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RESEARCH ARTICLE

Targeted Delivery of Paclitaxel Using Folic Acid-Conjugated Nanoliposomes for Enhanced Breast Cancer Therapy

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Abstract

Background: Targeted drug delivery systems have gained increasing attention as an effective approach to improve therapeutic efficacy while reducing systemic toxicity in cancer treatment. In this study, folic acid-conjugated nanoliposomes [FA-NLs] were engineered as an active tumor-targeting nanocarrier for the selective delivery of paclitaxel to folate receptor–overexpressing breast cancer cells. **Materials and Methods:** FA-NLs were prepared using the thin-film hydration technique and loaded with paclitaxel. The nanocarriers were comprehensively characterized by SEM, TEM, and DLS to evaluate morphology, particle size, and zeta potential. Encapsulation efficiency and in vitro drug release were assessed under physiological and acidic conditions. Cytotoxicity, cellular uptake, and apoptosis were analyzed using the MTT assay, fluorescence microscopy, and Annexin V/PI flow cytometry, respectively. **Results:** The optimized FA-NLs exhibited uniform spherical morphology, nanoscale size (< 210 nm), and favorable surface charge, ensuring colloidal stability. The formulation achieved high drug-loading capacity and sustained, pH-responsive release behavior. Compared with free paclitaxel and non-targeted liposomes, FA-NLs significantly enhanced intracellular accumulation and cytotoxic efficacy in MCF-7 cells. Flow cytometric analysis further demonstrated markedly elevated apoptosis induction, confirming improved therapeutic performance. **Conclusion:** Collectively, FA-conjugated nanoliposomes enabled receptor-mediated targeting, efficient cellular internalization, and controlled intracellular drug release, resulting in superior anticancer activity. These findings underscore the potential of FA-NLs as a promising precision nanomedicine platform for targeted breast cancer therapy.

Keywords: Nanoparticles- Drug delivery system- Breast cancer- Apoptosis- Paclitaxel

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Introduction

Breast cancer continues to represent one of the most prevalent malignancies and a leading cause of cancer-related mortality among women worldwide, posing a persistent clinical and socioeconomic burden despite improvements in screening and therapeutic modalities [1]. Although chemotherapy remains a fundamental component of treatment, its overall effectiveness is frequently limited by inadequate tumor selectivity, widespread systemic exposure, and dose-dependent toxicities [2]. Such drawbacks largely stem from the indiscriminate distribution of cytotoxic drugs, which damage healthy tissues alongside cancer cells, thereby diminishing therapeutic benefit and negatively affecting patient quality of life [2]. Paclitaxel, a widely used microtubule-stabilizing chemotherapeutic drug, has demonstrated potent antitumor activity against various solid tumors, including breast cancer [3]. Nevertheless, its clinical performance is constrained by poor water solubility, rapid elimination, multidrug resistance, and significant adverse effects, including neurotoxicity and hematological complications [4]. These limitations restrict optimal dosing and frequently compromise therapeutic outcomes [5]. Moreover, conventional solvent-based paclitaxel formulations are associated with hypersensitivity reactions, short circulation half-life, and low tumor bioavailability, often necessitating repeated high-dose administration [6]. As a result, considerable research efforts have focused on designing delivery strategies that enhance solubility, extend systemic circulation, and improve tumor-specific drug accumulation [7]. Nanotechnology-based drug delivery systems have emerged as promising tools to address these challenges by enabling controlled release, improved pharmacokinetics, and preferential tumor accumulation via the enhanced permeability and retention (EPR) effect [7]. Among the various nanoscale carriers, liposomes have gained particular attention due to their excellent biocompatibility, structural flexibility, and capacity to encapsulate both hydrophilic and hydrophobic therapeutics [8]. Furthermore, liposomal encapsulation can protect labile drugs, modulate biodistribution, and mitigate systemic toxicity, ultimately enhancing the therapeutic index of conventional chemotherapeutics [2]. However, many previously reported liposomal formulations still suffer from practical limitations such as low encapsulation efficiency, premature drug leakage during storage or circulation, physicochemical instability under physiological pH conditions, and uncontrolled burst release, which collectively reduce therapeutic consistency and limit clinical translation [9]. Beyond passive targeting, active targeting strategies have been introduced to further enhance tumor selectivity [7]. To further refine tumor specificity, ligand-mediated active targeting approaches have been developed to promote selective cellular recognition and uptake [10]. Folate receptor alpha (FRα), which is markedly overexpressed in many epithelial malignancies including breast cancer but minimally expressed in normal tissues, has emerged as an attractive molecular target [11]. Surface conjugation of folic acid to nanocarriers facilitates receptor-mediated endocytosis, thereby increasing intracellular drug delivery and improving anticancer efficacy while reducing off-target exposure [12]. Importantly, efficient receptor-mediated targeting may also overcome insufficient intracellular accumulation and subtherapeutic drug concentrations commonly observed with non-targeted systems [13]. Despite encouraging progress, the clinical translation of paclitaxel remains limited by formulation-related toxicities, the need for high systemic doses, hypersensitivity reactions associated with solvent-based preparations, and insufficient drug accumulation at tumor sites [6]. These challenges frequently necessitate dose reduction or treatment discontinuation, ultimately compromising patient outcomes [14]. Therefore, nanocarrier systems that enable selective tumor targeting, lower effective dosing, and controlled intracellular release may offer meaningful clinical advantages by improving therapeutic index and reducing adverse effects [7]. From a translational perspective, enhancing paclitaxel delivery efficiency could directly contribute to safer regimens, improved tolerability, and better long-term treatment compliance in breast cancer patients [15]. Moreover, although several liposomal and ligand-functionalized formulations have been reported, many existing systems suffer from suboptimal drug loading, limited targeting efficiency, or inadequate release control, and few studies comprehensively integrate physicochemical optimization with mechanistic cellular evaluation [16]. Despite extensive research on folate-targeted liposomes, few formulations have successfully combined high drug loading, long-term stability, and reproducible targeting performance. Therefore, developing a simplified yet robust FA-conjugated liposomal platform that addresses these practical limitations remains timely and may provide a strong preclinical foundation for future translational studies [7]. Consequently, further investigation of folate-conjugated nanoliposomes is timely and warranted to address these unmet needs and bridge current gaps in the literature [12]. From a formulation perspective, the physicochemical design of liposomal carriers critically determines their therapeutic performance. Specifically, lipid composition governs bilayer rigidity, membrane stability, and drug retention, while high–phase transition phospholipids such as DSPC enhance structural integrity and cholesterol improves membrane packing to minimize premature drug leakage [17]. In addition, PEGylation using DSPE-mPEG3350 provides steric stabilization, reduces opsonization and rapid clearance, and prolongs systemic circulation, thereby promoting tumor accumulation [18]. Maintaining comparable PEG density between targeted and non-targeted formulations is also essential to ensure similar particle size, colloidal stability, and pharmacokinetic behavior, enabling reliable evaluation of the true contribution of folate-mediated targeting [19]. Folic acid was introduced onto the liposomal surface using a post-insertion strategy, in which preformed DSPE–PEG–folate micelles were incubated with preassembled liposomes, allowing spontaneous micelle-to-membrane transfer and insertion into the outer lipid leaflet. This

approach enables stable surface presentation of folate ligands while preserving particle integrity, minimizing premature drug leakage, and maintaining optimal ligand orientation for receptor-mediated cellular recognition [18-19]. In this study, we developed folic acid-conjugated nanoliposomes [FA-NLs] as an actively targeted delivery system for paclitaxel and systematically evaluated their physicochemical characteristics, release behavior, cellular uptake, cytotoxicity, and apoptosis-inducing potential in folate receptor–overexpressing breast cancer cells. We hypothesized that FA functionalization would enhance receptor-mediated internalization and therapeutic efficacy compared with free drug and non-targeted liposomes, thereby offering an effective nanomedicine strategy for precision breast cancer therapy.

Methods and Materials

Materials

DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine) was supplied by Lipoid GmbH (Ludwigshafen, Germany). The lipid derivatives DSPE-mPEG3350 and DSPE-PEG3350 were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol, sodium pentanesulfonate, sodium edetate, and the MTT assay reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were purchased from Sigma-Aldrich (St. Louis, MO, USA). Analytical-grade organic solvents, including chloroform and methanol, were provided by Merck (Darmstadt, Germany). Cell culture supplements and media, namely penicillin/streptomycin, fetal bovine serum (FBS), RPMI-1640, and DMEM, were obtained from Invitrogen (Carlsbad, CA, USA). Paclitaxel hydrochloride was acquired from Ebewe Pharma (Unterach, Austria). The human breast cancer cell line MCF-7 was obtained from the National Cell Bank of Iran (Pasteur Institute of Iran, Tehran, Iran). Additional details regarding the chemicals employed and the experimental procedures are provided in the Supporting.

Development of Paclitaxel-Loaded Nanoliposomes

Nanoliposomes, either paclitaxel-loaded or blank, were prepared using the conventional thin-film hydration technique. Briefly, DSPC, cholesterol, and DSPE-mPEG3350 were mixed at a molar ratio of 65:16:3 (approximately 175, 20.6, and 31.4 mg, respectively) and dissolved in 20 mL of chloroform. The lipid solution was transferred to a round-bottom flask, and the organic solvent was evaporated under reduced pressure using a rotary evaporator at 50 °C and 100 rpm to form a thin lipid film. Residual solvent was completely removed by nitrogen purging. The dried lipid film was subsequently hydrated with phosphate-buffered saline (PBS, pH 7.2) at 50 °C to produce multilamellar vesicles. For drug-loaded formulations, paclitaxel (9 mg) was dissolved in the hydration medium, whereas PBS alone was used for blank liposomes. Folate-mediated targeting was introduced via a post-insertion approach. FA-PEG3350-DSPE (7 mg) was first dissolved in preheated PBS and then incubated with preformed liposomes at 50 °C, a temperature above the lipid phase transition point, under gentle stirring for 60 min. A non-targeted control formulation [L-Paclitaxel] was prepared similarly but without FA conjugation. To maintain comparable PEG surface density and physicochemical properties, the amount of DSPE-mPEG3350 was increased accordingly. To obtain a uniform particle size distribution, liposomal suspensions were extruded through polycarbonate membranes with a pore size of 200 nm. Unencapsulated paclitaxel was subsequently removed by gel filtration using a Sephadex G-50 column equilibrated with PBS (pH 7.2).

Characterization of liposomes

The mean particle size, zeta potential, and polydispersity profile of paclitaxel-loaded liposomes were determined by dynamic light scattering (DLS) using a Zetasizer Nano-ZS system (Malvern Instruments, UK; model ZEN3600) equipped with a 4 mW He–Ne laser operating at 633 nm and maintained at 25 °C. The morphological and structural properties of the liposomal vesicles were further characterized using transmission electron microscopy (TEM, ZEISS, Germany), scanning electron microscopy (SEM, ZEISS, Germany), and atomic force microscopy (AFM). For TEM imaging, a drop of diluted liposomal suspension was placed onto a carbon-coated copper grid, negatively stained with phosphotungstic acid, air-dried, and subsequently examined to evaluate vesicle shape and internal architecture. For SEM analysis, freeze-dried liposomes were spread as a thin layer onto a glass substrate, sputter-coated with a gold layer, and then visualized under the microscope. For AFM measurements, 10 μL of the liposomal dispersion was deposited onto freshly cleaved mica, dried at room temperature within a biological safety cabinet, and scanned using a Full Plus-Multi Mode AFM system (Ara Research Company, Iran). Surface morphology was recorded under ambient conditions in non-contact mode.

Encapsulation efficiency [EE %] and loading rate [LR %] of PTX in liposomes

For this purpose, 1 mL of each formulation was centrifuged (TOMY, GRX-220, Japan) at 45,000 × g for 45 min at 4 °C to separate unencapsulated drug from the liposomal fraction. The concentration of non-entrapped PTX present in the collected supernatant was quantified spectrophotometrically at 227 nm. Free PTX levels were calculated using a previously established calibration curve. Subsequently, encapsulation efficiency (EE%) and loading ratio (LR%) were determined according to the following equations.

EE % = [[initial PTX – unentrapped PTX] / initial PTX] × 100 (eq. 1)

Where initial PTX is the amount of drug present in 1 ml of formulation, and unentrapped PTX is the amount of the drug in the supernatant.

LR % = [entrapped PTX / weight of carrier] × 100 (eq. 2)

Where entrapped PTX is the amount of drug encapsulated in the liposomes and the weight of carrier

is the amount of liposomes used.

Release study

The in vitro drug release profile of folic acid–functionalized liposomal paclitaxel (FL-PTX) was assessed using a dialysis-based method. Briefly, 2 mL of either FL-PTX or free PTX solution was transferred into a dialysis bag (molecular weight cut-off: 12 kDa), securely sealed, and submerged in 25 mL of phosphate-buffered saline (PBS) at pH 7.4 or 5.2. The system was maintained at 37 °C under continuous agitation at 120 rpm for a total duration of 64 h. At predetermined time points, samples were withdrawn from the release medium and immediately replenished with an equal volume of fresh buffer to maintain sink conditions. The collected aliquots were analyzed spectrophotometrically at a maximum absorption wavelength of 227 nm using a UV–Vis spectrophotometer. Cumulative drug release was subsequently determined based on a pre-established calibration curve. The percentage of drug release was calculated according to the following equation:

$$\text{Release rate \%} = [Cs / Ct] \times 100$$

where Cs is the concentration of PTX in the collected sample at a given time, and Ct is the total PTX content initially encapsulated in the FL-PTX formulation prior to dialysis.

In vitro cytotoxicity studies

The cytotoxic activity of nanoliposomal PTX formulations against MCF-7 breast cancer cells was assessed using the MTT viability assay. Cells were maintained in culture medium supplemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin, 100 U/mL penicillin, and 0.25 µg/mL amphotericin B under humidified conditions at 37 °C with 5% CO_2. Approximately 1×10^4 cells per well were seeded into 96-well plates containing 200 µL of culture medium and allowed to adhere for 24 h. The medium was then replaced with either fresh medium (negative control) or medium containing varying concentrations of nanoliposomal PTX, followed by an additional 24 h incubation period. Subsequently, 180 µL of fresh medium together with 20 µL of MTT solution [5 mg/mL in PBS] was added to each well and incubated for 1 h to allow formazan formation. The reaction was stopped by adding 200 µL of DMSO to solubilize the resulting formazan crystals. Absorbance was recorded at 570 nm using a microplate reader [AccuReader M965, Metertech, Taipei, Taiwan]. The half-maximal inhibitory concentration [IC_{50}] values were calculated using GraphPad Prism 6 software (GraphPad Software, San Diego, CA).

Cellular uptake study

Cellular uptake of the nanoliposomal formulations was investigated in MCF-7 breast cancer cells. Briefly, cells were seeded in 24-well plates at a density of 3×10^5 cells per well and cultured either in folate-deficient medium or in medium supplemented with 1 mM folic acid. Following overnight incubation, receptor-associated folate was removed by washing the cells with cold PBS and an acidic saline buffer (pH 3.5; 130 mM NaCl, 20 mM sodium acetate). Subsequently, fresh medium containing 0.07 mg/mL PTX in various formulations (FL-PTX, L-PTX, or free PTX) was added, and the cells were incubated at 37 °C for 2 h. Unbound or non-internalized vesicles were removed by washing twice with PBS (pH 7.4). Cells were then harvested by centrifugation at 13,000 rpm for 5 min and lysed using 200 µL of 0.5% Triton X-100. The resulting lysates were combined with an equal volume of acetonitrile to precipitate cellular proteins, followed by centrifugation to separate the supernatant. The supernatant was filtered through a 0.22 µm membrane filter, and 20 µL of the filtrate was subjected to HPLC analysis. Cellular uptake efficiency (CUE%) was subsequently calculated using the following equation:

CUE%: Amount of PTX in the cells after the incubation time/Amount PTX added in liposomal formulations introduced into the cells × 100

Additionally, cellular internalization was qualitatively evaluated using Nile Red–loaded liposomes prepared according to the same formulation composition. Following treatment, intracellular fluorescence intensity was examined and imaged by confocal fluorescence microscopy to visualize liposomal uptake.

Apoptosis analysis by Annexin-V staining

Apoptotic cell death induced by the FL-PTX formulation in MCF-7 cells was assessed using a FITC Annexin V apoptosis detection kit (BioLegend Inc., San Diego, CA) in accordance with the manufacturer's instructions. Briefly, MCF-7 cells were seeded into six-well plates at a density of 5×10^5 cells per well and incubated overnight at 37 °C in a humidified atmosphere containing 5% CO_2 under folate-deprived conditions to allow cell attachment. Subsequently, the culture medium was replaced with fresh medium supplemented with 2 µM of either free PTX or FL-PTX, and the cells were treated in triplicate. After 48 h of incubation, cells were harvested, washed twice with ice-cold staining buffer, and resuspended in Annexin V binding buffer to achieve a final concentration of 1×10^6 cells/mL. A 100 µL aliquot of the cell suspension was transferred into 5 mL tubes, followed by the addition of 5 µL FITC Annexin V and 10 µL propidium iodide (PI). Samples were gently mixed and incubated for 15 min at room temperature in the dark. Finally, 400 µL of binding buffer was added, and apoptotic cell populations were quantified by flow cytometric analysis.

Statistical Analysis

Statistical analyses were conducted using SPSS software (version 18). All data are expressed as mean ± standard deviation (SD) based on three independent experiments. Comparisons between groups were performed using Student's t-test, and differences were considered statistically significant at $p < 0.05$.

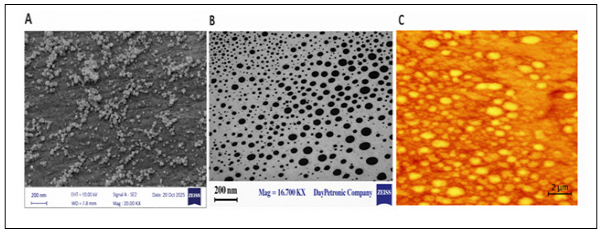

Figure 1. Morphological Characterization of PEGylated Liposomes Demonstrating Spherical Vesicular Architecture, as Observed by SEM (A), TEM (B), and AFM (C).

Results

Characterization of liposomes

The morphological features and physicochemical properties of the fabricated liposomes were characterized by SEM, TEM, and AFM analyses. SEM and TEM images demonstrated that the vesicles were mainly spherical and uniformly dispersed, whereas AFM observations verified their three-dimensional structure with smooth surface topology. In agreement with these morphological results, physicochemical measurements indicated an average particle diameter of 200.1 ± 18.2 nm and a polydispersity index (PDI) of 0.31 ± 0.22, suggesting a relatively homogeneous size distribution. The zeta potential was determined to be −25.6 ± 3.2 mV, reflecting substantial surface charge and good colloidal stability. Furthermore, the liposomes achieved encapsulation efficiency and drug loading values of 93.9 ± 2.6% and 5.27 ± 0.3%, respectively. Collectively, these findings (Figure 1) confirm that the prepared liposomes exhibited appropriate size, stability, and morphology, making them suitable candidates for subsequent biomedical applications

Release study

Free PTX solution exhibited rapid drug diffusion, achieving complete release within 10 h. In contrast, the folic acid–conjugated liposomal formulation (FL-PTX) demonstrated a markedly slower and sustained release behavior. The in vitro release profile was evaluated in PBS at pH 5.2 and 7.4 for up to 64 h. As illustrated in Figure 2, PTX release from FL-PTX proceeded in a controlled and prolonged manner, with no evident initial burst release. The cumulative drug release reached approximately 31% at pH 7.4 and 75% at pH 5.2, showing a statistically significant difference between the two media. These results suggest that FL-PTX effectively delays and regulates PTX release compared with the free drug, highlighting the suitability of folate-targeted liposomes as a controlled drug delivery platform.

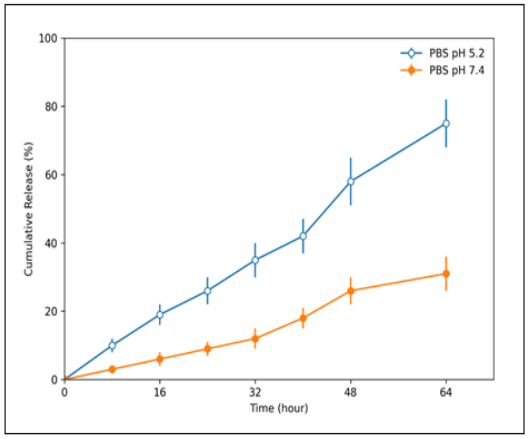

Figure 2. In Vitro Drug Release Kinetics from Liposomal Formulations Evaluated at 37 °C under Varying pH Conditions Over 64 h. Results are expressed as mean ± standard deviation from three independent experiments.

In vitro cytotoxicity assay

After 24 h of incubation at 37 °C, the cytotoxic effects of PTX-loaded nanoliposomes were evaluated and compared with those of free PTX at equivalent concentrations in MCF-7 cells (Figure 3). The findings demonstrated that FL-PTX produced a significantly greater inhibitory effect on cell viability than conventional PTX, particularly under folate-deprived conditions. In contrast, supplementation of the culture medium with 1 mM folate attenuated this enhanced cytotoxic activity.

Cellular uptake study

To elucidate the role of folate receptor–mediated targeting in cellular uptake, the internalization behavior of folate-functionalized nanoliposomes was examined in MCF-7 cells. As shown in Figure 4A, FL-PTX demonstrated significantly higher cellular accumulation compared with both non-targeted liposomes (L-PTX) and free PTX. Moreover, uptake efficiency increased in a time-dependent manner (data not shown), highlighting the contribution of folate conjugation to enhanced receptor-mediated internalization. These quantitative findings were further corroborated by confocal fluorescence microscopy (Figure 4B), where cells treated with Nile Red–loaded FL-PTX exhibited pronounced intracellular fluorescence signals. Collectively, these observations confirm the efficient internalization of the folate-decorated liposomal system and underscore its strong potential to improve targeted intracellular drug delivery.

Apoptosis Analysis by Annexin-V Staining

As shown in Figure 5 and Table 1, flow cytometric analysis demonstrated that exposure to FL-PTX significantly altered the cellular distribution, shifting the population from viable cells toward apoptotic and necrotic fractions compared with both free PTX and untreated controls. Notably, FL-PTX treatment resulted in a substantial elevation in late apoptotic cells, which constituted the predominant mode of cell death. By comparison, conventional PTX elicited only a moderate apoptotic effect, whereas the majority of control cells remained viable with negligible levels of apoptosis or necrosis. Collectively, these results suggest that folate-conjugated liposomal PTX potentiates anticancer activity in MCF-7 cells by effectively promoting apoptosis particularly at advanced stages thereby highlighting the therapeutic advantage of receptor-targeted liposomal delivery.

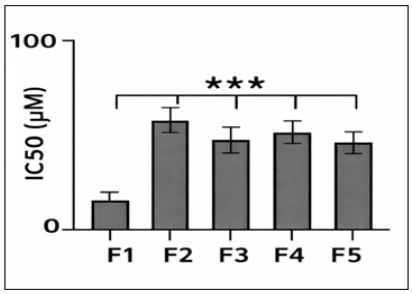

Figure 3. Half-maximal Inhibitory Concentration (IC_{50}, μM) Values of Various PTX Formulations Evaluated under Different Culture Conditions. Experimental groups included: F1, FL-PTX in folate-depleted medium; F2, FL-PTX in medium supplemented with 1 mM folate; F3, FL-PTX in standard culture medium; F4, liposomal PTX in standard medium; and F5, free PTX in standard medium. Results are reported as mean ± SD [n = 3, *** p = 0.0002].

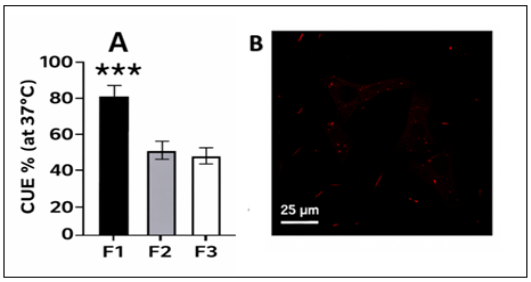

Figure 4. (A) Cellular Uptake Efficiency [CUE%] of Folate-functionalized Liposomes in MCF-7 Cells at 37 °C. The tested formulations were: F1, FL-PTX; F2, free PTX; and F3, L-PTX. Values are expressed as mean ± SD (n = 3; ***p < 0.0001; two-way ANOVA followed by Šidák's multiple comparison test). (B) Confocal fluorescence micrograph of MCF-7 cells exposed to Nile Red–loaded FL-PTX, demonstrating effective intracellular internalization.

Discussion

Conventional systemic chemotherapy remains fundamentally constrained by poor tumor selectivity, narrow therapeutic windows, dose-limiting toxicities, and the emergence of multidrug resistance [14]. Because most cytotoxic agents distribute nonspecifically throughout the body, substantial off-target accumulation in healthy tissues frequently leads to cardiotoxicity, neurotoxicity, and myelosuppression, thereby limiting the maximum tolerated dose and ultimately compromising therapeutic efficacy [15]. These clinical shortcomings have motivated the development of advanced drug-delivery systems designed to optimize pharmacokinetics, enhance intratumoral drug accumulation, and minimize systemic toxicity [7]. Among the various nanocarriers investigated, liposomes have emerged as one of the most clinically successful platforms due to their excellent biocompatibility, ability to encapsulate both hydrophilic and hydrophobic agents, protection of drugs from premature degradation, and passive tumor accumulation through the enhanced permeability and retention (EPR) effect [8]. Nevertheless, early-generation liposomes relied primarily on passive targeting and were rapidly cleared by the reticuloendothelial system (RES), particularly by hepatic and splenic macrophages [16]. This rapid opsonization and hepatic sequestration markedly reduced circulation half-life and limited therapeutic benefit, highlighting that passive accumulation alone is insufficient for efficient tumor targeting [9]. To address this limitation, PEGylation has been widely adopted as a critical surface-engineering strategy [18]. The incorporation of polyethylene glycol (PEG) creates a hydrophilic steric barrier that reduces protein adsorption, minimizes opsonization, and enables evasion of RES-mediated clearance [18]. As a result, PEGylated liposomes exhibit prolonged systemic circulation, improved stability, and a higher probability of tumor exposure [17]. Despite these pharmacokinetic advantages, PEGylation alone does not guarantee efficient cellular internalization, as extended circulation does not necessarily translate into enhanced tumor cell uptake [10]. Therefore, the integration of active targeting mechanisms has become essential to further improve therapeutic precision [13]. In recent years, ligand-mediated targeting strategies have gained substantial attention as an essential complement to passive nanocarrier delivery, as prolonged systemic circulation alone has proven insufficient to ensure efficient tumor cell internalization [20]. Folic acid (FA), a synthetic form of vitamin B9, has emerged as one of the most attractive targeting ligands owing to its small molecular size, chemical stability, low immunogenicity, low cost, and ease of conjugation to liposomes and other nanocarriers [21]. Importantly, the folate receptor alpha (FRα) is markedly overexpressed in many malignancies, including breast cancer, while remaining minimally expressed in normal tissues, thereby enabling receptor-mediated endocytosis and selective intracellular drug delivery [21]. Recent experimental advances further validate this strategy: Sachin et al (2025) reported that folic acid-anchored liposomal systems function as next-generation nanoplatforms capable of improving tumor selectivity, intracellular delivery, and overall therapeutic precision [20]. Similarly, Shengnan Liu et al. (2025) demonstrated that FA-targeted liposome-based nanoparticles significantly enhance tumor accumulation, promote receptor-mediated uptake, and reduce systemic toxicity compared with non-targeted formulations [21].

Table 1. Quantification of Annexin-V/PI Staining Analysis of MCF-7 Cells after 48 h of Treatment with FL-PTX, Conventional PTX, or Control. Data are expressed as mean ± SD (n = 3).

Formulations	Vital cells % An−/PI−	Early apoptosis % An+/PI−	Late apoptosis% An+/PI+	Necrotic cells % An+/PI−
FL-PTX	51.04 ± 5.2	7.33 ± 2.1	24.39 ± 3.1	13.24 ± 1.2
Conventional PTX	61.12± 6.1	7.09 ± 1.5	18.97 ± 2.4	7.82 ± 1.1
Control	76.68± 7.0	6.00± 1.3	8.87± 1.6	4.45± 1.0

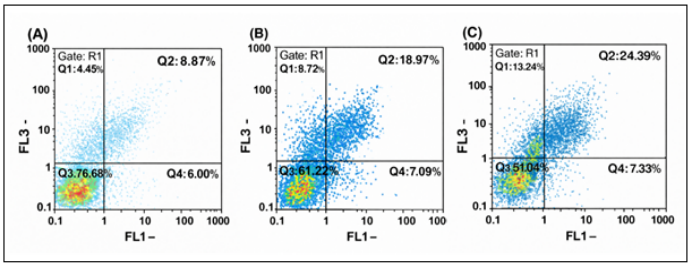

Figure 5. Flow Cytometric Evaluation of Apoptosis in MCF-7 Cells Following 48 h of Treatment: (A) Untreated Control, (B) Conventional PTX, and (C) FL-PTX.

These contemporary findings indicate that integrating PEGylation-mediated stealth properties with receptor-specific FA targeting provides a synergistic approach that simultaneously minimizes biological clearance and maximizes cellular internalization, ultimately improving the therapeutic index of liposomal chemotherapy [19]. Within this context, the present study was rationally designed to integrate these complementary mechanisms through the development of PEGylated, folic acid–conjugated paclitaxel-loaded liposomes (FL-PTX). Our results demonstrate that the engineered formulation exhibits favorable physicochemical stability, uniform morphology, controlled drug release, enhanced cellular internalization, and improved anticancer activity compared with non-targeted systems. Importantly, the observed improvements support the hypothesis that dual functionalization stealth behavior combined with receptor-mediated uptake can effectively address the key translational limitations of conventional chemotherapy and first-generation liposomes. The physicochemical characteristics of the developed liposomes position the formulation within the broadly accepted nanoscale window for tumor-oriented drug delivery [7]. The mean particle diameter of 200 nm falls within the size range (≈50–200 nm) frequently regarded as optimal for balancing prolonged systemic circulation with enhanced tumor accumulation through the enhanced permeability and retention (EPR) effect while minimizing rapid renal elimination [10]. This size is consistent with several contemporary liposomal systems reported in recent years. For instance, Jaradat et al. (2023) prepared PEGylated paclitaxel-loaded liposomes using microfluidic processing and reported particle diameters below 200 nm, demonstrating favorable pharmacokinetic stability and translational suitability [22]. Similarly, Mota Díaz et al. (2024) described PTX/DOX-loaded liposomes within a 150–230 nm range, which closely overlaps with the dimensions observed in our formulation [23]. Meanwhile, smaller folate-functionalized liposomes of approximately 152 ± 3 nm were reported by Pandey et al. (2024), suggesting that modest size reduction may further enhance tumor penetration. Collectively, these comparisons indicate that the present system resides at the upper boundary of the optimal size window while remaining fully compatible with current clinically relevant nanocarrier designs [24]. The polydispersity index (PDI = 0.31) confirms acceptable dispersity but indicates moderate heterogeneity compared with highly optimized platforms [22]. Notably, Jaradat et al. (2023) achieved PDI values below 0.25 through microfluidic synthesis, reflecting improved batch uniformity and tighter size control. Although our PDI remains within the acceptable threshold for liposomal drug delivery, this difference suggests that further process optimization such as controlled extrusion or microfluidic mixing could enhance homogeneity and potentially reduce variability in in vivo pharmacokinetics [22]. From a colloidal stability standpoint, the zeta potential of −25.6 ± 3.2 mV lies within the commonly cited stability guideline (approximately ±25 mV), indicating sufficient electrostatic repulsion to prevent particle aggregation during storage and systemic circulation [17]. Comparable surface charges have been reported in PEGylated and folate-targeted systems. For example, Lim et al. (2023) observed zeta potentials around −20 to −25 mV for folate-decorated liposomes, supporting the notion that moderately negative charge combined with PEG-mediated steric stabilization effectively maintains dispersion stability while minimizing rapid clearance [25]. Importantly, the formulation achieved a high encapsulation efficiency of 94%, demonstrating strong compatibility between the lipid bilayer and hydrophobic paclitaxel [24]. This loading performance closely matches recent high-performing targeted platforms; notably, Pandey et al. (2024) reported an encapsulation efficiency of 93% for folate-functionalized pH-sensitive liposomes. In comparison, other contemporary systems typically report efficiencies above 80%, indicating that the present formulation achieves loading performance at the upper end of the reported spectrum [24]. The release profile demonstrates a clear transition from the burst diffusion observed with free PTX to a sustained and controlled release pattern in the liposomal formulation [7]. This behavior confirms that encapsulation within the lipid bilayer effectively restricts premature drug leakage and improves temporal control over drug availability [8]. Free PTX solution exhibited rapid drug diffusion, reaching nearly complete release (~100%) within 10 h, reflecting the absence of any diffusional barrier and highlighting the limited capacity of the free drug to provide prolonged therapeutic exposure [14]. In contrast, the folic acid–conjugated liposomal formulation (FL-PTX) displayed markedly slower and sustained drug liberation over the entire study period [7]. The in vitro release behavior evaluated in PBS at pH 5.2 and 7.4 for up to 64 h revealed a controlled and prolonged release without any evident initial burst effect, indicating stable drug entrapment

within the vesicular bilayer [17]. Specifically, FL-PTX released only ~31% of PTX at physiological pH 7.4, whereas a significantly higher release of ~75% at acidic pH 5.2 was observed, demonstrating clear pH-responsive behavior [24]. This corresponds to an approximately 2.4-fold increase in drug liberation under acidic conditions, suggesting preferential payload release in tumor-like microenvironments or endo/lysosomal compartments while minimizing premature systemic leakage [24]. Comparable behavior has been reported in recent folate-targeted liposomal systems. For example, Prashant Pandey et al. (2024) developed FA-functionalized pH-sensitive liposomes and observed significantly accelerated drug release under acidic conditions compared with physiological media, with cumulative release values exceeding 70% at pH 5.2 while remaining substantially lower at neutral pH. The close agreement between their results and the present findings confirms that folate-decorated liposomes can effectively combine environmental responsiveness with controlled release kinetics [24]. The enhanced cytotoxicity of FL-PTX relative to free PTX indicates that liposomal encapsulation does not merely act as a carrier but significantly improves intracellular drug delivery efficiency [7]. The greater inhibitory effect under folate-deprived conditions suggests a folate receptor–dependent uptake mechanism, where receptor availability directly influences therapeutic performance [11]. Conversely, reduced efficacy in folate-supplemented media likely reflects competitive inhibition at FRα binding sites, further confirming receptor-mediated targeting [12]. These findings imply that the improved anticancer activity arises from enhanced cellular internalization rather than intrinsic drug potency, highlighting the functional contribution of folate conjugation to selective cytotoxicity, a mechanism consistent with the nanomedicine framework described by Liu Y et al. (2023), who emphasized that ligand-mediated nanocarriers enhance intracellular bioavailability by overcoming cellular and biological transport barriers [13]. The uptake data provide mechanistic confirmation for the superior cytotoxic performance observed with FL-PTX [7]. The significantly higher intracellular fluorescence intensity and quantitative accumulation indicate that folate decoration promotes receptor-mediated endocytosis rather than passive diffusion [11]. The time-dependent increase further suggests active internalization and intracellular retention, which likely enhances local drug concentration and prolongs therapeutic action [13]. Together, these results validate that FA conjugation effectively converts liposomes from passive carriers into actively targeted nanovehicles capable of preferential tumor cell internalization, consistent with the ligand-mediated transport mechanisms described by Liu Y et al. (2023) and further supported by Chaemin Lim et al. (2023), who demonstrated significantly enhanced cellular uptake and receptor-dependent accumulation in folate-functionalized PEGylated liposomes compared with non-targeted systems [12, 13, 25]. Flow cytometric analysis demonstrates that the enhanced cytotoxicity of FL-PTX translates into programmed cell death rather than nonspecific necrosis [26]. The marked increase in late apoptotic populations suggests that liposomal PTX effectively activates apoptotic pathways, likely through sustained intracellular drug exposure and microtubule stabilization mechanisms characteristic of paclitaxel [27]. Compared with free PTX, the higher apoptotic fraction indicates improved bioavailability and prolonged intracellular retention, thereby facilitating stronger mitotic arrest and apoptosis induction [7]. This apoptosis-dominant profile is therapeutically advantageous, as it minimizes inflammatory responses typically associated with necrotic cell death and promotes more controlled tumor cell elimination [28]. Similar apoptosis-enhancing effects of nanoparticle-mediated paclitaxel delivery have been reported by Nirav Desai, et al (2021) in solvent-free taxane formulations and more recently by Prashant Pandey, et al (2024) in folate-targeted liposomal systems, both demonstrating that improved intracellular delivery significantly increases apoptotic cell fractions relative to free drug treatment [6, 24]. Compared with previously reported FA-targeted liposomes, our formulation was designed to achieve higher encapsulation efficiency, improved physicochemical stability through DSPC/cholesterol optimization, controlled release behavior under acidic tumor-mimicking conditions, and uniform PEGylation to ensure prolonged circulation while preserving targeting efficiency. Therefore, folate-targeted liposomes appear to enhance not only the magnitude but also the quality of anticancer activity by preferentially inducing apoptosis over nonspecific cytotoxicity.

From a clinical standpoint, conventional paclitaxel therapy remains constrained by poor aqueous solubility, the need for Cremophor EL–based solvents, nonspecific biodistribution, and dose-limiting toxicities including neurotoxicity, myelosuppression, and hypersensitivity reactions that restrict the maximum tolerated dose and compromise therapeutic outcomes [14]. These limitations have motivated the development of nanocarrier-based drug delivery systems designed to optimize pharmacokinetics, enhance tumor accumulation, and minimize systemic toxicity [7]. Encapsulation of paclitaxel within liposomes improves drug solubility, protects the payload from premature degradation, and enables prolonged systemic exposure compared with free drug formulations [8]. PEGylation further enhances circulation half-life by reducing opsonization and reticuloendothelial clearance, thereby increasing the probability of tumor accumulation through the enhanced permeability and retention effect [18]. Active targeting through folate receptor alpha provides an additional layer of selectivity by promoting receptor-mediated endocytosis in FRα-overexpressing tumors while sparing normal tissues with low receptor expression [11]. The sustained and pH-responsive release profile observed for FL-PTX enables preferential drug liberation in acidic tumor microenvironments, reducing premature systemic leakage while maximizing intratumoral exposure [24]. Enhanced cellular uptake and apoptosis-dominant cytotoxicity indicate that therapeutic gains arise from improved intracellular bioavailability rather than simple dose escalation, thereby supporting

safer and more efficient chemotherapy [13]. Collectively, these translational advantages suggest that PEGylated folate-conjugated liposomal paclitaxel may lower effective therapeutic doses, reduce off-target toxicities, improve tolerability, and ultimately expand the therapeutic index compared with conventional paclitaxel or first-generation liposomal systems.

Limitations and Future Studies

Despite the promising in vitro findings demonstrating enhanced apoptosis and improved anticancer efficacy of folate-targeted liposomal paclitaxel (FL-PTX), several limitations should be acknowledged. First, the present study is confined to in vitro cellular models, which cannot fully recapitulate the complex biological environment of living organisms, including pharmacokinetics, biodistribution, tumor microenvironment heterogeneity, and systemic toxicity. Consequently, the therapeutic performance and safety profile of FL-PTX require validation in appropriate in vivo tumor models. Second, while flow cytometric analysis indicates apoptosis as the predominant mode of cell death, the study does not dissect the downstream molecular mechanisms involved, such as caspase activation, mitochondrial membrane potential disruption, or alterations in pro- and anti-apoptotic signaling pathways. Elucidation of these mechanisms would strengthen the mechanistic understanding of FL-PTX–induced cytotoxicity. Future studies will therefore focus on evaluating the antitumor efficacy, biodistribution, and safety of FL-PTX in relevant animal models, with particular attention to tumor targeting efficiency and off-target effects. In addition, mechanistic investigations at the molecular level, including apoptosis-related signaling cascades and intracellular trafficking pathways, are warranted. Such studies will be essential to support the translational potential of folate-targeted liposomal paclitaxel and advance its development toward clinical

In conclusion, this study demonstrates that dual-functionalized PEGylated, folic acid–conjugated paclitaxel-loaded liposomes represent a rational and effective strategy for overcoming key limitations of conventional paclitaxel therapy. By integrating prolonged systemic circulation with folate receptor–mediated active targeting, the developed FL-PTX formulation achieves controlled and pH-responsive drug release, enhanced cellular uptake, and apoptosis-dominant cytotoxicity compared with free paclitaxel and non-targeted systems. The findings highlight that improved therapeutic performance arises primarily from enhanced intracellular bioavailability rather than nonspecific dose escalation, supporting a safer and more precise chemotherapy paradigm. Although further in vivo validation is required, the present results provide a strong mechanistic and experimental foundation for the continued development of folate-targeted liposomal paclitaxel as a next-generation nanotherapeutic platform with the potential to expand the therapeutic index and improve clinical outcomes in folate receptor–overexpressing cancers.

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Competing interests

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Consent

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