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Intranasal Transfersosomal In Situ Gel for Enhanced Brain Targeting and Sustained Delivery of Paclitaxel

by

Ayesha Nasir

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CERTIFICATE OF APPROVAL

Intranasal Transferosomal In Situ Gel for Enhanced Brain Targeting and Sustained Delivery of Paclitaxel

by

Ayesha Nasir

(MPH243008)

THESIS EXAMINING COMMITTEE

S. No.	Examiner	Name	Organization
(a)	External Examiner	Dr. Shahiq uz Zaman	RIU, Islamabad
(b)	Internal Examiner	Dr. Muhammad Saalim	CUST, Islamabad

Dr. Nadia Shamshad Malik

Thesis Supervisor

August, 2026

Dr. Nadia Shamshad Malik

Head

Dept. of Pharmaceutics

August, 2026

Dr. Muzaffar Abbas

Dean

Faculty of Pharmacy

August, 2026

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(Ayesha Nasir)

Registration No: MPH243008

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(Ayesha Nasir)

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(Ayesha Nasir)

Abstract

Breast cancer brain metastasis (BCBM) is a devastating complication with limited therapeutic options, primarily due to blood-brain barrier (BBB) impermeability to paclitaxel (PTX). This study developed and optimized an intranasal PTX-loaded nano-transferosomal in situ gel (PTX-NTFG) to exploit the nose-to-brain pathway, circumventing the BBB and hepatic first-pass metabolism. Nano-transferosomes were fabricated by the thin-film hydration technique and optimized using a three-level, three-factor Box-Behnken design (BBD), with soy lecithin concentration (30-40 mg), Poloxamer 188 concentration (10-20 mg), and hydration volume (4-12 mL) as independent variables, and zeta potential, entrapment efficiency, PDI, and particle size as responses. The optimized transferosomal dispersion was incorporated into a thermoresponsive Poloxamer 407 in situ gel and characterized by FTIR, XRD, and DSC. The particle size of the optimized formulation (F15) was 166.3 nm, with a Polydispersity index (PDI) of 0.195, -35.9 mV zeta potential, and entrapment efficiency of 88.0 %. All BBD models showed high predictive accuracy ($R^2 \geq 0.969$). Solid-state investigation approved molecular dispersion of PTX inside the lipid-polymer complex with no drug-excipient incompatibilities. PTX-NTFG exhibited sustained release of $75.9 \pm 2.5\%$ at pH 7.4 over 24 h, best described by the Korsmeyer-Peppas model, which shows anomalous transport ($R^2 = 0.9953$; $n = 0.681$). The sol-to-gel transition occurred at $34.2 \pm 0.5^\circ\text{C}$ with gel viscosity of 2099.53 ± 18.40 cP at 37°C and $\text{pH } 5.5 \pm 0.05$. Ex vivo nasal permeation of PTX-NTFG ($945.92 \pm 25.13 \mu\text{g}/\text{cm}^2$) was 4.08-fold higher than conventional PTX gel ($237.56 \pm 24.84 \mu\text{g}/\text{cm}^2$) at 24 h. PTX-NTFG integrates nano-transferosome deformability with thermoresponsive mucoadhesive properties, achieving markedly enhanced transmucosal permeation and sustained drug release, representing a promising non-invasive nose-to-brain strategy for BCBM management.

Keywords: Breast cancer brain metastasis; Paclitaxel; Nano-transferosomes; Thermoresponsive, in situ gel; Intranasal drug delivery; Box-Behnken design

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Abbreviations

ABCB1	ATP-Binding Cassette Subfamily B Member 1
ANOVA	Analysis of Variance
BBB	Blood–Brain Barrier
BBD	Box–Behnken Design
BCRP	Breast Cancer Resistance Protein
BCS	Biopharmaceutics Classification System
CNS	Central Nervous System
DLS	Dynamic Light Scattering
DSC	Differential Scanning Calorimetry
EE	Entrapment Efficiency
ELS	Electrophoretic Light Scattering
ER	Enhancement Ratio
FTIR	Fourier Transform Infrared Spectroscopy
G2/M	Gap 2/Mitosis Phase
HER2	Human Epidermal Growth Factor Receptor 2
HPLC	High Performance Liquid Chromatography
ICH	International Council for Harmonisation
MWCO	Molecular Weight Cut-Off
nm	Nanometer
PBS	Phosphate Buffered Saline
PDI	Polydispersity Index
PLGA	Poly (Lactic-co-Glycolic Acid)
PTX	Paclitaxel
PTX-NTFG	Paclitaxel-Loaded Nano-Transferosomal In Situ Gel

PXRD	Powder X-Ray Diffraction
QbD	Quality by Design
RH	Relative Humidity
rpm	Revolutions Per Minute
RSM	Response Surface Methodology
SD	Standard Deviation
TEER	Transepithelial Electrical Resistance
TEM	Transmission Electron Microscopy
TNBC	Triple-Negative Breast Cancer
UV	Ultraviolet

Symbols

w/v	Weight per Volume
w/w	Weight per Weight
Y_1	Particle Size Response
Y_2	Polydispersity Index Response
Y_3	Zeta Potential Response
Y_4	Entrapment Efficiency Response
$ZO - 1$	Zonula Occludens-1
ZP	Zeta Potential
$^{\circ}C$	Degree Celsius
μg	Microgram
μm	Micrometer
R^2	Coefficient of Determination
kDa	Kilodalton
mg	Milligram
mL	Milliliter
mV	Millivolt

Chapter 1

Introduction

1.1 Overview of Breast Cancer Brain Metastasis

Breast cancer is still the most common cancer and second most common cause of cancer-associated death in women globally, responsible for about 2.3 million new cases and 685,000 deaths in 2020. Even after considerable progress in targeted drug development, immunotherapy, and multimodality therapy, brain metastasis remains one of the most difficult complications of breast cancer. Brain metastases occur in 10-16% of patients with metastatic breast cancer, but the percentage is highly elevated in the case of aggressive molecular breast cancers, such as triple-negative breast cancer (TNBC) and Human Epidermal Growth Factor Receptor-2 (HER2-positive) breast cancer, with the frequency of brain metastases being between 25-50%. Once brain metastases have been detected, the patient's prognosis becomes extremely poor, with life expectancy being three to six months even with conventional treatment. Moreover, with the use of novel treatment modalities, which lead to increased control of extracranial lesions, the issue becomes even more serious because these treatments rarely cross the blood-brain barrier [1, 2].

1.2 The Blood–Brain Barrier – a Key Challenge to Therapy

One key challenge to the therapeutic treatment of brain metastasis is the existence of the blood-brain barrier (BBB). The BBB is highly specialized and selectively permeable in nature and acts as an interface between neurovasculature. It consists of brain microvascular endothelial cells that are firmly attached to each other via junction proteins like claudin-5, occludin, and zonula occludens-1 (ZO-1).

Besides being a physical barrier, the BBB has an active transport mechanism, such as the expression of P-glycoprotein ATP-Binding Cassette Subfamily B Member 1 (ABCB1) and breast cancer resistance protein/ ATP-Binding Cassette Subfamily G Member 2 (BCRP/ABCG2), which serves as a system that actively expels toxins away from the brain. Overall, these factors lead to high resistance to drug entry, and thus the central nervous system bioavailability of most drugs becomes very low [3].

1.3 Paclitaxel: Therapeutic Value and Delivery Limitations

Paclitaxel is one of the commonly used drugs in chemotherapy that belongs to the taxane family; this drug is known to stabilize microtubules and inhibit mitosis leading to cell death in rapidly growing cancer cells. Even though paclitaxel is an effective agent for breast and other cancers, its use in brain metastases is highly limited.

Such limitations arise because of the low water solubility, high molecular weight, and ability to be a good substrate for the efflux transporters such as P-gp, thus not getting into the brain in any considerable amount. Moreover, traditional approaches for formulations add on to toxicity and hypersensitivity response of the body; therefore, dose optimization becomes a challenge [4, 5].

1.4 Intranasal Delivery as a Nose-to-Brain Approach

Intranasal drug delivery has emerged as a promising non-invasive strategy for targeting therapeutic agents to the central nervous system because it offers a direct pathway from the nasal cavity to the brain through the olfactory and trigeminal nerves, thereby bypassing the blood-brain barrier and avoiding first-pass metabolism; this route can provide rapid onset of action, improved brain availability, reduced systemic exposure, and better patient compliance, but its effectiveness is limited by several physiological and anatomical barriers, including mucociliary clearance, enzymatic degradation in the nasal mucosa, the small absorptive surface area of the olfactory region, restricted dosing volume, selective permeability of the nasal epithelium, and poor residence time of conventional formulations, which together reduce drug absorption and brain transport; therefore, there is a clear need for advanced intranasal delivery systems that can enhance mucosal permeation, prolong nasal retention, and improve direct nose-to-brain delivery for more effective treatment of central nervous system disorders and brain malignancies [6].

1.5 Transferosomes as Ultra-Deformable Vesicular Delivery Systems

Transferosomes have gained attention as an effective vesicular carrier system for brain-targeted drug delivery because of their unique structural and functional features.

These ultra deformable vesicles are composed mainly of phospholipids and edge activators, which give the lipid bilayer exceptional elasticity and allow the vesicles to squeeze through biological barriers that are much smaller than their own size without losing stability. This deformability improves their ability to interact with biological membranes and enhances penetration, making them particularly suitable

for intranasal delivery. Transferosomes can also entrap poorly water-soluble drugs, improving their dispersion and apparent solubility, while shielding the loaded drug from chemical breakdown and enzymatic degradation during transport. In addition, they may support sustained drug release and increase drug accumulation at the target site, which can improve overall bioavailability. These properties are especially valuable for paclitaxel, a hydrophobic anticancer drug with low aqueous solubility and poor penetration into the brain. By improving solubilization, stability, and membrane transport, transferosomes provide a promising platform for enhancing the nose-to-brain delivery of paclitaxel. [7].

1.6 Thermoresponsive In Situ Gel for Nasal Retention

Thermoresponsive in situ gels have been widely investigated as a strategy to overcome the short residence time associated with conventional nasal formulations. These systems are designed to remain in a liquid state during administration, allowing easy delivery into the nasal cavity, and subsequently undergo a sol-to-gel transition upon exposure to the physiological temperature of the nasal environment. The formation of a gel at the site of administration increases the residence time of the formulation, minimizes rapid mucociliary clearance, and enhances the contact of the drug with the nasal mucosa, thereby promoting drug absorption.

In addition, the gel matrix can provide controlled and sustained drug release, helping to maintain therapeutic drug levels over an extended period. When transferosomes are incorporated into a thermoresponsive in situ gel, the resulting system combines the advantages of both technologies. The transferosomes facilitate drug solubilization and permeation across the nasal epithelium, while the gel network retains the vesicles within the nasal cavity and protects them from premature elimination. This dual-function delivery system can improve both drug permeation and nasal retention, creating favorable conditions for enhanced nose-to-brain transport and more efficient localization of the drug within the central nervous system [8].

1.7 Quality by Design Approach as a Formulation Development Strategy

Quality by Design is a scientific approach that enables development of drug formulations through identification of critical quality attributes, critical material attributes, and critical process parameters. For the current study, Quality by Design is used to rationally optimize the in situ gel delivery system and determine the design space for critical formulation variables. Response surface methodology like the Box-Behnken design will be used to model the relationship between critical variables and drug delivery system performance [9].

1.8 Problem Statement

The treatment of brain metastases remains a major challenge due to the presence of the blood-brain barrier (BBB), which restricts the delivery of many therapeutic agents to the brain. Although paclitaxel is an effective anticancer drug, its clinical utility against brain tumors and brain metastases is limited by poor penetration into brain tissue, low aqueous solubility, and active efflux mechanisms that reduce its accumulation in the central nervous system.

Intranasal administration has emerged as a promising non-invasive approach for direct drug delivery to the brain through the olfactory and trigeminal pathways, bypassing the BBB. However, the effectiveness of this route is often compromised by rapid mucociliary clearance, limited residence time within the nasal cavity, and insufficient drug permeation across the nasal mucosa. These physiological barriers can significantly reduce the amount of drug reaching the brain.

Therefore, there is a need for a nasal drug delivery system that can enhance mucosal permeation, prolong nasal residence time, and provide sustained drug release. The combination of transferosomes and an in situ gelling system offers a potential strategy to address these challenges. Such a formulation may improve the

direct transport of paclitaxel from the nose to the brain, enhance drug availability at the target site, and support more effective management of brain metastases.

1.9 Rationale of the Study

Rationale of this research work lies in the assumption that an optimally designed intranasal formulation will provide better brain delivery of paclitaxel without having to use invasive methods.

The Transferosomes will be useful in enhancing membrane transport and thermoresponsive in situ gel will help in delaying the nasal retention of the drug and controlling its diffusion.

Using both systems together in a formulation development process guided by QbD principles provides a scientific justification for tackling several challenges at once and developing a more rational and translatable formulation.

1.10 Research Aim and Objectives

To design, formulate, optimize, and comprehensively evaluate a thermoresponsive paclitaxel-loaded transferosomal in situ gel for intranasal administration as a novel nose-to-brain drug delivery system. The developed formulation aims to overcome the limitations of conventional paclitaxel therapy, including poor aqueous solubility, limited blood-brain barrier permeability, rapid nasal clearance, inadequate drug localization, and systemic toxicity.

By enhancing nasal residence time, transmucosal permeation, sustained drug release, and brain targeting, this research seeks to improve the therapeutic potential of paclitaxel for the management of breast cancer brain metastasis.

- i) To formulate paclitaxel-loaded transferosomes using suitable phospholipids and edge activators, and to optimize the formulation for improved vesicular

stability, high entrapment efficiency, and desirable physicochemical characteristics.

- ii) To characterize the optimized transferosomal formulations in terms of particle size, polydispersity index, zeta potential, morphology, vesicle uniformity, and drug entrapment efficiency in order to assess their suitability for intranasal delivery.
- iii) To investigate the compatibility between paclitaxel and the selected excipients, and to evaluate the solid-state properties of the drug and formulation components using Fourier Transform Infrared Spectroscopy (FTIR), Differential Scanning Calorimetry (DSC), and X-ray Diffraction (XRD).
- iv) To evaluate the in vitro drug release behavior of the optimized transferosomal formulation and determine the release pattern and kinetics to assess its potential for sustained drug delivery.
- v) To incorporate the optimized paclitaxel-loaded transferosomes into a thermoresponsive and mucoadhesive in situ gel system designed for intranasal administration and improved nasal retention.
- vi) To characterize the developed transferosomal in situ gel with respect to gelation temperature, gelation time, rheological behavior, pH and drug content uniformity.
- vii) To assess the ex vivo permeation of paclitaxel across nasal mucosa and evaluate the ability of the formulation to enhance transmucosal transport and support nose-to-brain delivery.
- viii) To evaluate the stability of the optimized transferosomal in situ gel under appropriate storage conditions and determine its suitability for further development and potential therapeutic application.

1.11 Research Hypothesis

It is hypothesized that the development of a paclitaxel-loaded transferosomal in situ gel for intranasal administration will overcome the major limitations associated with conventional paclitaxel delivery to the brain. The ultra-deformable nature of transferosomes is expected to enhance the solubilization of paclitaxel and facilitate its permeation across the nasal mucosa, while the thermoresponsive in situ gel system is anticipated to prolong residence time within the nasal cavity by reducing mucociliary clearance and providing sustained drug release. The synergistic combination of these delivery platforms is expected to improve trans-mucosal transport and support more efficient nose-to-brain delivery of paclitaxel.

Furthermore, it is hypothesized that the application of a Quality by Design (QbD)-based optimization approach will enable the development of a robust and reproducible formulation with desirable physicochemical characteristics, including appropriate vesicle size, high drug entrapment efficiency, favorable stability, controlled release behavior, and enhanced permeation performance. Consequently, the optimized transferosomal in situ gel is expected to demonstrate superior suitability for intranasal brain-targeted delivery of paclitaxel compared with conventional formulations.

Chapter 2

Literature Review

2.1 Breast Cancer Brain Metastasis

2.1.1 Overview

Breast cancer continues to be one of the malignancies with significant clinical significance in women, not only due to its high prevalence but also due to its ability to spread beyond the primary lesion to other organs. The brain is one of the most difficult tissues for secondary colonization. Metastasis of cancer to the brain is not just an accidental process; it is a highly selective process whereby tumor cells develop the capability to survive in the bloodstream, migrate across blood vessel barriers, survive in the brain microenvironment, and receive proliferative signals from an environment completely different from the mammary gland. Advanced breast cancer patients whose brain is involved suffer from impaired neurologic functions, poor quality of life, limited treatment options, and bad prognosis. [10, 11]. The metastasis of breast cancer to the brain becomes more important when considering an aggressive molecular type of cancer, that is characterized by higher proliferation rate, increased motility, and a tendency for dissemination through blood circulation. After entering the brain metastasis, the therapy is complicated by numerous anatomical and physiological barriers [12]. Systemic chemotherapy

fails to deliver a therapeutically effective dose to the metastatic site, whereas radiotherapy and surgical techniques are limited by location of the tumor, number of tumors, and neurotoxicity. Thus, there is solid scientific justification for exploring other methods of drug delivery that will deliver cytotoxic drugs to the brain without damaging surrounding cells [13].

Another complication associated with brain metastasis therapy relates to its heterogeneity. The microenvironment of metastasis does not remain the same but changes depending on local cytokines, vascular conditions, glial support, and immune escape.

As a result, even if a drug can enter the brain, it should maintain sufficient concentration in the free form long enough to achieve an antitumor effect. Due to these reasons, the use of modern nanocarriers becomes important [14, 15].

2.1.2 Challenges in Brain Delivery

Drug effectiveness is not the only problem in dealing with brain metastasis, but also drug availability. The existence of the blood-brain barrier, tight inter - endothelial junctions, efflux transporters, enzymatic destruction, and poor paracellular diffusion all contribute to the limited brain availability of many drugs. [16].

Paclitaxel has been the most prominent example of a very powerful anticancer drug, but limited by a low ability to penetrate into the brain using standard administration protocols. Even with systemic delivery, the clinical utility of the drug in brain disorders remains compromised due to efflux pathways and dose-limiting systemic toxicity [17].

The brain is an organ with numerous physiological barriers which complicate effective delivery of drugs especially those with poor blood brain barrier permeation. There is selectivity of the blood-brain barrier, controlled interstitial fluid volume and extracellular matrix, which hinders diffusion of bigger particles or improper formulation of drugs.

Moreover, the brain is a very delicate organ and has limited tolerability; thus, a drug should not cause irritation, neuronal cell death, toxic effects on the mucosa or olfactory system dysfunction [18, 19].

These challenges have led to increased research into the use of nose-to-brain delivery systems. The nasal pathway provides a means by which drug delivery is able to bypass first pass effect and provide direct drug delivery to the brain via olfactory and trigeminal routes [20].

In the case of brain metastases, such an approach is especially promising, as it will provide an opportunity for multiple non-invasive administrations of an extremely toxic compound, optimizing its local delivery while reducing systemic toxicity [21].

2.2 Paclitaxel

Paclitaxel (PTX) is a diterpenoid anticancer agent originally isolated from the bark of the Pacific yew tree, *Taxus brevifolia*, and is currently produced through semisynthetic and biotechnological approaches. Since its approval for clinical use, paclitaxel has become one of the most widely prescribed chemotherapeutic agents for the treatment of various solid tumors, including breast, ovarian, non-small cell lung, and pancreatic cancers.

The broad-spectrum anti-tumoral effect along with high rates of improving survival in some cancers has made paclitaxel one of the pillars of current cancer chemotherapy.

Despite its clinical success, the therapeutic application of paclitaxel is challenged by its poor aqueous solubility, extensive systemic toxicity, and limited penetration across biological barriers such as the blood–brain barrier, which can restrict its effectiveness in treating central nervous system malignancies and brain metastases. It is a chemotherapeutic drug that is a microtubule stabilizer and belongs to the class of taxanes [22].

The anti-tumor effect of this substance occurs due to binding to the β -subunit of tubulin in microtubules, leading to an inhibition of dynamic instability. Paclitaxel interferes with mitosis by inhibiting microtubule depolymerization, leading to G2/M arrest and apoptosis [23].

It is particularly useful in the case of fast-growing cancers like metastatic breast cancer because mitosis plays an important role in tumor growth [24].

The benefits of paclitaxel are not limited to its ability to inhibit cell proliferation. In some cancers, it may even hinder the movement and invasion abilities of the cells, thus limiting their metastatic nature [25].

This factor is applicable in the case of brain metastasis because apart from proliferation, the cancer cells have to be able to adapt to the new environment. The same quality that makes paclitaxel effective is what makes it harsh on the body system because it can affect other dividing cells in the body [22].

2.2.1 Physicochemical Properties

Paclitaxel is highly lipophilic and has poor aqueous solubility, hence creating a lot of problems during formulation and accounting for the primary reason that makes its administration through aqueous medium difficult. According to the Biopharmaceutics Classification System (BCS), paclitaxel is generally categorized as a Class IV drug, characterized by low aqueous solubility and low permeability, resulting in poor and variable bioavailability.

These unfavorable biopharmaceutical properties necessitate the use of advanced drug delivery systems to enhance its pharmaceutical stability, solubility, and therapeutic performance. Due to its high lipophilicity, paclitaxel exhibits a strong affinity for lipid-based carriers and vesicular systems; however, its crystallization tendency and poor water solubility may limit drug loading efficiency and compromise formulation stability [26].

Paclitaxel's physicochemical properties have a significant influence on its tissue distribution. Its lipophilic nature facilitates interaction with biological membranes

and enhances cellular uptake, whereas its poor aqueous solubility may limit dissolution and absorption. In the context of intranasal administration, these characteristics necessitate a careful balance between adequate drug retention within the carrier system and efficient release at the target site.

Therefore, the carrier must exhibit sufficient affinity to encapsulate and transport paclitaxel effectively while also enabling controlled release upon reaching the nasal mucosa or brain tissue, thereby maximizing therapeutic efficacy [27].

2.2.2 Pharmacokinetics

Pharmacokinetics of paclitaxel depends on its extensive plasma protein binding, metabolism, and low oral absorption. If given via intravenous administration route, paclitaxel may enter systemic circulation successfully but will have a limited ability to enter the brain. There are physiological mechanisms that prevent penetration of drugs into the brain [28]. In other words, even if the levels of plasma concentration are quite high, the level of the free drug reaching brain tissue is still too low.

The pharmacokinetic properties of paclitaxel are similarly influenced by its formulation-dependent nature. Various carrier systems might affect the observed biological half-life, distribution volume, and tissue distribution pattern. This is particularly relevant to the nanomedicines that might affect the biodistribution of the drug in such a way that is favorable to tumor targeting but unpredictable when not appropriately designed. In the case of a brain-targeting formulation, one should expect from its reproducible delivery, storage stability, and in vivo delivery stability [29].

2.2.3 Limitations in Brain Targeting

Although paclitaxel is a potent anticancer agent, it is not optimized for delivery to the brain due to poor passage through the blood-brain barrier, excretion by

efflux transporters from the endothelial cells, and toxic effects of the compound at higher doses. Thus, the increase of the dose cannot be considered an optimal approach since the brain will receive relatively small amounts of the drug [30].

It is for this reason that paclitaxel is considered to be an ideal choice for such delivery systems. A suitable drug carrier, that is capable of protecting the drug during its delivery, increasing its duration of stay inside the nasal cavity, increasing the penetration through the mucous membranes, and also providing a route for nose-to-brain delivery, will make paclitaxel a smart drug targeting the brain. This is the rationale behind this thesis [31, 32].

2.3 Blood–Brain Barrier

Blood-brain barrier is one of the major physical and chemical controllers of access to the central nervous system. Blood-brain barrier is made up of special type of endothelial cells which are connected together by means of tight junctions along with other elements such as pericytes, astrocytes, basement membrane and their related transportation systems. Its physiological function is mainly to protect, and it maintains the cellular balance within neurons, prevents the entry of dangerous material, and also regulates the transport of nutrients, ions, and signaling molecules [33].

However, from the therapeutic point of view, the BBB poses a very difficult challenge. The BBB facilitates the passive transport of hydrophilic molecules or large molecules, but frequently blocks many anticancer drugs which could have been useful otherwise. The barrier is further strengthened by active efflux proteins, including transporters that reduce intracellular drug accumulation. The BBB in brain metastasis suffers from partial and heterogeneous dysfunction around the sites of tumors, but the level of permeability increase is irregular and incomplete, such that diffusion alone is inadequate for therapeutic purposes [34].

The significance of the BBB prompted scientists to investigate the possibility of utilizing carriers, receptors, temporary disruption of the BBB, and novel routes

of administration. All these methods have their own advantages; however, the intranasal route appears to be very promising since it is a route that can, to some extent, be independent of the BBB. The intranasal route uses the fact that there is anatomical continuity between the nasal cavity and the brain [35].

2.4 Intranasal Drug Delivery

Intranasal drug delivery systems have been identified as an effective means of drug targeting in central nervous system disorders. The nasal cavity provides a high surface area with extensive blood supply and pathways directly to the brain tissue. The administered formulations can diffuse across the respiratory mucosa and enter into systemic circulation, or they can follow olfactory and trigeminal pathways leading to the brain tissues. Such a dual option allows nasal delivery systems to be the most suitable way of delivering difficult-to-target drugs to the brain [36].

In case of paclitaxel, intranasal delivery is a very significant approach because it is a mechanism that helps in avoiding first-pass effect and dependency on the crossing of blood-brain barrier through the bloodstream. It is also an approach which is quite easy to use by patients and non-invasive. In conditions like brain metastases, where there is need for extended drug treatment, intranasal delivery becomes highly beneficial due to its non-invasive nature and potential of direct nose to brain drug delivery [37].

2.4.1 Nose-to-Brain Pathway

The nasal to brain route is an aggregate of anatomical and physiological mechanisms, with the olfactory being a direct route to the olfactory bulb and the trigeminal route being a mechanism that facilitates movement of agents across the nasal mucosa to brainstem or deeper brain regions. Perineural and perivascular transport mechanisms may be involved in delivery after crossing the epithelial layer [38].

The ideal design of the nasal formulation should take into account the constraints of this route of administration. The residence time in the nasal region is very short due to the constant removal of any substance by mucociliary clearance. The enzymes present in the mucosa are capable of breaking down any delicate chemical. The nasal mucosa can tolerate only limited levels of pH, tonicity, and viscosity [39].

Thus, nasal delivery systems have to be designed in a manner that ensures prolonged contact, protection, and permeation through the mucosa without damaging it.

2.4.2 Advantages of Intranasal Delivery

Intranasal administration of medications has several practical and physiological benefits. First of all, it prevents the first pass effect through the liver and lowers the risk of toxicity. Moreover, there is a possibility of fast onset of action. In terms of CNS delivery, there is an opportunity of transport to the brain without going through the highly selective BBB. It also enhances user acceptance, which is very essential especially for chronic treatments in oncology and neurological disorders [36, 38].

One more benefit is the ability to have flexible formulation strategies. Nasal dosage forms could be produced as solutions, suspensions, gels, nanoparticles, and combinations of different delivery approaches. This is particularly important in relation to paclitaxel as its incorporation in carrier systems providing solubilization and controlled release is beneficial. Thus, nasal nanogel could function as a solubilization vehicle as well as one increasing residence time [40].

2.4.3 Limitations of Intranasal Delivery

Despite its promise, intranasal delivery is not without limitations. The nasal cavity has limited volume capacity, and dosing must remain compatible with mucosal

tolerance. Clearance by mucus can rapidly remove formulations before full absorption occurs. Interindividual anatomical variation, nasal pathology, and disease-related congestion may further alter deposition patterns. In addition, achieving reproducible transport to the brain is challenging, especially for formulations that are too viscous, too dilute, or insufficiently stable [37].

Another limitation is formulation sensitivity. Drugs and carriers must remain stable during storage and after exposure to nasal conditions. If the system aggregates, crystallizes, or loses integrity upon administration, the intended nose-to-brain effect may be compromised.

This is where nanoscale vesicles and in situ gelling systems become valuable, because they can improve both physicochemical and biopharmaceutical performance [41].

2.5 Novel Drug Delivery Systems for Brain Targeting

Novel drug delivery systems aim to overcome the basic challenge in the treatment of the central nervous system, which is the delivery of a medicinal cargo to the brain. Such systems aim at enhancing solubility, protecting the active moiety, improving uptake, extending residence, and releasing the drug. In the context of brain targeting, the most pertinent delivery systems usually possess one or more of the following characteristics: small size, deformability, surface modification, mucoadhesion, and stimulation responsiveness [42].

2.5.1 Liposomes

The structure of liposomes allows these phospholipids to entrap hydrophilic drugs into the aqueous core and lipophilic drugs in the bilayer. Biocompatibility and structural analogy to biological membranes make them suitable candidates for

nasal and brain delivery. Liposomal technology may increase the apparent solubility of poorly soluble drugs and be tailored to interact with the mucosal membrane or neural tissue [43].

2.5.2 Niosomes

Niosomes are structures similar to liposomes that utilize non-ionic surfactants in place of phospholipids, thus combining some of the benefits of liposomes along with better physical stability. These vesicles have the potential to be cheaper and more chemically stable. They can be used in brain targeting if the drug formulation has to be in the form of vesicles but is not very physically stable due to phospholipid oxidation [44].

2.5.3 Ethosomes

Ethosomes are vesicles enriched with ethanol, which increases membrane fluidity and may enhance permeation. Their ability to deform and traverse biological barriers has made them attractive in transdermal and mucosal delivery research. In the nasal setting, ethosomal behavior may promote mucosal penetration and improve transport of hydrophobic drugs [45].

2.5.4 Solid Lipid Nanoparticles

Solid lipid nanoparticles combine the advantages of lipidic biocompatibility with the physical stability of a solid matrix. They are useful for protecting labile drugs and enabling controlled release. Their rigid internal structure can limit burst release, though this rigidity may also constrain loading or release if the system is not carefully engineered [46].

2.5.5 Polymeric Nanoparticles

Polymeric nanoparticles are known to provide good tuning capabilities. Materials like Poly (Lactic-co-Glycolic Acid) PLGA, chitosan, and similar polymers allow

modification of their size, degradation properties, surface charge, and release profile.

For instance, positively charged or mucoadhesive polymeric nanoparticles may increase their residence time in the nose and enhance interactions with epithelium for nose-to-brain applications. This class of nanoparticles is particularly valuable in oncology due to their ability to deliver cytotoxic drugs [47].

2.5.6 Nanoemulsions

Nanoemulsions are dispersed systems with extremely small droplet sizes, typically designed to enhance solubilization of lipophilic drugs. They may improve nasal absorption by increasing drug dispersion and facilitating contact with the mucosal surface.

However, nanoemulsions may require further stabilization when the therapeutic objective is prolonged residence or precise release [48].

2.5.7 Comparative Advantages and Limitations

Each delivery system has its own advantages. The liposomes have a wide range of applications and are highly compatible with the body; niosomes tend to be physically tough; ethosomes could offer improved permeability; lipid nanoparticles could help with stabilizing drugs that are hydrophobic; polymeric systems could afford great control over their release; and nanoemulsions would aid in solubilization. Their limitations are equally important. Some are too rigid, some too leaky, some are chemically unstable, and some may lack sufficient mucosal retention.

For a drug such as paclitaxel, the ideal system should combine high loading of a lipophilic payload, flexibility for mucosal transport, biocompatibility, and the ability to remain stable during nasal administration. These requirements explain the

evolution toward hybrid systems such as transferosomes and nano-transferosomes [49].

2.6 Transferosomes

Transferosomes are highly deformable vesicular carriers that have been constructed to be able to cross biological membranes effectively. Though they were initially constructed with the aim of improving transdermal delivery, their design makes them equally applicable for delivery to mucosal tissues or brain tissues. Elasticity is the key characteristic of transferosomes. Transferosomes are able to undergo deformation without losing their integrity when stressed [50].

This property is very important in nasal drug delivery because the mucosal barrier, mucus layer, and tight junctions of epithelial cells act as barriers to transportation. Deformable vesicles are able to overcome these barriers better compared to rigid carriers. In addition, transferosomes are capable of carrying lipophilic drugs like paclitaxel through phospholipid bilayer [51].

2.6.1 Composition of Transferosomes

The classical transferosome is made up of phospholipids, an edge activator, and an aqueous phase. In this case, the phospholipid forms the vesicular bilayer while the edge activator is a surfactant that decreases packing stability enough to make it flexible. It results in a vesicle that is well-structured yet highly flexible. [52].

Edge activators are very significant since they play a role in determining the size, elasticity, Entrapment efficiency (EE%) and stability. It should be elastic enough to be able to traverse the barrier, but stable enough not to leak the drug too early. For lipophilic drugs such as paclitaxel, the vesicular membrane can form a good microenvironment, whereas the aqueous phase contains hydrophilic components [53].

2.6.2 Mechanism of Permeation

Transfersome diffusion depends on the presence of a number of factors, which include hydration gradient, mechanical deformation, and membrane interactions. Transfersomes are able to pass through inter-cellular openings that are narrower than their original diameter due to their ability to change shape temporarily in the environment with many barriers present. It is very important in the nasal cavity [54].

The deformability of transfersomes also contributes to sustained performance. Rather than remaining fixed in one region, they can adapt to local structural constraints and maintain their integrity during transport. When coupled to a gel matrix, they may benefit from slower release at the nasal site while still preserving the ability to move toward the brain [55].

2.6.3 Advantages over Traditional Vesicular Systems

Compared with conventional liposomes, transfersomes generally offer greater flexibility and improved barrier traversal. Their deformable nature may enhance mucosal interaction, epithelial penetration, and in some cases, deeper tissue deposition. They are also advantageous for loading lipophilic molecules and for supporting controlled transport through challenging biological environments [56].

Transfersomes do not always offer an advantage, however, as their preparation must be optimized carefully, since excessive amounts of edge activator can destabilize the vesicles, whereas insufficient amounts may not provide enough deformability. It is for this reason, among others, that a Quality-by-Design approach is especially useful when developing transfersomes [57, 58].

2.6.4 Transfersomes in Brain Targeting

In regard to brain targeting, transfersomes have a promising outlook owing to their ability to improve transport across mucosa and help deliver drugs toward

nervous tissue following intranasal application. Deformability of these carriers might contribute to penetrating the mucosal barrier and entering through the olfactory region. Thus, improvement of transport will result in increased drug concentration for nose-to-brain delivery [49, 59].

In a brain metastasis setting, transferosomes may also reduce the need for high systemic doses. This is particularly important for paclitaxel, whose clinical utility is limited by peripheral toxicity and poor CNS penetration. Transferosomal systems can therefore serve as an enabling technology, converting a poorly brain-permeable anticancer drug into a more plausible nasal therapeutic [60].

2.6.5 Transferosomes for Anticancer Drug Delivery

Transferosomes have substantial potential in oncology because many anticancer drugs are hydrophobic, unstable, or poorly permeable. Their deformability and membrane affinity may support drug transport across difficult barriers and into tumor-associated tissues. For brain metastasis, this can be especially meaningful, as therapeutic success depends on reaching a protected site without causing undue systemic toxicity [61].

Paclitaxel is an ideal choice for this methodology. Paclitaxel is effective, hydrophobic, commonly used in breast cancer therapy, but lacks brain availability. Transferosomal carrier will help achieve increased solubilization and mucoadhesion properties and also controlled drug delivery. Conversion to nano-transferosomal in situ gel will further enhance residence time and convenience [62].

2.7 Nano-Transferosomes

Nano-transferosomes are a highly advanced type of transferosome system, where vesicle size is downscaled to the nanometer range to achieve improved transport properties and drug loading and biological performance. The benefits from being

downscaled include greater surface area, higher interactions with the mucosa and increased predictability of nasal absorption.

Small vesicles might be able to penetrate biological environments easier than larger vesicular systems. Nano-transferosomes are referred to as an ultra-deformable nanosized vesicle, which follows the basic principles of transferosomes but with improved colloid properties. In case of intranasal administration, the benefit of having vesicles downscaled to the nanoscale becomes even more important because of the improved deposition and transport through the nasal architecture [63].

The combination of nanosize and deformability is highly strategic. A vesicle that is both small and flexible is more likely to move through mucosal barriers while preserving the integrity of a lipophilic cargo. In the case of paclitaxel, nano-transferosomes may improve payload retention and controlled release, while simultaneously lowering the risk of local irritation compared with less refined delivery systems [64].

2.8 In Situ Gels

In situ gels are aqueous preparations that change from a sol to a gel state post-administration under certain biological conditions, like changes in temperature, pH, ion concentration, and enzymatic action [65].

The application of in situ gels is quite useful in nasal administration since these formulations can be delivered as liquids, easily spread over the mucosal surface, and then converted to a gel phase that prevents their immediate removal [66].

The rationale for combining in situ gelation with nanoparticulate carriers is straightforward. Nanovesicles provide transport and release control, while the gel matrix improves residence time, reduces drainage, and enhances local availability. This synergy is especially relevant for paclitaxel, where both retention and controlled exposure are needed [67].

2.8.1 Thermoresponsive In Situ Gels

Thermoresponsive in situ gels are designed to remain fluid at room temperature or during handling and to gel at nasal or body temperature. This behavior is extremely useful because it supports convenient administration and immediate in vivo structuring. A thermoresponsive system can be formulated so that it is easy to instill but becomes more viscous after contact with the nasal environment [68]. This property is particularly important for intranasal brain delivery. The nasal cavity is a dynamic environment in which materials can be cleared rapidly. When a thermoresponsive gel sets in situ, it can reduce the speed of mucociliary clearance and increase the duration over which the drug remains accessible for absorption or neuronal transport [69].

2.8.2 Advantages of In Situ Gels in Nasal Delivery

In situ gels offer several advantages in nasal drug delivery. They prolong residence time, improve patient compliance, reduce the frequency of administration, and may increase the overall fraction of dose available for absorption. They also protect the drug from immediate washout and can reduce the irritancy associated with repeated application of free drug solutions [70].

For brain targeting, these advantages are especially meaningful. A formulation that remains longer at the nasal site has more opportunity to interact with the olfactory and trigeminal pathways. In effect, the gel acts as a depot that stores the vesicles close to the absorbing surface while preserving the liquid convenience of the dosage form [71].

2.8.3 Combination of Transferosomes with In Situ Gels

The integration of transferosomes with in situ gel systems creates a multilevel delivery strategy. The transferosome contributes deformability, membrane interaction, and drug transport capability, while the gel improves residence time and

modulates release. Together they may produce a formulation that is both biologically efficient and practically usable [72].

This hybrid design is particularly suited to paclitaxel. Because paclitaxel is poorly soluble and benefits from controlled exposure, a transferosomal gel can supply a protected vesicular environment and a nasal depot in one formulation.

It is also suitable for Quality-by-Design development because multiple variables can be systematically optimized to yield the desired product profile [73].

2.9 Poloxamer 407 as a Thermoresponsive In Situ Gelling Polymer

Poloxamer 407 is a non-ionic triblock copolymer widely used as a thermo-gelling agent in pharmaceutical formulations. It has a poly (ethylene oxide) – poly (propylene oxide) poly (ethylene oxide) (PEO–PPO–PEO) structure that allows micelle formation in aqueous media and temperature-dependent sol–gel transition, making it useful in nasal in situ gels and other drug delivery systems.

Its thermogelling behavior is due to dehydration of the PPO block and micellar aggregation at higher temperatures. At low temperatures, it remains a low-viscosity liquid, while near physiological temperature it forms a gel network with increased viscosity and strength, helping the formulation stay at the site of administration [74].

In intranasal delivery, this property allows easy administration as a liquid followed by gelation in the nasal cavity, which prolongs residence time, reduces mucociliary clearance, and may improve drug absorption and nose-to-brain transport.

In the present study, Poloxamer 407 is important because it provides the thermal responsiveness needed for intranasal retention of paclitaxel-loaded nanotransfersomes. It also supports formulation stability and sustained drug release through the gel network formed after administration. [75].

Poloxamer 407 is also valued for its biocompatibility and compatibility with other excipients. It can be combined with mucoadhesive polymers such as chitosan, carbopol, or HPMC to further improve nasal residence time and drug delivery.

However, its concentration must be optimized carefully. Low concentrations may lead to poor gelation, while high concentrations may make the formulation too viscous for comfortable administration. Therefore, a QbD-based approach is useful for identifying the optimal formulation design space [76]. A QbD-based approach is therefore essential to identify the optimal design space.

2.10 Enhanced Nasal Permeation

Enhanced nasal permeation refers to the deliberate improvement of drug transport across the nasal mucosa without causing unacceptable irritation or toxicity. This can be achieved through size reduction, surface modification, mucoadhesion, permeation enhancers, surfactant selection, and formulation pH adjustment. In a complex system such as paclitaxel-loaded nano-transferosomes thermoresponsive in situ gel, enhanced permeation is not the result of a single mechanism but of multiple reinforcing mechanisms acting together [21, 67].

First, nanosizing enhances the surface area for interaction with the mucosa. Secondly, the use of transferosomes creates deformability which allows the delivery system to conform to the biological barrier.

Thirdly, the use of gel enhances the contact period thus providing sufficient time for absorption. Fourthly, appropriate excipients might enhance permeation or prevent the formulation from degrading prematurely.

Lastly, the delivery route itself circumvents first pass effect and minimizes the distance to the target tissue. An appropriate enhanced permeation technique should also take into consideration safety. Over enhancement of permeation could disrupt the mucosal membrane integrity, lead to irritation, or change the mucociliary clearance.

Hence, an optimal formulation would increase permeability in a controlled manner, preserving mucosal health while improving therapeutic exposure [37].

2.11 Current Advances and Limitations in Paclitaxel Brain Delivery

TABLE 2.1: Previous Studies on Paclitaxel-Loaded Novel Drug Delivery Systems for Enhanced Delivery and Targeting.

Literature Evidence	Formulation Strategy	Principal Findings	Research gap	Novelty of the Present Research	Ref
Transferrin-Conjugated NLCs	Brain cancer targeting	Achieved high entrapment efficiency and sustained release with enhanced brain-targeting potential.	Focused on receptor-mediated targeting; did not address intranasal delivery, nasal retention, or direct nose-to-brain transport.	Development of an intranasal transferosomal system with prolonged nasal residence and enhanced transmucosal permeation.	[77]
Folic Acid/PEG Functionalized NLCs	Cancer targeting	Improved cellular uptake and cytotoxicity against cancer cells.	Emphasis was placed on cellular targeting rather than overcoming physiological barriers associated with brain delivery.	Investigates a non-invasive nose-to-brain approach for improved brain localization of paclitaxel.	[78]
Magnetic PLGA Nanoparticles	Glioblastoma	Enhanced accumulation of paclitaxel	Requires external magnetic targeting and	Provides a simpler and potentially more	[79]

Table 2.1 continued from previous page

Literature Evidence	Formulation Strategy	Principal Findings	Research gap	Novelty of the Present Research	Ref
Loaded with Paclitaxel	Glioblastoma	within brain tumors through magnetic guidance.	does not offer a practical non-invasive delivery route.	patient-friendly intranasal delivery platform.	
Dual-Targeting Peptide Functionalized Nanoparticles	Brain glioma	Improved BBB transport and controlled drug release.	Relied on BBB-mediated transport and did not exploit direct neural pathways from the nasal cavity to the brain.	Utilizes the nose-to-brain route to bypass the BBB and improve drug transport.	[80]
Dual-Modified Cationic Liposomes (Paclitaxel + Survivin siRNA)	Brain glioma stem cells	Enhanced therapeutic efficacy through co-delivery of paclitaxel and siRNA.	Complex multi-functional system with limited focus on nasal delivery challenges such as rapid clearance and short residence time.	Combines permeation enhancement with prolonged nasal retention through thermoresponsive gelation.	[81]
Intranasal Paclitaxel-Loaded PLGA Nanoparticles	Glioblastoma	Demonstrated improved brain uptake and anti-glioblastoma activity following intranasal administration.	Did not incorporate a strategy to significantly prolong residence time within the nasal cavity.	Integrates transferosomes into a thermoresponsive in situ gel to reduce mucociliary clearance.	[82]
RGD-Modified Paclitaxel	Glioblastoma	Achieved targeted delivery and	Focused primarily on ligand-mediated targeting;	Provides both enhanced permeation and	[83]

Table 2.1 continued from previous page

Literature Evidence	Formulation Strategy	Principal Findings	Research gap	Novelty of the Present Research	Ref
Nanoparticles (Intranasal)	Glioblastoma	substantial reduction in tumor burden.	sustained retention and controlled release within the nasal cavity were not addressed.	sustained release through a transferosomal in situ gel system.	
Intranasal Paclitaxel Solution	Neurodegenerative disease model	Demonstrated successful transport of paclitaxel from the nasal cavity to the brain.	Conventional solution formulations are susceptible to rapid nasal clearance and poor retention.	Employs transferosomes and thermoresponsive in situ gel technology to improve drug retention and absorption.	[84]
Paclitaxel in Date Palm Lipid Droplets	Brain tumor targeting	Increased brain accumulation and reduced systemic exposure after intranasal administration.	Lacked a controlled-release mechanism and did not address formulation residence time.	Designed to provide sustained release and prolonged contact with the nasal mucosa.	[85]
Ascorbic Acid-Conjugated Paclitaxel Polymeric Nanoparticles	Brain targeting	Significantly increased brain concentration of paclitaxel after intranasal administration.	Although brain delivery was enhanced, the formulation did not combine permeation enhancement with thermoresponsive nasal retention technology.	Integrates transferosomal permeation enhancement with thermoresponsive gel-based retention in a single platform.	[86]

2.12 Research Gap

Previous research has indicated the positive influence of incorporating paclitaxel in several innovative drug delivery systems such as liposomes, nanostructured lipid carriers (NLCs), solid lipid nanoparticles (SLNs), polymeric nanoparticles, nanoemulsion, micelles, and targeted nanocarriers on the solubility, stability, bioavailability, and anti-cancer effects of paclitaxel. In addition, many studies have examined the brain targeting of paclitaxel through the use of ligand functionalized nanoparticles, transferrin conjugates, and polymeric formulations via nasal route for the treatment of glioblastoma and other CNS diseases [87]. Recent advancements in the field indicate the positive influence of administering paclitaxel-polymeric nanoparticles and lipid-based carriers through intranasal route on brain drug delivery. However, there are still considerable drawbacks in spite of these developments. The vast majority of formulations described are based on inflexible nanoparticulate carriers which can demonstrate inadequate permeability in nasal mucosa and may fail to fully utilize the potential of nose-to-brain transport mechanism [88]. Moreover, rapid clearance in the nasal cavity may limit the time of retention for the drug formulation leading to decreased drug bioavailability. Also, most of the scientific literature is related to glioblastomas, and only little data about breast cancer brain metastasis is provided although it is one of the serious challenges in modern medicine due to the inability to penetrate into the brain [89].

However, after conducting an exhaustive search of the literature, it was found that there is no research work available on paclitaxel loaded transferosomes incorporated in a thermoresponsive in situ nasal gel for improved drug delivery to the brain. Transferosomes are flexible vesicles that can pass through biological membranes more efficiently than conventional liposomes and rigid vesicles. In addition, the use of transferosomes in Poloxamer 407 based in situ gel can prolong the residence time of the medication in the nasal cavity, decrease mucociliary clearance, deliver a controlled dose of the drug, and facilitate drug absorption through olfactory and trigeminal routes. It therefore becomes clear that the current formulation

of an intranasal transferosomal in situ gel of paclitaxel is a novel formulation technology, combining the benefits of ultra deformable vesicular carriers along with a thermosensitive mucoadhesive delivery system. The current formulation strategy is therefore anticipated to solve all the limitations associated with existing formulations of paclitaxel, along with increasing brain bioavailability, providing sustained drug delivery, and becoming a much better choice for treating patients suffering from breast cancer brain metastasis. It is evident, therefore, that there is no scientific literature available in the field of paclitaxel loaded transferosomal in situ gels for nose to brain delivery.

Chapter 3

Materials and Methods

3.1 Materials

Paclitaxel (purity $\geq 98\%$) was obtained from Bio-Labs Private Limited (Islamabad, Pakistan). Soy lecithin (phosphatidylcholine $\geq 94\%$) was obtained from Pharma Excipients (Islamabad, Pakistan). Poloxamer 407 (Pluronic F-127) and Poloxamer 188 (Pluronic F-68), methanol (HPLC-grade), potassium chloride, sodium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate, and dipotassium hydrogen phosphate were procured from Sigma-Aldrich in St. Louis, Missouri, USA. Employing a Merck KGaA, Darmstadt, Germany, Milli-Q purification system, ultrapure water was produced fresh and utilized throughout. Unless otherwise stated, all materials were utilized as received, without any further purification.

3.2 Methodology

3.2.1 Preparation of Paclitaxel - Loaded Nano - Transferosomes

PTX loaded nano-transferosomes were prepared using the method of thin-film hydration technique that is a highly recognized technique in the preparation of

deformable lipid-based vesicles, having good EE% for hydrophobic drug delivery. In brief, an accurately weighed amount of soy lecithin (as bilayer forming phospholipid), Poloxamer 188 (as edge activator), and PTX were co-dissolved in methanol (HPLC grade) in 250 mL round bottom flask. The obtained clear organic phase was probe sonicated (30-60 seconds, 40% amplitude).

Solvent removal was achieved by rotary evaporation (RE-201D, 220 V/50 Hz) under reduced pressure until a thin, homogeneous lipid film was formed on the inner flask walls at 40–45°C and 60–100 rpm [90, 91].

Residual solvent traces were eliminated by maintaining the flask under high vacuum for 45 min with nitrogen gas purging (Figure 3.1).

The dried lipid film was subsequently hydrated with the rotating flask that was dropwise filled with preheated phosphate buffer saline (PBS, pH 7.4, 45°C) at 60 rpm for 5-8 min, generating a milky transferosomal dispersion comprising predominantly multilamellar vesicles.

The dispersion obtained was sonicated using a probe-type sonicator (20 kHz, 30% amplitude, 5 minutes, pulse-mode) to minimize the vesicle size and lamellar nature, producing transferosomes having unilamellar or oligolamellar vesicles [92].

All the preparative processes were conducted under the nitrogen environment so that oxidative deterioration of the phospholipids could be avoided.

3.2.2 Preparation of Thermoresponsive In Situ Gel

The cold dispersion method was used to produce thermoresponsive in situ gel. Poloxamer 407 (20% w/v) was gradually added to ice cold PBS (pH 5.5, 4°C) under constant magnetic stirring at 200 rpm in the precooled vessel (Figure 3.1), maintaining the system below 5°C in order to prevent immediate gelling of the formulation. The polymer was allowed to hydrate overnight under refrigeration (4°C) to yield a clear, homogeneous sol.

The polymer was hydrated for an entire night at refrigerated temperatures (4 °C) to obtain a homogenous sol. After complete dissolution of the polymer, the PTX-loaded nano-transferosomes dispersion, which was prepared separately and stored at refrigerated temperatures, was slowly added to the chilled sol of the polymer with continuous stirring (100 rpm; 15 minutes; 4 °C) to ensure even distribution of vesicles without causing premature gel formation [93].

The pH formulation was determined and confirmed to be 5.5 ± 0.1 , which is equal to the pH level of the nasal mucosa. The PTX nano-transferosomal gel (PTX-NTFG) was kept in sealed and light-proof containers at 4°C prior to testing [94]. Preparation was carried out under sterile conditions in a laminar air flow cabinet.

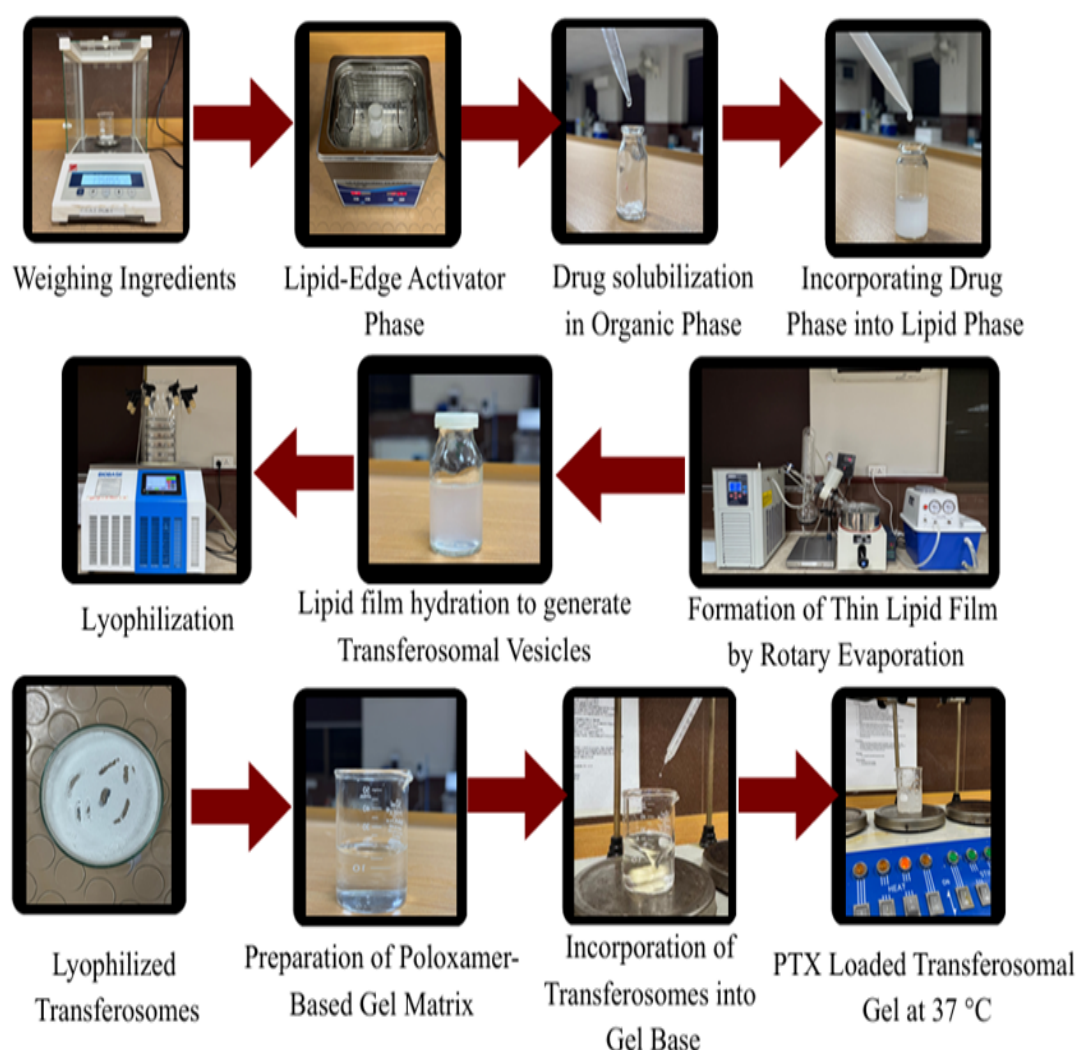


FIGURE 3.1: Schematic Representation of the Preparation Process of PTX-Loaded Nano-Transferosomal in Situ Gel

3.3 Box-Behnken Experimental Design and Statistical Optimization

For the study of effect of formulation variables on critical quality attributes of the nano-transferosomal system, a three-factor and three-level Box-Behnken design (BBD) was utilized in conjunction with Design-Expert[®] software v13.0 (State-Ease Inc., Minneapolis, USA). This design was preferred over the central composite design because of its efficiency in the presence of lack of axial points, thereby avoiding any unfeasible combinations of factors [95]. Independent variables along with their levels were determined using initial screening experiments as follows: soy lecithin (A: 30 to 40 mg); Poloxamer 188 (B: 10 to 20 mg); and hydration medium volume (C: 4 to 12 mL). Response/Dependent variables included particle size (Y_1 , nm); polydispersity index (Y_2); zeta potential (Y_3 , mV); and entrapment efficiency (Y_4 , %). For the purpose of evaluating pure experimental error and model curvature, the BBD generated 17 experimental runs, involving five center-point replicates [95, 96]. All runs were performed in randomized order to minimize systematic bias.

Quadratic polynomial models were fitted to each response using multiple linear regression. Model adequacy was assessed by the analysis of variance (ANOVA), adjusted R^2 , predicted R^2 , lack-of-fit test, and coefficient of determination (R^2). The significance value was defined at $p < 0.05$. Numerical optimization was executed by the desirability function approach, simultaneously minimizing Y_1 and Y_2 , and maximizing Y_3 and Y_4 [97]. The predicted optimal formulation was experimentally verified, and observed values were compared with model predictions to confirm robustness.

TABLE 3.1: Independent variables and dependent responses used in the Box-Behnken Design for optimization of PTX-loaded nano-transferosomes.

Factor / Response	Variable	Low (-1)	Medium (0)	High (+1) / Goal
A	Soy lecithin (mg)	30	35	40
B	Poloxamer 188 (mg)	10	15	20
C	Hydration volume (mL)	4	8	12

Table 3.1 continued from previous page

Factor / Response	Variable	Low (-1)	Medium (0)	High (+1) / Goal
Y ₁	Particle size (nm)	-	-	Minimize
Y ₂	Polydispersity index (PDI)	-	-	Minimize
Y ₃	Zeta potential (mV) (ZP)	-	-	Maximize
Y ₄	Entrapment efficiency (%) (EE%)	-	-	Maximize

Plots of three-dimensional response surfaces were produced using Design-Expert[®] software v13.0 and are presented as a composite (Figure 4.1A-L) to illustrate the influence of each variable pair on the respective response at the mid-level of the third variable.

3.4 Physicochemical Characterization

3.4.1 Particle Size, Polydispersity Index, and Zeta Potential

A Malvern Zetasizer Nano ZS (Malvern Panalytical, Worcestershire, UK) fitted with a 633 nm He-Ne laser at a back-scattering angle of 173° was used to measure the hydrodynamic particle size, polydispersity index (PDI), and zeta potential of the nano-transfersomes using dynamic light scattering (DLS) and electrophoretic light scattering (ELS), respectively [98, 99].

The measurements were carried out at 25°C following appropriate dilution with ultrapure water (1:100 v/v). Each sample was equilibrated for 120s prior to data acquisition [100].

The zeta potential of the nanotransfersomal formulations was measured using a Zetasizer equipped with folded capillary cells. The analysis was performed based on the electrophoretic mobility of the vesicles to evaluate their surface charge and colloidal stability.

3.4.2 Entrapment Efficiency

An indirect ultracentrifugation approach was used to calculate entrapment efficiency (EE%). Nano-transferosomes dispersions were centrifuged at 20,000 $\times g$ for 60 min at 4°C (Beckman Coulter Optima MAX-XP, Brea, CA, USA) to sediment the vesicles and collect the supernatant containing free (untrapped) PTX. UV-visible spectrophotometry was employed to determine the amount of free PTX in the supernatant at $\lambda_{\text{max}} = 227$ nm after appropriate dilution with methanol to disrupt any residual vesicular structures [101, 102]. EE% was calculated as shown in equation 3.1:

$$EE(\%) = [(W_{\text{total}} - W_{\text{free}})/W_{\text{total}}] \times 100 \quad (3.1)$$

where W_{total} is the total PTX added during formulation, W_{free} is the amount of untrapped PTX recovered in the supernatant.

3.4.3 Transmission Electron Microscopy

Internal vesicular architecture and lamellarity were assessed by transmission electron microscopy (TEM, HT7800, Hitachi High-Tech Corp., Japan). A single drop of diluted transferosomal dispersion was applied to a carbon-coated copper grid (300 mesh), blotted, negatively stained for 60 seconds with a 1% (w/v) phosphotungstic acid solution (pH 7.0), allowed to air dry for 15 minutes, and then inspected at 80–120 kV [103].

3.4.4 Fourier-Transform Infrared Spectroscopy

The Fourier Transform Infrared (FTIR) spectra of pure paclitaxel (PTX), soy lecithin, Poloxamer 188, and the optimized PTX-loaded nano-transferosomal formulation (F15) were recorded using a PerkinElmer Spectrum Two FTIR spectrometer fitted with an ATR accessory (Waltham, MA, USA; PerkinElmer Inc.). Spectra were collected over the wavenumber range of 4000–400 cm^{-1} at a resolution of 4 cm^{-1} with 32 accumulated scans per sample [104]. The obtained spectra were

baseline-corrected and examined for the presence, shifting, broadening, or disappearance of characteristic absorption bands to evaluate drug–excipient compatibility and identify potential molecular interactions within the nano-transferosomal formulation.

3.4.5 Powder X-Ray Diffraction

The crystallinity of PTX, excipients, and PTX-NTFG after lyophilization was evaluated through PXRD analysis performed on a Cu K α source-equipped X'Pert PRO diffractometer (PANalytical B.V., Almelo, The Netherlands) (45 kV, 30 mA). The measurements were conducted from 2θ values between 4° and 80° at $2^\circ/\text{min}$ with 0.02° intervals. The samples were carefully powdered and refilled into zero-background Si sample holders [105]. The absence of crystalline PTX diffraction peaks in the formulation was interpreted as indicative of amorphization or molecular-level drug dispersion within the lipid matrix.

3.4.6 Differential Scanning Calorimetry

Thermal studies were carried out with a Shimadzu DSC-60 Plus differential scanning calorimeter (Shimadzu Corporation, Kyoto, Japan) calibrated with indium and zinc standards. Accurately weighed samples (~ 5 mg) of pure PTX, Poloxamer 188, and lyophilized PTX-loaded nano-transferosomes (prepared with a Vertical Freeze Dryer BK-FD18) were enclosed in aluminum pans (crimped, non-hermetic) and heated in a dynamic nitrogen environment (50 mL/min flow rate) from 25°C to 300°C at a steady rate of $10^\circ\text{C}/\text{min}$ [106].

3.4.7 In Vitro Drug Release and Release Kinetic Analysis

In vitro release of PTX from PTX dispersion and PTX-loaded transferosomes was assessed using the membrane dialysis diffusion technique. Accurately measured

volumes for every formulation (which is equal to 1 mg PTX) were loaded into pre-soaked cellulose dialysis bags (MWCO 12-14 kDa; Sigma-Aldrich) and engrossed in 500 mL of Phosphate Buffer Saline (PBS) at pH 5.5 and pH 7.4 separately, maintained via continuous magnetic stirring at $37 \pm 0.5^\circ\text{C}$ (100 rpm) in jacketed vessels. The sink's conditions were maintained throughout by assuring the maximum PTX concentration within the receptor medium was less than 20% of its saturation concentration. Aliquots (5 mL) were withdrawn at specified times (0.25, 0.5, 1, 2, 4, 6, 8, 12, 18, and 24 h) and immediately exchanged with an equivalent volume of fresh pre-warmed medium. PTX concentration in each sample was quantified by UV-visible spectrophotometry at $\lambda_{\text{max}} = 227 \text{ nm}$ (Shimadzu UV-1800, Kyoto, Japan), following appropriate dilution with methanol [107]. Every experiment was conducted in triplicate ($n = 3$), and the results are shown as mean $\pm$ SD.

Release data were fitted to Higuchi, Korsmeyer-Peppas, zero-order, and first-order models mathematical models using nonlinear regression analysis in OriginPro 2021 (OriginLab, Northampton, MA, USA). The Korsmeyer-Peppas model ($M_t/M_\infty = k t^n$) was applied to elucidate the release mechanism, where the release exponent n discriminates Fickian diffusion ($n \leq 0.45$), anomalous (non-Fickian) transport ($0.45 < n < 0.89$), and Case II (polymer relaxation-controlled) release ($n \geq 0.89$) for slab geometries. The model yielding the highest coefficient of determination (R^2) was considered to best describe the release mechanism [108].

3.4.8 Rheological Characterization of PTX-NTFG

Rheological properties of PTX-NTFG were assessed using a calibrated Brookfield DV3T viscometer (AMETEK Brookfield, Middleborough, MA, USA) equipped with spindle No. 3. Viscosity was measured over a shear rate range of 10-100 rpm at $25 \pm 1^\circ\text{C}$ and $37 \pm 0.5^\circ\text{C}$. The gelation temperature (T_{gel}) was determined by gradually increasing the temperature (rate of $2^\circ\text{C}/\text{min}$ from 20 - 40°C) while measuring the change in viscosity with respect to temperature; the gelation temperature was the temperature where a minimum increase in viscosity of 10-fold

occurred over 2°C. A calibrated pH meter (Mettler Toledo FiveEasy, Columbus, OH, USA) was used to determine the pH of formulation. Thixotropic behavior was evaluated by constructing ascending and descending shear rate ramps, and the thixotropic loop area was calculated as an index of structural recovery [109, 110]. Triplicate measurements were made ($n = 3$).

3.4.9 Ex Vivo Nasal Permeation Study

Ex vivo transmucosal permeation of PTX was assessed using Franz diffusion cells (effective diffusion area: 1.77 cm²; receptor volume: 12 mL). Fresh sheep nasal mucosa was obtained from a local certified abattoir within 2 h of slaughter, transported on ice in cold Krebs-Ringer buffer (pH 7.4), cleaned of connective tissue, and stored at -20°C in cryoprotective medium until use. Prior to each experiment, tissue integrity was assessed by visual inspection for macroscopic damage, and by monitoring transepithelial electrical resistance (TEER; Millicell ERS-2, Merck Millipore) as a relative barrier indicator; the mean TEER of intact mucosa prior to freezing was $65 \pm 12 \Omega \cdot \text{cm}^2$; after thawing and mounting, tissues with TEER $< 45 \Omega \cdot \text{cm}^2$ (i.e., $>30\%$ below mean) were excluded, consistent with the approach used in previous ex vivo nasal permeation studies [94]. This conservative screening ensured functional epithelial continuity without imposing an absolute threshold that is not established for frozen-thawed excised mucosa. The mucosal membrane was placed in between the donor and receptor compartments, with the epithelial side oriented towards the donor compartment. PTX-Gel and PTX-NTFG (each standardized to 1 mg/mL PTX) were applied to the donor compartment, with the receptor chamber filled with PBS pH 5.5 at $37 \pm 0.5^\circ\text{C}$ under continuous stirring (300 rpm). Samples (0.5 mL) were withdrawn from the receptor at predetermined intervals (0.5, 1, 2, 4, 6, 8, 12, 18, 24 h) and replaced with equal volumes of fresh pre-warmed buffer [111]. PTX concentrations were determined by UV spectrophotometry at 227 nm. Permeation parameters (cumulative permeation, steady-state flux J_{ss} and enhancement ratio ER) were determined from the linear segment of the cumulative permeation versus time curves. All experiments were conducted

using mucosa from four independent animals ($n = 4$), with data expressed as mean $\pm$ SD.

The formula used for steady state flux J_{ss} is given as 3.2:

$$J_{ss} \left(\frac{\mu g}{cm^2} \right) = \frac{(Q_{18} - Q_0)}{18} \quad (3.2)$$

The formula used for enhancement ratio ER is given as 3.3:

$$\text{Enhancement ratio ER} = \frac{J_{ss}(NTFG)}{J_{ss}(Gel)} \quad (3.3)$$

3.4.10 Physicochemical Stability Studies

Accelerated and long-term stability studies were performed following the ICH Q1A(R2) guidelines. PTX-NTFG was stored in sealed, light-resistant amber glass vials under three storage conditions: refrigerated ($4 \pm 1^\circ\text{C}$ / $60 \pm 5\%$ RH), ambient ($25 \pm 2^\circ\text{C}$ / $60 \pm 5\%$ RH), and accelerated ($40 \pm 2^\circ\text{C}$ / $75 \pm 5\%$ RH). Samples were analyzed at 0, 1, 2, and 3 months for pH, drug content, and visual appearance (color, homogeneity, phase separation) [112, 113].

3.4.11 Statistical Analysis

Most of the experiments were carried out in triplicate ($n = 3$). Mean $\pm$ standard deviation (SD) is used to express data. One-way analysis of variance (ANOVA) was used to statistically compare various formulations, and Tukey's post-hoc test was used for pairwise comparisons (GraphPad Prism v9.4, San Diego, CA, USA). The ex vivo permeation data of PTX-NTFG versus PTX-Gel have been compared using an independent-samples Student's t-test. Two-way ANOVA was applied to assess the effects of formulation type and pH on in vitro release profiles and their interaction. A p-value < 0.05 was regarded as statistically significant.

Chapter 4

Results and Discussion

4.1 Box-Behnken Design Optimization and Statistical Model Evaluation

A three-factor BBD comprising 17 experimental runs was employed to systematically evaluate the influence of soy lecithin concentration (A), Poloxamer 188 concentration (B), and hydration medium volume (C) on the critical quality attributes of PTX-loaded nano-transferosomes. Particle sizes of the 17 formulations ranged from 142.09 to 177.40~nm, PDI from 0.152 to 0.219, EE from 85.7 to 89.0%, and zeta potential from -30.9 to -36.4 ~mV (Table 4.1). The narrow experimental variability at center-point replicates (F1, F5, F7, F8, F17) confirmed high reproducibility and low random error across the design space. All four responses were adequately described by quadratic polynomial models with high predictive accuracy (Table 4.2). ANOVA confirmed that all models showed statistically significant differences ($p < 0.0001$ for Y_1, Y_2, Y_4 ; $p = 0.0002$ for Y_3), with a non-significant lack-of-fit ($p > 0.05$ for all responses), confirming absence of model inadequacy and suitability for navigating the design space. Coefficients of determination were: Y_1 ($R^2 = 0.9869$, Adj $R^2 = 0.9701$), Y_2 ($R^2 = 0.9907$, Adj $R^2 = 0.9788$), Y_3 ($R^2 = 0.9695$, Adj $R^2 = 0.9302$), and Y_4 ($R^2 = 0.9835$, Adj $R^2 = 0.9623$).

TABLE 4.1: Box–Behnken Design parameters with observed and predicted responses for PTX- Loaded Nano-Transferosomes.

Independent Variables				Dependant Variables									
Run	Soy Lecithin (mg) (A)	Poloxamer 188 (mg) (B)	Hydration Medium (mL) (C)	Observed Responses					Predicted Responses				
				Particle Size (nm)	Poly sity (PDI)	Disper- Index	Zeta Poten- tial (ZP) mV	Entrapment Efficiency (EE%) %	Particle Size nm	Poly sity (PDI)	Disper- Index	Zeta Poten- tial (ZP) mV	Entrapment Efficiency (EE%) %
				Y1	Y2		Y3	Y4	Y1	Y2		Y3	Y4
F1	35	15	8	156.8	0.181		-35.7	86.7	157.58	0.1806		-35.52	86.82
F2	35	10	12	172.1	0.208		-34	87.1	172.14	0.2105		-34	87.11
F3	35	10	4	150.2	0.167		-33.4	87.3	150.19	0.167		-33.35	87.39
F4	40	15	4	152.4	0.172		-32.4	89	152.36	0.1732		-32.3	88.9
F5	35	15	8	156.2	0.181		-35.4	86.6	157.58	0.1806		-35.52	86.82
F6	30	10	8	157.45	0.1738		-33.1	85.7	157.36	0.1725		-33	85.59
F7	35	15	8	156.7	0.181		-36.4	87.1	157.58	0.1806		-35.52	86.82
F8	35	15	8	161.4	0.177		-35.4	86.9	157.58	0.1806		-35.52	86.82
F9	30	15	4	142.09	0.152		-31	85.8	142.19	0.1533		-31.15	85.82
F10	35	20	12	177.4	0.219		-33.4	86.7	177.41	0.219		-33.45	86.61
F11	40	20	8	171.62	0.2034		-30.9	87.7	171.7	0.2047		-31	87.81
F12	30	15	12	165.2	0.1942		-34.3	85.8	165.24	0.193		-34.4	85.9
F13	30	20	8	161.4	0.1863		-35.8	86	161.35	0.1875		-35.65	85.99
F14	40	15	12	174.51	0.2127		-31.9	87.1	174.41	0.2114		-31.75	87.07
F15	40	10	8	166.3	0.195		-35.9	88	166.35	0.1938		-36.15	88.01
F16	35	20	4	154.3	0.187		-31.4	88.1	154.26	0.1845		-31.4	88.09
F17	35	15	8	156.8	0.183		-34.7	86.8	157.58	0.1806		-35.52	86.82

The close agreement between predicted R^2 and adjusted R^2 (difference < 0.2) confirmed absence of overfitting and adequate signal-to-noise ratios. These findings demonstrate the reliability of the BBD-response surface methodology (RSM) framework for optimization of nano-transferosomal systems, consistent with published literature [96, 114].

TABLE 4.2: Statistical parameters for Box-Behnken Design quadratic models.

Parameter	Particle Size (nm)	PDI	Zeta Potential (mV)	EE (%)
R^2	0.9869	0.9907	0.9695	0.9835
Adjusted R^2	0.9701	0.9788	0.9302	0.9623
Predicted R^2	0.9791	0.9096	0.9161	0.9035
Model p-value	<0.0001	<0.0001	0.0002	<0.0001
Lack-of-fit p-value	0.9997	0.2974	0.9440	0.6676
SD	1.63	0.0025	0.485	0.173
%CV	1.01	1.35	1.43	0.199

R^2 = Coefficient of determination; Adjusted R^2 = adjusted coefficient of determination; Predicted R^2 = predicted coefficient of determination; p-value < 0.05 indicates a significant model; Lack of fit p-value > 0.05 indicates a non-significant lack of fit.

4.1.1 Formulation Variables on Critical Quality Attributes

4.1.1.1 Particle Size

Particle size was significantly affected by all three independent variables ($p < 0.05$). Increasing soy lecithin concentration (A) initially reduced particle size by improving lipid packing density and promoting smaller unilamellar vesicle formation (Figure 4.1A-C); however, at supraoptimal concentrations, size increased, attributable to elevated lipid phase viscosity and bilayer thickening.

Poloxamer 188 (B) had a significant size reduction influence because of the reduction of interfacial surface tension and steric stability at the water-vesicle interface.

Hydration volume (C) had a high positive correlation (+11.28) coefficient, meaning that higher hydration volumes enabled vesicle swelling and aggregative growth by diluting the stabilizing surfactant layer.

The quadratic equation for Y_1 is 4.1:

$$\begin{aligned} Y_1 = & 157.58 + 4.84A + 2.33B + 11.28C \\ & + 0.3425AB - 0.2500AC + 0.3000BC \\ & + 0.8312A^2 + 5.78B^2 + 0.1388C^2 \end{aligned} \quad (4.1)$$

4.1.1.2 Polydispersity Index

All PDI values were below 0.22, suggesting good colloidal homogeneity. The greatest reduction in PDI value was achieved by poloxamer 188, with a coefficient of +0.0065, due to the ability of poloxamer 188 to sterically stabilize the vesicles and avoid any size heterogeneity.

Soy lecithin concentration slightly enhanced PDI values, which may be explained by higher system viscosity and generation of larger vesicle sizes (Figure 4.1D-F). Optimal uniformity was achieved at intermediate hydration volumes, whereas high hydration volumes led to polydispersity.

The quadratic equation for Y_2 is 4.2:

$$\begin{aligned} Y_2 = & 0.1806 + 0.0096A + 0.0065B + 0.0195C \\ & - 0.0010AB - 0.0004AC - 0.0022BC \\ & - 0.0018A^2 + 0.0108B^2 + 0.0039C^2 \end{aligned} \quad (4.2)$$

4.1.1.3 Zeta Potential

The absolute zeta potential value in all of the formulations was observed to be between -30.9 mV to -36.4 mV, meeting the electrostatic stability criterion for a colloidal system. The surface charge is negative due to the presence of the

phosphate head group on soy lecithin, which becomes ionized under physiological conditions. An increase in the soy lecithin concentration (A) and Poloxamer 188 concentration (B) led to an increase in the negative zeta potential.

The former increases the phospholipid density while the latter provides steric stabilization to the vesicles by adsorption of the non-polar Poloxamer 188 chains, preventing electrostatic screening without introducing new surface charges. The hydration volume had a negative impact on the zeta potential because of its diluting effects on charged surface species (Figure 4.1G-I).

The quadratic model for Y_3 is 4.3:

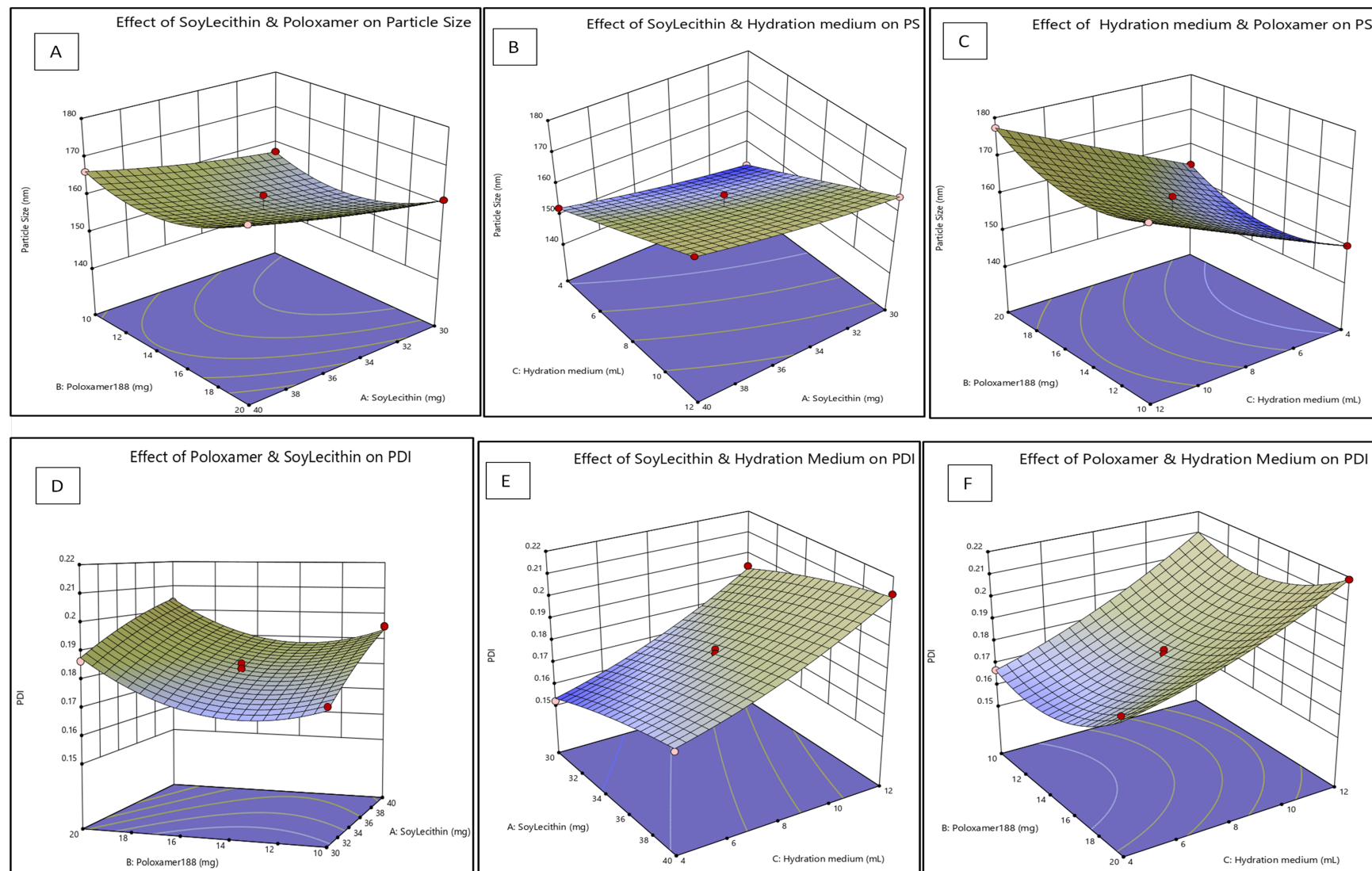
$$\begin{aligned} Y_3 = & -35.52 + 0.3750A + 0.6250B - 0.6750C \\ & + 1.95AB + 0.9500AC - 0.3500BC \\ & + 1.11A^2 + 0.4600B^2 + 2.01C^2 \end{aligned} \quad (4.3)$$

4.1.1.4 Entrapment Efficiency

EE varied between 85.7 and 89.0%, due to the highly lipophilic nature of PTX towards the phospholipid bilayer matrix. The highest contribution to EE was made by soy lecithin concentration (+1.06 coefficient value) as per the larger hydrophobic space created for PTX solubilization with increased lecithin concentrations [115].

Poloxamer 188 had an indirect effect on enhancing EE via stabilization of the drug-filled vesicles and reduction of drug loss through membrane defects. Excess hydration volumes lowered EE by increasing the water volume fraction and favoring drug solubilization in the continuous phase (Figure 4.1J-L). The quadratic model for Y_4 is 4.4:

$$\begin{aligned} Y_4 = & 86.82 + 1.06A + 0.0500B - 0.4375C \\ & - 0.1500AB - 0.4750AC - 0.3000BC \\ & - 0.1725A^2 + 0.2025B^2 + 0.2775C^2 \end{aligned} \quad (4.4)$$



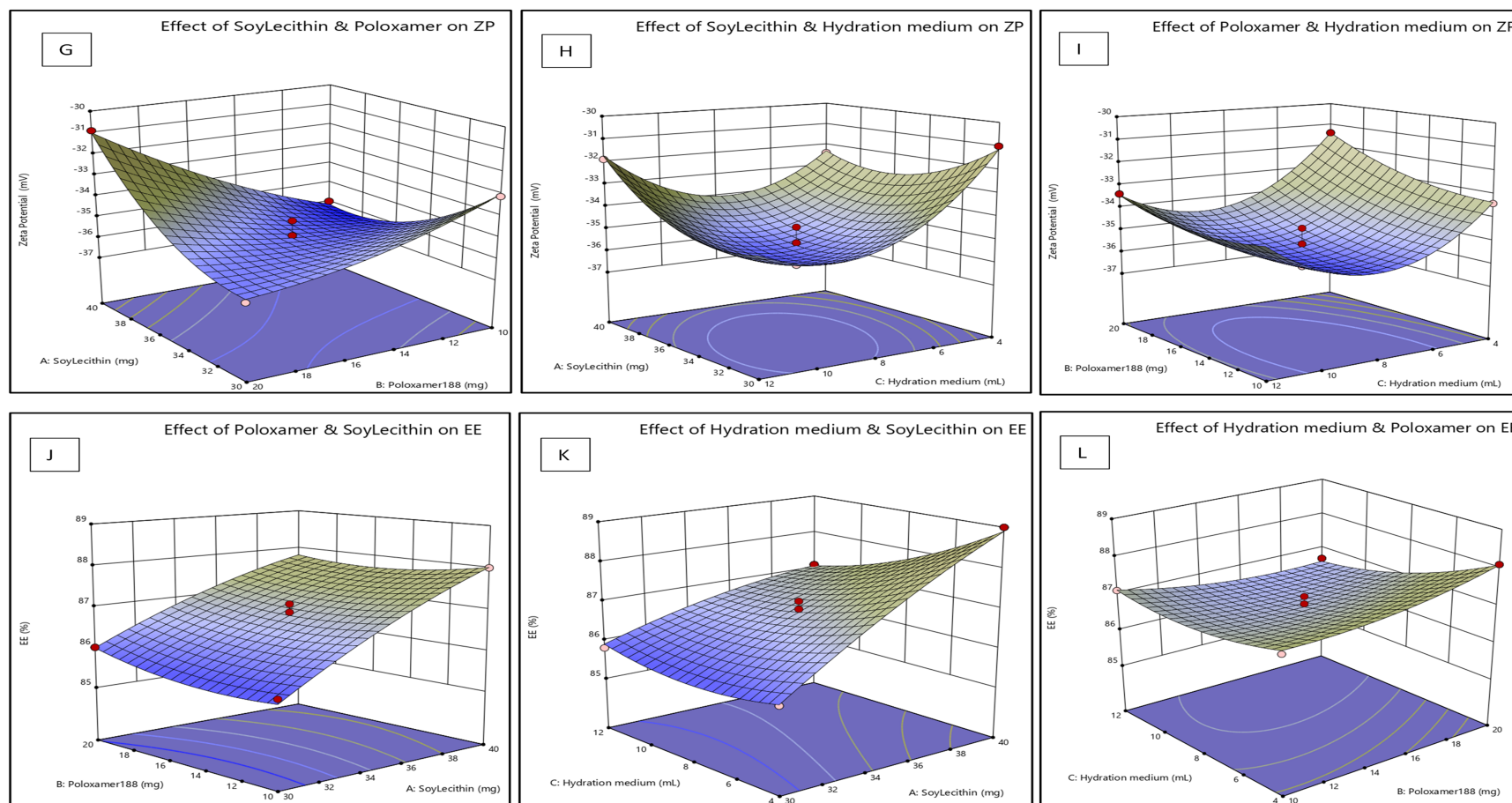


FIGURE 4.1: Three-dimensional response surface plots showing the influence of formulation variables on the critical quality attributes of PTX-loaded nano-transferosomes. Rows correspond to responses: particle size (Y_1 , 2A–C), polydispersity index (Y_2 , 2D–F), zeta potential (Y_3 , 2G–I) and entrapment efficiency (Y_4 , 2J–L). Columns are variable interactions: soy lecithin (A) vs. Poloxamer 188 (B) at a fixed hydration volume ($C = 8$ mL); soy lecithin (A) vs. hydration volume (C) at a fixed Poloxamer 188 ($B = 15$ mg); Poloxamer 188 (B) vs. hydration volume (C) at a fixed soy lecithin ($A = 35$ mg). All plots were generated using Design-Expert[®] v13.0.

4.1.2 Model Validation and Selection of Optimized Formulation

Optimization of the numerical parameters by use of the composite desirability function yielded Run F15 [soy lecithin: 40 mg; Poloxamer 188: 10 mg; hydration volume: 8 mL; phospholipid-edge activator (ratio): 80:20 w/w] as the optimal formula, giving $D = 0.891$.

Although some formulations exhibited superior individual responses (e.g., F9 showed the smallest particle size and PDI, whereas F16 and F4 exhibited slightly higher entrapment efficiency), none simultaneously satisfied all the predefined optimization criteria. F15 was selected because it provided the best overall balance among all critical quality attributes (CQAs), achieving an appropriate nanoscale particle size (166.3 nm), low PDI (0.195), highly stable zeta potential (-35.9 mV), and high entrapment efficiency (88.0%). The composite desirability function simultaneously optimized all responses rather than maximizing a single parameter, making F15 the formulation with the highest overall desirability ($D = 0.891$). Therefore, F15 represented the most balanced and robust formulation for further characterization and evaluation. The predictions and experimental data were in excellent agreement (Table 4.3) as there were deviations less than 1% in all response values, which established the predictive power of the quadratic model developed from BBD analysis. The optimized formulation F15 had the following properties: particle size 166.3 nm (predicted: 166.35 nm; 95% PI: 161.26–171.44 nm), PDI 0.195 (predicted: 0.194), zeta potential -35.9 mV (predicted: -36.15 mV), and EE 88.0% (predicted: 88.01%).

TABLE 4.3: Model validation compares the predicted responses with the observed results for the optimized formulation F15.

Response	Predicted Mean	Observed	SD	95% PI (Low– High)	n
Particle size (nm)	166.35	166.3	1.627	161.26–171.44	1
PDI	0.1938	0.195	0.00251	0.1859–0.2016	1
Zeta potential (mV)	-36.15	-35.9	0.484	-37.67 to -34.63	1
EE (%)	88.01	88.0	0.173	87.47–88.55	1

4.2 Particle Size, PDI, and Zeta Potential

The Dynamic Light Scattering (DLS) evaluation of the optimized PTX-NTFG (F15) resulted in a hydrodynamic diameter of 166.3 nm, PDI of 0.195, and zeta potential of -35.9 mV (Figure 4.2). The size parameters obtained for the nanovesicles are ideal for intranasal administration because nanoparticles with diameters <200 nm can penetrate the olfactory epithelium, pass through the nasal mucus layer, and efficiently interact with the mucosa for enhanced permeation [115, 116].

A PDI of 0.195 is significantly lower than the recommended value of 0.3 for homogeneous nanoformulations, suggesting uniformity in particle size. This is advantageous in developing formulations suitable for drug delivery applications [117].

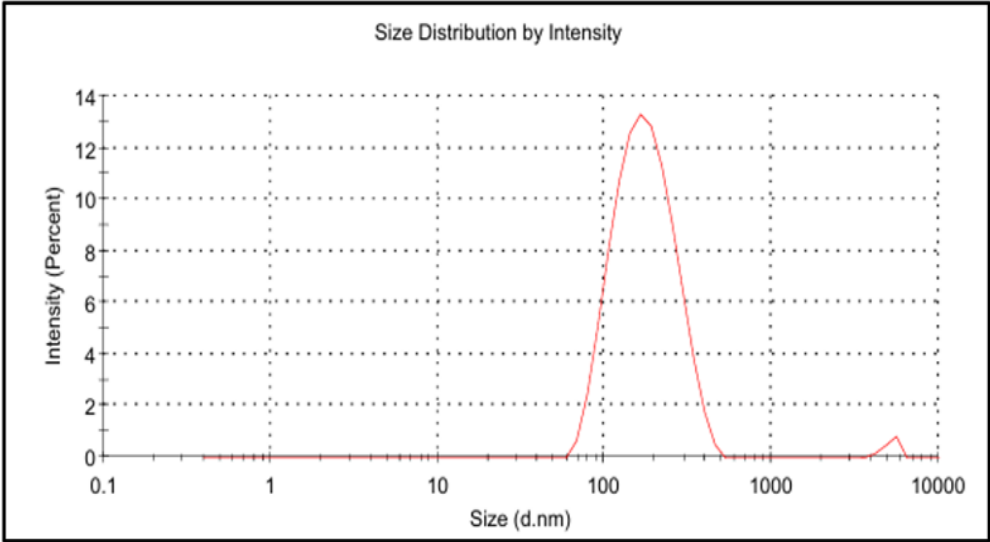
Zeta potential of -35.9 mV was higher than the critical value $\zeta = 30$ mV, indicating sufficient electrostatic interactions between particles to ensure colloidal stability of the vesicles [118, 119]. A negative surface charge can lead to an enhanced ability to interact with positively charged amino acid residues of nasal mucin glycoprotein complex, thus possibly enhancing mucoadhesion and sustained nasal residence time.

4.3 Transmission Electron Microscopy

The transmission electron microscopy (TEM; HT7800, Hitachi High-Tech Corp., Japan) technique was applied for the study of the vesicle structure and internal composition of the optimized nano-transferosome formulation loaded with paclitaxel.

The micrograph was obtained using an electron microscope under 10,000x magnification with a scale bar of 1.0 μm . TEM imaging confirmed that uniform spherical vesicles with smooth surfaces and clear margins had been formed successfully, indicating the formation of transferosomes at nanoscale level (Figure 4.3) [120]. This formulation had minimal aggregation, suggesting excellent colloid stability due to steric stabilization conferred by Poloxamer 188.

	Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 166.3	Peak 1: 184.7	98.5	74.92
Pdl: 0.195	Peak 2: 5133	1.5	520.2
Intercept: 0.944	Peak 3: 0.000	0.0	0.000
Result quality : Good			



	Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -35.9	Peak 1: -35.9	100.0	11.0
Zeta Deviation (mV): 11.0	Peak 2: 0.00	0.0	0.00
Conductivity (mS/cm): 0.180	Peak 3: 0.00	0.0	0.00
Result quality : See result quality report			

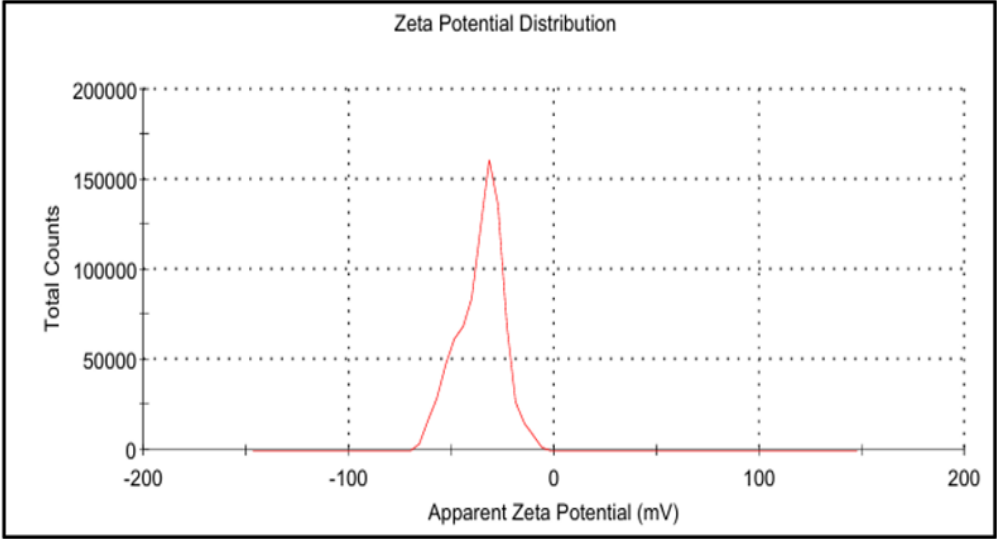


FIGURE 4.2: Particle Size Distribution & Zeta Potential of PTX-Loaded Nano-Transfersomes. Particle size and surface charge were determined through dynamic light scattering method. Red texts indicate significant values of Z-average & Zeta Potential of PTX-Loaded Nano-Transfersomes, which prove that it is a nano form of PTX having moderate stability.

The TEM results confirmed that the dimensions of the vesicles were within the range of the nanometer-size distribution acquired from DLS measurement. Additionally, some vesicles exhibited internal lamellarity characterized by unilamellar and oligolamellar forms (Figure 4.3). This structural heterogeneity is a recognized feature of transferosomal systems and arises from the self-assembly of phospholipids and edge activators into flexible bilayered vesicles [121]. Such architecture is known to enhance vesicular deformability and membrane permeability, which is critical for transmucosal transport applications.

No visible crystalline drug particles were observed, suggesting successful encapsulation of paclitaxel within the lipid bilayer system. The observed vesicle size was within the nanometric range (<200 nm), supporting their suitability for intranasal brain-targeted delivery [103, 120].

Overall, TEM confirmed a uniform spherical morphology, nanoscale vesicular structure, and intact integrity, supporting the appropriateness of the system for thermoresponsive in situ nasal delivery and brain-targeted therapy in breast cancer metastasis.

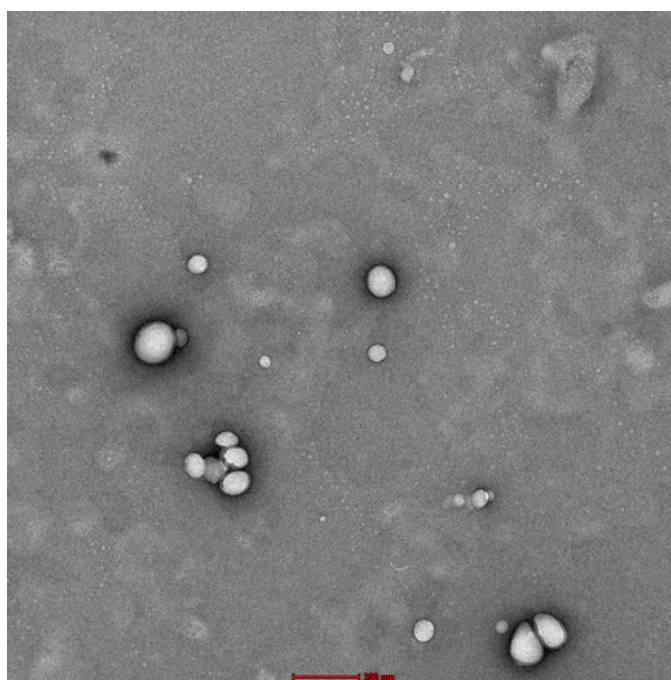


FIGURE 4.3: Transmission electron microscopy (TEM, HT7800, Hitachi High-Tech Corp., Japan) of PTX-NTF.

4.4 FTIR Spectroscopic Analysis

FTIR spectra of pure PTX, soy lecithin, Poloxamer 188, and the optimized PTX-loaded nano-transferosomal formulation (F15) are presented in Figure 4.4. The characteristic FTIR profile of pure PTX was identified by: a broad O-H stretching band ($3200 - 3600\text{ cm}^{-1}$), carbonyl ester stretches at 1732 and 1701 cm^{-1} , aromatic C = C deformations at 1641 cm^{-1} , strong C – O – C asymmetric and symmetric stretches ($1247 - 1050\text{ cm}^{-1}$), aromatic C – H out-of-plane bending ($777 - 707\text{ cm}^{-1}$), and skeletal vibrations ($530 - 650\text{ cm}^{-1}$) [122].

Soy lecithin exhibited characteristic asymmetric C – O – C stretches at 1736 cm^{-1} , aliphatic C – H stretches at 2923 and 2853 cm^{-1} , asymmetric PO_2^- stretch at 1239 cm^{-1} , and symmetric P – O – C stretches at $1089 - 1060\text{ cm}^{-1}$ [123]. The Poloxamer 188 spectrum was characterized by a broad O-H stretch ($3360 - 3390\text{ cm}^{-1}$), aliphatic C – H stretches ($2850 - 2950\text{ cm}^{-1}$), prominent C – O – C ether stretches ($1060 - 1150\text{ cm}^{-1}$), and methylene bending ($\sim 1460\text{ cm}^{-1}$) [124].

The FTIR spectrum of the optimized transferosomal formulation retained all characteristic absorption bands of the individual components, with no evidence of new covalent bond formation (no new peaks), confirming physicochemical compatibility between PTX, soy lecithin, and Poloxamer 188. Notably, the ester carbonyl peak at 1734 cm^{-1} (from both PTX and lecithin) showed no significant displacement, confirming structural integrity of PTX within the formulation. Minor spectral modifications were observed: broadening of the O-H band ($3360 - 3400\text{ cm}^{-1}$) indicated hydrogen bonding between PTX hydroxyl moieties and the phosphate and ether groups of lecithin and Poloxamer 188; slight expansion of the C – O – C/ PO_2^- band was consistent with close-range molecular association in the condensed lipid matrix without covalent modification. Furthermore, the characteristic aliphatic C–H stretching bands of Poloxamer 188 ($2850\text{--}2950\text{ cm}^{-1}$) became less distinct in the optimized formulation due to overlap with soy lecithin alkyl chain vibrations and incorporation of the polymer into the transferosomal bilayer, rather than chemical degradation.

Similarly, slight broadening and reduced intensity of the methylene bending ($\sim 1460\text{ cm}^{-1}$) and $\text{PO}_2^-/\text{P-O-C}$ bands indicate intermolecular interactions among PTX, soy lecithin, and Poloxamer 188, leading to a stable and compact vesicular structure. The C-O-C ether bands ($1060 - 1150\text{ cm}^{-1}$) were retained with slight overlap in the fingerprint region, confirming successful incorporation of Poloxamer 188 without altering its chemical structure.

Likewise, the characteristic PTX peaks were retained with slight reduction in intensity, indicating molecular encapsulation within the lipid bilayer without chemical modification. The retention of all PTX-characteristic peaks at 964 , 823 , 720 , 1463 , and 1361 cm^{-1} confirmed successful encapsulation without chemical degradation [122, 125].

Overall, the absence of new peaks and the slight broadening or overlap of characteristic bands confirm that PTX was successfully incorporated through non-covalent interactions without altering the chemical integrity of the formulation components.

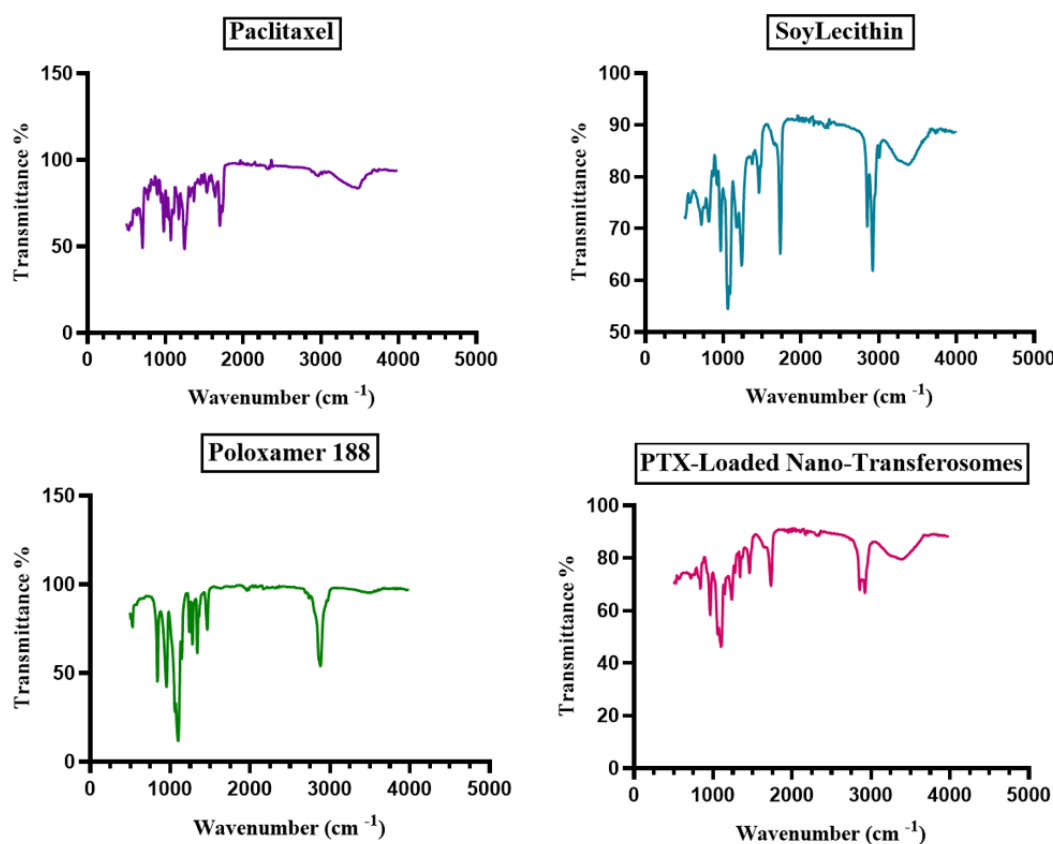


FIGURE 4.4: FTIR Spectra of pure Paclitaxel, Soy lecithin, Poloxamer188 and PTX-Loaded Nano-Transfersomes Formulation.

4.5 Powder X-Ray Diffraction Analysis

The crystalline PTX's PXRD pattern showed several sharp, intense diffraction peaks at $2\theta \approx 5.5^\circ$, $9 - 10^\circ$, $12.5 - 13^\circ$, $15 - 16^\circ$, and $20 - 23^\circ$, with the principal reflection at $12 - 13^\circ$ (2θ) consistent with its well-defined monoclinic crystal lattice structure (Figure 4.5) [126]. These characteristic sharp reflections are directly attributable to the high degree of long-range molecular order in crystalline PTX and are correlated with its characteristically poor aqueous solubility (intrinsic dissolution rate $\sim 0.3 \mu\text{g/mL}$).

In marked contrast, the diffractogram of the lyophilized PTX - loaded nano - transferosomes formulation (F15) demonstrated complete attenuation of all PTX crystalline reflections, with the diffraction pattern dominated by a broad, asymmetric halo centered at $2\theta \approx 21.88^\circ$ ($\sim 3,680$ counts) attributable to the amorphous/semi-crystalline contributions of the Poloxamer 188 and soy lecithin matrix. A secondary diffuse reflection at 23.87° and a low-intensity feature at 18.28° were also observed. The complete disappearance of crystalline PTX reflections in F15 provides unequivocal evidence that PTX exists in a molecularly dispersed, amorphous state within the lipid-polymer matrix, a solid-state transformation associated with dramatically enhanced dissolution rate and bioavailability compared to the crystalline form [127, 128]. The reduction in crystallinity within the transferosomal matrix is also expected to translate into higher in vivo drug availability at the site of absorption.

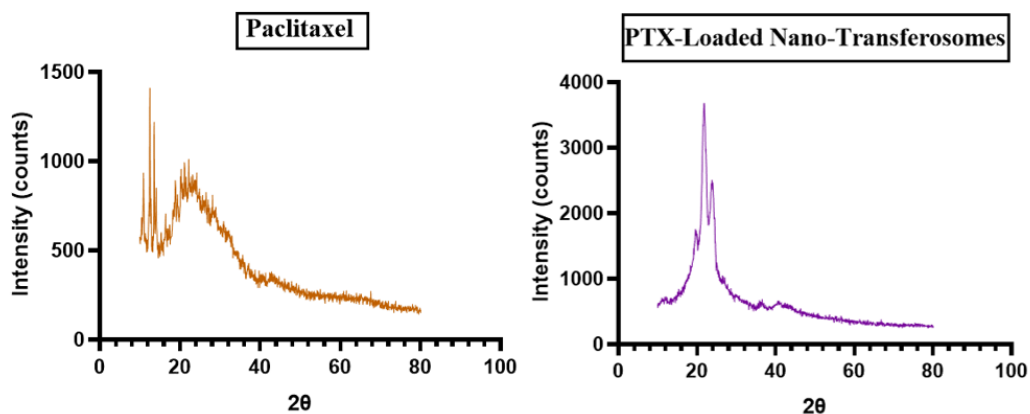


FIGURE 4.5: XRD Patterns of Pure Paclitaxel and PTX-Loaded Nano - Transferosomes Formulation.

4.6 Differential Scanning Calorimetry

DSC thermograms of pure PTX, Poloxamer 188, and lyophilized PTX-loaded nano-transferosomes (F15) are presented in Figure 4.6. Pure PTX displayed a broad endothermic transition spanning 200–230°C (peak heat flow: $\sim 8\text{--}10\text{ mW}$), consistent with a partially disordered crystalline state at reduced crystallinity.

Poloxamer 188 exhibited a sharp endothermic melting peak with onset at $\sim 45^\circ\text{C}$ and maximum at $\sim 55^\circ\text{C}$ (heat flow: $\sim 16\text{--}18\text{ mW}$), characteristic of its semicrystalline the triblock copolymer configuration of polyethylene oxide–polypropylene oxide–polyethylene oxide (PEO–PPO–PEO) [125].

DSC thermogram of lyophilized PTX-loaded nano-transferosomes showed no discrete endothermic events corresponding to either PTX melting or the Poloxamer crystalline transition. Only a minor baseline deviation was observed in the 50–60°C region ($\sim 5\text{--}6\text{ mW}$), consistent with residual Poloxamer microcrystalline domains within the vesicular matrix.

The complete suppression of the PTX melting endotherm confirms molecular-level dispersion of PTX within the lecithin-Poloxamer matrix, corroborating the amorphization evidenced by PXRD.

he relatively broad and low-enthalpy peak of the commercial PTX sample indicates partial crystallinity, which further highlights the amorphization achieved in the formulation.

This molecular dispersion is attributed to strong non-covalent interactions, including hydrophobic interactions, van der Waals forces, and hydrogen bonding between PTX and lipid bilayer components, which disrupt crystal lattice cohesive energy and maintain the drug in an energetically disordered, higher-free-energy amorphous state favorable for dissolution and absorption [124].

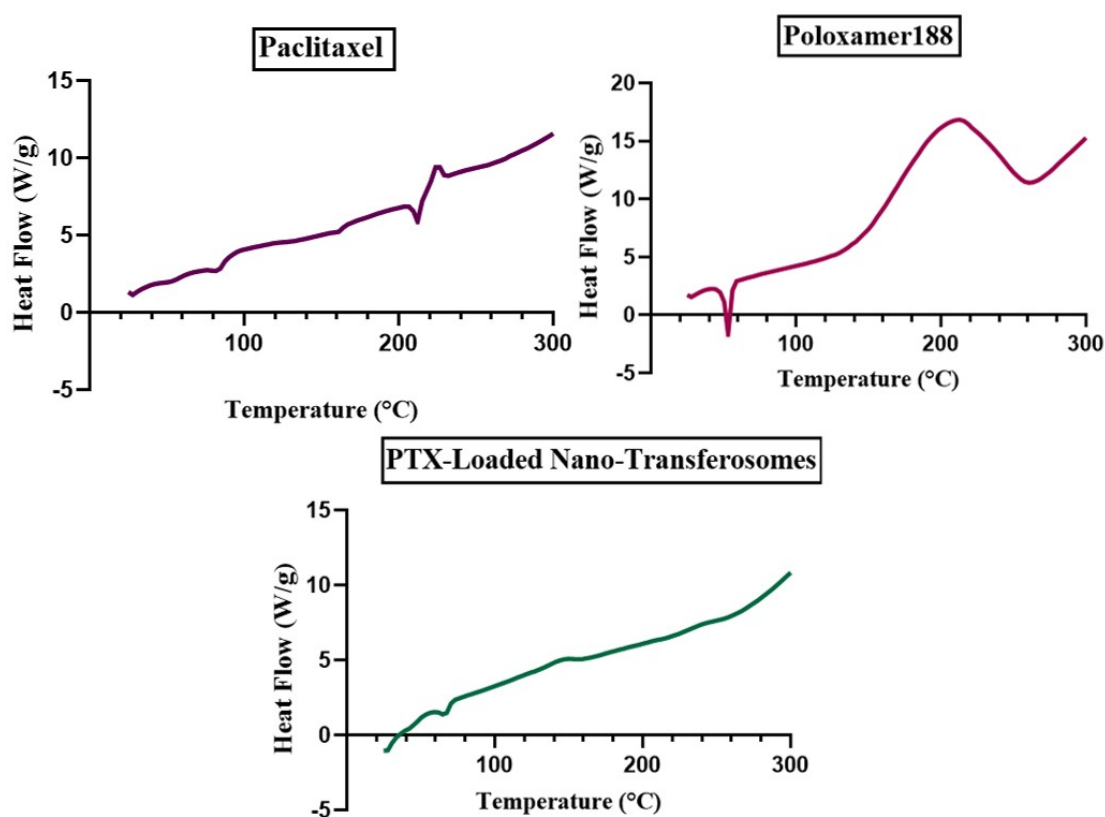


FIGURE 4.6: DSC Thermogram of Pure Paclitaxel, Poloxamer 188 and PTX-Loaded Nano-Transferosomes Formulation.

4.7 In Vitro Drug Release and Kinetic Analysis

The cumulative in vitro release profiles of PTX from PTX dispersion, PTX-loaded transferosomes, and PTX-NTFG at pH 5.5 and 7.4 over 24h are illustrated in Figure 4.7. Two-way ANOVA confirmed statistically notable primary impacts of both formulation type ($p < 0.001$) and pH ($p < 0.001$), as well as a significant formulation-by-pH interaction ($p < 0.001$), indicating that the pH dependence of drug release was formulation-specific [129].

PTX dispersion exhibited rapid, uncontrolled release characteristic of passive diffusion without a rate-limiting membrane, achieving $89.5 \pm 1.0\%$ (pH 5.5) and $90.0 \pm 2.5\%$ (pH 7.4) cumulative release within 6 h, plateauing thereafter. This clearly indicates that there is no formulation-dependent kinetic control mechanism

present, and supports the diffusion of drug molecules through dialysis membrane by purely diffusional means.

However, in the case of PTX-containing transferosomes, significant modification to the release rate was observed; cumulative release after 24 hours was $78.6 \pm 2.4\%$ at pH 5.5 and $85.6 \pm 2.5\%$ at pH 7.4. The PTX-NTFG formulation showed the best prolonged release profile, releasing $66.8 \pm 2.2\%$ (pH 5.5) and $75.9 \pm 2.5\%$ (pH 7.4) in 24 h.

The initial burst release from PTX-NTFG was significantly suppressed ($\sim 75\%$ reduction compared to PTX dispersion at 6 h) because of the Poloxamer 407 gelation-mediated reduction in effective diffusion coefficient and prolongation of drug diffusion mechanisms [130]. The enhanced release at pH 7.4 compared to pH 5.5 for vesicular formulations is because of higher membrane fluidity at higher pH, leading to lower inter-molecular interaction energies among lecithin molecules and enhanced PTX partitioning into the release medium.

Kinetic analysis confirmed the non-Fickian and uncontrolled release as PTX dispersion showed poor fit for all models ($R^2 < 0.5$; $n = 0.391$). The Korsmeyer-Peppas model ($R^2 = 0.9868$; $n = 0.623$) best described the PTX-loaded transferosomes at pH 7.4, suggesting an anomalous transport governed by a combination of diffusion processes and vesicle structural relaxation.

PTX-NTFG fitted best with the Korsmeyer-Peppas model at pH 7.4 ($R^2 = 0.9953$, $n = 0.681$) confirming the coupled diffusion-polymer relaxation mechanism in agreement with anomalous (non-Fickian) transport in a thermoresponsive gel network [110]. The Korsmeyer-Peppas model at pH 5.5 was also a good fit ($R^2 = 0.977$, $n = 0.612$), indicating anomalous transport with relatively smaller contribution of polymer relaxation, which is due to the decrease in the fluidity of lecithin bilayer and gel network swelling under acidic conditions.

All kinetic parameters (R^2 and n) were determined by nonlinear regression of mean release data ($n = 3$). Inter-batch variation in n was small ($SD \leq 0.02$) which confirmed the reproducibility of the model. The n values of $0.612 - 0.681$ ($0.45 < n < 0.89$) for PTX-NTFG confirm anomalous (non-Fickian) transport,

indicating that the permeation of the drug is controlled by both Fickian diffusion through the gel matrix and polymer chain relaxation of the Poloxamer network [131, 132].

This type of mechanism-dependent sustained release behavior appears optimal for nose-to-brain delivery, as it facilitates prolonged contact with olfactory epithelium and sustained CNS input of the drug, while minimizing nasal mucosal irritation due to high concentrations.

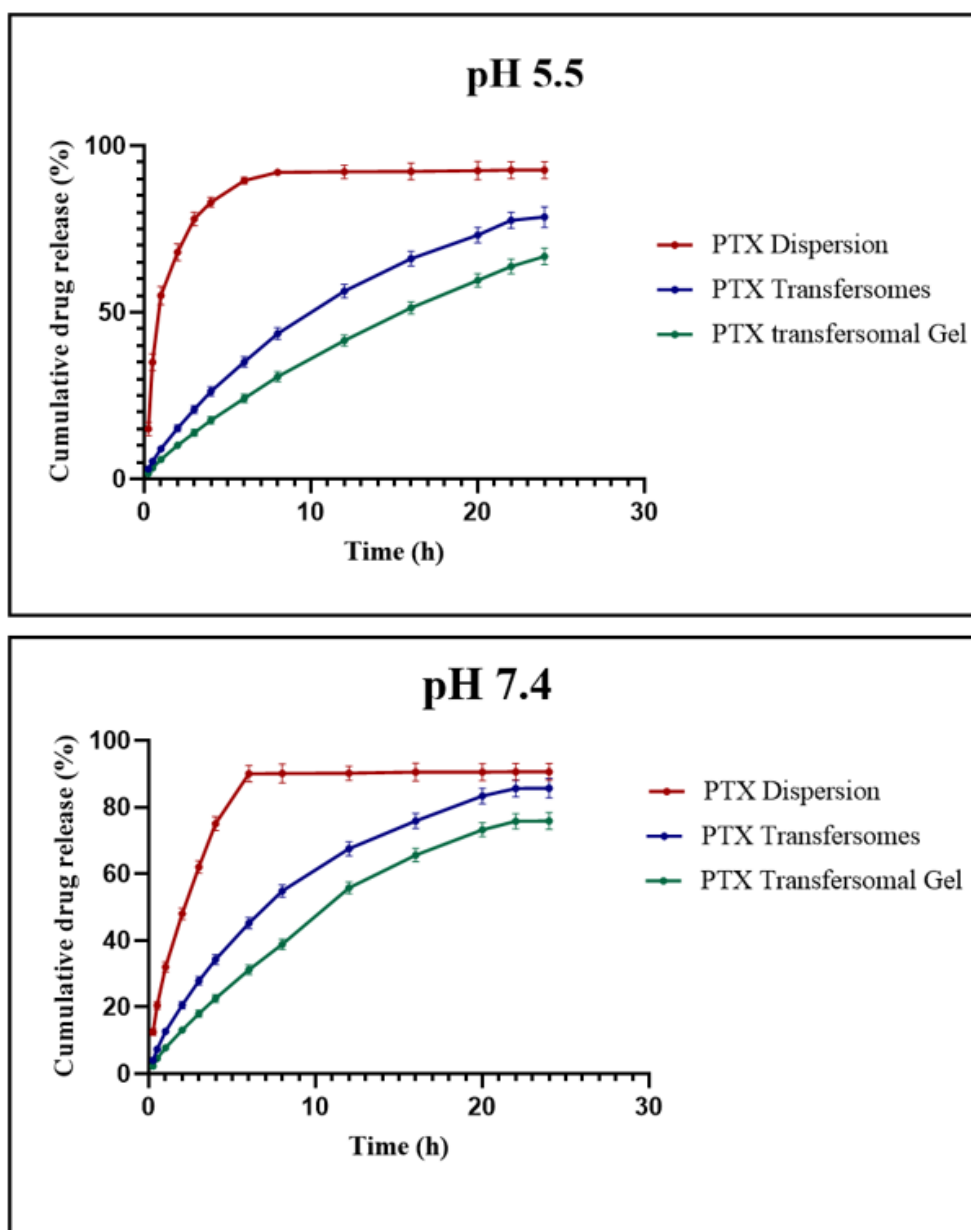


FIGURE 4.7: In vitro release profile of PTX dispersion, PTX-loaded transfersomes and PTX-loaded transfersosomal in situ gel at pH 5.5 and pH 7.4 for 24 h. Data are mean $\pm$ SD ($n = 3$).

TABLE 4.4: Kinetic model fitting for Paclitaxel release at pH 5.5 and 7.4

Release Model	Para-meter	PTX Dispersion		PTX Transferosomes		PTX-NTFG	
		pH 5.5	pH 7.4	pH 5.5	pH 7.4	pH 5.5	pH 7.4
Zero-order	R ²	0.4699	0.5676	0.9295	0.924	0.8763	0.9648
First-order	R ²	0.6371	0.4988	0.7212	0.5449	0.7486	0.6551
Higuchi	R ²	0.6622	0.7568	0.9241	0.9532	0.8923	0.9907
Korsmeyer	R ²	0.7954	0.7903	0.9868	0.9868	0.977	0.9953
–Peppas							
Korsmeyer	n	0.2941	0.391	0.412	0.623	0.612	0.681
–Peppas							

4.8 Rheological Properties of PTX-NTFG

The physicochemical properties of PTX-NTFG in terms of intranasal thermoresponsive administration were confirmed by rheological evaluation (Table 4.5). The formulation was a clear, homogeneous, colorless translucent sol at ambient temperature (25°C; viscosity < 200 cP) and was effortlessly administered as a uniform nasal instillation.

The sol-to-gel transition was determined at $34.2 \pm 0.5^\circ\text{C}$, the temperature at which a minimum 10-fold increase in viscosity was recorded over a 2°C increment during controlled temperature ramping. The gelation temperature exists in the physiological temperature range of the nasal mucosa ($32\text{--}37^\circ\text{C}$), which enables the formulation to change from a freely flowing sol to a cohesive gel depot in situ after administration thus extending the duration of nasal residence.

The viscosity of the formulation at 37°C showed a considerable increase to 2099.53 ± 18.40 cP (CV < 1%), suggesting the fast micellization and micellar network formation of Poloxamer 407 [133]. This viscosity range ensures proper retention while also allowing for easy syringing. The pseudoplastic nature of the gel was established through the decrease in viscosity with an increase in shear rate, which is highly favorable during delivery (shear thinning during instillation) and after delivery (viscosity recovery due to low shear rate in the nasal cavity).

The formulation pH was 5.5 ± 0.05 , closely matching the slightly acidic nasal mucosal pH (5.5–6.5), minimizing mucosal irritation and supporting membrane integrity [134, 135].

TABLE 4.5: Physicochemical and Rheological Properties of PTX-Loaded Nano-Transfersosomal In Situ Gel (PTX-NTFG).

Parameter	PTX-NTFG
pH	5.5 ± 0.05
Visual appearance	Clear, translucent, uniform
Color	Colorless
Homogeneity	Uniform; no aggregates or phase separation
Viscosity at 37°C (cP)	2099.53 ± 18.40
Gelation temperature, Tgel (°C)	34.2 ± 0.5
Flow behavior	Pseudoplastic (shear-thinning)

4.9 Ex Vivo Nasal Permeation Analysis

Ex vivo transmucosal permeation profiles for PTX-NTFG determined from Franz diffusion cells (effective diffusion area: 1.77 cm^2 ; receptor volume: 12 mL) with sheep nasal mucosa are shown in Figure 4.8, whereas their permeation profiles are presented in Table 4.6.

PTX-NTFG demonstrated significantly superior permeation compared to conventional PTX-Gel at all time points from 0.5 h onward ($p < 0.001$ by independent-samples t-test at each time point), exhibiting a progressive, sustained permeation profile without evidence of plateau within 24 h. Cumulative permeation at 24 h was $945.92 \pm 25.13 \text{ } \mu\text{g}/\text{cm}^2$ for PTX-NTFG versus $237.56 \pm 24.84 \text{ } \mu\text{g}/\text{cm}^2$ for PTX-Gel (approximately 4-fold difference; $p < 0.001$), representing an extremely large effect size given the magnitude of the between-group difference relative to within-group variability (pooled SD $\approx 25 \text{ } \mu\text{g}/\text{cm}^2$).

The permeation enhancement ratio (ER = 4.08) was consistent across time points (ER = 3.3 – 3.5 at early time points; ER ≈ 4.1 at 18–24 h), confirming sustained and time-stable superiority of the nano-transfersosomal system [136, 137].

Steady-state flux (J_{ss}) of PTX-NTFG ($51.70 \pm 1.3 \mu\text{g}/\text{cm}^2/\text{h}$) was 4.08-fold higher than PTX-Gel ($12.66 \pm 1.4 \mu\text{g}/\text{cm}^2/\text{h}$), and the apparent permeability coefficient K_p was correspondingly elevated (0.05170 ± 0.0013 vs. $0.01266 \pm 0.0014 \text{ cm}/\text{h}$).

PTX-Gel exhibited early plateauing of cumulative permeation after 18h, consistent with diffusion-limited, formulation-concentration-dependent permeation from a non-deformable conventional gel matrix without nanocarrier permeation enhancement [138]. In contrast, PTX-NTFG maintained a near-linear permeation rate throughout 24 h, indicative of reservoir-sustained delivery from the transferosomal depot within the gel matrix.

TABLE 4.6: Ex vivo permeation parameters of PTX-Gel and PTX-NTFG across sheep nasal mucosa (mean $\pm$ SD, n = 4).

Parameter	Formula	PTX-Gel	PTX-NTFG
J_{ss} ($\mu\text{g}/\text{cm}^2/\text{h}$)	$(Q_{18} - Q_0) / 18$	12.66 ± 1.4	$51.70 \pm 1.3^*$
K_p (cm/h)	J_{ss} / C_0 (1000 $\mu\text{g}/\text{mL}$)	0.01266 ± 0.0014	$0.05170 \pm 0.0013^*$
Enhancement Ratio (ER)	$J_{ss} \text{ (NTFG)} / J_{ss} \text{ (Gel)}$	-	4.08
Cumulative permeation at 24 h ($\mu\text{g}/\text{cm}^2$)	-	237.56 ± 24.84	$945.92 \pm 25.13^*$

* $p < 0.001$ versus PTX-Gel (independent-samples t-test)

The markedly superior permeation of PTX-NTFG is attributed to the synergistic contributions of the nano-transferosomes carrier and the in situ gel matrix. The ultra-deformable phospholipid bilayer of transferosomes enables permeation through tight intercellular junctions of the nasal epithelium via reversible deformation, exploiting the transcellular and paracellular routes [136, 137].

Poloxamer 188 as an edge activator provides steric stabilization and reduces interfacial energy barriers at the vesicle-mucosa interface, facilitating intimate vesicular contact with epithelial membranes [110, 124]. Furthermore, the lipophilic PTX-lecithin interactions promote membrane fusion and partition-based transmucosal drug transfer.

The thermoresponsive in situ gel prolongs nasal residence time and maintains intimate formulation-mucosa contact, further amplifying the permeation advantage

of the transferosomal carrier. Collectively, these mechanisms account for the approximately 4-fold enhancement in transmucosal PTX delivery across the nasal epithelium, which may translate into improved drug bioavailability within the brain *in vivo*, pending confirmation through pharmacokinetic and brain distribution studies.

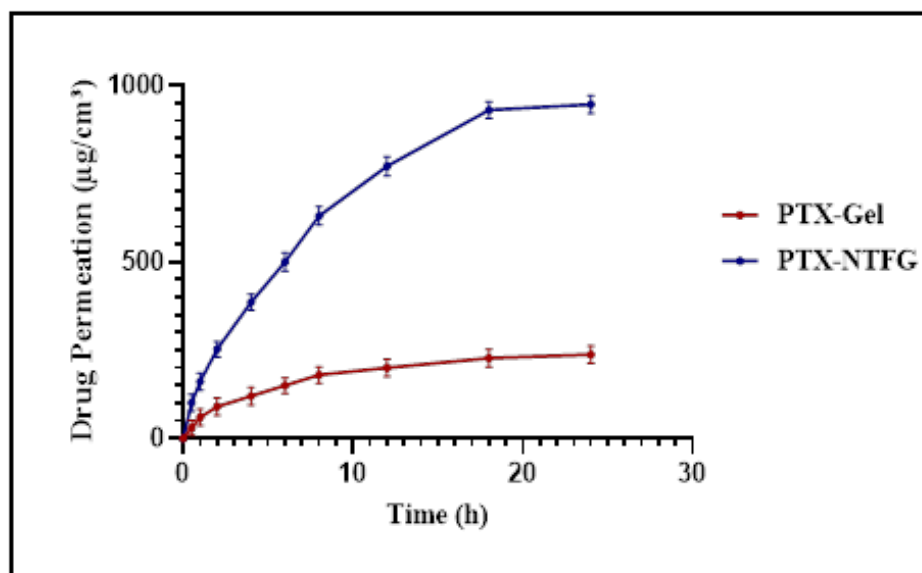


FIGURE 4.8: Ex vivo drug permeation from PTX-GEL and PTX-NTFG at pH 5.5 over the nasal mucosal membrane. Mean $\pm$ SD ($n = 4$) is used to express the data.

4.10 Stability Studies

Accelerated and long-term stability tests were performed on the optimized intranasal PTX-NTFG (F15) in sealed amber vials at 4°C/60% RH, 25°C/60% RH, and 40°C/75% RH (ICH Q1A(R2) conditions) for up to 3 months [139]. At each time point (0, 1, 2, 3 months) the samples were inspected for visual appearance and assayed for pH, drug content and visual appearance (color, homogeneity, phase separation).

Throughout the study, the gel remained clear and homogeneous with no observable color change, turbidity or phase separation under any condition. Likewise, the pH stayed essentially constant (near physiological range), indicating no significant

chemical degradation of gel components [140]. No evidence of precipitation or turbidity in PTX-NTFG proved that both the nano-colloidal system and polymer matrix were stable [140, 141].

Importantly, PTX levels were kept within pharmacopoeia range during the entire study period. There was no statistically significant difference observed in assay values; the level of PTX obtained was relatively unchanged (within 90–110%) even at accelerated storage condition [142]. In case of PTX-NTFG, any small variations that occurred in assay results (below 2–5%) were due to analytical variations only.

Therefore, PTX-NTFG proved to be chemically stable: there was almost no decrease in drug content even after 3 months storage at 40°C/75% RH, just like most other nanoparticles-based drug delivery formulations. Taking into account the data obtained on stability of the PTX-NTFG formula, it can be stated that the intranasal PTX-NTFG remains physically and chemically stable.

Under both normal and accelerated storage conditions, slight yellowish coloring could be observed after 3 months of storage without decreasing the drug content and homogeneity. Drug content in the gel remained constant, which shows its high stability both under normal and accelerated conditions [143, 144]. Summarizing, the PTX-NTFG was found to be stable based on all the stability tests performed, as shown in Table 4.7 below.

TABLE 4.7: PTX-NTFG stability study at $4 \pm 1^\circ\text{C}/60 \pm 5\%$ RH, $25 \pm 2^\circ\text{C}/60 \pm 5\%$ RH, and $40 \pm 2^\circ\text{C}/75 \pm 5\%$ RH.

Parameter	Storage Condition	0 Month	1 Month	2 Months	3 Months
pH	Refrigerated ($4 \pm 1^\circ\text{C}$, ambient RH)	5.50 ± 0.05	5.48 ± 0.04	5.47 ± 0.05	5.45 ± 0.06
	Room Temperature ($25 \pm 2^\circ\text{C}$ / $60 \pm 5\%$ RH)	5.50 ± 0.05	5.47 ± 0.05	5.45 ± 0.06	5.43 ± 0.07
	Accelerated ($40 \pm 2^\circ\text{C}$ / $75 \pm 5\%$ RH)	5.50 ± 0.05	5.44 ± 0.06	5.39 ± 0.07	5.34 ± 0.08
Drug Content (%)	Refrigerated	100.0 ± 1.2	99.2 ± 1.5	98.6 ± 1.8	97.9 ± 2.0

Table 4.7 continued from previous page

Parameter	Storage	Condi- tion	0 Month	1 Month	2 Months	3 Months
Color/ Ap- pearance	Room Temperature		100.0 ± 1.2	98.8 ± 1.7	97.4 ± 2.1	95.8 ± 2.5
	Accelerated		100.0 ± 1.2	96.9 ± 2.3	95.2 ± 2.8	94.9 ± 3.1
	Refrigerated		Clear, col- orless	Clear, col- orless	Clear, col- orless	Clear, col- orless
		Room Temperature	Clear, col- orless	Clear, col- orless	Clear, col- orless	Slightly pale yel- low*
	Accelerated		Clear, col- orless	Clear, col- orless	Clear, col- orless	Slightly pale yellow [®]
Homogeneity	All conditions		Homogeneous	Homogeneous	Homogeneous	Homogeneous
Phase Sepa- ration	All conditions		None	None	None	None

Chapter 5

Conclusion and Future Recommendations

5.1 Conclusion

The present investigation successfully optimized the intranasal delivery of nano transferosomal in situ gels loaded with paclitaxel using an innovative Quality-by-Design method employing Box-Behnken response surface analysis. The optimized formulation exhibited nanometric vesicular sizes (166.3 nm), minimal polydispersity index ($PDI = 0.195$), superior colloidal stability (ζ potential -35.9 mV), and high Entrapment efficiency (EE%) (88.0%), meeting all essential quality attributes. Solid state evaluation using FTIR, PXRD, and DSC indicated molecular level loading of PTX into the lipid-polymer system, ensuring complete amorphization of the crystalline PTX and no excipient-drug incompatibility. The thermoresponsive gel formulation showed a sharp sol-gel phase transition at $34.2 \pm 0.5^\circ\text{C}$, within the physiologically relevant temperature range for the nose, proper pH (5.5 ± 0.05), and adequate gel viscosity (2099.53 ± 18.40 cP at 37°C) to ensure long residence time in the nasal cavity without causing discomfort during administration.

The in vitro release profile of PTX-NTFG exhibited a prolonged, anomalous transport pattern (Korsmeyer-Peppas equation, $n = 0.681$), releasing about 75.9% drug within 24 hours at pH 7.4 with no significant burst phenomenon.

The ex vivo study on nasal permeability revealed that PTX-NTFG had 4.08 times greater cumulative absorption than PTX-Gel and higher steady-state flux due to its ultra-deformable vesicle structure, which facilitated translocation across tightly packed nasal epithelial junctions along with thermoresponsive gel for prolonged mucosal residence.

All of the above observations indicate that PTX-NTFG is a very promising, minimally invasive intranasal drug delivery system which may lead to improved transport of drugs through the nasal mucosa and facilitate nose-to-brain delivery of paclitaxel for the treatment of breast cancer brain metastasis.

Further investigations are required for in vivo pharmacokinetic and brain distribution study, in vitro cytotoxicity activity against BCBM cells, and nasal tolerability testing.

5.2 Future Recommendations

The encouraging results obtained with the developed PTX-NTFG system indicate that this formulation deserves further investigation for the treatment of breast cancer brain metastases (BCBMs). Although the present work successfully demonstrated formulation development, physicochemical characterization, and ex vivo permeation behavior, several important aspects were not addressed and should be examined in future studies.

In particular, detailed in vivo pharmacokinetic, bioavailability, and biodistribution studies are needed to determine the extent of nose-to-brain transport, measure paclitaxel levels in different brain regions, and compare its delivery performance with conventional formulations. These studies would provide stronger evidence of the formulation's ability to improve drug availability at the intended site of action.

In addition, mucoadhesive strength studies should be carried out to evaluate how effectively the formulation adheres to the nasal mucosa and how well it can withstand mucociliary clearance. Such data would help clarify the role of mucoadhesion in prolonging nasal residence time and enhancing drug absorption. Since these evaluations were not included in the present study, they remain essential for a more complete understanding of the formulation's nasal performance.

The biological activity of PTX-NTFG should also be confirmed through in vitro cytotoxicity testing, including MTT assays, using appropriate HER2-positive and triple-negative breast cancer (TNBC) brain metastatic cell lines. These experiments would help determine the anticancer potential of the formulation, assess cellular response, and compare its effectiveness with free paclitaxel and other conventional delivery systems. The absence of such assays in the current study limits conclusions regarding the formulation's direct therapeutic effect, making them an important priority for future work.

Furthermore, preclinical in vivo studies in suitable animal models of breast cancer brain metastasis are necessary to evaluate safety, tolerability, therapeutic response, and possible toxic effects after repeated intranasal administration. Long-term stability studies under both storage and physiological conditions should also be performed to confirm the robustness of the formulation and its suitability for further development.

Overall, these additional investigations would generate essential information on the pharmacokinetic behavior, nasal retention, biological activity, safety, and translational value of PTX-NTFG. Together, they would support the advancement of this system as a promising non-invasive nose-to-brain delivery approach for targeted paclitaxel therapy in breast cancer brain metastases.

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