to a smaller particle size, a smaller particle size distribution, and homogeneous surface properties. These conditions increase adsorption of CAP and coating uniformity which results in an improved diffusion barrier. Thus, formulations with surfactant systems that were favorable showed better protection of the stomach than those with less efficient interfacial stabilization [138].

For all formulations, cumulative drug release gradually increased in the dissolution time. NLC10 released only 0.50% drug during the first 30 min and reached 8.10% after 24 h, whereas NLC7 increased from 2.54% at 0.5 h to 19.42% at 24 h. The slow and controlled release pattern is in agreement with the fact that most of the encapsulated azathioprine was protected in the CAP-coated lipid matrix during the simulated gastric phase [139].

The dissolution data was analyzed using four different models to give an insight into the mechanism and kinetics of azathioprine release under the gastric condition, which are zero order, first order, Higuchi and Korsmeyer-Peppas model. The most suitable model for describing the release behaviour for each sample was determined by comparing the correlation coefficient (R^2) for each model. The release exponent (n) was used to describe the release mechanism, and values of $n \leq 0.43$ are indicative of Fickian diffusion, $0.43 < n < 0.85$ are indicative of anomalous (non-Fickian) transport and $n \geq 0.85$ are indicative of Case-II transport for spherical geometry for Korsmeyer-Peppas model [140].

The Korsmeyer-Peppas model gave the best correlation coefficient for all the formulations ($R^2 = 0.847-0.990$), suggesting that this model was the most appropriate model describing drug release at acidic pH. The dominance of the Korsmeyer-Peppas model indicates that the drug release from the CAP-coated NLCs was a diffusion and matrix related process and not a simple concentration dependent release process. But the release mechanism was different with the formulations. The NLC1 ($n = 0.428$), NLC4 ($n = 0.378$), NLC7 ($n = 0.336$), NLC9 ($n = 0.418$), and NLC12 ($n = 0.396$) formulations showed Fickian diffusion behavior implying that the drug release occurred mainly by diffusion through the intact CAP barrier. In these formulations, the main rate limiting barrier was the insoluble CAP coating that limited drug transport to the dissolution medium. The Korsmeyer-Peppas correlation coefficient for NLC7 was 0.847 and the release exponent was 0.336 indicating diffusion-controlled release and less matrix relaxation under acidic condition [141].

Of particular interest was a release exponent of 1.312 with an R^2 of 0.988 for the NLC10, which corresponds to super Case-II transport, which in turn suggests

that predominantly relaxation-controlled drug release occurred in the intact CAP barrier with swelling driven drug expulsion. The release pattern of NLC10 is unique and it is in harmony with the very low gastric drug release and may indicate the formation of a highly effective and stable CAP coating. This is desirable since it helps to retain the drug in the gastrointestinal tract and reduce early exposure to the system.

The Higuchi model also showed high correlation coefficients for several formulations ($R^2 = 0.715 - 0.986$) especially for NLC2, NLC6, NLC8, NLC10 and NLC11 but the good fit of Korsmeyer - Peppas model implies that drug release is not only explained as diffusion. Rather, both diffusion and matrix relaxation processes were involved in the overall release process under acidic conditions [142].

Upon transferring it to simulated colonic fluid (pH 7.4), there was a significant release of the drug in all the formulations. The cumulative release at 24 h was found to be 31.28% for NLC6 and 91.84% for NLC10 showing the pH responsive nature of CAP-coated NLCs. Above the pKa of CAP, the ionization of the phthalate groups leads to the dissolution of the polymer coating, and the direct contact of the lipid matrix with the dissolution medium [143].

When the CAP barrier is dissolved, the hydration, swelling and erosion of the lipid matrix begin, which causes the azathioprine that is trapped in the matrix to diffuse into the outside medium. Therefore, drug release depends on the structural properties of the lipid matrix, such as the lipid composition, lipid matrix crystallinity, distribution of the surfactant and the morphology of the nanoparticles. NLC10 formulation showed the highest cumulative drug release as 91.84%, which revealed the most favourable colon-targeting characteristics among all formulations. Drug release from NLC10 increased progressively from 8.93% at 0.5 h to 11.95% at 1 h, 19.69% at 2 h, 32.43% at 4 h, 39.90% at 6 h, 53.42% at 8 h, 65.72% at 12 h, 78.17% at 16 h, 88.00% at 20 h, and ultimately 91.84% after 24 h. This prolonged release profile suggests a high efficiency in the drug retention during the passage through the stomach and a high efficiency in the release in the colonic environment. NLC6, on the other hand, exhibited the lowest release ($31.28 \pm 1.72\%$) following 24 h. The decrease in release may be explained by the fact that the

lipid matrix was very rigid, which hindered dissolution medium penetration and the drug's diffusion even after CAP coating was dissolved. Similar trends were observed for NLC12 ($32.84 \pm 1.81\%$), NLC2 ($50.37 \pm 2.77\%$), and NLC4 ($57.38 \pm 3.16\%$), indicating incomplete drug liberation from these formulations [144].

Formulations NLC1, NLC3, NLC5, NLC7, NLC8, NLC9, NLC11, and NLC13 exhibited intermediate release behavior, achieving cumulative release values of 62.47%, 69.47%, 70.94%, 65.28%, 68.73%, 55.84%, 72.37%, and 60.17%, respectively. These formulations exhibited sustained release properties; however, they were not as efficient as NLC10 to deliver the bulk of the encapsulated drug to the desired/designed environment. The progressive rise in release of NLC10 when compared to other formulations indicate the presence of favorable internal matrix architecture which allows the controlled penetration of water and diffusion of the drug. The matrix stability and permeability in this formulation seem to have been in a favorable balance, thus leading to an efficient formulation with no undesirable burst effect [145].

The release data was obtained at pH 7.4 was fitted to zero order, first order, Higuchi and Korsmeyer-Peppas model to investigate the release mechanism after the CAP dissolution. The correlation coefficients showed that Korsmeyer - Peppas model was the best fit for all formulations with R^2 value range of 0.966 to 0.999. The model-fitting results showed that Korsmeyer-Peppas model was predominant, suggesting that the drug release from CAP dissolution was not only diffusion- but also matrix relaxation controlled. The release exponent values were in between 0.451 to 0.944, which means most of the formulations demonstrated anomalous (non-Fickian) transport behavior, while NLC10 exhibited Case-II transport characteristics [146].

Formulations NLC1 ($n = 0.642$), NLC2 ($n = 0.681$), NLC3 ($n = 0.557$), NLC4 ($n = 0.703$), NLC5 ($n = 0.487$), NLC7 ($n = 0.600$), NLC8 ($n = 0.451$), NLC11 ($n = 0.548$), and NLC13 ($n = 0.682$) exhibited moderate anomalous transport, indicating that drug release occurred through the simultaneous contribution of diffusion and matrix relaxation. The release behaviour of these formulations indicated that there was progressive hydration of the lipid matrix and controlled diffusion of

the drug through the newly formed aqueous channels. Formulations NLC6 ($n = 0.842$), NLC9 ($n = 0.748$), and NLC12 ($n = 0.797$) showed higher effect of matrix relaxation processes which were closer to the upper limit of anomalous transport but they don't meet other CQAs. The higher n values indicate higher swelling and restructuring of the lipid matrix during the dissolution process, which played a major role in the release behavior observed [147].

Interestingly, NLC10 showed the highest release exponent ($n = 0.944$) and an excellent correlation coefficient ($R^2 = 0.995$); the transport behavior was at the border of the anomalous and Case-II regime and the transport was dominated by the matrix relaxation. This indicates that the release of the drug after the dissolution of the CAP-coating has been primarily influenced by matrix relaxation and structural reorganization. According to the spherical geometry, the n value falls in the top of the Case-II regime, suggesting that matrix relaxation and lipid restructuring processes are important, and a secondary diffusion process occurs through the hydrated lipid matrix. This behavior is similar to that of NLC10, which is highly controlled drug release profile and sustained release, and accountable for the near complete release of the drug in 24 h dissolution. The Korsmeyer-Peppas model revealed high correlation coefficient for all formulations indicating that simple diffusion can't describe the release from the developed NLC system. However, the release process was a complex mechanism, comprising of drug diffusion, matrix hydration, relaxation of lipids and reorganization of structure after dissolution of the CAP coating [148]. The formulation NLC10 had the best release profile of all the formulations investigated. There was little gastric release at pH 1.2 (8.10% in 24 h), suggesting good gastric protection, which is important to prevent premature upper gastrointestinal absorption of azathioprine. The release of the encapsulated drug at pH 7.4 was found to be sustained and extensive for NLC10 with 91.84% drug released in 24 h suggesting its efficient delivery at target colonic environment. The release of NLC10 at pH 1.2 showed to follow the Korsmeyer-Peppas model with super Case-II transport characteristics ($n = 1.312$ and $R^2 = 0.988$), suggesting that the release was mainly controlled by polymer relaxation of the non-destructed CAP barrier.

TABLE 4.3: Drug Release Kinetic Parameters of Azathioprine-Loaded NLC Formulations

Formulation	pH	Zero - Or- der R^2	Zero - Or- der K_0	First - Or- der R^2	First - Or- der K_1	Higuchi R^2	Higuchi KH	Korsmeyer - Peppas R^2	Korsmeyer - Peppas KKP	Release Exponent (n)
NLC1	1.2	0.338	1.025	0.431	0.012	0.922	4.297	0.939	5.154	0.428
NLC1	7.4	0.876	3.103	0.977	0.048	0.958	12.342	0.99	8.565	0.642
NLC2	1.2	0.728	0.634	0.76	0.007	0.986	2.574	0.989	2.343	0.537
NLC2	7.4	0.916	2.37	0.969	0.032	0.949	9.35	0.994	5.86	0.681
NLC3	1.2	0.599	0.903	0.659	0.01	0.976	3.715	0.976	3.805	0.491
NLC3	7.4	0.755	3.581	0.948	0.063	0.973	14.506	0.98	12.533	0.557
NLC4	1.2	0.031	1.121	0.161	0.013	0.833	4.779	0.895	6.49	0.378
NLC4	7.4	0.928	2.772	0.989	0.04	0.938	10.899	0.994	6.446	0.703
NLC5	1.2	0.507	1.008	0.583	0.011	0.961	4.177	0.965	4.565	0.465
NLC5	7.4	0.591	3.72	0.882	0.07	0.972	15.313	0.972	15.808	0.487
NLC6	1.2	0.744	0.531	0.769	0.006	0.986	2.154	0.99	1.925	0.544
NLC6	7.4	0.985	1.394	0.997	0.016	0.889	5.358	0.999	2.187	0.842
NLC7	1.2	0.313	1.159	0.152	0.014	0.715	5.011	0.847	7.538	0.336

Table 4.3 continued from previous page

Formulation	pH	Zero - Or- der R^2	Zero - Or- der K_0	First - Or- der R^2	First - Or- der K_1	Higuchi R^2	Higuchi KH	Korsmeyer - Peppas R^2	Korsmeyer - Peppas KKP	Release Exponent (n)
NLC7	7.4	0.824	3.298	0.963	0.053	0.968	13.236	0.886	10.261	0.6
NLC8	1.2	0.661	0.851	0.712	0.009	0.984	3.48	0.984	3.388	0.511
NLC8	7.4	0.464	3.63	0.804	0.068	0.958	15.073	0.966	17.074	0.451
NLC9	1.2	0.284	1.077	0.388	0.012	0.906	4.531	0.929	5.565	0.418
NLC9	7.4	0.954	2.625	0.995	0.036	0.924	10.239	0.997	5.378	0.748
NLC10	1.2	0.984	0.384	0.92	0.036	0.979	1.179	0.988	0.237	1.312
NLC10	7.4	0.988	4.874	0.795	0.143	0.882	14.051	0.995	6.221	0.944
NLC11	1.2	0.713	0.748	0.752	0.008	0.986	3.042	0.988	2.817	0.533
NLC11	7.4	0.732	3.752	0.95	0.069	0.969	15.239	0.974	13.499	0.548
NLC12	1.2	0.154	1.096	0.271	0.013	0.873	4.643	0.915	6.03	0.396
NLC12	7.4	0.975	1.483	0.991	0.018	0.907	5.74	0.999	2.647	0.797
NLC13	1.2	0.544	0.955	0.613	0.011	0.967	3.948	0.969	4.208	0.475
NLC13	7.4	0.912	2.931	0.986	0.043	0.945	11.568	0.992	7.234	0.682

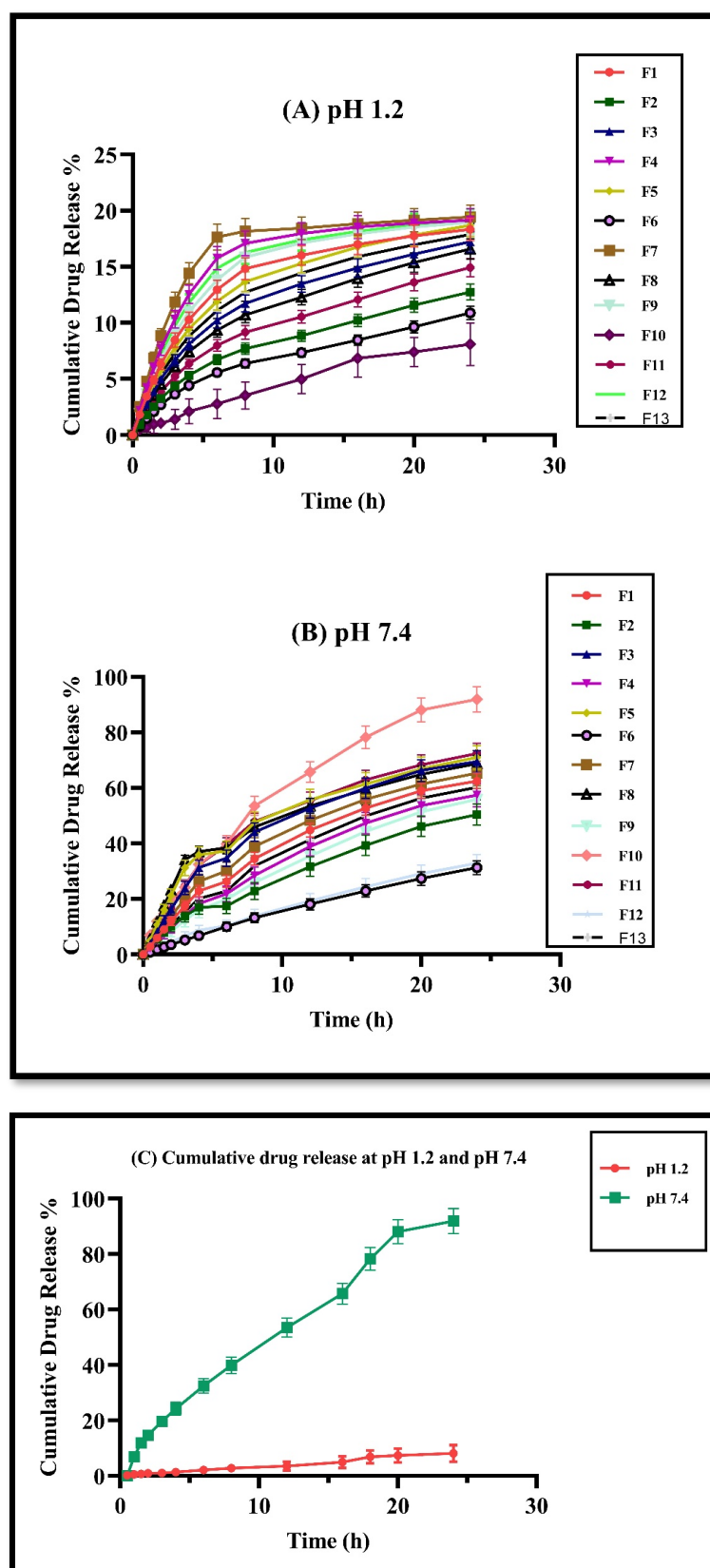


FIGURE 4.2: In Vitro drug release profiles of azathioprine-loaded NLCs formulations at (A) pH 1.2, (B) pH 7.4, and (C) Mean Cumulative drug release at both pH 1.2 and pH 7.4. Data are presented as mean $\pm$ SD ($n = 3$). Statistical significance between groups was assessed by one-way ANOVA ($p < 0.05$)

4.4 FTIR Spectroscopy

In order to ensure structural integrity of the components of the formulations and to examine any possible drug-excipient interactions, FTIR spectroscopy was used. The FTIR spectrum of azathioprine exhibited characteristic absorption peaks at 913 cm^{-1} (C-H bending), 1227 cm^{-1} (C-N stretching), 1378 cm^{-1} (CH_3 bending), 1525 cm^{-1} (C- NO_2 stretching), and 3113 cm^{-1} (N-H stretching), consistent with its well-defined crystalline structure [151]. Compritol[®] 888 ATO showed distinct bands of aliphatic C-H stretching at 2920 and 2850 cm^{-1} and a characteristic ester carbonyl (C=O) stretching at 1735 cm^{-1} which confirmed its glyceride structure. Precirol[®] ATO 5 showed similar aliphatic C-H stretching (2920 - 2850 cm^{-1}) and an ester carbonyl at $\sim 1735\text{ cm}^{-1}$ [53, 152, 153].

The optimized NLC10 formulation was seen to have both drug and lipid related absorption bands in its FTIR spectrum. Characteristic lipid peaks were maintained with same intensities and some peaks of the drugs were slightly widened and weakened comparing with the pure drug spectrum. Significantly, no new absorption bands were noticed and no major peaks were missing, which meant there was no alteration in the chemical structure of the drug and confirmed the successful incorporation of the drug and their compatibility with the excipients in the NLC formulation. The observed peak broadening is consistent with molecular dispersion of azathioprine within the lipid matrix rather than chemical interaction, consistent with reports for lipid-based nanoparticulate systems incorporating poorly soluble drugs [154, 155].

4.5 Differential Scanning Calorimetry

DSC analysis was performed to investigate the thermal behavior, crystallinity, and physicochemical interactions between azathioprine and lipid components of the optimized formulation. The DSC thermogram of pure azathioprine exhibited a sharp and intense endothermic melting peak at approximately 243 - 244°C , confirming its crystalline nature and high thermal purity [156]. Compritol[®] 888 ATO displayed

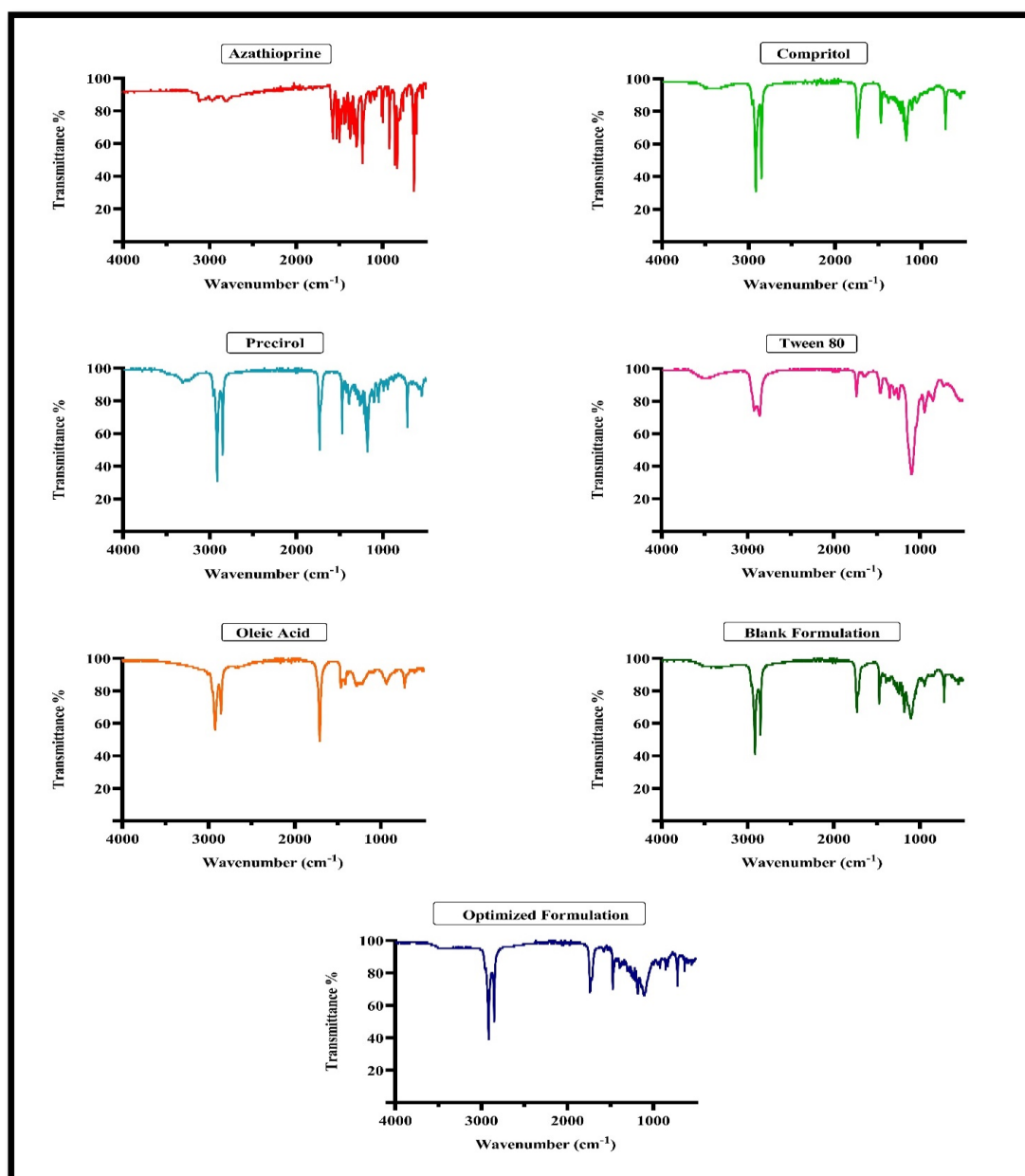


FIGURE 4.3: FTIR spectra of pure azathioprine, Compritol[®] 888 ATO, Precirol[®] ATO 5, Tween[®] 80, blank NLC, and optimized NLC10 formulation.

a broad endothermic melting transition characteristic of its heterogeneous glyceride composition. The DSC thermogram of the optimized NLC10 formulation showed a significantly altered thermal profile in which the characteristic sharp melting endotherm of azathioprine was completely absent. The thermogram was dominated by broad lipid-related thermal transitions, suggesting that azathioprine was no longer present in its crystalline form within the formulation matrix [157].

The disappearance of the drug's melting peak confirms molecular dispersion, amorphization, or solubilization within the lipid core of the NLCs. No additional thermal events were observed, confirming the absence of chemical incompatibility during formulation processing. These findings are consistent with successful incorporation of azathioprine into an amorphous or molecularly dispersed state within the NLC matrix, a feature known to enhance both solubility and formulation stability [158].

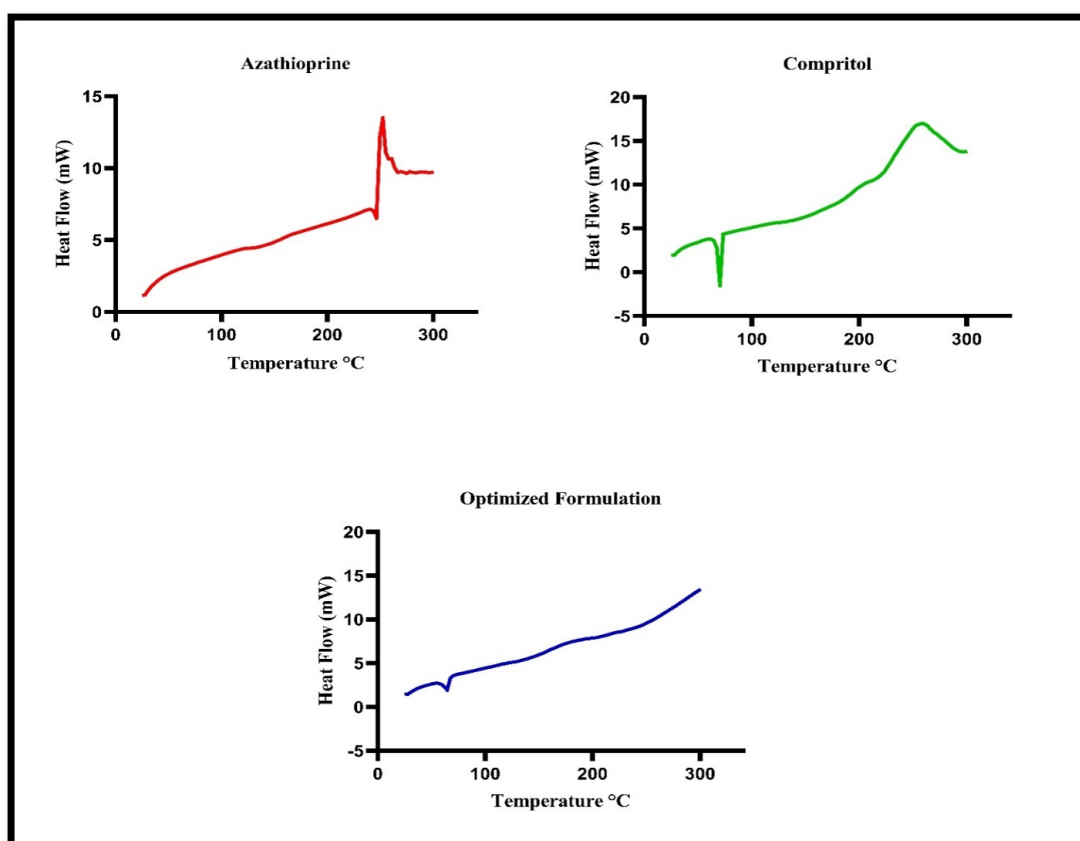


FIGURE 4.4: DSC thermograms of pure azathioprine, Compritol[®] 888 ATO and optimized NLC10 formulation. Disappearance of the azathioprine melting endotherm in NLC10 confirms successful drug amorphization within the lipid matrix.

4.6 Selection of Optimized Formulation

NLC10 was uniquely identified as the optimized formulation where all the six CQAs were met simultaneously, based upon comprehensive evaluation against all the six CQAs. Table 4.4 shows the entire CQA evaluation.

TABLE 4.4: Evaluation of NLC1-NLC13 against six critical quality attributes (CQAs)

Code	EE% ($\geq 80\%$)	Size (100 – 250)	PDI (< 0.30)	ZP ($\geq \pm 30$ mV)	Gastric R% ($\leq 10\%$)	Colonic R% ($\geq 80\%$)	Meets CQA
NLC1	<i>X</i>	<i>X</i>	<i>X</i>	<i>X</i>	<i>X</i>	<i>X</i>	No (0/6)
NLC2	<i>X</i>	<i>X</i>	<i>X</i>	<i>X</i>	<i>X</i>	<i>X</i>	No (0/6)
NLC3	✓	<i>X</i>	<i>X</i>	<i>X</i>	<i>X</i>	<i>X</i>	No (1/6)
NLC4	<i>X</i>	✓	✓	<i>X</i>	<i>X</i>	<i>X</i>	No (2/6)
NLC5	<i>X</i>	<i>X</i>	<i>X</i>	<i>X</i>	<i>X</i>	<i>X</i>	No (0/6)
NLC6	<i>X</i>	✓	✓	<i>X</i>	<i>X</i>	<i>X</i>	No (2/6)
NLC7	✓	<i>X</i>	<i>X</i>	<i>X</i>	<i>X</i>	<i>X</i>	No (1/6)
NLC8	✓	✓	✓	<i>X</i>	<i>X</i>	<i>X</i>	No (3/6)
NLC9	✓	✓	✓	<i>X</i>	<i>X</i>	<i>X</i>	No (3/6)
NLC10	✓	✓	✓	✓	✓	✓	YES (6/6)
NLC11	<i>X</i>	✓	✓	<i>X</i>	<i>X</i>	<i>X</i>	No (2/6)
NLC12	<i>X</i>	✓	✓	<i>X</i>	<i>X</i>	<i>X</i>	No (2/6)
NLC13	<i>X</i>	✓	✓	<i>X</i>	<i>X</i>	<i>X</i>	No (2/6)

✓ = Criterion met; *X* = Criterion not met”

The optimal combination of all the critical parameters was achieved by NLC10: entrapment efficiency ($93.47 \pm 0.42\%$), particle size (110.3 ± 5.1 nm), polydispersity index (0.285 ± 0.01), zeta potential (-40.4 ± 0.8 mV), gastric release ($8.10 \pm 0.4\%$) and colonic release ($91.84 \pm 1.8\%$) after 24 hours. The drug entrapment efficiency (93.47%) is high enough to provide sufficient drug payload for therapeutic effect at the colon target site. The particle size of 110.3 nm allows the particle to penetrate the mucosa and the PDI of 0.285 is considered acceptable because the distribution of particle size is moderately monodisperse and appropriate for steady drug delivery. A zeta potential of -40.4 mV shows a very good colloidal stability and the electrostatic repulsion is sufficient to prevent any aggregation of particles during storage. The drug release profile of NLC10 is most clinically favorable among all formulations with very low gastric release (8.10%) which is indicative of good gastric protection that is crucial in preventing non-selective upper gastrointestinal absorption and any associated systemic toxicity. NLC10 exhibited progressive sustained release of 91.84% after 24 hours at colonic pH which ensured

therapeutic concentrations of azathioprine at the colonic mucosa for a longer period. The release kinetics at pH 7.4 followed the Korsmeyer-Peppas model ($n = 0.944$, $R^2 = 0.9950$), suggesting that the transport of the drug molecule occurs at the upper limit of the anomalous and Case-II regimes, and the matrix relaxation is dominant [159].

NLC10 is a significant improvement over Imuran[®] (azathioprine 50 mg, conventional immediate-release tablet) which produces a rapid and non-selective release of azathioprine in the gastrointestinal tract, and which is systemically converted to 6-mercaptopurine with oral bioavailability of about 47% and dose-limiting myelosuppression and hepatotoxicity. By ensuring that only 10% of the NLC is released in the stomach and that 91.84% of azathioprine reaches the colon, NLC10 can overcome the fundamental shortcomings of the conventional azathioprine drug delivery system [160, 161].

After thorough evaluation of all the six CQAs, NLC10 was chosen as an optimized formulation for further characterization in solid state (FTIR, DSC, XRD, SEM), stability studies and acute oral toxicity studies. These results make NLC10 a good candidate as a nanocarrier for colon delivery of AZA in the treatment of IBD.

4.7 X-Ray Diffraction Analysis

To understand the crystalline state of azathioprine and its state of change after incorporation into the NLC matrix, the X-ray diffraction analysis was performed. The XRD diffractogram of pure azathioprine showed intense and sharp diffraction peaks at characteristic 2θ angles of about 11.2° , 15.4° , 19.8° and 24.3° , indicating highly ordered crystallinity of pure azathioprine [162]. Compritol[®] 888 ATO gave a diffraction peak pattern which corresponded to the β -polymorphic form, whereas Precirol[®] ATO 5 gave a semi-crystalline pattern with lower peak intensity.

However, the XRD pattern of the optimized NLC10 formulation showed a broad diffuse halo, with much lower intensity of the peaks with no distinct crystalline

diffraction peaks of pure azathioprine. This reduction in the crystalline reflections indicates effective molecular dispersion of the drug in the heterogeneous lipid matrix of the NLCs and consequently the amorphization of the drug. One of the well-known benefits of NLC systems is the change of crystal structure to the amorphous form, which increases the apparent drug solubility and its dissolution rate.

Lipid related peaks were partially retained, which indicate that the solid lipid matrix has still some degree of crystallinity and the drug is distributed in the liquid lipid domains in an amorphous way, which is consistent with the structure model of imperfect type NLCs [163].

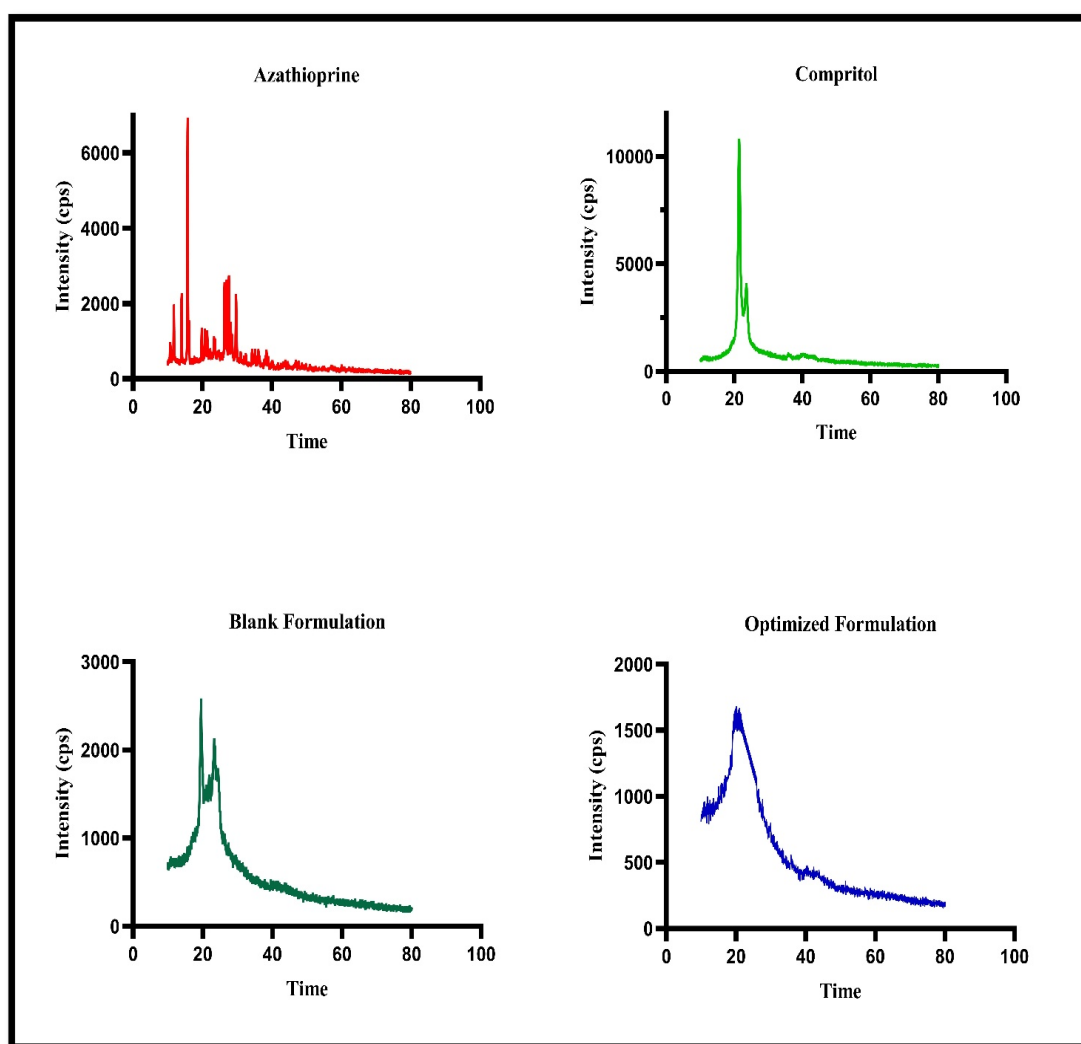


FIGURE 4.5: XRD diffractograms of pure azathioprine, Compritol[®] 888 ATO, Precirol[®] ATO 5, and optimized NLC10 formulation. Complete absence of azathioprine diffraction peaks in NLC10 confirms amorphous drug dispersion.

4.8 Scanning Electron Microscopy

The optimized NLC10 was morphologically characterized by scanning electron microscopy (SEM) at different magnifications ranging from 1000x to 100,000x. The SEM micrographs showed a homogeneous population of nanosized particles with the majority of the particles being spherical to quasi-spherical in shape with well-defined boundaries and discrete spatial distribution.

The SEM micrographs showed a homogeneous population of nanosized particles with the majority of the particles being spherical to quasi-spherical in shape with well-defined boundaries and discrete spatial distribution. Generally, the particle surfaces were found to be smooth, but some surface irregularities and slight textural variations were noted in some of the particles. The regularity in shape and boundaries of the particles indicates that the particles are well formed nanoparticles, as the lipids have been solidified during the hot homogenization process [164].

The results at higher magnifications revealed the surface irregularities more clearly which could be attributed to molecular level interaction of azathioprine with the heterogeneous solid-liquid lipid matrix.

The results at higher magnifications revealed the surface irregularities more clearly which could be attributed to molecular level interaction of azathioprine with the heterogeneous solid-liquid lipid matrix. The lack of sharp-edged, needle-shaped, or plate-like crystalline domains is in line with the DSC and XRD results, showing that azathioprine is not in a free crystalline form, but rather is molecularly dispersed or amorphously incorporated in the lipid core. Some particle clustering was observed in some areas.

The particle clustering observed in some areas is likely an artifact of the drying process during SEM sample preparation rather than evidence of instability in the aqueous dispersion, as confirmed by the high zeta potential (-40.4 mV) and stable DLS measurements. However, some degree of aggregation during sample drying cannot be entirely excluded [165].

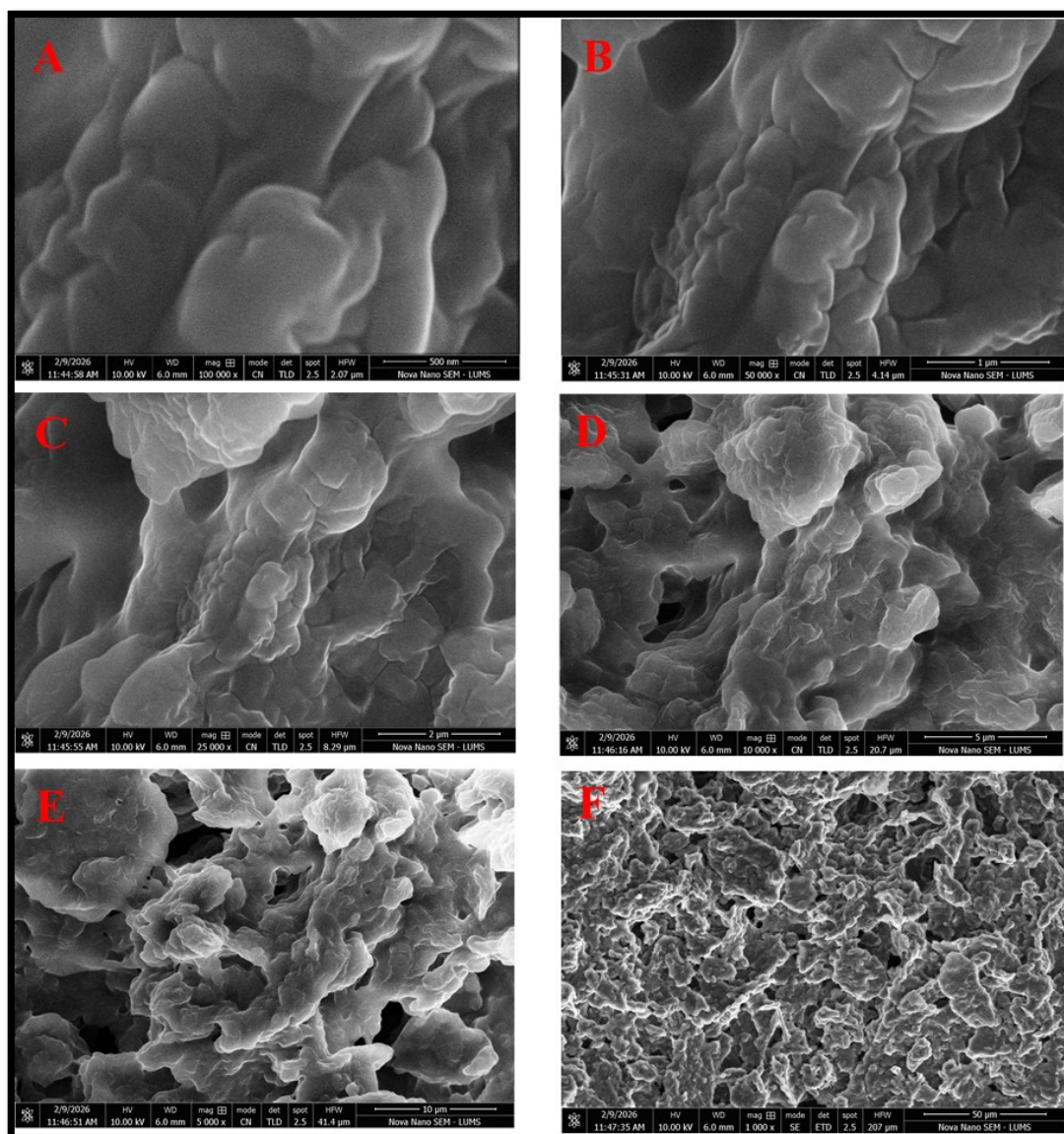


FIGURE 4.6: SEM micrographs of optimized NLC10 formulation at (A) 100,000 $\times$, (B) 50,000 $\times$, (C) 25,000 $\times$, (D) 10,000 $\times$, (E) 5,000 $\times$, and (F) 1,000 $\times$ magnification. Spherical morphology and absence of drug crystalline deposits confirm successful NLC formation and drug encapsulation

4.9 Stability Studies

The physical stability of the optimized CAP-coated NLC10 formulation was evaluated over a period of 90 days under refrigerated ($4 \pm 2^\circ\text{C}$), room-temperature ($25 \pm 2^\circ\text{C}/60 \pm 5\% \text{ RH}$), and accelerated ($40 \pm 2^\circ\text{C}/75 \pm 5\% \text{ RH}$) storage conditions in accordance with ICH Q1A(R²) guidelines [166]. The formulation was characterized at predetermined intervals for particle size, polydispersity index (PDI),

and entrapment efficiency (EE%) to assess the effect of storage conditions on its physicochemical stability. The results are presented in Table 4.5.

TABLE 4.5: SEM micrographs of optimized NLC10 formulation at (A) 100,000 $\times$, (B) 50,000 $\times$, (C) 25,000 $\times$, (D) 10,000 $\times$, (E) 5,000 $\times$, and (F) 1,000 $\times$ magnification. Spherical morphology and absence of drug crystalline deposits confirm successful NLC formation and drug encapsulation

Parameter		0	30	60	90	30	60	90	30	60
		Days	Days	Days	Days	Days	Days	Days	Days	Days
			(4 °C)	(4 °C)	(4 °C)	(25 °C)	(25 °C)	(25 °C)	(40 °C)	(40 °C)
Particle Size (nm)		110 $\pm$	113 $\pm$	117 $\pm$	121 $\pm$	125 $\pm$	132 $\pm$	154 $\pm$	142 $\pm$	168 $\pm$
		5.1	5.4	5.6	6.0	5.1	6.5	7.1	6.8	7.4
PDI		0.285	0.285	0.285	0.31	0.29	0.31	0.34	0.34	0.38
EE (%)		93.47	91.8	91.3	90.5	92.2	90.1	88.2	89.8	86.4

Data are presented as mean $\pm$ standard deviation (SD) ($n = 3$). Stability was evaluated by monitoring changes in particle size, polydispersity index (PDI), and entrapment efficiency (EE%) under refrigerated ($4 \pm 2^\circ\text{C}$), room-temperature ($25 \pm 2^\circ\text{C}/60 \pm 5\% \text{ RH}$), and accelerated ($40 \pm 2^\circ\text{C}/75 \pm 5\% \text{ RH}$) storage conditions over 90 days

Under refrigerated storage conditions ($4 \pm 2^\circ\text{C}$), the optimized NLC10 formulation exhibited excellent physical stability throughout the 90-day study period. The particle size increased only marginally from $110 \pm 5.1 \text{ nm}$ at the initial time point to $113 \pm 5.4 \text{ nm}$ after 30 days, $117 \pm 5.6 \text{ nm}$ after 60 days, and $121 \pm 6.0 \text{ nm}$ after 90 days. Also, the PDI also did not change significantly, going from 0.285 to 0.31, signifying that a small and uniform particle size distribution was preserved. Entrapment efficiency decreased modestly from 93.47% to 91.8%, 91.3%, and 90.5% after 30, 60, and 90 days, respectively. The small changes seen when stored under refrigerated conditions indicate a good suppression of the molecular mobility of the lipid, minimal diffusion of the drug from the lipid matrix and no significant aggregation of the nanoparticles, thus preserving the structural integrity of the formulation [37].

Under room-temperature conditions ($25 \pm 2^\circ\text{C}/60 \pm 5\% \text{ RH}$), gradual physicochemical changes were witnessed during the storage period. Particle size increased from $125 \pm 5.1 \text{ nm}$ initially to $132 \pm 6.5 \text{ nm}$ after 60 days and further to $154 \pm 7.1 \text{ nm}$ after 90 days. The PDI rose from 0.29 to 0.31 and 0.34 during the same time,

suggesting a moderate broadening of particle size distribution. After 60 days, the entrapment efficiency reduced from 92.2% to 90.1% and after 90 days, it was reduced to 88.2%. These results indicate that there was some degree of growth of the nanoparticles and a slight decrease in drug retention which would be due to the increased freedom of the lipid molecules and the ongoing reorganization of the lipid matrix at room temperature. However, the formulation still had a nanoscale particle size of less than 200 nm and high entrapment efficiency of over 88% and no visual precipitation, phase separation or irreversible instability was observed [167].

More prominent changes were witnessed under accelerated storage conditions ($40 \pm 2^\circ\text{C}/75 \pm 5\% \text{ RH}$). The particle size increased progressively from $110 \pm 5.1 \text{ nm}$ to $142 \pm 6.8 \text{ nm}$ after 30 days, $168 \pm 7.4 \text{ nm}$ after 60 days, and $196 \pm 8.3 \text{ nm}$ after 90 days. At the same time, the PDI value rose from 0.285 to 0.34, 0.38 and 0.43, which reflects a gradual increase in particle size distribution and heterogeneity of the nanoparticles. Entrapment efficiency declined from 93.47% initially to 89.8%, 86.4%, and 82.7% after 30, 60, and 90 days, respectively. The increased degree of alteration seen under the accelerated conditions is likely due to more mobility of the lipid molecules, more chance for aggregation of the particles and faster diffusion of the drug from the lipid matrix at higher temperature and humidity. Such conditions can lead to partial recrystallization of lipids and/or structural changes in the nanocarrier system, which could increase particle size and decrease drug retention. The formulation experienced a gradual change in its physicochemical properties under accelerated conditions. After 90 days PDI rose to 0.43 which is above the pre-defined CQA value of 0.30, thus showing that accelerated storage had a negative effect on the colloidal homogeneity [168]. The particle size was below 200 nm and EE was above 80%, refrigerated storage at $4 \pm 2^\circ\text{C}$ is recommended as the best long term storage condition. The stability data were statistically analysed with one-way ANOVA and Tukey's post hoc test to determine the time point at which the difference was statistically significant ($p < 0.05$) from the Day 0 value. The particle sizes were increased and the EE decreased progressively over 90 days, all under refrigerated conditions ($4 \pm 2^\circ\text{C}$);

however, all changes were not statistically significant ($p > 0.05$) reflecting the excellent stability. On the other hand, the increase in particle size (196 ± 8.3 nm) and PDI (0.43) at 90 day was statistically significant as compared to Day 0 ($p < 0.05$) and showed that accelerated storage affects the colloidal homogeneity [169].

The overall results of the stability studies for all the formulations indicated that NLC10 formulation was most stable when stored under refrigerated conditions followed by room temperature while accelerated conditions showed the maximum changes in the physico-chemical parameters. However, the formulation showed acceptable particle size and size distribution and drug entrapment properties under all the storage conditions. This binary lipid matrix, CAP coating and non-ionic surfactant system were able to effectively minimize the drug leakage and maintained the integrity of the nanoparticles during storage period, which further indicates that NLC10 is suitable as a stable colon-targeted azathioprine delivery system.

Under refrigerated conditions, the changes in particle size (121 nm at 90 days), PDI (0.31), and EE (90.5%) were not statistically significant compared to Day 0 ($p > 0.05$). However, under accelerated conditions (40°C), the increase in particle size from 110 nm to 196 nm and PDI from 0.285 to 0.43 were statistically significant ($p < 0.05$) at the 90-day time point.

4.10 Acute Oral Toxicity Study

There was no death in both control and treated animals during 14 days observation period, which is an acute safety profile of azathioprine loaded NLC10 formulation. During the study all animals in both groups maintained normal activity, alertness and grooming. Toxicities symptoms, including lethargy, tremors, convulsions, salivation, piloerection, or abnormal behavioural patterns were not observed. There was no difference in food and water consumption among groups and they remained within normal range. Details of clinical observations are summarised in Table 4.6.

TABLE 4.6: Clinical observations in acute oral toxicity study (14-day observation period).

Parameter	Group I (Control)	Group II (Treated)	Interpretation
Body Weight	Normal growth	Normal growth	No toxicity
Food Intake	Normal	Normal	No reduction
Water Intake	Normal	Normal	No dehydration
Locomotor Activity	Normal	Normal	No CNS effect
Grooming Behavior	Normal	Normal	Physiological
Salivation	Absent	Absent	No toxicity
Piloerection	Absent	Absent	No stress
Convulsions/Tremors	Absent	Absent	No neurotoxicity
Lethargy	Absent	Absent	Normal activity

Change in body weight was used as a measure of systemic toxicity. During the 14-day observation, the physiological weight gain of both control and treated groups were normal with control group animals gaining 33.5 ± 0.5 g weight and the treated group 35.0 ± 1.0 g body weight at day 14. There was no significant difference in body weight between the groups ($p > 0.05$, Student's t-test), which means that there was no metabolic or physiological disturbance caused by the treatment [170].

Liver function tests (ALT, AST, ALP) and kidney function tests (creatinine and urea) did not differ significantly ($P > 0.05$) between control and treated groups suggesting normal hepatic and renal function and a lack of hepatotoxicity and nephrotoxicity at the dose used. The hematological parameters such as hemoglobin, WBC, and platelet count remained in the normal physiological range proving that NLC10 administration did not cause hematopoietic abnormalities. Biochemical and hematological parameters are shown in the table 4.7.

Results are expressed as mean $\pm$ SD ($n = 4$ /group). Abbreviations: WBC, white blood cells; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase. All $p > 0.05$ (Student's t-test).

At the end of the 14 days of the study period, the histopathological examination of major vital organs namely liver, kidneys, stomach, small intestine, large intestine,

heart and spleen was carried out. Histological examination of all organs of both the treated and control groups did not show any abnormal tissue structure.

TABLE 4.7: Biochemical and hematological parameters in acute oral toxicity study (mean $\pm$ SD, n = 4 per group).

Parameter	Group I (Control)	Group II (Treated)	p-value
Hemoglobin (g/dL)	13.5 $\pm$ 0.4	13.7 $\pm$ 0.3	>0.05
WBC ($10^3/\text{mm}^3$)	7.2 $\pm$ 0.5	7.4 $\pm$ 0.4	>0.05
Platelets ($10^3/\text{mm}^3$)	250 $\pm$ 12	255 $\pm$ 10	>0.05
ALT (U/L)	32.4 $\pm$ 2.3	31.8 $\pm$ 2.1	>0.05
AST (U/L)	28.5 $\pm$ 1.8	29.0 $\pm$ 1.7	>0.05
ALP (U/L)	72.0 $\pm$ 3.4	73.2 $\pm$ 3.1	>0.05
Creatinine (mg/dL)	0.80 $\pm$ 0.05	0.81 $\pm$ 0.04	>0.05
Urea (mg/dL)	22.1 $\pm$ 1.2	21.8 $\pm$ 1.3	>0.05
Glucose (mg/dL)	92.3 $\pm$ 3.5	91.5 $\pm$ 3.2	>0.05

Liver parenchyma was normal without any sign of hepatocellular necrosis, fatty changes and inflammatory infiltration. No degeneration of the tubular structures or glomeruli was seen in the kidneys.

There was normal organization of myocardial fibers with no disruption of cardiac tissue. Macroscopic examination of the stomach showed an intact gastric mucosa without any erosion or ulceration [171].

The lack of histological changes of any of the examined organs of the treated group compared with the non-treated group is a strong indicator of the non-toxic nature of the formulation when tested at the dose levels.

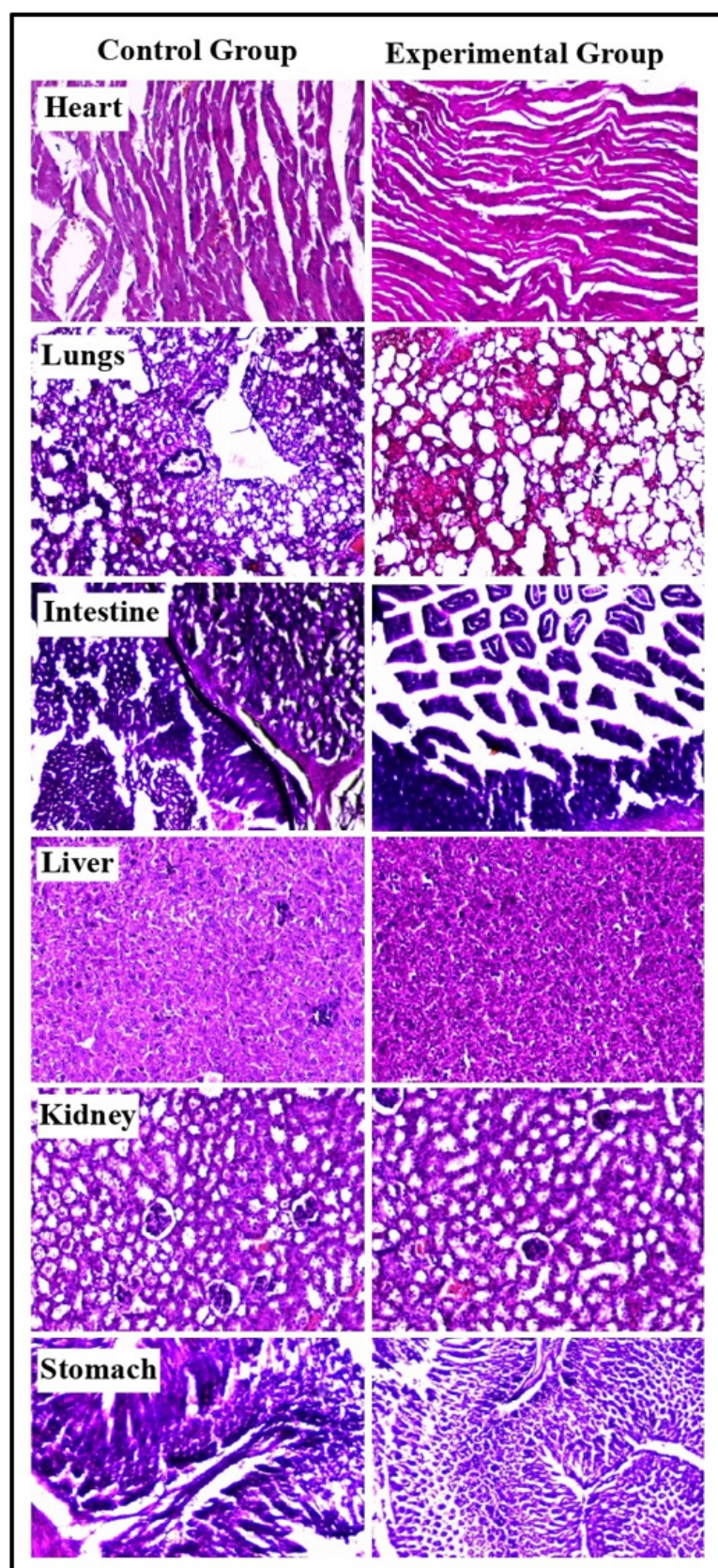


FIGURE 4.7: Histopathological evaluation of vital organs from control and treated groups. All organs showed preserved tissue architecture without cellular damage, inflammation, or structural abnormalities.

The acute oral toxicity results are indicative of the preliminary safety profile of the azathioprine-loaded NLC10 formulation, at the doses investigated. The lack of mortality, normal behavioral parameters, normal body weight progression, absence of significant biochemical changes ($p > 0.05$) and normal tissue architecture of all the vital organs by themselves, all confirm that the formulation is not acutely orally toxic at 2 g/kg body weight [172]. These data serve as preliminary safety data and sub-chronic and chronic toxicity studies with larger numbers of animals are indicated for regulatory advancement

Chapter 5

Conclusion and Future Recommendations

5.1 Conclusion

In the current study, cellulose acetate phthalate coated nanostructured lipid carriers containing azathioprine were successfully fabricated, and pH-sensitive drug delivery system targeting colon for the management of IBD. All thirteen formulations were systematically optimized and the NLC10 formulation was found to be the only one that simultaneously satisfies all six critical quality attributes such as EE (93.47 ± 0.42 %), particle size (110.3 ± 5.1 nm), PDI (0.285 ± 0.01), zeta potential (-40.4 ± 0.8 mV), gastric release (8.10 ± 0.4 %) and colonic release (91.84 ± 1.8 %) within 24 h. Chemical compatibility and the lack of drug-excipient interactions were confirmed by FTIR spectroscopy, while DSC and XRD analyses showed successful amorphous dispersion of azathioprine in the lipid matrix, which will increase the drug solubility and stability as expected. The SEM micrographs showed that the nanoparticles were spherical in shape and evenly distributed, indicating the formation of NLCs. The pH-dependent release profile was governed by super Case-II transport at pH 1.2 ($n = 1.312$, $R^2 = 0.988$) and upper-boundary anomalous/Case-II transport at pH 7.4 ($n = 0.944$, $R^2 = 0.995$). This results in

minimal leakage of the drug into the stomach and maximum prolonged release into the colon at the site of inflammation in the bowel. Stability studies showed excellent physicochemical stability of the particles during refrigerated storage ($4 \pm 2^\circ\text{C}$) for 90 days, with marginal increase in particle size (from 110 nm to 121 nm) and minimal decrease in EE (93.47% to 90.5%). Moderate particle growth (154 nm at 90 days) and some EE loss (88.2%) were observed at ambient conditions ($25 \pm 2^\circ\text{C}/60 \pm 5\% \text{ RH}$), but no immediate or irreversible precipitation, phase separation or instability was observed, and the formulation maintained nanoscale size dimensions, acceptable PDI (0.34 at 90 days) and high entrapment ($> 85\%$). PDI rose to 0.43 after 90 days under accelerated storage conditions ($40 \pm 2^\circ\text{C}/75 \pm 5\% \text{ RH}$), surpassing the CQA limit of 0.30, suggesting that refrigerated storage at $4 \pm 2^\circ\text{C}$ is advisable for extended storage to prevent loss of physicochemical integrity.

Acute oral toxicity studies in healthy BALB/c mice at 2 g/kg (OECD 423, 14-day observation) demonstrated complete biocompatibility with no mortality, normal clinical parameters, stable body weight, and no significant hematological, biochemical, or histopathological alterations (all $p > 0.05$). Collectively, these findings establish azathioprine-loaded CAP-coated NLCs as a promising, safe, and effective platform for site-specific IBD therapy, warranting further investigation in pharmacodynamic and in vivo efficacy models.

5.2 Future Recommendations

While the present study establishes the successful formulation, optimization, and preliminary safety evaluation of azathioprine-loaded CAP-coated NLCs (NLC10) as a colon-targeted delivery platform, several avenues warrant further investigation to strengthen the translational potential of this system and address the limitations inherent to a formulation-focused thesis.

- i. The therapeutic efficacy of the optimized NLC10 formulation should be evaluated in a validated murine model of colitis, such as the DSS-induced or

TNBS-induced colitis model, to confirm anti-inflammatory activity and mucosal healing relative to free azathioprine and marketed Imuran[®] tablets. Endpoints should include disease activity index, colon length, histopathological scoring, and inflammatory biomarkers (TNF- α , IL-6, myeloperoxidase activity).

- ii. Comparative pharmacokinetic studies (plasma concentration-time profiles, AUC, C_{max}, T_{max}) between the CAP-coated NLC formulation and conventional azathioprine should be conducted in an appropriate animal model to quantitatively confirm the anticipated reduction in systemic drug exposure and corresponding decrease in off-target 6-TGN accumulation in bone marrow, liver, and lymphoid tissue.
- iii. Ex vivo permeation studies using excised colonic tissue (e.g., Franz diffusion cells or everted gut sac technique) and in vivo colonic tissue drug quantification would help confirm the extent of mucosal drug accumulation and the mucus-penetrating behaviour attributed to the nanoscale particle size of the CAP-coated NLCs.
- iv. Given that PDI exceeded the critical quality attribute limit under accelerated conditions ($40 \pm 2^\circ\text{C}/75 \pm 5\% \text{ RH}$) by 90 days, longer-duration real-time stability studies (12-24 months) under ICH-recommended long-term and intermediate conditions are recommended to establish a definitive shelf-life and to evaluate whether lyophilization or spray-drying into a stable solid dosage form (e.g., NLC-loaded pellets or capsules) could improve storage robustness beyond refrigerated conditions.
- v. Translation from laboratory-scale hot high-speed homogenization to pilot- and industrial-scale manufacturing (e.g., high-pressure homogenization or microfluidization) should be explored, along with Quality by Design (QbD) approaches to identify critical process parameters and ensure batch-to-batch reproducibility under GMP-compliant conditions.
- vi. While acute oral toxicity findings were reassuring, sub-chronic and chronic toxicity studies (28-day and 90-day repeated-dose studies per OECD 407 /

- 408) are recommended to evaluate the safety of long-term administration, given that azathioprine therapy in IBD is typically maintained over months to years.
- vii. Studies employing fluorescently labelled NLCs and confocal microscopy or flow cytometry in intestinal epithelial or macrophage cell lines (e.g., Caco-2, RAW 264.7) would help elucidate the specific endocytic pathways (clathrin- or caveolae-mediated) responsible for colonic mucosal and macrophage uptake at inflamed tissue sites.
 - viii. Head-to-head comparison of CAP-coated NLCs with Eudragit S100- or HP-MCP - coated counterparts would help quantitatively validate the site-specificity advantage attributed to CAP's lower pH dissolution threshold in the context of this specific drug-lipid system.
 - ix. Incorporation of the optimized NLC10 formulation into a solid oral dosage form, such as CAP-coated pellets, minitabets, or capsules filled with NLC-loaded granules, may improve patient compliance, dosing accuracy, and manufacturability compared with a liquid nanosuspension.
 - x. Following successful completion of preclinical efficacy and toxicology packages, Phase I clinical studies in healthy volunteers would be the logical next step to evaluate human safety, tolerability, and preliminary pharmacokinetics prior to progression toward Phase II proof-of-concept trials in IBD patients.
 - xi. In addition to the proposed *in vivo* pharmacokinetic evaluation, *in vitro* release studies at pH 6.8 should be performed, as this pH more closely approximates the terminal ileum/ileocecal junction and the lower end of physiological colonic pH variability than pH 7.4 alone. Assessing azathioprine release at pH 6.8 would confirm whether the CAP coating remains sufficiently intact under this borderline condition or undergoes premature dissolution, thereby providing a more complete and clinically realistic release profile to support the formulation's intended colon-targeting performance.

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