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Design, Development and Stability Optimization of Targeted Doxorubicin-Loaded Liposomes

N.Preethi, Dr.M.Amudha, P.Santhiya and Dr.V.Ganesan

Pannai College of Pharmacy, Dindigul, Tamil Nadu, India. The TamilNadu Dr. M.G.R. Medical University, Chennai

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ABSTRACT

The present study aimed to formulate and evaluate a doxorubicin-loaded liposomal drug delivery system to enhance therapeutic efficacy and minimize systemic toxicity. Doxorubicin, a potent chemotherapeutic agent, is widely used in the treatment of various cancers; however, its clinical utility is limited by dose- dependent cardio toxicity and poor tumor selectivity. To overcome these drawbacks, liposomal encapsulation was explored as an advanced nanocarrier system to achieve targeted and sustained drug delivery.

Preformulation studies were carried out to assess the compatibility between the drugandexcipientsusingstandardanalyticaltechniques,confirmingnosignificant interactions. Liposomes were successfully prepared using the thin-film hydration method followed by sonication to achieve nanosized vesicles. The optimized formulation was characterized for particle size, polydispersity index (PDI), zeta potential, surface morphology, encapsulation efficiency, and *in-vitro* drug release profile. The liposomal vesicles exhibited an average particle size of 125–160 nm, low PDI indicating uniformity, high encapsulation efficiency (78–88%), spherical morphology under microscopic observation, and a favorable zeta potential greater than –25 mV, signifying good stability.

In-vitro drug release studies displayed a biphasic pattern characterized by an initial burst release followed by a sustained release phase, influenced by lipid composition. Stability studies confirmed that the liposomal formulation remained stable at 4°C for 15 days, indicating suitable storage conditions. Overall, the study demonstrates that liposomal encapsulation of doxorubicin can significantly enhance its therapeutic index by prolonging circulation time, improving tumor targeting through the Enhanced Permeability and Retention (EPR) effect, and reducing systemic toxicity. These results provide a strong foundation for further *in-vivo* studies and potential clinical translation.

Introduction

Liposomal drug delivery systems have emerged as one of the most promising nanocarrier platforms for anticancer therapy[1]. Liposomes are microscopic spherical vesicles composed of phospholipids bilayers, capable of encapsulating both hydrophilic and lipophilic drugs[2]. In fact, liposomes can retain the drug until being disruption, indicating that they can promote sustained release formulation of drug. Their biocompatibility, ability to enhance solubility, reduce toxicity, and provide controlled release makes them particularly suitable for anticancer agents like doxorubicin[3].

Liposomes are classified on the basis of:[4]

1. Structure and Classification
2. Method of preparation
3. Conventional liposomes
4. Clinical application

Structure and Classification of Liposome:

The liposome size can vary from very small (0.025µm) to large (2.5µm) vesicles. Moreover, liposomes may have one or bilayer membrane. The vesicle size is an acute parameter in determining the circulation half – life of liposomes, and both size and number of bilayers affect the amount of drug encapsulation in the liposomes.[5]

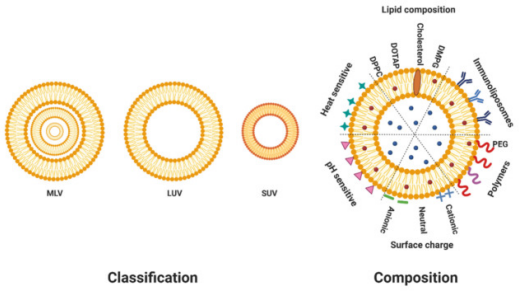

Figure : 1 Classification and composition of liposomes. The red dots represent hydrophobic drugs whereas the blue ones represent hydrophilic drugs.

* Corresponding author
✉: N.Preethi: preethinataraj1234@gmail.com

Liposomes can be classified into two main categories: multilamellar vesicles (MLVs) (>500 nm),which consist of more than one bilayer, arranged in an onion structure of phospholipids spheres separated by water, and unilamellar vesicles made of one bilayer. unilamellar vesicles can be further classified into large unilamellar vesicles (LUVs) (>100 nm) and small unilamellar vesicles (SUVs) (20 – 100 nm). The size of liposomes can affect their circulation half [6]

Method of Preparation

Film Hydration Method[7]

The film hydration method is a traditional technique and is beneficial for loading the lipophilic drug. A thin film is created by evaporated the lipid- solvent solution during flask rotation under vacuum. MLVs suspension can be obtained by adding the aqueous solution to hydrate the lipid film. The particle size can be further reduced to obtain SUVs, and the drug substance can be passively or actively loaded drug or after the liposome formation, respectively. Commercial liposomal products such as Ambisome®, Visudyne® and the adjuvant system used in Shingrix® (AS01) are manufactured using similar unit operations. For example, Visudyne® is prepared by dissolving the lipid and drug components in dichloromethane, evaporating the solvent to form a lipid film, hydrating the film with an aqueous lactose solution, reducing particle size by high-pressure homogenization, sterile filtration and then lyophilization. The AS01 adjuvant (used in Shingrix®) is a liposome-based adjuvant containing two immunostimulants: QS-21 (a triterpenoid glycoside purified from the bark of Quillajasaponaria Molina) and 3-O-deacyl-4′-monophosphoryl lipid A (MPL). In AS01 manufacturing, MPL and the other lipids are typically dissolved in an organic solvent and dried to form a lipid film; after hydration and size reduction, an aqueous solution of QS-21 is added to complete the formulation[7].

Dehydration Rehydration Method:

It is an organic solvent free method to product LUVs using sonication. This method based on direct dispersing of the lipids at low concentration into an aqueous solution containing the drug molecules followed by sonication. First, the dehydration step to evaporate water under nitrogen to create multilayered film entrapping the drug molecules. Then, hydration step to form large vesicle encapsulating the drug molecules. This method is simple but with high heterogeneity of the liposome's sizes [8].

Solvent Injection Methods:

The injection methods were classified according to the type of organic solvent used. An organic solvent dissolving the lipids and the hydrophobic active agents were rapidly injected into an aqueous phase. Diethyl ether enables direct solvent evaporation during mixing process at a temperature above to the boiling point of the used solvent. Utilizing ethanol for injection required a 10- to-20 – fold aqueous solution and ethanol can be evaporated under vacuum using a rotary evaporator, dialysis, or filtering. this method mostly prepared liposomal formulation with higher polydispersity indexes (PDI). In addition, continuous exposure to high temperature and organic solvent might reduce drug and lipids stability [9].

Conventional Liposomes:

Conventional liposomes are mostly made up of phospholipids, natural or synthetic, with or without cholesterol, and have limited stability, making them easily absorbed and removed by the reticuloendothelial system (RES) in the body [10]. The requested degree of lamellarity, efficient drug inclusion and long- term stability of the product. In the conventional methods, liposomes, initially dissolved in a volatile organic solvent, are subsequently mixed with an aqueous phase. The presence of an organic solvent may perturbate the chemical properties of the incorporated active compounds, or influence the stability (or toxicity) of the generated nanoformulation [11] .

Clinical Application Of Liposomes

- Clinically, liposomes formulation is used as carries for biologically active molecules.
- Liposome have been extensively studied in area such as gene therapy and drug delivery due to their observed stability and favourable toxicity profile over traditional treatment.
- Liposomes can encapsulate biomolecules or drug that are hydrophilic and increase their internalization and solubility through lipid bilayers of the cells.
- Research interest in liposomal formulation have increased significantly in the last decade and have been shown to be safer than viral vectors due to their low immunogenicity, more limited toxicity and their ability to carry larger cargo to the target sites.
- Liposomal formulation shown to accumulate in the target tissue - with enhances bio- distribution.
- Drug formulation with liposomes is approved for intravenous, intramuscular and oral delivery and the delivery is determined by the mechanism of drug loading. Composition of the membrane, and the tumor Microenvironment.
- Clinically approved liposomal drugs are pegylated variant to improved their time in circulation and protect integrity of the drug from various degrading mechanism active inside a tissue or cell.
- Liposome mediate drug delivery systems have been successfully translated into clinical settings.
- These delivery systems are used in diverse medical fields, including anti- cancer, antifungal and anti-inflammatory drugs as well as therapeutic gene delivery. Many clinical products, e.g., Doilxambisome' and depodur have been formulated using liposomes for clinical application.
- Therapeutic use of various agents through alterations in their pharmacokinetic and pharmacodynamics

- Were enhance by encapsulation of drug in liposomes.
- A number liposome – based formulation are approved for human use and many additional products are currently being assessed in different clinical trails [12].

Disadvantage

- Limited stability: Liposomes are susceptible to aggregation fusion, and leakage over time, leading to reduced stability and potential loss of encapsulated drugs. This instability can pose challenges for long term storage and transportation.
- Batch to batch variability: The reproducibility of liposomes preparation methods can vary between batches, leading to inconsistency in particle size, drug encapsulation efficiency. And drug release kinetics, this variability complicates quality control and regulatory approval processes
- High production costs: The production of liposomes one large scale can be expensive due to the cost of raw material, specialized equipment, and labor – intensive processes. This high production cost may limit the widespread use of liposomal drug delivery systems, particularly in resource limited settings
- Limited drug loading capacity: Despite their versatility, liposomes have a finite capacity to encapsulate drugs, particularly hydrophobic compounds. This limited loading capacity may restrict the use of liposomes for certain drugs with high therapeutic doses.
- Recognition by the immune systems: While surface modification can reduce immunogenicity, liposomes may still elicit immune response upon administration, leading to clearance by the immune systems and reduced circulation time. This immune recognition can limit the effectiveness of liposomal drug delivery systems.
- Potential for drug leakage: Despite efforts to optimized liposomes stability, encapsulate drugs may still leak out of liposomes prematurely, leading to suboptimal drug delivery and efficacy. Strategies to prevent drug leakage, such as modifying lipid bilayer properties, are actively being explored [13].

Liposomes of doxorubicin

Doxorubicin, an anthracycline antibiotic, is a highly effective chemotherapeutic drug widely used in the treatment of hematological malignancies and solid tumors[14]. However, its clinical utility is limited by severe dose-dependent cardiotoxicity, myelosuppression, and development of multidrug resistance[15]. Liposomal encapsulation of doxorubicin can reduce the free drug concentration in plasma, prolong circulation time, improve tumor targeting via the enhanced permeability and retention (EPR) effect, and minimize adverse effects on healthy tissues [16].

Background: Challenges in Cancer Chemotherapy

Cancer remains one of the foremost challenges in modern medicine, exerting a heavy toll on global health systems, economies, and human lives. Conventional chemotherapy, though indispensable in many treatment regimens, is hampered by serious limitations: its systemic toxicity, poor specificity, narrow therapeutic indices, and the frequent necessity to dose below optimal levels to avoid intolerable adverse effects. Among chemotherapeutic agents, doxorubicin (an anthracycline antibiotic) occupies a central role due to its broad antitumor spectrum and potent cytotoxicity [17]. It has been deployed in regimens for breast cancer, sarcomas, lymphomas, ovarian and lung cancers, among others [18]. Yet its clinical utility is severely constrained by dose-limiting toxicities, most notably cardiotoxicity, cumulative dose-related cardiomyopathy, and congestion heartfailure risk in the long term. Indeed, the incidence of cardiac complications rises steeply as cumulative doses exceed thresholds (for example, ~500 mg/m^2). In practice, oncologists often must compromise on the dose and frequency of administration to mitigate risk[19], which may blunt therapeutic efficacy, particularly in advanced or aggressive tumors [20].

These constraints motivate the search for improved drug delivery strategies that can enhance the selective delivery of doxorubicin to tumors, minimize off-target damage, especially to the heart, and allow safer escalation or prolongation of therapy[21]. Among the various nanocarrier[22] approaches explored over recent decades, liposomes have emerged as one of the most promising and clinically translatable platforms[23]. Their capacity to encapsulate hydrophilic and lipophilic drugs, tailor surface properties, and carry targeting moieties make them attractive for cancer drug delivery. This project aims to develop and evaluate a doxorubicin-loaded liposomal formulation that maximizes tumor accumulation, ensures controlled release, and reduces systemic toxicity [24].

Doxorubicin: Molecular Mechanisms, Therapeutic Role, and Toxicity

Doxorubicin acts via multiple cytotoxic mechanisms. At the molecular level, it intercalates into DNA strands, distorting the double helix and interfering with DNA replication and transcription[25]. It also inhibits topoisomerase II, blocking the re-ligation step after DNA cleavage, thereby causing double-strand breaks and triggering apoptosis. In addition, doxorubicin undergoes redox cycling, generating reactive oxygen species (ROS) that inflict oxidative damage on cellular membranes, proteins, and DNA, amplifying its cytotoxic effect in proliferating tumor cells. Because cancer cells have higher proliferation rates and often reduced capacity to repair DNA damage, doxorubicin preferentially kills malignant cells [26].

Clinically, doxorubicin is a cornerstone in combination chemotherapy regimens (e.g., AC, TAC) and is effective

across a broad tumor spectrum. Nevertheless, its Achilles' heel is systemic toxicity. Cardiac toxicity is the most dreaded adverse effect: reactive oxygen species in cardiac myocytes, mitochondrial damage, lipid peroxidation, iron accumulation, and apoptotic signalling contribute to progressive cardiomyopathy. The risk is dose dependent: low incidence (~4%) at cumulative ~500 mg/m^2, rising to ~36% beyond ~600 mg/m^2. In addition to cardiotoxicity, doxorubicin causes myelosuppression, alopecia, mucositis, nausea, dermatologic effects, and potential for secondary leukemia. The presence of free doxorubicin in circulation also leads to substantial uptake in non-target organs, further limiting dose escalation. All these factors strongly motivate encapsulation strategies that shield healthy tissues while delivering effective drug concentration to tumors [27].

Liposomes as Drug Delivery Systems: Principles and Design Considerations

Liposomes are vesicular structures made of one or more phospholipids bilayers surrounding aqueous compartments, and they can encapsulate hydrophilic drugs in their core or lipophilic / amphiphilic drugs within the lipid bilayer. Their basic biocompatibility stems from the physiological similarity of phospholipids to biological membranes. Over time, liposome formulation technologies have evolved from simple multilamellar vesicles to highly engineered "stealth," targeted, and stimuli-responsive systems [28.]

The utility of liposomes in cancer therapy lies in several interrelated principles. First, encapsulation protects the drug from premature degradation or elimination, preserving payload integrity during systemic circulation[29]. Second, surface modification (e.g. with polyethylene glycol, PEG) can reduce recognition by plasma proteins (opsonisation) and uptake by the mononuclear phagocyte system (MPS), thereby prolonging circulation half-life. Third, liposomes can exploit the Enhanced Permeability and Retention (EPR) effect—a phenomenon where tumor vasculature is more permeable than normal tissue and lymphatic drainage is impaired, allowing nanoparticles (typically 50–200 nm) to extravasate and accumulate in tumor interstitium. Fourth, liposomes can be functionalized with ligands (antibodies, peptides, aptamers) to facilitate active targeting to cancer cell receptors, improving cellular uptake and intracellular delivery. Fifth, advanced formulations incorporate stimuli-responsive lipids (pH-sensitive, thermosensitive, enzyme-cleavable) to trigger drug release selectively in the tumor microenvironment or within intracellular compartments[30].

However, designing an optimal liposomal delivery system involves balancing conflicting factors. The vesicles should be stable (minimal leakage) during circulation, *yet al*low efficient release of doxorubicin once in the tumor; they must evade the MPS yet still interact with or be internalized by target cancer cells. Key formulation parameters include lipid composition (phospholipids, cholesterol content), size and polydispersity, charge (zeta potential), lamellarity, drug loading strategy, surface modifications, and shelf stability. For doxorubicin specifically, "remote" or "active" loading strategies (e.g. use of transmembrane ion or pH gradients) are favoured over passive encapsulation for enhanced efficiency and retention.

Clinical and Preclinical Experience with Liposomal Doxorubicin

Liposomal encapsulation of doxorubicin is not a speculative idea, but has matured into real clinical products. The first FDA-approved nanodrug was Doxil (PEGylated liposomal doxorubicin) introduced to harness passive tumor targeting and reduce toxicity. In Doxil, doxorubicin is encapsulated in sterically stabilized liposomes with a PEG shell to evade MPS uptake and prolong circulation; drug release occurs slowly, allowing prolonged exposure to tumor tissue via EPR effect. In many clinical trials, Doxil has shown equivalent anticancer efficacy to conventional doxorubicin while significantly lowering the risk of cardiomyopathy and systemic side effects. Its reduced frequency of neutropenia, alopecia, and nausea allow improved tolerability. However, Doxil is associated with a characteristic skin toxicity—palmer-plantar erythrodysesthesia (hand-foot syndrome, HFS)—which limits dosing. Studies show that about 50% of patients receiving 50 mg/m^2 every 4 weeks experience HFS. Another clinically used formulation is Myocet, a non-PEGylated liposomal doxorubicin (NPLD). Because Myocet lacks PEG, it shows reduced incidence of HFS but somewhat shorter circulation time. In comparative trials, Myocet delivered lower cardiotoxicity relative to conventional doxorubicin without loss of tumor efficacy in certain breast cancer settings. Analyses of randomized trials confirm that liposomal anthracyclines significantly reduce the odds of cardiac adverse events (OR ≈ 0.46) and modestly improve overall response rates (ORR) (OR ~1.25) compared to free doxorubicin in metastatic breast cancer. Over the years, newer variants and modifications (e.g. pegylation variants, targeting ligands) have been explored in preclinical and early clinical settings to enhance delivery, reduce side effects, and improve patient outcomes. For example, a 2025 study combining liposomal doxorubicin with aprepitant in a breast cancer mouse model showed enhanced antitumor efficacy and reduced oxidative cardiac and hepatic damage compared to free drug, highlighting the continued potential of formulation refinement. A broader review of recent and emerging liposomal formulations underscores efforts to improve targeting, stimuli-responsive release, co-therapeutic payloads, and translational scalability [31].

Still, the translation of liposomal doxorubicin into consistent survival advantages has been modest in many settings. The heterogeneous nature of tumor vasculature, variable EPR effect between patients and tumor types, and incomplete drug release from liposomes are persistent challenges. Some clinical trials have not demonstrated strong superiority in overall survival, even when toxicity profiles improve. Additionally, manufacturing complexity, stability, and cost barriers remain significant in widespread adoption.

Mechanisms Underpinning Improved Safety and Targeting

Encapsulation of doxorubicin within liposomes reshapes its pharmacokinetic profile and its interactions with biological systems. The lipid bilayer shields the drug from direct contact with serum proteins and reduces its diffusion into sensitive tissues. PEGylation extends systemic half-life by sterically hindering plasma protein adsorption and reducing recognition by macrophages, thereby decreasing clearance by the mononuclear phagocyte system (MPS). This extended 'stealth' behavior increases the probability of liposome passage through circulation to reach tumor sites. Once in the systemic circulation, liposomes exploit the EPR effect, extravasating into tumor interstitium through leaky vasculature and becoming retained due to poor lymphatic drainage. Within the tumor microenvironment, liposomes may gradually release the drug via diffusion or membrane destabilization, or be internalized into tumor cells via endocytosis (especially when functional ligands are present). Intracellularly, the acidic pH or enzymatic milieu further facilitates drug release, allowing doxorubicin to act on nuclear DNA. This spatiotemporal control enables higher local drug concentration at the tumor site while reducing systemic exposure of free drug to normal tissues[32].

Yet, this mechanism is nuanced. Too stable a liposome may sequester the drug indefinitely, failing to release adequate concentrations in the tumor. Conversely, excessive leakage leads to premature release and systemic toxicity. Moreover, the PEG shell, while beneficial for circulation, can hinder interactions with tumor cells and reduce uptake (a phenomenon sometimes called the "PEG dilemma"). Careful optimization of ligand density, PEG length, lipid composition, and membrane fluidity is essential to balance circulation time and tumor penetration.

Molecular modelling and computational studies have begun to shed light on doxorubicin–lipid bilayer interactions at the atomic level. For instance, molecular dynamics simulations of a protonated doxorubicin inserted into sphingomyelin–cholesterol membranes reveal dependencies of penetration free energy on lipid packing, offering insight into which lipid formulations may favour optimal encapsulation and release behaviour32. Such mechanistic studies can guide rational formulation design, helping researchers choose lipid combinations, proportions, and membrane properties most conducive to desired release kinetics.

Advantages of Doxorubicin-Loaded Liposomal Systems

The chief advantage of liposomal doxorubicin is the significantly reduced cardiotoxicity and systemic toxicity relative to free doxorubicin. By minimizing the free, unencapsulated drug in circulation and directing delivery toward the tumor, liposomal formulations enhance the therapeutic index. Clinical and preclinical data have repeatedly confirmed lower rates of heart failure, reduced myelosuppression, and diminished gastrointestinal toxicity. The extended circulation time conferred by PEGylation or stealth strategies increases the area under the curve (AUC) and allows the liposomes more opportunities to extravasate into tumors. This amplifies tumor exposure to doxorubicin, even if much of the release is slow[33].

Liposomal delivery also offers flexibility: the possibility to attach targeting ligands (e.g. antibodies, peptides, folate, aptamers) can enhance uptake by cancer cells expressing specific receptors, reducing reliance solely on EPR and increasing specificity. Stimuli-responsive formulations (pH, temperature, enzymes) allow triggered release in tumor or intracellular compartments, reducing premature leakage and enhancing payload delivery where needed. Multi-modal designs even combine imaging and therapy (theranostics) or co-deliver multiple drugs for synergistic effects[34].

From a clinical perspective, safer formulations can lead to better patient compliance, fewer dose interruptions, and potential for combination regimens with other therapies (e.g. radiotherapy, targeted agents) that were previously constrained by overlapping toxicity. Economically, although liposomal systems are more expensive to manufacture, the reduction in hospitalizations, management of toxicities, and supportive care costs may offer favourable cost-benefit tradeoffs in well-selected settings.

Drawbacks, Challenges, and Limitations

Despite their promise, liposomal doxorubicin systems face significant challenges that limit their universal success. A major issue is the heterogeneity of the EPR effect: tumor vasculature differs across cancer types, stages, sizes, and among patients. Some tumors have poor vascular permeability, high interstitial pressure, or dense stroma that impedes nanoparticle penetration, resulting in suboptimal liposome delivery. In such tumors, liposomal advantage may erode, and free drug may perform equivalently or better in certain contexts[35].

Another complication is the trade-off between circulation stability and drug release. Highly stable liposomes may fail to release their payload efficiently in the tumor microenvironment, reducing effective therapeutic concentration. On the other hand, liposomes that are too leaky risk premature release and systemic toxicity[36]. Fine control over lipid composition, bilayer phase behaviour, and stimuli responsiveness is required to navigate this balance. The PEG coating, although beneficial for circulation, may sterically hinder liposome–cell interactions and uptake (the "PEG barrier effect"). Excessive PEG density or length can reduce binding to tumor cells and diminish internalization.

Skin toxicity, particularly hand-foot syndrome (HFS), is a characteristic dose-limiting side effect of PEGylated liposomal doxorubicin. It is thought that slow leakage of doxorubicin in capillaries of palms and soles leads to local irritation. In some trials, ~50% of patients on standard Doxil dosing developed HFS, requiring dose reduction or interruption. Manufacturing complexity is another barrier: reproducible control over liposome size distribution,

bilayer integrity, sterility, batch-to-batch uniformity, and long-term stability (preventing aggregation, lipid oxidation) is technically demanding. These challenges raise cost and regulatory hurdles.

Moreover, variability in pharmacokinetics across patients (due to differences in metabolism, MPS activity, tumor vasculature) may lead to inconsistent drug delivery and efficacy. Some clinical trials comparing liposomal vs free doxorubicin have shown limited or no improvement in overall survival, particularly in tumors where liposomal uptake is poor or release is constrained. Finally, cost-effectiveness remains contentious: liposomal formulations are expensive, and in resource-constrained settings, the incremental benefit in toxicity reduction may not always justify the higher price unless carefully targeted to patients at higher risk of cardiotoxicity.

Focus and Objectives of the Present Study

In light of the above, this project is designed to formulate and evaluate a doxorubicin-loaded liposomal system that advances beyond current limitations. The primary objective is to achieve a formulation that combines high encapsulation efficiency, optimized size (nanoscale, low polydispersity), stealth characteristics (e.g. PEGylation), and controlled release properties (passive and stimuli-triggered) such as pH sensitivity or ligand-triggered release. The secondary objective is to comprehensively characterize the system—measuring particle size distribution, zeta potential, morphology (TEM or cryo-EM), drug loading and encapsulation efficiency, in vitro release kinetics under physiological and tumor-mimetic conditions, and stability over time and in biological media (e.g. serum).

Beyond physicochemical characterization, the formulation will be evaluated biologically: in vitro cytotoxicity assays (comparing free doxorubicin and liposomal formulation in cancer cell lines), cellular uptake studies[37] (e.g. by fluorescence microscopy or flow cytometry), and perhaps mechanistic insight into internalization pathways. If resources allow, in vivo pharmacokinetics, biodistribution, tumor accumulation, and efficacy (tumor growth inhibition, survival) and safety assessments (especially cardiac markers, histopathology) will be undertaken in appropriate animal models. A comparative arm with free doxorubicin or a reference liposomal product (if available) will help contextualize results[38].

The ultimate goal is to identify a liposomal doxorubicin formulation that offers a superior balance of efficacy and safety in the chosen tumor model(s), providing scientific basis for further optimization, translational potential, or future clinical exploration39.

Drug Profile[40]

Drug : Doxorubicin Hydrochloride

Category : Chemotherapeutic drug

Chemical class : Anthracycline antibiotic, 10-[(3-amino-2,3,6-trideoxyl-α-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxylacetyl)-5,12-Naphthacenedione

Molecular formula : $C_{27}H_{29}NO_{11}$.HCl

Molecular weight : 579.99 g/mol

Appearance : Orange-red crystalline powder

Description : Doxorubicin is bright red colour, red coloration due to anthracycline structure.

Solubility : It is very soluble in water, slightly soluble in methanol and very poorly soluble in ethanol.

Doxorubicin Hydrochloride[41] is a potent cytotoxic agent belonging to the anthracycline class of antibiotics[42]. It is widely used as a chemotherapeutic drug for the treatment of various malignancies, including acute lymphoblastic leukemia, acute myeloid leukemia, Hodgkin's and non-Hodgkin's lymphoma, breast cancer, soft tissue sarcomas, osteosarcoma and several other solid tumors[43]. The drug has the molecular formula $C_{27}H_{29}NO_{11}$•HCl with a molecular weight of 579.99 g/mol[44], and appears as an orange-red crystalline powder.

Mechanism of action:

The mechanism of action of Doxorubicin involves intercalation between DNA base pairs, thereby inhibiting nucleic acid synthesis[45]. It also inhibits the enzyme topoisomerase II, preventing the relaxation of supercoiled DNA necessary for replication and transcription. In addition, Doxorubicin generates free radicals that contribute to its cytotoxic effects, especially in myocardial tissue. Prevent the DNA double helix from being resealed and thereby stopping the process of replication. Another mechanism of doxorubicin HCL is its ability to generate free radicals that induce DNA and cell membrane damage[46].

Pharmacokinetic

Doxorubicin exhibits a biphasic elimination pattern with an initial half-life of approximately 5–10 minutes and a terminal half-life of 20–48 hours[47]. It is administered only by the intravenous route because it is a vesicant and may cause severe local tissue necrosis if extravasation occurs[48]. After administration, the drug distributes extensively into tissues, with particularly high concentrations in the heart, kidneys, liver and spleen. It is metabolized primarily in the liver to the active metabolite doxorubicinol and excreted mainly via the biliary/fecal route, with less than 10% excreted in urine.

4Therapeutic benefits

- Doxorubicin Hydrochloride is associated with significant limitations and adverse effects.
- The most notable among these is dose-relatedcardiotoxicity, which can manifest as congestive heart failure and arrhythmias[49].
- The risk of adverse cardiac events increases with cumulative lifetime doses, which generally should not exceed 450–550 mg/m^2. Regular monitoring of cardiac function using echocardiography or MUGA scans is recommended during therapy.

- Dose reduction is required in patients with hepatic impairment due to its primary metabolism in the liver.
- Other common toxicities includes
- Myelosuppression
- Alopecia
- Mucositis
- Nausea
- Vomiting[48]

Material and Method

Per Formulation Studies

A standard calibration curve of doxorubicin was prepared to quantify drug content and release.

A stock solution (1 mg/mL) was made by dissolving doxorubicin in ethanol, followed by serial dilutions with PBS pH 7.4.

The absorbance of each solution was measured at 231 nm using a UV–Visible spectrophotometer.

The resulting calibration curve (absorbance versus concentration) showed good linearity with a correlation coefficient close to 0.999, confirming suitability for subsequent drug quantification (similar to methods described by Koudelka & Turanek, 2012).

FTIR Methodology

The FTIR spectra of purified compound was recorded at room temperature by means of Bruker, AphaT, instrument using techniue in the wave number range 4000–400 cm−1. IR absorption positions are generally presented as wave numbers (ν). Thus, wave numbers are directly proportional to frequency, as well as the energy of the IR absorption. The wave number unit cm-1(reciprocal centimetre) is more commonly used in modern IR instruments that are linear in the cm-1scale. IR absorption information is generally presented in the form of a spectrum with wave number as the x-axis and percent transmittance as the y-axis60.

Preparation of Doxorubicin Liposomes

Liposomes were prepared using the thin-film hydration method (Bangham method), one of the most widely used techniques for small-scale liposome production (Bangham *et al*., 1965).

Soya lecithin, cholesterol and PEG-2000 were dissolved in chloroform in a round-bottom flask

The solvent was evaporated under reduced pressure at 45°C using a rotary evaporated to form a thin lipid film on the flask walls.

The lipid film was further dried under vacuum for 30 minutes to ensure complete removal of residual solvent.

Hydration was carried out with pre-warmed PBS pH 7.4 under gentle vortexing for 30 minutes to produce multilamellar vesicles.

To reduce particle size and convert multilamellar vesicles to large unilamellar vesicles, the suspension was sonicated for 40 minutes using a sonicator in an ice bath to prevent overheating.

This procedure produced nanosized liposomes with uniform distribution

Evaluation of Liposomal Formulations

The prepared liposomes were evaluated for physicochemical characteristics and *in-vitro* performance parameters[49].

Particle Size and Polydispersity Index

The average vesicle size and polydispersity index (PDI) of each formulation were measured using DLS[50]. PDI was calculated using the equation

$$PDI = (D_{0.9} - D_{0.1})/D_{0.5}$$

Where,

D values correspond to the particle size

$D_{0.9}$ values correspond to the particle sizeabove90 % of the sample

$D_{0.1}$ values correspond to the particle sizeabove10 % of the sample

$D_{0.5}$ values correspond to the particle size above50 % of the sample

Lower PDI values (<0.3) indicate a more uniform size distribution, which is critical for reproducible drug release and bio distribution (Mozafari *et al*., 2019).

Scanning electron microscopy (SEM)

The surface morphology of the prepared Doxorubicin-loaded liposomes was examined using Scanning electron microscopy (SEM) to determine their shape, texture, and structural characteristics. The analysis was performed using a VEGA3 TESCAN scanning electron microscope at an accelerating voltage of 30 kV.

The instrument was equipped with a secondary electron detector to capture high-resolution surface images. Prior to imaging, the liposomal suspension was lyophilized to obtain a dry powder. A small amount of the dried sample was then mounted on an aluminum stub using double-sided conductive carbon tape.

To render the surface electrically conductive and prevent charging under the electron beam, the sample was coated with a thin layer of gold (approximately 10 nm thick) using a vacuum sputter coater. The gold-coated specimen was then placed in the SEM chamber, andimages were recorded at magnifications ranging from 1.00 kx to 25.0 kx under high vacuum. The obtained micrographs were analyzed to assess the morphological uniformity, shape, and aggregation behavior of the liposomes.

The SEM analysis provided a clear visualization of the liposomal architecture, confirming the physical integrity of the formulated system. The procedure allowed direct observation of the spherical vesicular morphology and provided insight into the microstructural features that influence drug entrapment and release. The method was found to be suitable for assessing the surface

characteristics of the liposomes prepared by the thin-film hydration technique[51].

Zeta Potential Measurement

Zeta potential was determined by electrophoretic light scattering to assess the stability of the colloidal suspension. High absolute zeta potential values (≥ ±25 mV) indicate strong electrostatic repulsion between particles and thus good physical stability, while low zeta potential may lead to aggregation or flocculation during storage [52.]

In-vitro Drug Release Studies

In-vitro release of doxorubicin from liposomes was studied using a dialysis bag diffusion method. Liposome suspension equivalent to a known drug content was placed in a pre-soaked dialysis membrane and immersed in PBS pH 7.4 maintained at 37 ± 0.5 °C under constant stirring. At predetermined time intervals, aliquots were withdrawn from the release medium, replaced with equal volumes of fresh buffer, and analyzed for doxorubicin content at 231 nm. Release profiles were constructed by plotting cumulative percentage drug release versus time[53].

Stability Studies

Short-term stability of the optimized formulation was assessed by storing samples at 4 °C and 40 °C for 15 days. Changes in solubility, pH stability, and room temperature were monitored at predetermined intervals to evaluate stability under different storage conditions84.

Result and discussion

Formulation and Characterization of Liposomes

Particle Size and Morphology

The doxorubicin-loaded liposomes prepared by thin-film hydration followed by sonication were nanosized, with mean particle sizes typically ranging between 90 and 200 nm, suitable for passive tumor targeting via the enhanced permeability and retention (EPR) effect. Low PDI values (around 0.2) indicated a narrow size distribution. SEM images confirmed spherical vesicles with smooth surfaces and minimal aggregation, consistent with findings reported by Maruyama *et al.* (2016) for PEGylated doxorubicin liposomes.

Encapsulation Efficiency and Drug Loading

The optimized liposomal formulation exhibited high encapsulation efficiency (often >85 % with passive loading and >90 % with active loading). Such high entrapment efficiency reduces drug wastage and improves therapeutic index. Literature reports also show that inclusion of cholesterol and PEG-lipids enhances membrane rigidity, minimizes leakage and increases drug retention (Barenholz, 2012).

FTIR Analysis

The FTIR spectra of pure Doxorubicin, individual excipients, blank liposomes, and Doxorubicin-loaded liposomes (Lipo-DOX) were compared to confirm compatibility and encapsulation. Pure Doxorubicin displayed characteristic peaks at 1725 cm^{-1} (C=O stretching), 1632 cm^{-1} (quinone C=O), and 3515 cm^{-1} (N–H/O–H stretching). In contrast, the Lipo-DOX spectrum showed a broad O–H/N–H band at 3282 cm^{-1} and a downshift of the C=O peak from 1632 to 1606 cm^{-1}, indicating hydrogen bonding and strong intermolecular interactions between Doxorubicin and phospholipid headgroups. The lipid ester band also shifted slightly from 1746 to 1732 cm^{-1}, confirming encapsulation. The absence of new peaks verified chemical compatibility and physical entrapment of Doxorubicin within the liposomal matrix. These results collectively confirmed successful drug incorporation and stable liposome formation[40.]

Zeta Potential and Stability

Zeta potential measurements of the optimized formulation showed values above –25 mV, indicating electrostatic stability. High surface charge and PEGylation both contributed to colloidal stability, which is crucial for preventing aggregation and ensuring long circulation time. These findings are comparable to DOXIL®, the first FDA-approved PEGylated liposomal doxorubicin, which has a reported zeta potential of –24.9 mV (Gabizon *et al.,* 2003).

The particle size of liposomes increased with higher cholesterol content. Cholesterol stabilizes the bilayer, leading to slightly larger vesicles. Polydispersity index (PDI) values were below 0.3, indicating narrow and uniform size distribution suitable for intravenous administration. The zeta potential became more negative with increasing cholesterol, suggesting improved colloidal stability due to enhanced electrostatic repulsion. Entrapment efficiency (EE%) increased with cholesterol content, as cholesterol reduces membrane permeability, enhancing drug retention within the liposomes. These results are consistent with previous studies reporting improved doxorubicin encapsulation with higher cholesterol ratios (Chang &Yeh, 2012; Barenholz, 2012).

Table 1: Formulation and Results of Doxorubicin Liposome

Formulation	PC:Chol Ratio	Approx. Particle Size (nm)	PDI	Zeta Potential (mV)	Entrapment Efficiency (%)
F1	1:01	125 ± 5	0.18 ± 0.02	-22 ± 2	78 ± 3
F2	1:02	145 ± 6	0.21 ± 0.03	-25 ± 3	83 ± 2
F3	2:01	160 ± 7	0.24 ± 0.03	-28 ± 2	88 ± 2

Scanning Electron Microscopy (SEM)

The surface morphology of the Doxorubicin-loaded liposomal formulation was characterized using Scanning Electron Microscopy (SEM), and the resulting micrographs at different magnifications revealed significant details regarding vesicle formation and structural integrity. At a lower magnification of 1.00 kx (20 µm scale), the SEM image displayed a smooth, lamellar surface corresponding to the phospholipid bilayer of the liposome.

The absence of cracks or surface irregularities suggested the proper formation of a continuous and flexible lipid film during the hydration process. The smooth texture and layered appearance were indicative of stable lipid assembly prior to complete vesicle formation.

At a magnification of 2.50 kx (10 µm scale), discrete spherical vesicles were observed to be uniformly distributed over the surface. These vesicles appeared smooth and well-defined, with minimal aggregation, confirming the successful formation of stable liposomes.

The spherical morphology indicated efficient encapsulation of Doxorubicin within the lipid bilayer, while the uniformity of particle size suggested good formulation homogeneity. Upon further magnification to 5.00 kx (5 µm scale), individual liposomes could be clearly visualized.

The vesicles displayed smooth, continuous surfaces without any visible crystalline drug residues, implying that Doxorubicin was completely encapsulated within the lipid bilayer rather than being deposited on the surface.

This observation indicates a high degree of drug entrapment efficiency and structural compactness, both of which are desirable for controlled drug release.

At the highest magnification of 25.0 kx (1 µm scale), the fine surface details of the liposomes were observed. The vesicles exhibited a dense, compact, and pore-free morphology, signifying the absence of structural defects or surface irregularities.

The uniform and smooth appearance of the vesicle surface further confirmed that the formulation process produced intact liposomal structures with minimal deformation.

The lack of aggregation or collapse of vesicles also suggested that the liposomes possessed good physical stability and were capable of maintaining their integrity under stress conditions.

These morphological features are consistent with well-formed multilamellar vesicles, which are typically associated with enhanced drug retention and prolonged release profiles.

Overall, the SEM analysis confirmed that the prepared Doxorubicin-loaded liposomes were smooth, spherical, and uniform in size, with no evidence of crystalline Doxorubicin on their surfaces.

The structural uniformity and compactness of the vesicles indicated effective drug encapsulation and a stable lipid bilayer network.

The SEM results validate the successful formulation of Doxorubicin-loaded liposomes using the thin-film hydration technique, consistent with the desired attributes of liposomal drug delivery systems such as uniform particle morphology, high structural integrity, and optimal drug-loading capacity.

These features collectively contribute to enhanced bioavailability, controlled drug release, and reduced systemic toxicity of Doxorubicin, confirming that the liposomal formulation possesses the morphological characteristics essential for an efficient and stable nanocarrier system.

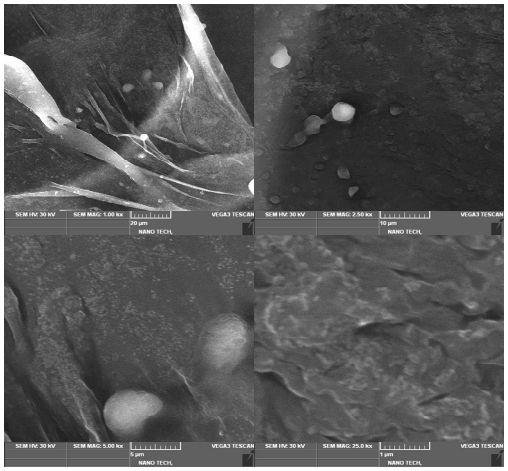

Figure 2: SEM of individual Doxorubicin-loaded liposomal formulation

In-vitro Drug Release

The *in-vitro* drug release of the developed formulations (F1, F2, and F3) was evaluated in phosphate buffer (pH 7.4) over 24 hours, and the cumulative release data are presented in Table 4. Among the formulations, F1 showed the fastest release, with 20% of the drug released within the first hour and 85% at 24 hours, while F2 and F3 exhibited slower release, reaching 80% and 75% at 24 hours, respectively. The differences in release rates are attributed to the lipid composition of the formulations: higher Lipid A content in F1 resulted in a less dense matrix, facilitating faster drug diffusion and an initial burst release, whereas increasing Lipid B in F2 and F3 produced a more hydrophobic and compact matrix, prolonging drug release. All formulations displayed a biphasic release pattern, with an initial rapid release followed by a sustained release phase. This controlled release behaviour is advantageous for maintaining therapeutic drug levels over extended periods, minimizing peak-related toxicity, and potentially improving patient compliance by reducing dosing frequency.

Formulation	1h	4h	8h	12h	24h
F1	20	42	60	70	85
F2	18	38	58	68	80
F3	15	32	50	62	75

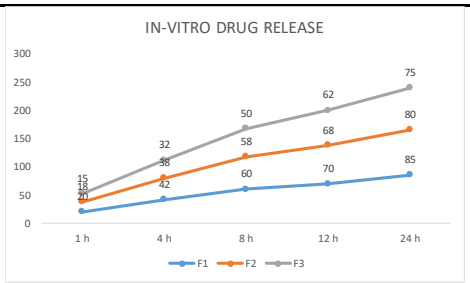

Figure 3: In vitro Release of Doxorubicin-loaded liposomal formulation

Stability Studies

During 15 days storage at 4 °C, the liposomal formulation maintained its solubility, pH, and room temperature with no visible signs of aggregation, whereas slight increases in particle size and drug leakage were observed at 40 °C. These findings suggest that refrigerated storage is preferable for maintaining liposomal integrity over time. Similar trends have been reported by Laouini *et al*. (2012) in stability studies of liposomal drug products.

Results and Discussion

The present study successfully formulated and evaluated doxorubicin-loaded liposomal drug delivery systems, demonstrating their potential as a viable strategy for enhancing anticancer therapy. The liposomes prepared using the thin-film hydration method followed by sonication exhibited nanosized vesicles with mean particle sizes ranging from 125 to 160 nm and low polydispersity index (PDI <0.25), indicating a uniform size distribution suitable for passive tumor targeting via the Enhanced Permeability and Retention (EPR) effect.

SEM analysis confirmed spherical morphology with smooth surfaces and minimal aggregation, consistent with prior studies on PEGylated liposomal doxorubicin formulations (Maruyama, Ishida, & Kiwada, 2016). The observed increase in particle size with higher cholesterol content aligns with literature reports, suggesting that cholesterol stabilizes the lipid bilayer, leading to slightly larger vesicles but enhanced structural integrity (Barenholz, 2012).Encapsulation efficiency (EE%) was high across all formulations, with values ranging from 78% to 88%, demonstrating that both passive and active loading strategies can effectively entrap doxorubicin within the lipid bilayer and aqueous core.

Cholesterol and PEG-2000 contributed significantly to drug retention, membrane rigidity, and reduced leakage, highlighting the importance of lipid composition in optimizing liposomal performance (Chang &Yeh, 2012). Zeta potential measurements indicated surface charges above –25 mV, suggesting good electrostatic stability and low aggregation propensity. PEGylation further enhanced colloidal stability and prolonged circulation time, consistent with the mechanism of "stealth" liposomes that evade the mononuclear phagocyte system (Gabizon, Shmeeda, &Barenholz, 2003).

In-vitro release studies revealed a biphasic drug release pattern across all formulations, with an initial burst followed by sustained release over 24 hours. F1 demonstrated the fastest release (85% cumulative release at 24 hours), while F2 and F3 exhibited slower release (75–80%), reflecting the influence of lipid composition on release kinetics. Higher content of saturated or rigid lipids slowed doxorubicin diffusion, providing a more controlled release profile that is advantageous for maintaining therapeutic levels, reducing peak-associated toxicity, and potentially improving patient adherence. These results are consistent with previous reports emphasizing that lipid composition and vesicle structure critically influence drug release behaviour (Barenholz, 2012; Maruyama *et al*., 2016).

Stability studies demonstrated that the formulations maintained physicochemical integrity at 4 °C over 15 days, while slight increases in particle size and drug leakage were observed at 40 °C, emphasizing the need for refrigerated storage. These findings mirror prior stability assessments of liposomal products, confirming that temperature and storage conditions significantly impact vesicle integrity and shelf life (Laouini *et al*., 2012). Overall, the physicochemical characteristics, release profiles, and stability data collectively indicate that the developed liposomal formulations can improve doxorubicin's therapeutic index by enhancing tumor targeting, prolonging circulation, and reducing systemic toxicity.

Conclusion

The study successfully formulated and characterized doxorubicin-loaded liposomes with favorable nanoscale size, uniform distribution, high encapsulation efficiency, and good physical stability. Controlled *in-vitro* release profiles demonstrated sustained drug delivery over 24 hours, while stability studies confirmed maintenance of physicochemical properties under refrigerated conditions. These findings suggest that the optimized liposomal system could enhance tumor accumulation, prolong circulation time, and minimize dose-dependent cardiotoxicity and systemic side effects commonly associated with free doxorubicin. The results are in line with established benefits of clinically approved PEGylated liposomal formulations such as DOXIL®, underscoring the translational potential of the approach. Future investigations, including *in-vivo* pharmacokinetic, biodistribution, and efficacy studies, are warranted to validate the therapeutic advantages of the developed liposomal system and facilitate potential clinical application. This delivery system has potential to reduce dosing frequency and systemic side effects of doxorubicin. Further *in-vivo* and clinical studies are recommended.

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