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RESEARCH ARTICLE

Liposomes and Chitosan-Coated Liposomes (Chitosomes) for the Delivery of Letrozole: A Comparative Study

Tahir Emre YALCIN[*°], Nihal Tugce OZAKSUN[**], Cigdem YUCEL[***], Tuba INCECAYIR[****]

Liposomes and Chitosan-Coated Liposomes (Chitosomes) for the Delivery of Letrozole: A Comparative Study

SUMMARY

Letrozole (LTZ) is a first-line aromatase inhibitor for hormone-sensitive breast cancer in postmenopausal women, but its poor aqueous solubility and limited tissue selectivity reduce therapeutic efficacy. This study aimed to develop liposome and chitosan-coated liposome (chitosome) formulations as lipid-chitosan-based nanocarriers to enhance LTZ delivery, enable sustained release, and minimize side effects. LTZ-loaded liposomes were prepared by the thin-film hydration method, and the optimal formulation was selected for chitosan coating. The impact of chitosan on physicochemical properties was assessed through particle size, zeta potential, encapsulation efficiency, and stability studies. In vitro drug release was evaluated via dialysis at 37°C, and cytotoxicity was tested on MCF-7 breast cancer cells. Optimized liposomes and chitosomes exhibited mean particle sizes of 112 nm and 155 nm, respectively, with high encapsulation efficiency (>90%). Chitosan coating led to a slight reduction in the net negative zeta potential, decreasing it from –48.9 mV to –40.0 mV. Both formulations demonstrated sustained LTZ release and maintained their particle size stability after one month of storage at 2–8°C (p>0.05). Cytotoxicity assays indicated that both formulations exhibited similar effects to free LTZ on MCF-7 cells. In conclusion, LTZ-loaded liposome and chitosome formulations were successfully developed, offering a favorable particle size, high encapsulation efficiency, and sustained drug release. Chitosan coating modified surface properties and influenced formulation stability. These findings suggest that lipid-chitosan nanocarriers are promising platforms for LTZ delivery. Further in vivo studies are warranted to confirm their potential in breast cancer therapy.

Keywords: *Letrozole, liposome, chitosome, MCF-7, cytotoxicity.*

Letrozol İletimi için Lipozom ve Kitosan Kaplı Lipozomlar (Kitozomlar): Karşılaştırmalı Çalışma

ÖZ

Letrozol (LTZ), postmenopozal kadınlarda hormon duyarlı meme kanserinin tedavisinde birinci basamak aromataz inhibitörüdür; ancak düşük suda çözünürlüğü ve sınırlı doku seçiciliği terapötik etkinliğini azaltmaktadır. Bu çalışmada, LTZ iletimini artırmak, kontrollü salım sağlamak ve yan etkileri en aza indirmek amacıyla lipit-kitosan bazlı nanotaşıyıcılar olan lipozom ve kitosan kaplı lipozom (kitazom) formülasyonlarının geliştirilmesi hedeflenmiştir. LTZ yüklü lipozomlar ince film hidrasyon yöntemi ile hazırlanmış ve en uygun formülasyon kitosan ile kaplanmıştır. Kitosan kaplamanın fizikokimyasal özelliklere etkisi; partikül boyutu, zeta potansiyeli, enkapsülasyon etkinliği ve stabilite çalışmaları ile değerlendirilmiştir. İn vitro ilaç salımı, 37°C'de diyaliz yöntemi ile; sitotoksisite ise MCF-7 meme kanseri hücrelerinde test edilmiştir. Optimum lipozom ve kitazom formülasyonlarının ortalama partikül boyutları sırasıyla 112 nm ve 155 nm olup, her iki formülasyon da yüksek enkapsülasyon etkinliği göstermiştir (>%90). Kitosan kaplaması, net negatif zeta potansiyelinde hafif bir azalmaya neden olmuş ve bu değer –48,9 mV'den –40,0 mV'ye düşmüştür. Her iki formülasyon da sürekli LTZ salımı sergilemiştir ve 2–8°C'de bir ay süreyle depolama sonrasında parçacık boyutunda anlamlı bir değişim göstermemiştir (p>0,05). Sitotoksisite testleri, her iki formülasyonun da serbest LTZ ile benzer etki gösterdiğini ortaya koymuştur. Sonuç olarak, LTZ yüklü lipozom ve kitozom formülasyonları başarıyla geliştirilmiş olup; bu formülasyonlar uygun bir parçacık boyutu, yüksek enkapsülasyon verimi ve sürekli ilaç salımı sunmuştur. Kitosan kaplama, yüzey özelliklerini değiştirmiş ve formülasyon stabilitesini etkilemiştir. Bulgular, lipit-kitosan bazlı nanotaşıyıcıların LTZ taşınmasında umut verici olduğunu ve potansiyellerinin doğrulanması için ileri in vivo çalışmalar gerektiğini göstermektedir.

Anahtar Kelimeler: *Letrozol, lipozom, kitozom, MCF-7, sitotoksisite.*

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[*] ORCID: 0000-0001-5834-2335, Department of Pharmaceutical Technology, Faculty of Pharmacy, Gazi University, Ankara, Turkiye
[**] ORCID: 0000-0001-6276-9227, Department of Pharmaceutical Technology, Faculty of Pharmacy, Gazi University, Ankara, Turkiye
[***] ORCID: 0000-0002-0622-5150, Department of Pharmaceutical Technology, Faculty of Pharmacy, Erciyes University, 38039 Kayseri, Turkiye
[****] ORCID: 0000-0002-7106-7929, Department of Pharmaceutical Technology, Faculty of Pharmacy, Gazi University, Ankara, Turkiye

[°] Corresponding Author; Tahir Emre YALÇIN
E-mail: emreyalcin@gazi.edu.tr

INTRODUCTION

Breast cancer is the most common type of cancer among women worldwide and accounts for the majority of cancer-related deaths in this population (Maajani et al., 2019). Estrogens have been shown to play a critical role in the development and progression of breast cancer (Samavat & Kurzer, 2015). Consequently, reducing circulating estrogen levels is a well-established therapeutic strategy aimed at limiting the growth of estrogen-dependent tumors (Simon et al., 2002). Several pharmacologically active compounds are used for this purpose, among which letrozole (LTZ) is a widely utilized agent.

LTZ is a third-generation non-steroidal aromatase inhibitor and has been approved for both primary and secondary treatment of breast cancer (Fenton et al., 2004). Clinical studies have demonstrated that LTZ significantly reduces circulating estrogen levels (Bhatnagar, 2007). However, LTZ is associated with a range of adverse effects, including hot flashes, headaches, dizziness, agitation, anxiety, insomnia, dry skin, and hypertension (Iqbal et al., 2019). Its limited therapeutic efficacy is primarily attributed to its excessive partitioning into red blood cells and insufficient accumulation within tumor tissues following intravenous administration (Ijaz et al., 2024). Moreover, LTZ's clinical utility is further constrained by dose-dependent toxicities, including neurotoxicity. The systemic delivery of LTZ and other chemotherapeutic agents often results in off-target effects, as these drugs impact both malignant and healthy cells. Considering these limitations, there is a clear need for an alternative drug delivery strategy that enhances antitumor efficacy while minimizing toxicity to normal tissues. One promising approach to overcome these challenges involves the use of nanocarriers for the targeted delivery of chemotherapeutic agents.

Various strategies have been developed to reduce the side effects of LTZ and improve its targeted delivery to tumor tissues. One such approach involves encapsulating LTZ within liposomes. Liposomes are spherical vesicles composed of a phospholipid bilayer surrounding an aqueous core. They exhibit several advantageous properties, such as small particle size (PS), high encapsulation efficiency (EE%) of hydrophobic drugs, and biocompatibility with human cells (Liu et al., 2016; Maja et al., 2020). Despite these favorable characteristics, liposomes are associated with certain stability issues (Sogut et al., 2021). To address this limitation, surface modification with different polymers has been explored to improve their physicochemical stability.

Chitosan is a natural aminopolysaccharide derived from the deacetylation of chitin (Motiei et al., 2017). Owing to its positive charge and bioadhesive properties, chitosan readily interacts with negatively charged drug delivery systems (Yuan et al., 2006). Chitosomes, a hybrid system formed by coating liposome surfaces with chitosan, can enhance the physicochemical stability of liposomes and promote improved drug retention and tumor-targeting efficiency (Esposto et al., 2021; Zhou et al., 2018).

In this study, LTZ-loaded liposomes were prepared using soy phosphatidylcholine (SPC) in combination with two different excipients: cholesterol (CHOL) and cholesteryl hemisuccinate (CHEMS). Based on PS and zeta potential (ZP) measurements, the most suitable liposome formulation was selected and subsequently coated with chitosan. The EE%, *in vitro* release profiles, and physicochemical stability of the prepared formulations were systematically evaluated. The cytotoxicity of the optimized liposome and chitosome formulations was further evaluated in MCF-7 cells via the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. In this context, LTZ-loaded chitosome formulations were developed and characterized for the first time, contributing novel findings to the literature.

MATERIAL AND METHODS

Materials

LTZ, the active pharmaceutical ingredient, was kindly provided by Deva Holding (Istanbul, Türkiye). CHOL, CHEMS, low molecular weight (MW) chitosan, and dialysis tubing cellulose membranes were purchased from Sigma-Aldrich (St. Louis, MO, USA). L-α-soy phosphatidylcholine (95%) (SPC) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Polysorbate 80 and sucrose were procured from Merck (Darmstadt, Germany). All other chemicals were of analytical grade, and all solvents were of high-performance liquid chromatography (HPLC) grade.

The MCF-7 human breast cancer cell line (ATCC CCL-222) was obtained from the American Type Culture Collection (Manassas, VA, USA) for cytotoxicity studies. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% penicillin–streptomycin and 10% fetal bovine serum (FBS) (Gibco-Invitrogen, Grand Island, NY, USA).

Preparation of liposomes

LTZ-loaded liposomes were prepared using the thin-film hydration method. Briefly, LTZ (10 mg), SPC, and either CHOL or CHEMS were dissolved in a chloroform: methanol mixture (2:1, v/v) (Vu et al., 2020). The organic solvents were subsequently removed under reduced pressure at 60°C using a rotary evaporator (Rotavapor® R-3, Büchi, Switzerland), and a thin lipid film was formed. This film was hydrated with phosphate buffer solution (PBS, pH 7.4) and subjected to probe sonication (Sonics VCX 130 FSJ, Newtown, CT, USA) for 15 minutes with a 2-second /3-second on/off pulse cycle to reduce PS. The resulting liposomal dispersions were then extruded through polycarbonate membranes with a pore size of 200 nm using an extruder (Avanti Polar Lipids Inc., Alabaster, AL, USA) to obtain uniform vesicle sizes.

Preparation of chitosomes

Different concentrations of low molecular weight chitosan were dissolved in a 1% v/v aqueous solution of glacial acetic acid. The liposome suspension was then added dropwise into an equal volume of the chitosan solution under continuous magnetic stirring at 500 rpm for 1 hour (Lee et al., 2019). Subsequently, both liposomes and chitosomes were transferred into dialysis bags (molecular weight cut-off: 14,000 Da) to remove unencapsulated LTZ prior to lyophilization. The dialysis bags were immersed in 400 mL of PBS (pH 7.4) and stirred at 100 rpm for 2 hours at room temperature. Following dialysis, the formulations were collected from the bags and lyophilized. The compositions of all formulations are detailed in Table 1.

Table 1. Composition of various liposome and chitosome formulations.

Formulation code	SPC (mg)	CHOL (mg)	CHEMS (mg)	Chitosan concentration (%)
LP1	140	60	-	-
LP2	140	-	60	-
CCL1	140	-	60	0.01
CCL2	140	-	60	0.025
CCL3	140	-	60	0.05

*SPC: Soy phosphatidylcholine, CHOL: Cholesterol, CHEMS: Cholesteryl hemisuccinate

Lyophilization of formulations

All formulations (liposomes and chitosomes) containing sucrose as a cryoprotectant were transferred into glass vials and frozen at −80°C. The frozen samples were then lyophilized at −55°C for 40 hours using a freeze dryer (Christ Alpha 1-2 LD plus, Osterode am Harz, Germany) (Yalcin et al., 2021). The resulting lyophilized powders were stored at 2-8°C for further analysis.

Characterization of formulations

PS, polydispersity index (PDI), and ZP measurements

The lyophilized formulations were dispersed in deionized water, and the PS, PDI, and ZP of both li-

posomes and chitosomes were measured using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) (Yalcin et al., 2022; Yalcin et al., 2025) (n = 3).

Determination of EE%

The EE% of both liposomes and chitosomes was determined using an indirect method. Briefly, the formulations were placed in dialysis bags to separate the unencapsulated LTZ and stirred magnetically for 1 hour. Following dialysis, 1 mL of the external medium was collected and analyzed by HPLC (n = 3). The EE% was calculated using the following equation:

$$EE\% = \frac{total\ added\ drug - free\ drug}{total\ added\ drug} \times 100 \quad (1)$$

HPLC assay for quantification

The amount of LTZ in the samples was quantified using HPLC (Agilent 1200 Series, Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was achieved on a reverse-phase C18 column (250 mm × 4.6 mm, 5 µm; XSelect, Waters, Ireland). The ultraviolet (UV) detection and column temperature were 242 nm, and 30°C, respectively. The mobile phase consisted of acetonitrile and water (65:35, v/v) under isocratic conditions, with a flow rate of 1 mL/minute (Hooshyar et al., 2021). The injection volume was 20 µL. The retention time was approximately 11 minutes. The standard deviation (SD) of the y-intercepts (σ) and the slopes (S) of the calibration curves were obtained from three independent replicate experiments. These values were then used to calculate the limits of detection (LOD) and quantification (LOQ) using the formulas: LOD = 3.3 (σ/S) and LOQ = 10 (σ/S), respectively. The LOD and LOQ were 0.035 and 0.106 µg mL^{-1}, respectively.

***In vitro* drug release studies**

Drug release from the formulations was evaluated by measuring the drug concentration in the release medium using the HPLC method. For this purpose, a specified amount of lyophilized LTZ-loaded formulations, adjusted based on the EE% to contain 0.5 mg of LTZ, was dispersed in PBS containing 2% Polysorbate 80 and transferred into dialysis bags (cellulose membranes, MW cut-off: 14,000 Da). Polysorbate 80 was included to maintain sink conditions during the release process (Kaplan et al., 2022), as LTZ exhibits poor water solubility in aqueous media (Mohammadi et al., 2022). The dialysis bags were then immersed in 200 mL of release medium composed of PBS (pH 7.4) with 2% Polysorbate 80, maintained at 37 ± 0.5°C. At predetermined time points (0.5, 1, 2, 4, 6, 8, 10, 12, and 24 hours), 1 mL aliquots were withdrawn and immediately replaced with an equal volume of fresh medium. All experiments were performed in triplicate.

Differential scanning calorimetry (DSC)

Approximately 2 mg of each sample was accurately weighed and placed into standard aluminum pans, which were then sealed. Differential Scanning Calorimetry (DSC) measurements were performed using a DSC-60 instrument (Shimadzu, Japan) under a continuous nitrogen purge at a flow rate of 20 mL/min. Samples were heated from 20°C to 250°C at a controlled rate of 10°C/min. An empty aluminum pan was used as the reference to ensure baseline correction and accurate thermal signal detection. The analysis was conducted to investigate possible interactions between the active pharmaceutical ingredient and excipients, as well as to evaluate their thermal behavior in physical mixtures.

Transmission electron microscopy (TEM)

Liposome and chitosome morphologies were examined using transmission electron microscopy (TEM). For sample preparation, a drop of each sample was placed onto a carbon-coated copper grid and negatively stained with 1% (w/v) phosphotungstic acid. The grids were air-dried and kept at room temperature until analysis. TEM observations were performed under low vacuum using a FEI Tecnai G2 Spirit BioTwin CTEM microscope (Hillsboro, OR, USA) at 120 kV.

Stability of liposome and chitosome formulations under different temperature conditions

The stability of the formulations (liposomes and chitosomes) was assessed by monitoring PS, PDI, and ZP. The stability tests for both liposome and chitosome formulations were performed on samples in dis-

persion form (non-lyophilized). Samples were stored at 2-8°C and 25°C for one month prior to analysis. Before measurements, the samples were diluted with deionized water (n = 3).

***In vitro* cytotoxicity studies**

The estrogen receptor (ER)-positive MCF-7 (ATCC CCL-222, Human Breast Cancer Cell Series) cell line model, which is often used to demonstrate the estrogen dependence of breast cancer development (Xu et al., 2017), was cultured in DMEM supplemented with 1% penicillin, streptomycin mix solution (Gibco-Invitrogen, Grand Island, NY, USA), and 10% FBS.

Cell viability was assessed with MTT assay. MCF-7 cells were seeded at a density of 1 x 10^4 cells/well in 96-well plates and allowed to attach overnight. Control groups containing only culture medium without substance were determined. Then, the solutions of the test substances in DMEM (100 µL) were added to the wells, and after 24 hours of incubation, MTT solution (13 µL/well) in fresh DMEM (100 µL/well) (Mitropoulou et al., 2003) was added to all wells whose contents were emptied and kept for 4 hours. Then, 100 µL of DMSO was added to the wells to solubilize formazan crystals. The resulting purple color was determined spectrophotometrically at 570 nm. The mean absorbance of the control wells was considered as corresponding to 100% cell viability. Different absorbance values were obtained for different test substances, and viability percentages were calculated (Yalcin et al., 2022).

Statistical analysis

Results were expressed as mean ± SD for all parameters. A paired t-test was used to test the statistical significance when comparing data between two groups, and more than two groups were compared using one-way analysis of variance (ANOVA) with post hoc Tukey's test. A difference was considered statistically significant at $p<0.05$. For this purpose, GraphPad Prism 9.3.0 (GraphPad Software, Inc.) was used (Yalcin et al., 2025).

RESULTS AND DISCUSSION

PS, PDI, and ZP measurements

The mean PS of the LTZ-loaded liposomes were found to be 164 nm for the CHOL-containing formulation (LP1) and 112 nm for the CHEMS-containing formulation (LP2). The ZP values were −14.9 mV and −48.9 mV for LP1 and LP2, respectively (Table 2). Chitosan-coated liposomes (chitosomes) prepared using LP2 formulation, which had a smaller PS and higher surface charge, exhibited increased PS values, indicating successful coating. The mean particle sizes of the chitosomes were 155 nm (CCL1, 0.01% chitosan), 198 nm (CCL2, 0.025% chitosan), and 1088 nm (CCL3, 0.05% chitosan). Chitosomes exhibited less negative ZP values ranging from −40.0 mV to −27.5 mV, confirming surface modification. All formulations, except CCL3, exhibited low PDI values (<0.5), indicating monodisperse particle distribution.

Table 2. Characteristic properties of various liposome and chitosome formulations (mean ± SD, n=3).

Formulation code	PS (nm)	PDI	ZP	EE%
LP1	164 ± 10	0.283 ± 0.028	-14.9 ± 2.1	95.6 ± 0.1
LP2	112 ± 3	0.286 ± 0.010	-48.9 ± 1.4	95.1 ± 0.2
CCL1	155 ± 19	0.302 ± 0.056	-40.0 ± 2.3	93.7 ± 0.5
CCL2	198 ± 12	0.245 ± 0.007	-27.5 ± 1.7	93.8 ± 0.7
CCL3	1088 ± 323	0.876 ± 0.175	-29.3 ± 2.0	92.7 ± 1.5

*LP1 contains 140 mg SPC and 60 mg CHOL. LP2 contains 140 mg SPC and 60 mg CHEMS. CCL1, CCL2, and CCL3 are liposomal formulations based on LP2, coated with 0.01%, 0.025%, and 0.05% chitosan solutions, respectively. SPC: Soy phosphatidylcholine, CHOL: Cholesterol, CHEMS: Cholesteryl hemisuccinate. For PS and PDI value, CCL3 was significantly different from LP1, LP2, CCL1, and CCL2 ($p<0.05$), while no significant differences were observed among the other formulations. For ZP, CCL2 and CCL3 did not differ significantly from each other ($p>0.05$), while all other pairwise comparisons showed significant differences. For EE%, significant differences were observed between LP1 and CCL3; and between LP2 and CCL3 ($p<0.05$), while no significant differences were found among the other formu-

lations ($p>0.05$).

The smaller PS observed in the CHEMS-containing formulation (LP2) compared to LP1 may be attributed to the increased net negative surface charge provided by the carboxylic acid group of CHEMS, enhancing repulsion between particles and thus preventing aggregation. These findings align with previous reports (Kulig et al., 2015). The chitosan coating process was performed on LP2 due to its favorable physicochemical properties (smaller size and higher ZP). The subsequent increase in PS after chitosan coating further supports the formation of a polymeric layer on the liposome surface. As the chitosan concentration increased, the PS also increased significantly—particularly in CCL3—suggesting that chitosan self-assembled into thicker or possibly aggregated structures (Park et al., 2014). The observed decrease in the magnitude of negative ZP upon chitosan coating—from −48.9 mV (LP2) to values between −40.0 mV and −27.5 mV—supports the interaction between the positively charged chitosan molecules and the negatively charged liposomal surface (Table 2). While CCL1 and CCL2 remained within monodisperse limits (PDI<0.5), CCL3 exhibited a markedly higher PDI value, likely due to aggregation induced by the excess polymer concentration. This finding suggests that high chitosan concentrations may compromise colloidal stability by promoting interparticle bridging or aggregation (Soo et al., 2016).

Determination of EE%

Drug EE% in liposomal formulations is influenced by several physicochemical factors, including liposome size, surface charge, bilayer rigidity, aqueous core volume, and the preparation method (Hooshyar et al., 2021). In this study, the EE% of LTZ in uncoated liposomes was found to be high and not significantly affected by the presence of either CHOL (LP1) or CHEMS (LP2). The average EE% ranged between 92.7% and 95.6% for all tested formulations (Table 2). However, a slight decrease in EE% was observed as the chitosan concentration increased during the coating process. Notably, a statistically significant reduction in EE% was recorded for CCL3 compared to LP2 ($p<0.05$), while the EE% of CCL1 and CCL2 remained comparable to the uncoated liposome formulation (LP2).

The consistently high EE% (92.7–95.6%) across all liposomal and chitosomal formulations indicates the favorable incorporation of LTZ within the lipid bilayer. This is likely due to the lipophilic nature of LTZ and the optimized lipid composition, which promotes drug retention. The lack of significant difference in EE% between LP1 and LP2 suggests that the inclusion of CHOL or CHEMS did not disrupt bilayer packing to an extent that would affect drug loading. This finding aligns with previous studies indicating that CHOL and CHEMS, although they influence membrane rigidity and surface charge, do not necessarily affect the EE% of hydrophobic drugs (Haeri et al., 2017). The slight reduction in EE% with increasing chitosan concentration may be attributed to partial drug leakage during the coating step. This phenomenon is likely due to the mechanical stress introduced by stirring and the potential disruption of the lipid bilayer interface during the formation of the chitosan shell (Trapani et al., 2018). The statistically significant drop in EE% in the CCL3 formulation (0.05% chitosan) supports this explanation, and aligns with earlier research suggesting that excessive polymer concentration can destabilize vesicle structure or displace encapsulated drug molecules (Haeri et al., 2017).

***In vitro* drug release studies**

The *in vitro* release profile of LTZ from conventional liposomes (LP1 and LP2) and chitosomes (CCL1 and CCL2) was evaluated over 24 hours. Due to its significantly large PS, the CCL3 formulation was excluded from the release study. As shown in Figure 1, CCL1 and CCL2 exhibited a markedly lower cumulative release than the uncoated liposomes at all measured time points. At 6 hours, cumulative LTZ release from LP1 and LP2 was 70.2% and 58.7%, respectively. In contrast, CCL1 and CCL2 showed release rates of 46.9% and 38.2%, respectively. The overall maximum

release observed for CCL1 and CCL2 was 53.5% and 46.6%, respectively. Additionally, all formulations displayed a biphasic release pattern characterized by an initial burst phase followed by a sustained release phase. Within the first 2 hours, LTZ release was 40.2% for LP2, 35.7% for CCL1, and 23.6% for CCL2.

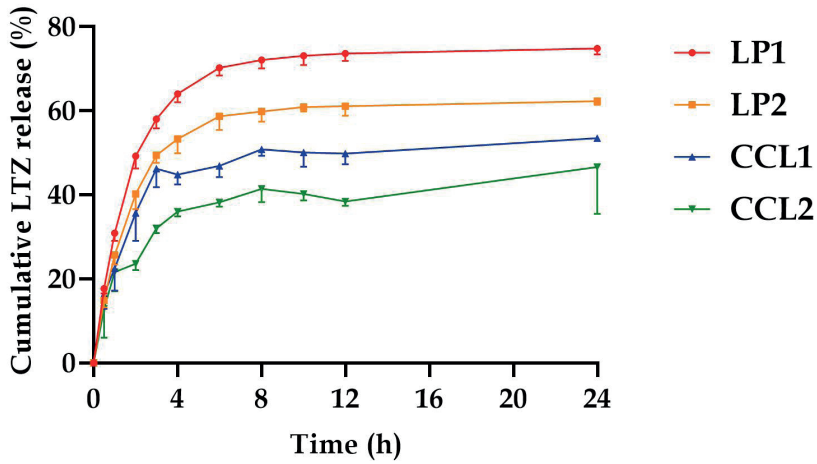

Figure 1. *In vitro* drug release profiles of LTZ-loaded liposomes and chitosomes (mean ± SD, n=3).

*LP1 contains 140 mg SPC and 60 mg CHOL, while LP2 contains 140 mg SPC and 60 mg CHEMS. CCL1 and CCL2 are LP2-based liposomes coated with 0.01% and 0.025% chitosan solution, respectively.

The higher release rates observed in uncoated liposomes (LP1 and LP2) suggest a more rapid diffusion of LTZ, potentially due to the absence of a polymeric barrier. The difference between LP1 and LP2 may stem from the influence of CHEMS on bilayer rigidity. As previously reported (Xu et al., 2017), CHEMS enhances membrane stability more effectively than CHOL, potentially limiting drug diffusion and resulting in slower release from LP2. Similar trends were reported by Ding et al (Ding et al., 2005). The reduced release rates from chitosomes, especially CCL2, suggest that higher chitosan concentrations result in greater restriction of drug diffusion. The polymeric chitosan coating likely decreases bilayer fluidity and acts as an additional diffusional barrier (Alshamsan et al., 2019). This effect is more pronounced at higher chitosan concentrations, as demonstrated by the lower release rate of CCL2 compared to CCL1. The initial burst release observed in all formulations may be attributed to the release of LTZ adsorbed on or near the surface of the liposomes and chitosomes (El-Hammadi et al., 2017). Following the burst phase, the sustained release is likely governed by a combination of drug diffusion through the lipid bilayer and the gradual degradation or swelling of the chitosan coating (Hasan et al., 2019). These findings support the hypothesis that chitosan coating modulates drug release from liposomal carriers, enabling controlled delivery profiles, which depend on polymer concentration.

Differential scanning calorimetry (DSC)

The DSC thermogram of pure LTZ exhibited a sharp endothermic peak at 187.14°C, while sucrose and CHEMS showed endothermic peaks at 190.94°C and 187.11°C, respectively (Figure 2A and B). These thermal analysis results were found to be consistent with previous studies (Hegde et al., 2023; Zhang et al., 2020; Verma et al., 2020). In the physical mixtures, all combinations except the mixture with CHEMS showed shifts of less than 5°C, indicating minimal interactions between LTZ and the excipients (Yalcin et al., 2025). In the LTZ–CHEMS mixture, however, the endothermic peaks of both LTZ and CHEMS shifted by more than 5°C, which was attributed to the solubilization of LTZ into CHEMS or a solid-state interaction induced by the input of energy and dilution during heating, although the interaction was not complete. Similar findings have been reported in the study by Zhang et al (Zhang et al., 2020).

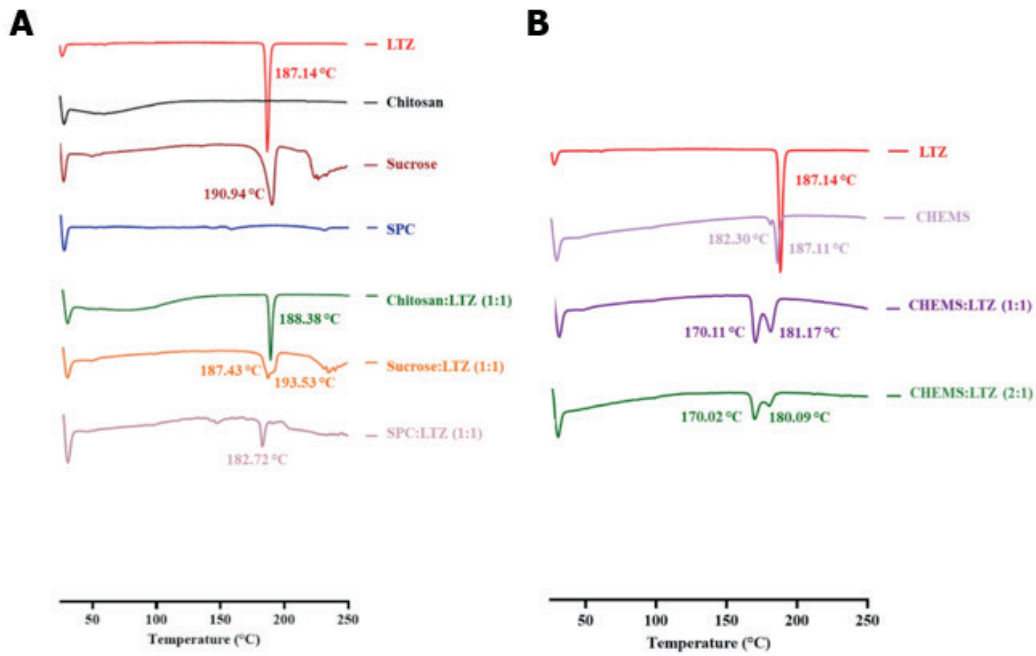

Figure 2. DSC thermograms of (A) LTZ, chitosan, sucrose, SPC, and their physical mixtures with LTZ; (B) LTZ, CHEMS, and their physical mixtures.

Transmission electron microscopy (TEM)

TEM images (Figure 3) revealed that both the liposome (LP2) and chitosome (CCL1) formulations exhibited predominantly spherical morphology. The vesicles appeared well-dispersed with smooth surfaces, indicating uniform particle formation. No significant aggregation or deformation was observed, suggesting that the preparation methods successfully produced stable and structurally intact vesicles. These morphological characteristics align with the expected behavior of lipid- and chitosan-based vesicular systems, confirming their suitability for potential drug delivery applications.

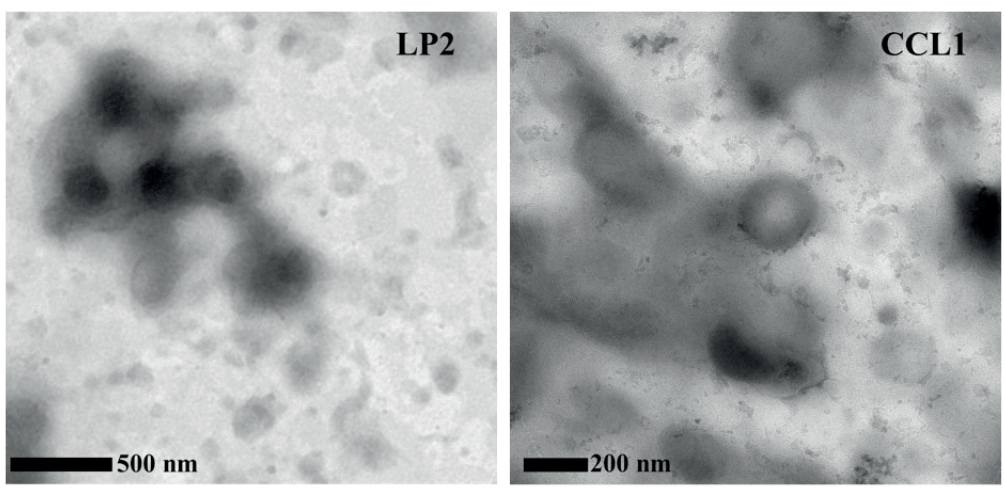

Figure 3. TEM images of liposome (LP2) and chitosome (CCL1) formulations,

Stability of liposome and chitosome formulations under different temperature conditions

The stability profiles of liposome (LP2) and chitosome (CCL1) formulations stored at 2-8°C and 25°C for one month are summarized in Table 3. The LP2 formulation showed no statistically significant changes in mean PS at either storage condition compared to its initial measurements ($p>0.05$). The changes in ZP and PDI were also minimal, indicating good physical stability over time. In contrast, the CCL1 formulation exhibited a noticeable increase in PS after storage, particularly at 25°C. The observed increase was accompanied by a corresponding decrease in the net negative ZP value. Notably, PS analysis could not be performed for CCL1 stored at 25°C due to severe aggregation and poor PS distribution.

Table 3. Characteristic properties of LP2 and CCL1 formulations after a month of storage at 2-8°C and 25°C (mean ± SD, n=3).

Formulation code	Time and storage conditions	PS (nm)	PDI	ZP
LP2	Initial	112 ± 3	0.286 ± 0.010	-48.9 ± 1.4
	1 month (2-8°C)	112 ± 3	0.244 ± 0.027	-55.1 ± 1.4
	1 month (25°C)	117 ± 13	0.285 ± 0.054	-48.6 ± 3.2
CCL1	Initial	155 ± 19	0.302 ± 0.056	-40.0 ± 2.3
	1 month (2-8°C)	206 ± 39	0.322 ± 0.060	-32.1 ± 0.8
	1 month (25°C)	Remarkable aggregation observed		

*LP2 contains 140 mg SPC and 60 mg CHEMS. CCL1 is an LP2-based liposome coated with 0.01% chitosan solution.
SPC: Soy phosphatidylcholine, CHEMS: Cholesteryl hemisuccinate

The stability results indicate that the uncoated liposome formulation (LP2) retained its physicochemical integrity over the storage period, with minimal variations in PS, ZP, and PDI. These findings suggest that the incorporation of CHEMS contributes to the structural stability of the liposomal bilayer, even at elevated temperatures. In contrast, the chitosome formulation (CCL1) demonstrated instability during storage, especially at 25°C. The observed increase in PS and decrease in ZP may indicate weakened electrostatic repulsion between particles, leading to aggregation. The inability to measure PS for CCL1 after storage at 25°C further supports this conclusion. Similar observations were reported by Ezzat et al. (Ezzat et al., 2019), who noted that elevated temperatures could compromise the integrity of chitosan-coated vesicles. The high purity of SPC used in this formulation may have contributed to weak chitosan adhesion on the vesicle surface (Guo et al., 2003), thus reducing the stability of the chitosan coating. As temperature increases, this fragile interaction may further deteriorate, promoting the formation of large aggregates.

***In vitro* cytotoxicity studies**

The cytotoxic potential of LTZ and its nanoformulations was evaluated using the MTT assay on MCF-7 breast cancer cells. The IC_{50} value of free LTZ was determined to be 61.3 nM. Drug-free liposome and chitosome formulations showed cell viability rates above 90% after 24 hours of treatment, indicating minimal cytotoxicity in the absence of the active drug. LTZ-loaded LP2 and CCL1 formulations, when administered at an LTZ concentration of 61.3 nM, reduced cell viability to 48.9% and 48.5%, respectively, indicating a relatively higher cytotoxic effect compared to the free LTZ solution (Figure 4).

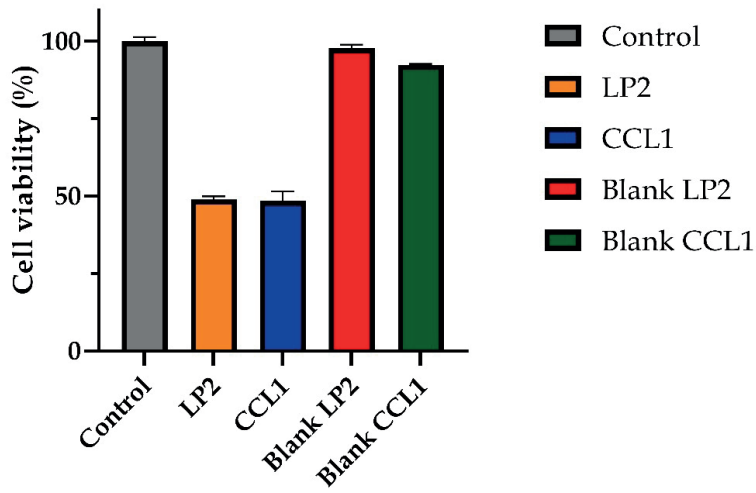

Figure 4. The percent cell viability of the MCF-7 cells after 24 h of incubation (mean ± SD, n=6).

*LP2 contains 140 mg SPC and 60 mg CHEMS. CCL1 is a liposomal formulation based on LP2, coated with 0.01% chitosan solution.

The IC_{50} value observed for LTZ (61.3 nM) was consistent with previous studies, confirming the reproducibility and reliability of the assay (Kijima et al., 2005). The high cell viability observed with drug-free formulations aligns with literature findings that demonstrate the biocompatibility of liposomal and chitosomal carriers (Patel et al., 2018). The slightly enhanced cytotoxicity observed in LTZ-loaded liposome (LP2) and chitosome (CCL1) formulations suggests improved intracellular delivery of LTZ. This may result from endocytosis-mediated uptake, a well-documented advantage of nanoparticulate drug delivery systems (Hassanzadeganroudsari et al., 2020; Kadari et al., 2017). Encapsulation within nanocarriers can also protect the drug from degradation and facilitate its accumulation in tumor cells, thus amplifying its therapeutic effect.

CONCLUSION

In this study, LTZ-loaded liposomal (LP2) and chitosomal (CCL1) formulations were successfully developed, demonstrating high EE% (>90%) and suitable particle sizes (<200 nm) suitable for passive targeting in cancer therapy. The chitosan coating process significantly altered key physicochemical characteristics of the formulations, including PS, ZP, and drug release profiles. *In vitro* stability assessments revealed that the chitosome formulation (CCL1) was more sensitive to temperature variations than the uncoated liposome (LP2). The observed instability may be attributed to the use of highly pure phospholipids, potentially resulting in weaker interactions with the chitosan layer. To improve thermal stability, future studies may consider using phospholipid grades with lower purity or incorporating stabilizing agents during formulation development. Cytotoxicity studies performed on MCF-7 breast cancer cells demonstrated that LTZ-loaded liposomal and chitosomal formulations exhibited comparable IC_{50} values to the free LTZ solution. However, both LP2 and CCL1 induced a relatively higher cytotoxic effect, suggesting enhanced intracellular delivery of LTZ via nanocarrier-mediated uptake. Overall, these findings support the potential of LTZ-loaded liposome and chitosome systems as effective nanocarriers for the delivery of anticancer drugs. Further, *in vivo* studies are warranted to evaluate the pharmacokinetic profile, therapeutic efficacy, and safety of these formulations in animal models.

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AUTHOR CONTRIBUTION STATEMENT

Concept, design, supervision, resources, materials, data collection and/or processing, analysis and/or interpretation, literature search, writing, critical reviews (TEY), data collection and/or processing, analysis and/or interpretation, literature search, writing, critical reviews (NTO), Supervision, resources, materials, data collection and/or processing, analysis and/or interpretation, literature search, writing, critical reviews (CY), Concept, design, supervision, resources, materials, data collection and/or processing, analysis and/or interpretation, literature search, writing, critical reviews (TI).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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