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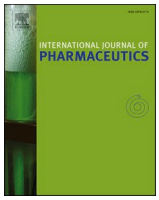

Conventional *vs* PEGylated loaded liposomal formulations by microfluidics for delivering hydrophilic chemotherapy

Eman Jaradat [a], Adam Meziane [b], Dimitrios A. Lamprou [a,*]

[a] School of Pharmacy, Queen's University Belfast, 97 Lisburn Road, Belfast BT9 7BL, UK
[b] Fluigent, 94270 Le Kremlin-Bicêtre, France

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ABSTRACT

Developing drug delivery systems (DDSs) is one of the approaches used to improve cancer treatment, with the main goal of loading cancer drugs into a carrier targeting a specific organ and avoiding the distribution to healthy tissues. Nanoparticles (NPs) have been shown to be one of the optimum carriers that can be used as DDSs. Lipid-based NPs, such as liposomes, have been investigated in the current study due to their low toxicity and ability to carry hydrophilic and hydrophobic molecules. In the current studies, conventional liposomes composed of DPPC, and cholesterol and PEGylated liposomes composed of DPPC, cholesterol, and DSPE-PEG2000 are manufactured and loaded with Carboplatin. The study focused on investigating and comparing the impact of modifying the carboplatin-loaded liposomes with different concentrations of DSPE-PEG2000 on the NP diameter, polydispersity, ζ-potential, encapsulation efficiency (EE%), and drug release. The hydrodynamic microfluidic system was used to investigate any possible improvement in the EE% over other conventional methods. The results showed the microfluidic system's promising effect in enhancing the EE% of the Carboplatin. Moreover, the results showed a smaller diameter and higher stability of the PEGylated liposome. However, conventional liposomes represent better homogeneity and higher encapsulation efficiency for hydrophilic molecules.

1. Introduction

Through years of research, cancer treatments have been under continuous development to overcome major concerns, such as the effect of cytotoxic drugs, inadequate drug accumulation in targeted tissues, and elevated risk and toxicity. Studies focused on investigating the ability to control the delivery of anticancer molecules to a specific location and enhance accumulation in the tissues without affecting other healthy tissues in the area, which characterise a drug delivery system. Nano-drug delivery systems (NDDS) are highly promising approaches that have generated significant interest in cancer research due to their superior capacity to overcome the challenges associated with conventional anticancer treatments. The distinctive characteristics of the nanoparticles (NPs) are the main reason for their superiority, including the high surface-to-mass ratio that offers a large "functional" surface and the high capability to adsorb to different molecules and function as a carrier (Jaradat et al., 2021). Moreover, the nanometre size of the carriers allows them traverse intercellular and physiological barriers, facilitating their accumulation within cancerous cells and tissues due to the enhanced permeability and retention (EPR) effect, which is predominantly observed in cancerous tissues (Sindhwani et al., 2020). Various types of NPs have been investigated for encapsulating anticancer molecules such as, metal, lipid-based (e.g., liposomes, niosomes, solid lipid NPs), and polymeric (e.g., PLGA) (Xu et al., 2022, Afsharzadeh et al., 2018, García-Pinel et al., 2019). Lipid-based NPs, including nanoliposomes (NLs), have superior features in encapsulating cancer drugs and creating NDDSs. Multiple studies have shown the positive impacts of encapsulating anticancer molecules into NLs, such as lowering the drugs' toxicity, increasing their uptake by tumour cells, and inhibiting tumour cell growth (Tsermentseli et al., 2018, Gkionis et al., 2020). The nano diameter of the NLs allows them to passively target the cancerous tissues, promoting their accumulation in the cancerous tissue rather than the healthy one. The primary essential feature of NLs is their high safety and low toxicity as carriers due to their natural composition of phospholipids, which is consistent with the human cell membrane. Besides safety, NLs can encapsulate either hydrophilic or hydrophobic anticancer molecules; the hydrophobic molecules are encapsulated within the lipid's bilayer, and the hydrophilic molecules are loaded in the core of the NL. However, loading the hydrophilic drugs into liposomes is still challenging due to the low

* Corresponding author.
E-mail address: d.lamprou@qub.ac.uk (D.A. Lamprou).

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encapsulation efficiency. Several studies reported the low encapsulation efficiency of hydrophilic drugs in NL (Eloy et al., 2014, Xu et al., 2012, Trang Le et al., 2020).

The limited encapsulation efficiency of hydrophilic drugs in NL is due to the hydrophobic nature of the NL bilayer, which limits the encapsulation into the NL core only. Moreover, due to the high aqueous solubility of the hydrophilic drugs, it mainly dissolved into the external aqueous phase during the NL manufacturing process and only small quantities entrapped inside the aqueous core of the formed NL (Eloy et al., 2014, Xu et al., 2012). The encapsulation of the hydrophilic drugs can be affected by several parameters such as liposome size and type, charge on the liposome surface, bilayer rigidity, method of preparation, loading type, and the ionic strength of the medium (Xu et al., 2012). The researchers made several efforts to enhance the encapsulation of hydrophilic drugs through several approaches, particularly by the loading method (Guimarães et al., 2021). Passive or active loading can be used for loading hydrophilic drugs. The active loading has been developed to enhance the encapsulation of hydrophilic drugs by pH-induced *trans*-membrane transfer of the drug (Rahim et al., 2021). The theory of this method is based on fabricating the NL at a specific pH, then changing the external phase pH to drive the uncharged drug molecule to diffuse into the NL and become protonated; thus, the protonated drugs will be entrapped inside the core, and their passage through the NL bilayer will be prevented. The active loading of the hydrophilic drugs shows promising EE and successful examples, such as the active loading of Doxorubicin, which reached 95 % EE and was processed successfully to the market as Doxil®. Still, the effectiveness of the active loading of hydrophilic drugs depends on the pH conditions of the drugs, which limit the suitability for specific drugs only, such as weak acids and bases (Rahim et al., 2021). Due to the mentioned limitations, the efforts to enhance the passive loading are increased to enhance the EE of various hydrophilic molecules. Multiple studies investigate the enhancement of hydrophilic molecules passive loading by optimizing the NL composition, charge, and manufacturing method parameters (Kępczyński et al., 2008, Villasmil-Sánchez et al., 2013, Eloy et al., 2014).. In general, the NL composition affects the EE of hydrophobic drugs; as the hydrophobic drugs EE depends mainly on the solubility of the drug in the NL bilayer. However, the lipid composition and the presence of additives also affect the fluidity, thickness, and polarity of the bilayer membrane, which might affect the EE of the hydrophilic drugs (Kępczyński et al., 2008). For example, some studies reported a noticeable improvement in EE's hydrophilic molecules after incorporating cholesterol in the lipid phase (Glavas-Dodov et al., 2005, Manojlovic et al., 2008). The studies show that the integration of cholesterol organises the construction of the hydrocarbon chains and controls the dynamics of the bilayer, which reduces the phospholipid chains' movement and provides some rigidity and stability to the lipid bilayer, which prevents the loss and leakage of the hydrophilic drugs in consequence (Manojlovic et al., 2008). Moreover, the modification of the NL bilayer with polyethene glycol (PEG) was reported to impact the liposome size, EE%, drug distribution, and drug release (Yalcin et al., 2018). The NL PEGylation provides a hydration effect to the lipid surface and decreases the hydrophobicity of the vesicle, which may lead to attracting the hydrophilic drug accumulation in the NL (Andresen et al., 2005). Also, The PEG-lipid incorporation reduces the incidence of NL aggregation and enhances the physical stability of the formulation (Kowalska et al., 2021, Sriwongsitanont and Ueno, 2004).

In addition to the lipid composition, the manufacturing method of the NL was reported to impact the EE of the hydrophilic drugs (Eloy et al., 2014). The most common method used for liposome manufacturing is thin film hydration. The main drawback of this method is the low EE of the hydrophilic molecules; as hydrophilic drugs should be dissolved in the aqueous hydration medium to be encapsulated in the inner core of the NL, thus a high quantity of the drug stayed in the external aqueous medium and small amounts entrapped inside the NL. The studies investigate other manufacturing methods to enhance the EE, such as reverse phase evaporation, cycles of freeze–thaw (Bernal-Chávez et al., 2023, Petrilli et al., 2017, Joshi et al., 2016, Jaradat et al., 2023).

Recently, several studies have reported the effectiveness of hydrodynamic microfluidics (MF) in manufacturing PEGylated NL (Arduino et al., 2021, Cheung and Al-Jamal, 2019Joshi et al., 2016). MFs is a leading-edge method that controls small amounts of fluids (ranging from 10^{-9} to 10^{-18} L) by utilising micrometre-sized channels, microvalves, and micromixers. The MF approach's outstanding properties provide a consistent laminar flow that enhances the quality of mixing for NL formulations, increasing the control of NL size and enhancing homogeneity (Jaradat et al., 2021). Controlling a constant TFR and FRR between the phases, ensures continuous monodisperse and homogeneous NL production. TFR and FRR are considered parameters of the NL manufacturing method; changing the TFR and FRR impacts the NL size and PDI. Several studies represented the impact of changing the TFR and FRR on the NL properties (Jaradat et al., 2022, Aghaei et al., 2021, Kastner et al., 2014). The determination of the most optimum FRR and TFR can contribute to producing a high-quality NL.

In this study, a novel investigation is conducted to determine the impact of NL Type,composition, and MF parameters on enhancing the passive EE of Carboplatin. Carboplatin (*cis*-diamine (1,1-cyclobutane dicarboxylate) platinum (II)) is classified as a second-generation platinum drug that was developed to overcome the toxicity of cisplatin. Carboplatin has become a preferred agent over cisplatin due to its higher safety and fewer side effects (Nguyen et al., 2019). It has also been approved for multiple types of cancers, including ovarian, testis, cervical, and small-cell lung cancer (Pourmadadi et al., 2023). Like cisplatin, carboplatin is an alkylating agent that disrupts the division and growth of cancer cells by binding to a specific region of the DNA (Herath et al., 2019). However, a significant concern was reported about the carboplatin's unselective effect that attacks and destroys the healthy and cancer cells together. Moreover, the low cellular uptake of the drug requires higher doses to reach the therapeutic index of the drugs. These limitations drove the search for an efficient DDS to target drug delivery and enhance cellular uptake. Various studies demonstrate the potential of encapsulation in carboplatin in PEGylated NP and lipid-based NP (Trang Le et al., 2020, Ebrahimifar et al., 2017). The encapsulation of carboplatin as a hydrophilic drug with poor solubility in organic solvents and limited aqueous solubility in NL is challenging; the main challenge was the low passive EE of carboplatin as a small hydrophilic drug in both conventional and PEGylated NL (Trang Le et al., 2020, Ebrahimifar et al., 2017). Therefore, optimum NDDs with suitable particle size and EE for effective carboplatin delivery are still in demand. Most of the previous studies have attempted to enhance the passive encapsulation of the hydrophilic drug by modifying the manufacturing method parameters or the composition of the liposomes. For example, some of these studies investigate the effect of altering the thin film hydration parameters, such as hydration time and volume or tried to modify the composition of PEGylated and conventional liposomes separately, such as varying the PEG lipid concentration or the cholesterol concentration in the conventional NL (Xu et al., 2012, Yamamoto et al., 2007, Kępczyński et al., 2008, Zamboni et al., 2004, Chaudhury et al., 2012b). In the microfluidic area, one study investigated the impact of MF parameters (TFR and FRR) on enhancing the EE of hydrophilic drugs into different compositions of PEGylated liposomes (Aghaei et al., 2021). The research gap between these studies was examining the effects of utilising various types of NL including conventional and PEGylated NL, on the EE of hydrophilic drugs. This work includes a novel comparative investigation between conventional and PEGylated NL and how this impacts the EE of the Carboplatin. Both types were manufactured based on optimised lipid compositions and manufacturing method parameters (TFR and FRR). The study thoroughly compared various aspects of conventional and PEGylated NL, including the particle size, PDI, ζ-potential, morphology, EE, and release profile. Moreover, the study highlighted the efficiency of the MF system in enhancing the passive encapsulation of hydrophilic molecules into NL

compared to the traditional methods.

The main aim of this work is to optimise the nano-carrier for drug delivery. Conventional and PEGylated NL were fabricated using similar TFR and FRR and loaded with equal carboplatin concentrations to compare the conventional and PEGylated NL efficiency in encapsulating hydrophilic drugs.

2. Materials and methods

2.1. Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was purchased form Tokyo Chemical Industry Co., Ltd (TCI) (Portland, USA). 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[carbonyl-amino(polyethylene glycol)-2000] (DSPE-PEG2000) was purchased from Avanti. Cholesterol, phosphate-buffered saline (PBS, pH 7.4) tablets, Tween 80, ethanol (≥99.8 %,) and acetonitrile (≥99.9 %), were purchased from Sigma Aldrich. Carboplatin was purchased from TCI. The chemical structure of the materials used can be seen in Fig. 1.

2.2. Methods

2.2.1. Liposomal formulation preparations

The NL manufactured using the FLUIGENT MFCS-EZ (Paris, France) microfluidic flow control device and software. The conventional carboplatin NL prepared by mixing lipid phase composed of DPPC and cholesterol, and aqueous phase composed of PBS water and carboplatin. The lipid phase prepared by mixing DPPC and cholesterol with 2:1 mass ratio and 0.52: 0.48 M ratio respectively; the utilised lipid to cholesterol ratio has been determined as the optimal ratio in previous research (Briuglia et al., 2015, Weaver et al., 2022). The total lipid concentration was 1 mg/ml and the final molarity is 0.00176 mol/L The calculated masses of the lipid weighted and dissolved in particular amount of ethanol then sonicated to ensure the complete dissolution of the lipids. The aqueous phase prepared by mixing 0.5 mg/ml or 1 mg/ml of carboplatin with PBS water then sonicated until the drug dissolved. The utilised concentrations of carboplatin were determined based on the previously established concentrations range 0.25–2 mg/ml of carboplatin in liposomal formulation and final molarity 0.00135 mol/L and 0.00269 for 0.5 and 1 mg/ml formulations respectively (Zhang et al., 2014, Chaudhury et al., 2012a). The lipid phase is placed in the first microfluidic reservoir and the aqueous phases are placed in the second reservoir (Fig. 2). Both phases are injected to Y shaped microfluidics chip at TFR 1 ml/min and processed at 1:2 and 1:4 FRR. Every formulation is prepared nine times to allow for statistical analysis and reproducibility data.

The PEGylated carboplatin NL prepared using the same method as above with some variations. The DSPE-PEG 2000 lipid is added to the lipid phase with 2:0.9:0.1 and 2:2:2 DPPC to cholesterol to DSPE-PEG2000 mass ratio and 0.58:0.41:0.02 and 0.32:0.6:0.08 M ratio respectively. The calculated masses of lipids were weighted and dissolved in ethanol to produce lipid phase with total lipid concentration 1 mg/ml. Each ratio prepared and processed separately as two different formulations of PEGylated liposomes (Table 1). For the aqueous phase, two different concentrations of 0.5 and 1 mg/ml of carboplatin loaded to the PEGylated NL. The lipid and aqueous phases of each group injected to Y shaped microfluidic chip with 1:2, 1:4 FFR and TFR 1 ml/min. Every formulation prepared nine times to allow for statistical analysis and reproducibility.

2.2.2. Characterization of liposomes

2.2.2.1. Particle sizing and ζ-potential. The particle size and PDI were determined using dynamic light scattering (DLS) analysis using the Nanobrook Omni particle sizer (Brookhaven Instruments, Holtsville, NY, USA). 20 μL of the liposomal formulation was diluted into 2 ml of PBS. The identical approach was also employed to measure the ζ-potential. The measurement of each sample was repeated three times, utilising samples that were initially prepared in sets of three (n = 9).

2.2.2.2. Spectroscopic analysis. An attenuated total reflection (ATR) Fourier transform infrared (FTIR) spectrometer (Thermo Fisher Scientific, Nicolet is 50 FTIR with built-in ATR) was used to perform the analysis for the conventional and PEG NL. The purpose was to investigate the effects of modifying the liposome surface with PEGylated lipids on stretching, bending, or forming additional chemical bonds. The liposomal formulations were prepared by centrifuging them at 14,800 rpm per minute for 30 min. The resulting pellets were then collected for examination. The liposome pellets were analysed under controlled conditions using an inert atmosphere. The analysis included a range of wavelengths from 4000 to 600 cm^{-1}, with 64 scans performed at a resolution of 4 cm^{-1} and an interval of 1 cm^{-1}. Each sample underwent three tests, and all samples were evaluated on day zero to minimize the occurrence of any potential formulation degradation.

2.2.2.3. Atomic force microscopy (AFM). The AFM TT-2 AFM (AFM-Workshop, US) was used to study the morphology of the conventional and PEG NL. Each sample is diluted with 2 ml of PBS water, using 20 μL of the sample. Then, 20 μL of the resulting solution, applied onto a cleaved mica of 1.5 cm × 1.5 cm (G250–2 Mica sheets 1 in. × 1 in. ×

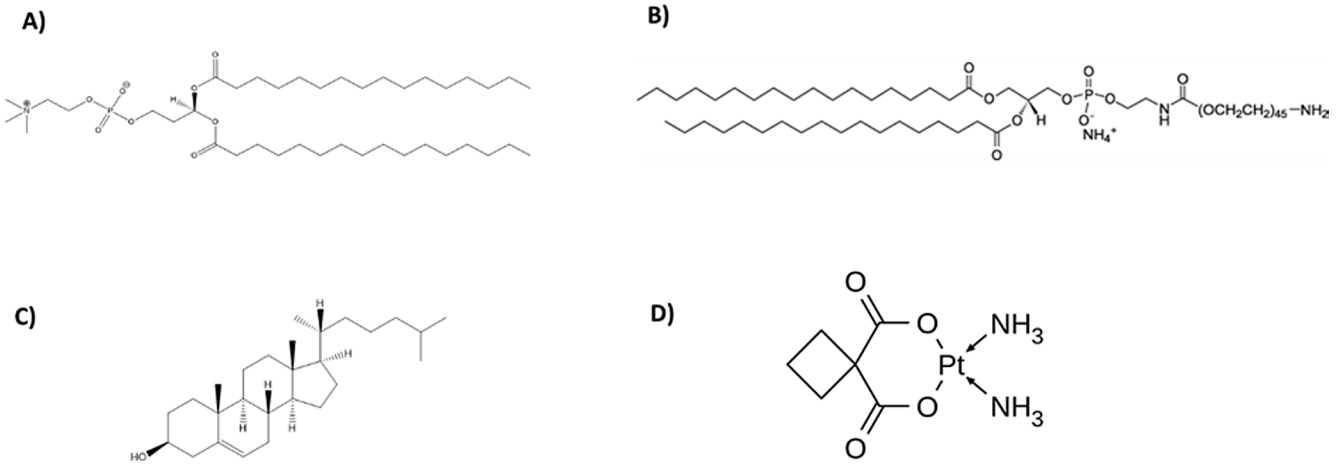

Fig. 1. Chemical structure of: A) 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), B) 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG200), C) Cholesterol, and D) Carboplatin.

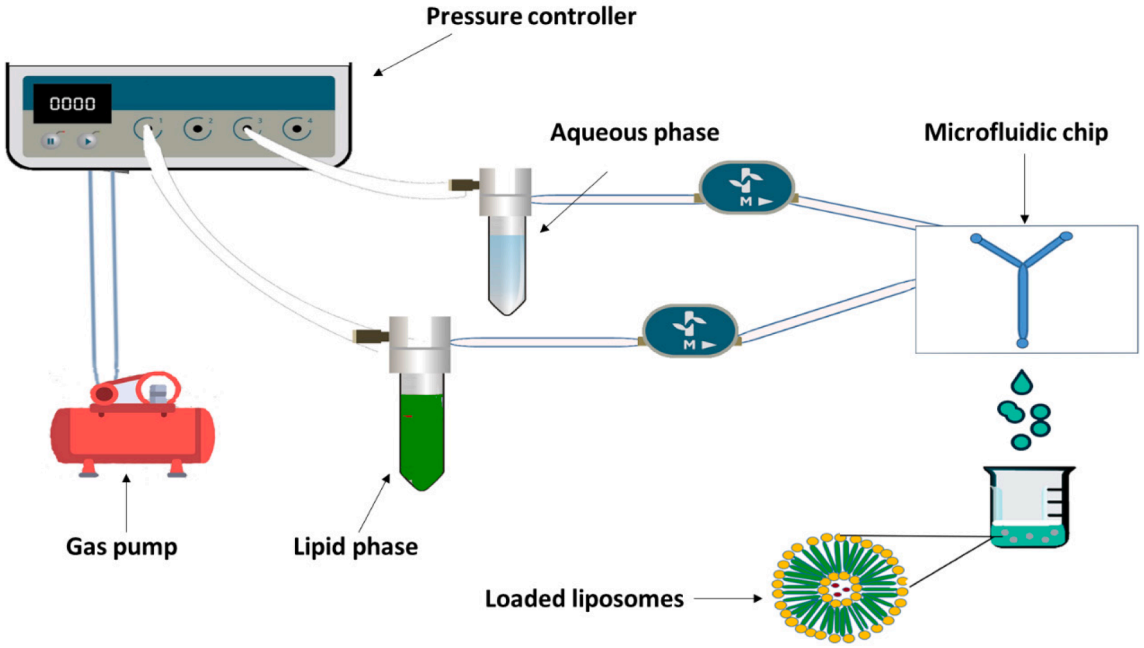

Fig. 2. Graphic presentation for the MF system and the process of the NL manufacturing. The system composed of gas pump for providing the pressure, pressure controller, two compartments one of them for the lipid phase and the other one for the aqueous phase, and the MF chip as the mixer of the system.

Table 1
Lipid ratio, drug concentration, and FRR of different PEGylated formulations.

Formulations Code	DPPC: chol: DESP-PEG200 ratio	Drug concentration (mg/ml)	FRR
P1	2:2:2	0.5	1:2
P2	2:2:2	0.5	1:4
P3	2:2:2	1	1:2
P4	2:2:2	1	1:4
P5	2:0.9:0.1	0.5	1:2
P6	2:0.9:0.1	0.5	1:4
P7	2:0.9:0.1	1	1:2
P8	2:0.9:0.1	1	1:4

0.006 in.; Agar Scientific ltd., Essex, UK). The solution is allowed to dry for a duration of 30 min in a room temperature. The samples then underwent a thorough wash using 1 ml of PBS to eliminate any liposomes that were not adherent to the mica surface. The solution was allowed to dry for further 30 min prior to examination by AFM. AFM images obtained with Ohm-cm Antimony doped Si probes, operating within a 50–100 kHz frequency range, a 512 × 512 pixels resolution, and a scan rate of 0.6 Hz.

2.2.3. Stability studies

The stability study of the formulations conducted at two temperatures to investigate the stability and compatibility of the particles under storage settings (4 °C) and body temperature (37 °C), in order to simulate the conditions experienced during shelf life and within the body after administration. Each sample underwent three analyses, and the samples were divided into two groups, with each group being stored at 4 and 37 °C. Diameter, PDI, and ζ-potential were assessed on a weekly basis for up to 1-month. The study conducted to establish the impact of the liposomes PEGylation on the physical stability of the liposomes. The conventional and PEGylated formulations were stored as liquid suspensions and set in place to assess the physical stability of the formulations during storage.

2.2.4. Encapsulation efficiency (EE%)

A two-step centrifugation process was carried out. Initially, 1 ml of the liposomal suspension underwent centrifugation at 14,800 rpm for 30 min. Following the initial centrifugation, the resulting supernatant was withdrawn for subsequent analysis. Subsequently, 1 ml of fresh PBS was added to the precipitate, and a second centrifugation round was conducted to extract any drug that may have been physically adsorbed onto the liposomal surface. Both supernatants obtained from these centrifugation steps were subjected to analysis using Ultraviolet High-Performance Liquid Chromatography (UV-HPLC). The free drug analysis was performed utilizing a C18 column (250 mm × 4.6 mm) sourced from ThermoFisher Scientific, with detection at 227 nm. The samples processed with isocratic elution flow using acetonitrile and water as a mobile phase with 50:50 gradient. The volume of injected sample was 50 μL and the overall flow rate was 1 ml/min. The EE% calculated for the conventional and PEGylated carboplatin NL using equation (1).

$$\textbf{Encapsulationefficiency}(\textbf{EE}\%) = \frac{\boldsymbol{Totalamountoftheadddrug}(\boldsymbol{mg}) - \boldsymbol{freedrugamount}(\boldsymbol{mg})}{\boldsymbol{Totalamountofthedrug}(\boldsymbol{mg})} \times 100$$

(EQ 1) Equation used to calculate encapsulation efficiency for carboplatin-loaded NL.

2.2.5. In vitro drug release study

The in vitro release profile of carboplatin from conventional and PEG NL was investigated using the dialysis tubing method. Dialysis bags with a cellulose membrane (average flat width of 10 mm, 0.4 in., MWCO 14,000, Sigma Aldrich) were employed for this study. Prior to analysis,

the dialysis bags were sterilized by boiling them in deionized (DI) water and subsequently rinsing them with DI water. The samples prepared by centrifuging the liposomes for 30 min at 14,800 rpm. The resulting supernatant is removed, and the liposomal pellets from the participants are hydrated with PBS water and introduced into the dialysis bags. The dialysis bags submerged in a release media are composed of PBS water 7.4 pH to mimic in vivo environment conditions. The release study performed for the conventional and PEG NL.

2.2.6. Statistical analysis

All experiments were performed in triplicate trials, and standard deviation and mean were calculated when required. Ordinary-one-way (ANOVA) tests were performed for the ζ-potential of the PEGylated and conventional NL, for the different drug concentration and % of the released drug. As well as, T-test performed for the PEGylated NL diameter at the stability study temperatures 4 and 37 °C.

3. Results and discussion

The hydrodynamic MF system utilized for manufacturing both conventional and PEGylated carboplatin loaded-NL. The liposomes manufactured by mixing the lipid phase and aqueous phases at 1 ml/min TFR and 1:2 / 1:4 FRR. The optimum MF parameters have been determined based on our previous work that studied multiple TFRs and FRRs (Jaradat et al., 2022). The promising role of MFs, lies in its capacity to regulate several factors of the production process, such as the FRR, which can greatly impact the size and uniformity of liposomes. This work aims to study the efficiency of the MF system in encapsulating a hydrophilic chemotherapy drug (Carboplatin) into conventional and PEGylated NL, and to investigate the impact of the PEGylation on liposomes' diameter, EE% and drug release, comparing to the conventional ones.

3.1. Carboplatin-loaded conventional liposomes

The empty conventional NL created by coupling DPPC with cholesterol using a MF system at TFR 1 ml/min and 1:2/1:4 FRR. Lipid type and MF parameters, were determined based on our previous work that studied 4 different phospholipids and coupled them with cholesterol in various ratios (Jaradat et al., 2022). The previous work results showed that 1:2 and 1:4 FRR at TFR 1 ml /min provide liposomes with smallest size, lowest PDI, and high stability at 37 ℃ and 4℃ that makes them optimum for carboplatin encapsulation. The empty conventional liposomes' diameter was measured to found 216 ± 8 nm and 168 ± 4.5 nm, for 1:2 and 1:4 FRR respectively. The PDI was 0.2 ± 0.04 for the 1:2 FRR and 0.18 ± 0.02 for the 1:4 FRR and the ζ-potential was −5.7 ± 2.4 mV for 1:2 and − 6.5 ± 1.1 mV for 1:4. The carboplatin encapsulated into the liposomes by dissolving it in the aqueous phase; due to the hydrophilic nature of the drug. The diameters of the resultant conventional carboplatin-loaded NLs (Fig. 3), was comparable and no significant different. In depth look, the NLsdiameter slightly increase after carboplatin encapsulation, especially with 1 mg/ml concentration, which mimics the trend of other reported data (Shi et al., 2018). The reason for this increase is due to the hydrophilicity of the encapsulated drug; the hydrophilicity of the carboplatin it might lead to an increase in the water uptake into the NL, resulting in a swell of the bilayer and an increase in the overall diameter. The diameter of the liposomes after encapsulating 1 mg/ml of carboplatin was ≥ 200 nm, meanwhile the encapsulation of 0.5 mg/ml of carboplatin kept the diameter < 200 nm. For example; the diameter of NLsat 1:2 FRR increased to 242 nm after loading 1 mg/ml of carboplatin and the diameter of the NLsat 1:4 FRR raised to 200 nm. Also, the increase of the drug concentration from 0.5 to 1 mg/ml at the same FRR increased the NLs diameter specially at 1:4 FRR. In general, the liposomal formulations with 1:4 FRR were smaller, more homogenous, and reproducible. The increase of the FRR from 1:2 to 1:4 have a positive impact on the liposomes diameter and PDI; the raise in the FRR decrease the liposomes diameter and PDI, which have been reported previously (Jaradat et al., 2022). The inverse-relationship between the FRR and liposome diameter can be explained by the decreasing of the liposomes final solvent concentration when the FRR increased, which reduce the prevalence of particle fusion and the decrease the incidence of the "Ostwald ripening" phenomenon (Leung et al., 2018).

In general, The liposomes with average diameter of ~ 200 nm considered as the optimum range for drug delivery purposes, based on the FDA "Guidance for industry" (U.S, 2018). The small diameter of the liposomes is important to keep the DDSs away from any immune attack and sustained their life in the circulation system (Jaradat et al., 2021). Moreover, the small diameter of the liposomes is preferable to enhance the diffusion of the liposomes into the cancerous tissue.

The PDI measurements is another key attribute that can directly affect the stability, efficacy, and final appearance of the pharmaceutical liposomes (Duong et al., 2021). The low PDI of the formulations is not less important than the small diameter of liposomes; the formulations with PDI < 0.3 indicate a homogeneous population of liposomes and are considered acceptable for DDs (Guimarães et al., 2021). Both, low PDI

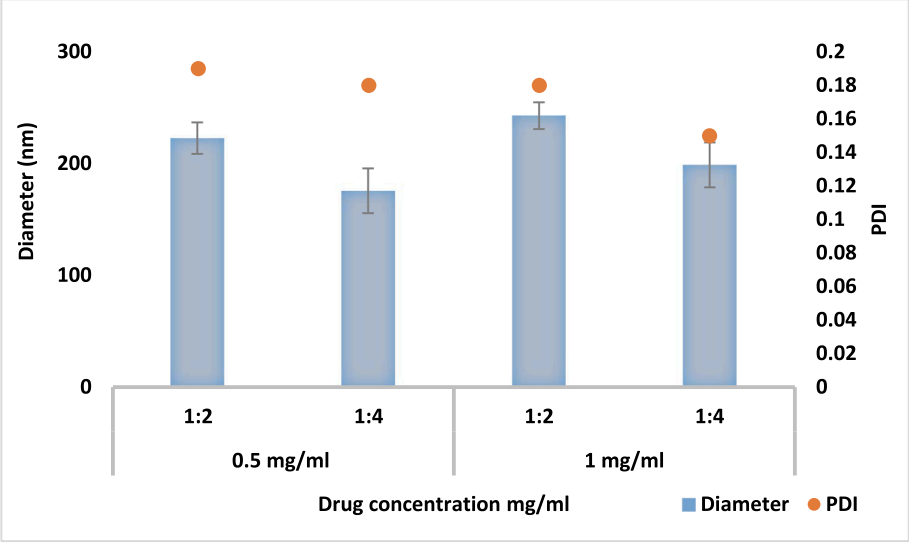

Fig. 3. Average diameter and PDI of conventional carboplatin-loaded NLs using 1:2 and 1:4 FRR at 0.5 and 1 mg/ml concentration.

and the small diameter, are main physicochemical characteristics that improve the endocytosis cellular uptake and considered as critical quality attributes (CQAs) (Maherani and Wattraint, 2017). The PDI results of the carboplatin-loaded NLs were < 0.2 representing high homogeneity of the liposomal formulations. The average PDI for the 1:2 FRR formulated NLs was 0.185 and decreased to 0.16 when the FRR increased to 1:4. The impact of the FFR on the PDI was inverse; increasing the FRR decreases the PDI and produced more homogenous formulations. The positive impact of raising the FRR on the PDI results was reported in previous studies (Sedighi et al., 2019, Jaradat et al., 2022).

The ζ-potential is an indicator of the surface charge of liposomes and a key factor of the stability of the liposomal formulations. More negative or positive ζ-potential values induce a strong repulsion forces between the liposomes that decrease the incidence of liposomes aggregates and enhance the liposomal formulations stability (Gonzalez Gomez and Hosseinidoust, 2020). The ζ-potential results of the conventional NLs represent a neutral or slightly anionic charge (Fig. 4). Despite using neutral lipids, the liposome's slightly anionic charge is owed to the negative phosphate group orientation at the end of the phospholipid acyl chain. The assumption supposes that the phosphate group is oriented toward the surface of the liposomes instead of the partially hydrophobic choline group; the nature of the choline group drives it toward the liposome's lipid bilayer interphase to avoid any possible contact with the surrounding aqueous. However, natural liposomes possess superior qualities in vivo compared to anionic or cationic liposomes. The neutral charge of liposomes bypasses any immunological responses by the RES cells, preventing early elimination and extending the carboplatin liposomes' circulation time (Zhen and Li, 2020).

The 1:4 FRR liposomal formulations have been determined for performing stability study due to their superior characteristics over 1:2 FRR formulations. The physical stability of the conventional NLs studied for up to 4-weeks at 37 °C and 4 °C, to investigate the particle size, homogeneity, and incidence of aggregation of the formulations at the physiological body and storge temperature. The results show high stability at 4 °C for both formulation with 0.5 and 1 mg/ml carboplatin; the particle sizes were comparable over the 4 weeks and PDI kept < 0.2, which indicate low incidence of aggregation and homogenies formulations. An increase in particle sizes and PDI noticed at 37 °C (Figure S1); as the PDI of the 0.5 mg/ml formulation increased from 0.19 to 0.23 and the PDI of the 1 mg/ml formulation increased from 0.15 to 0.20. The increase in NLs diameter and PDI after incubating the NLs at 37 °C might happened due to the effect of increasing the temperature on the lipid bilayer packing (Morini et al., 2015). The same trend of liposomes size increase at 37 °C have been reported previously (Jaradat et al., 2022, Morini et al., 2015).

3.2. PEGylated carboplatin liposomes

The conventional carboplatin-loaded NLs were modified with PEGylated lipid to create PEGylated NLs or as been called in the literature, "stealth liposomes". Several studies emphasize the PEGylation of liposomes effect in enhance the liposomes stability, prolong the circulation time and improve the passive targeting to the cancerous tissues (Milla et al., 2012, Jaradat et al., 2023, Kowalska et al., 2021). Furthermore, the PEGylation of liposomes results in a reduction of the drugs toxicity and improvement in the therapeutic effects (Milla et al., 2012). The main aim of this modification is to study the impact of the PEGylation on the conventional liposome's characteristics including particle size, PDI, stability, EE, and drug release. The PEGylated lipid added to the lipid phase of the liposomes; 2 different mass ratios 2:0.9:0.1 and 2:2:2 DPPC to cholesterol to DSPE-PEG2000. The utilized ratios have been determined based on our previous work that studied various PEGylated lipid ratios incorporated with DPPC and cholesterol to manufacture empty PEGylated liposomes (Jaradat et al., 2023). The result of the study shows a high stability and compatibility of the prepared liposomes with the forementioned ratios which make them suitable for manufacturing carboplatin loaded NLs. The same MF parameters, drug concentrations, and manufacturing process conditions were used to validate the comparison between the conventional and PEGylated carboplatin loaded NLs.

3.2.1. Particle size, PDI, ζ-potential

The DLS measurements show a variation between the diameter of the PEGylated and conventional NLs. The NL sizes were remarkably

Table 2
Empty conventional and PEGylated liposomes diameter and PDI.

FRR	Liposomes composition	Diameter (nm)	PDI
1:2	DPPC: chol 2:1	216 ± 8	0.2 ± 0.03
1:2	DPPC:chol: PEG 2:0.9:0.1	163 ± 22	0.24 ± 0.02
1:2	DPPC: chol: PEG 2:2:2	137 ± 21	0.25 ± 0.03
1:4	DPPC: chol 2:1	168 ± 4.5	0.18 ± 0.01
1:4	DPPC: chol: PEG 2:0.9:0.1	144 ± 21	0.23 ± 0.04
1:4	DPPC: chol: PEG 2:2:2	137 ± 17	0.24 ± 0.03

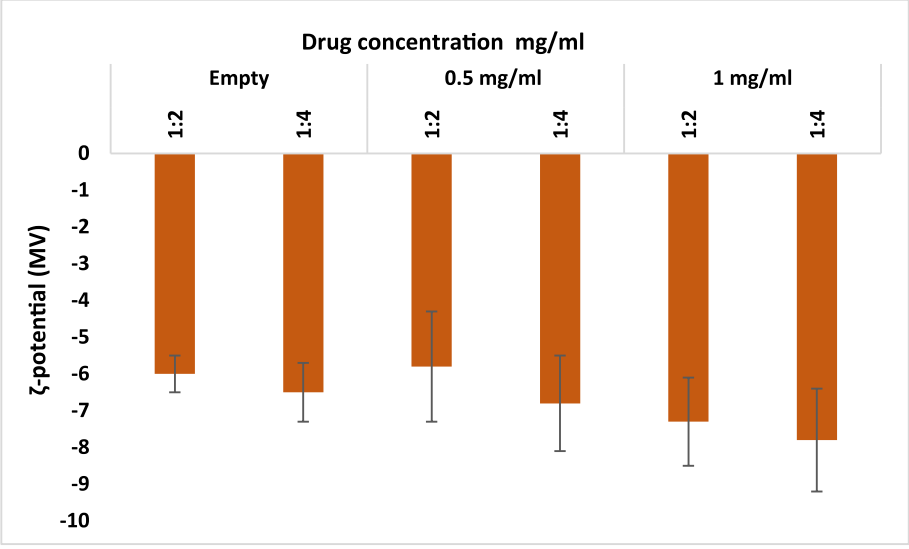

Fig. 4. Average ζ-potential of conventional NLs at different drug loading concentrations.

decreased after incorporating DSPE-PEG2000 lipid into the lipid bilayer. For example, the empty PEGylated NLs with 2:0.9:0.1 lipid ratio and 1:2 FRR, the diameter of the NLs measured to be 163 ± 22 nm compared to the conventional NLs which was 216 ± 8 nm (Table 2). The same trend of liposome diameter decreasing was noticed with the loaded NLs; for example, the particle size of the conventional NLs formulated at 1:2 FRR and loaded with 0.5 mg/ml carboplatin was 222 ± 14 nm compared to P5 NLs that prepared at the same FRR and loaded with the same drug concentration the diameter was 169 ± 13 nm. The decline in NL diameter after incorporating PEG lipids can be explained by the slightly negative charge of the PEG lipid that promotes the lateral repulsion intensity. The repulsion forces result in pushing and curving the bilayer lipid, which diminishes the overall liposome diameter (Sriwongsitanont and Ueno, 2004, Najlah et al., 2019, Tsermentseli et al., 2018). Moreover, the incorporation of PEG-lipid leads to increased interlamellar repulsion, which subsequently causes a reduction in the liposome's lamellarity (Tsermentseli et al., 2018).

Increasing the PEG lipid ratio in liposomal formulation from 2:0.9:0.1 to 2:2:2 results in a reduction of the size of the NL(Figs. 6 & 7). For example, the particle size of formulation P7, was 221 ± 12 compared to 180 ± 27 for P3. The reduction in NLsize can be explained by the impact of the high PEG-lipid concentration around the liposomes outer shell; the increase of the PEG-lipid ratio lead to dehydrate and remove the water from the lipid Bilayer which reduces the polar head-group size, and enhance the lateral packing of the lipids chains (Garbuzenko et al., 2005). Moreover, the reduction of NLs diameter can be explained by understanding the correlation between the PEG lipid concentration and the configuration of the PEG lipid chains around the liposomes surface. The hypothesis assume that at low PEG-lipid concentration (formulations P5 to P8) the lipid chains configuration is a mushroom-like shape (Nosova et al., 2019). At higher concentration of the PEG lipid (formulations P1 to P4), the chains moieties extended and start converting from mushroom-like structure to a brush-like shape (Nele et al., 2019). This conversion lead to increasing the liposome's surface coverage and enhances the lateral repulsion of the PEG chains, which curve the lipid bilayer and decrease the liposomes size consequently (Nele et al., 2019). The FRR is additional parameter affects the PEGylated NLs diameter. The increase of the FRR form 1:2 to 1:4 decrease the NLs diameter which reported also with the PEGylated NLs (Figs. 5 and 6). The 1:4 FRR provide liposomal formulations with smaller diameter and lower PDI which make them more suitable for drug delivery purposes.

The PEGylation of liposomes not only Impacted the size of the liposomes but also affect the PDI of the formulation. The results showed a variation in the PDI values of the conventional and PEGylated NLs; the PEGylated NLs had higher PDI values. The average PDI of empty conventional NLs was calculated to be 0.19, and the PDI of the PEGylated NLs were 0.24. In the meantime, increasing the PEG-lipid concentration in the PEGylated NLs from 2:0.9:0.1 to 2:2:2 increases the PDI values (Figs. 5 & 6). Several studies reported a correlation between the PDI value growth and the increased PEG-lipid concentration (Cheung and Al-Jamal, 2019, Shenoy et al., 2011, Jaradat et al., 2023). For example, Cheung et al, studied the effect of incorporating different PEG-lipid concentration on the liposome's characteristics. The results show a decrease in the liposome diameter and an increase in the PDI values with higher PEG-lipid concentrations, which support the trend of our results (Cheung and Al-Jamal, 2019). This study's findings are also consistent with our previous work, which investigated the liposome size and PDI changing after incorporating different molar ratios of DSPE-PEG2000 from 0.6 mol % to 9 mol % and showed increased PDI values with higher molar ratios (Jaradat et al., 2023).

The ζ-potential was measured for both conventional and PEGylated NLs to study the impact of incorporating PEG-lipids on the electrostatic charge of liposomes. The PEG-lipid alters the charge of the liposome's surface to be more anionic or cationic; the surface charge alteration depends mainly on the PEG-lipid charge. Here, the PEGylated NLs showed more anionic charge than conventional NLs (Figs. 4 and 7). The average charge of the conventional NLs was −6.7 mV compared to the average charge of the PEGylated NLs, which was −9.6 mV. This can be explained by the negative charge of the DSPE-PEG2000, which is considered a relatively anionic lipid. The increasing of PEG-lipid ratio from 2:0.9:0.1 to 2:2:2 show a slight increase in the negative charge, but no significant different notice between the two formulations ($P > 0.05$). The ζ-potential of the liposomes is a significant factor affecting the stability of the liposomal formulations as well as the adsorption of the liposomes into the cell membranes. The ionization of the liposomes to be more anionic or cationic increases the electrostatic repulsion between the liposomes and limits liposome aggregation, which enhance the liposomal formulation stability. The increase of the anionic charge of the liposomes after the surface PEGylation might be a major factor in improving the formulations stability. Several studies reported a major improvement in the stability of the liposomal formulation after modifying the liposome surface with PEG-lipids (Hassanzadeganroudsari et al., 2019, Honary and Zahir, 2013, Ebrahimifar et al., 2017). Some of these studies, found a direct relationship between the ζ-potential and the stability (Honary and Zahir, 2013). The stability study was performed

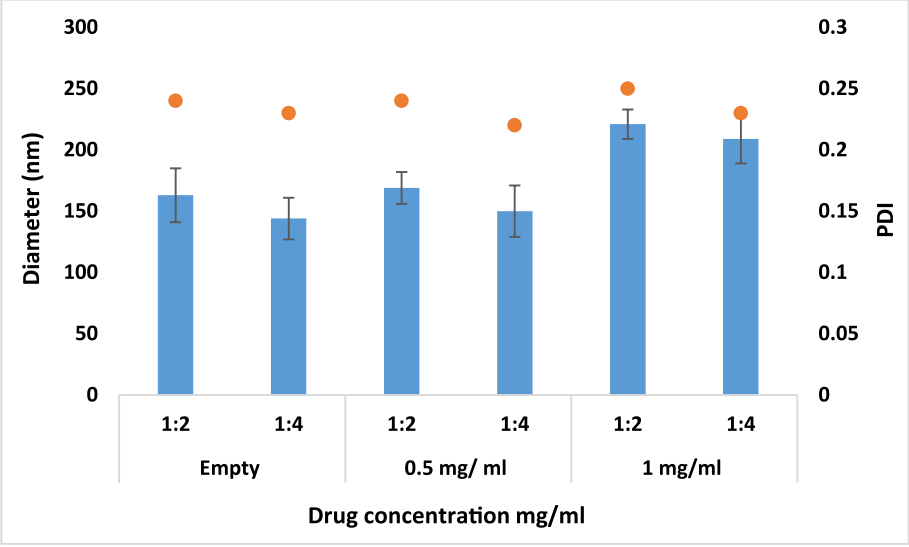

Fig. 5. Carboplatin-loaded PEGylated NLs (2:0.9:0.1) at 0.5 and 1 mg/ml concentration using TFR 1 ml/min and 1:4 FRR.

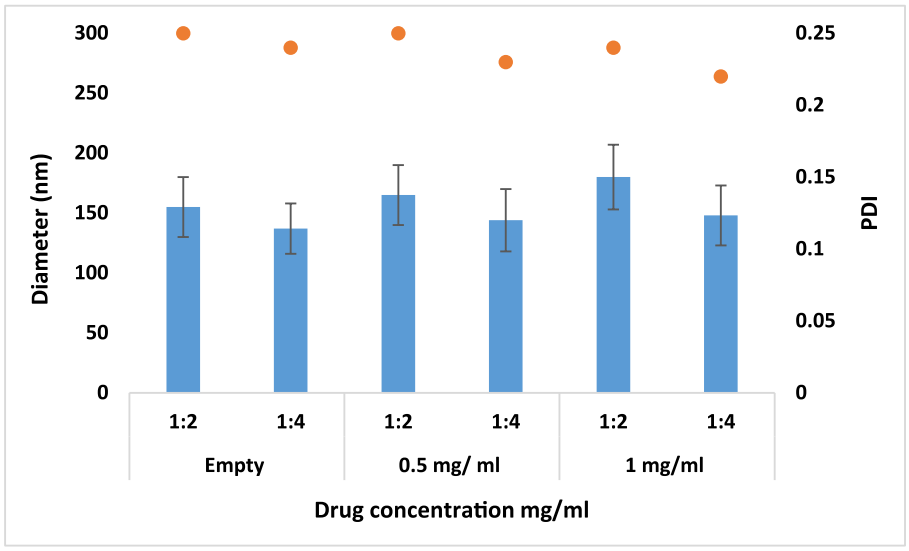

Fig. 6. Carboplatin-loaded PEGylated NLs (2:2:2) at 0.5 and 1 mg/ml concentration using TFR 1 ml/min and 1:4 FRR.

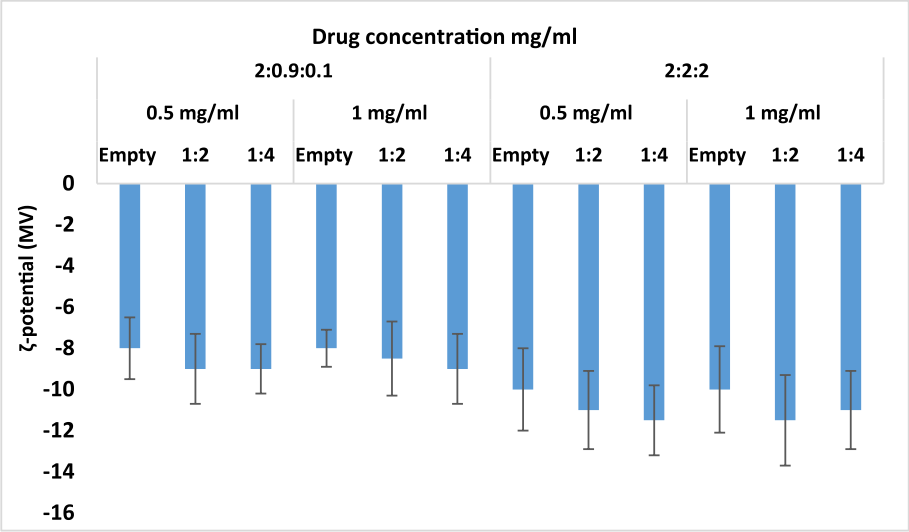

Fig. 7. Average ζ-potential of conventional NLs at different drug loading concentrations.

for the PEGylated lipid to investigate the ζ-potential effect and ensure the impact of PEGylation on the liposomes on enhancing the liposome's stability.

3.2.2. *Stability study*

The stability study was performed for the superior size and PDI liposomal formulations, which were the formulations at 1:4 FRR. The physical stability of the PEGylated NLs was studied over 4 weeks at different temperatures, including the physiological temperature of 37 °C and the storage temperature of 4 °C. The liposome's diameter was affected by increasing the incubating temperature to 37 °C; a significant increase in the diameter of liposomes was noticed at 37 °C ($P < 0.05$). In contrast to the 4 °C incubated formulations, the liposome diameter kept consistent (Figures S2 & S3). The PEG-lipid incorporation significantly affects the liposomes' PDI; the PDI of the PEGylated NLs was lower than that of conventional NLs. For example, the PDI of conventional NLs loaded with 0.5 mg/ml carboplatin result was 0.23 at 37 °C compared to P6 PEGylated NLs the PDI was 0.19. The same trend of PDI decreasing was noticed with the P8 and P4 formulations, carboplatin-loaded PEGylated NLs. The low PDI, is one of the primary stability parameters that ensure the low incidence of aggregation and the high homogeneity of the formulation, which support the ζ-potential results. Several studies reported improved liposome formulation stability after incorporating PEG lipids (Immordino et al., 2006, Cao et al., 2022, Lin et al., 2019, Jaradat et al., 2023). The stability improvement is owed to the increased steric hindrance by the PEG chains that create a space between the liposomes and prevent liposome aggregation (Cheung and Al-Jamal, 2019). Moreover, the creation of repulsion forces between the liposomes due to the negative charge increase the liposomes stability, as discussed before. The results of this study support the trend of our previous work, as the PEGylation of liposomes loaded with hydrophobic drug (Paclitaxel) improved the stability of the liposomes. By looking at both the results of this work and the previous work on liposome PEGylation, the liposomes were loaded with either hydrophilic or hydrophobic drugs, enhanced their stability by PEGylation.

The different PEG-lipid concentrations exhibited a discrepancy in improving the stability of the liposomes. The liposomal formulation with higher PEG-lipid concentration 2:2:2 represents higher stability and comparable sizes of the liposomes at both temperatures, especially at 37 °C, during the study. The contrast in the PEGylated liposomal

formulations' stability might be related to the steric hindrance and charge. The more elevated PEG-lipid concentration in the 2:2:2 PEGylated liposomes increases the steric hindrance of the liposomes, which might preserve the liposome's diameter comparable and stable (Cao et al., 2022). Also, it provides a more anionic charge to the formulations, which can enhance the formulation's stability, as discussed before.

3.3. FTIR analysis

The FTIR study was performed for conventional and PEGylated NLs to study any alteration in the number, type, or vibration of chemical bonds due to incorporating PEG-lipids. The conventional carboplatin-loaded NLs are used as a control to investigate any shifts in the peaks or any newly generated chemical bonds. Some peaks were noticed for both formulations representing specific chemical bonds, including the O–H bond that appeared at the 3218–3349 cm^{-1} and indicated a primary alcohol group. The presence of an O–H stretching bond properly exists due to alcohol traces from the alcohol that have been used during the formulation process. The bonds noticed at 2958 and 2862 cm^{-1} correspond to the symmetric C–H stretching vibrations of the alkyl chain (Figure S4). In the PEGylated NLs spectra, the C–H stretching vibrations peak appeared more intense at 2919 and 2850 cm^{-1} which represents a more striking vibration of the -CH_2 group of the PEG lipid (Yang et al., 2020). The peaks at 1610 and 1640 cm^{-1} indicate the stretching vibrations of the amine group NH_3. The NH_3 peaks appeared in both formulation's spectra; the carboplatin structure in (Fig. 1) shows two amine groups in the structure. The stretching vibration of the Pt-NH_3 bond of carboplatin appeared at 971 cm^{-1} in the PEGylated spectra compared to the conventional NLsspectra; the band was less sharp and intense and appeared at 876 cm^{-1} (Azhar et al., 2017). The Pt-NH_3 stretching vibration varied depending on the other bonds' strength. The possible reason for the NH_3 appearance at 876 cm^{-1} is the rocking vibration mode of the NH_3 in the conventional NLs (Table 3) (Yang et al., 2020). The hypothesis assumed the addition of PEG lipids consisting of an amine group can change the vibrational mode of the NH_3 (Yang et al., 2020) (Iliescu et al., 2011). The peaks at 1040–1080 cm^{-1} correlate to the PO_4 group. The PO_4 group is one of the special groups located at the end of the DPPC structure, which explains the appearance of this peak in both formulations (Masaki et al., 2017). A newly generated band was noticed at 1222 cm^{-1} in the PEGylated NLs spectra. This peak is correlated to the extra C-N bond vibration of the amine group, which is the main chemical-characterized group of the PEG-lipid. The stretching vibration of the C-N bond is usually presented at the range of 1176 to 1230 cm^{-1} (Rozenberg et al., 2004).

3.4. AFM result

The AFM images can provide a visual indication of liposome morphological and structural properties. The AFM is used to image the conventional NLs as a standard and study any alteration in the morphological characteristics in the PEGylated NLs. The images show the semicircular and semi-organized shapes of the carboplatin-loaded conventional NLs (Fig. 8). After PEGylation, the liposomes look more spherical and uniform at the 2:0.9:0.1 or 2:2:2 ratio. The results show that incorporating PEG-lipid into the liposome composition not only results in smaller liposomes but also makes the liposome morphology more organized. The more circular and uniform shape is possibly due to the increase of the lateral repulsion between the PEG chains that results in curving the lipid bilayer lipid and enhances the packing of the lipid chains, which provide smaller and more spherical liposomes (Najlah et al., 2019, Tsermentseli et al., 2018). The results support the DLS analysis about the decrease in liposome diameter after incorporating PEG-lipids. The impurities and unorganized shape of some liposomes in the AFM images is due to the drying steps affecting the liposome's shapes and uniformity, which is well noticed in the literature (Weaver et al., 2022).

Table 3
A comparison between the resulting frequency ranges of functional groups and the literature frequency ranges.

Frequency range (cm^{-1})	Functional group	Literature frequency range (cm^{-1})	References
3218–3349 cm^{-1}	O–H stretching	3385 cm^{-1}	(Aisha et al., 2014)
2919 and 2850 cm^{-1}	CH2 striking vibration	2850 ± 1 cm^{-1} and 2919 ± 1 cm^{-1}.	(Le-Deygen et al., 2022)
1610 and 1640 cm^{-1}	NH3 stretching vibrations	1600–1700 cm^{-1}	(Chatterley et al., 2022)
971 and 876 cm^{-1}	Pt-NH3 rocking vibration mode	842 and 970 cm^{-1}	(Yang et al., 2020) (Nakamoto, K., 2008)
1040–1088 cm^{-1}	PO4 stretching vibration	1088 cm^{-1}	(Le-Deygen et al., 2022, Masaki et al., 2017)

3.5. Encapsulation efficiency (EE%)

The EE% of the formulations is studied to investigate any impact of liposomes' PEGylation on the EE% of carboplatin. The result represents a variation in the EE% between the conventional and PEGylated NLs and between the different ratios of PEGylated NLs. The conventional NLs show higher EE% of the carboplatin than the PEGylated NLs; the average EE% at both drug concentrations was 59.5 %, while the average EE% of the diverse PEGylated NLs was 47.5 %. The passive hydrophilic drug encapsulation procedure should be explained to understand the higher efficacy of conventional liposomes in encapsulating a higher percentage of carboplatin. The hydrophilic drugs are encapsulated mainly in the aqueous core of the liposomes. The amount of the encapsulated drug relies on the volume of the aqueous phase that is encapsulated during the lipid assembly and liposome formation; the increased encapsulated aqueous volume increases the amount of the encapsulated drug. The studies show a direct relationship between the volume of liposomes' internal core and the liposome's diameter; the larger liposome diameter has a higher inner core volume (Xu et al., 2012, Nsairat et al., 2022). This can explain the higher EE% of carboplatin in conventional liposomes since the diameter of conventional liposomes was larger than that of PEGylated liposomes (Table 2). The PEGylation of liposomes impacts the diameter positively by decreasing it, but this reduction in liposome diameter affects the EE% negatively. The different ratios of incorporated PEG-lipid affect the EE%; the results show that P6 and P8 with lower PEG-lipid content have higher EE% (Figure S5). The inverse relationship between the PEG lipid ratio and EE% might be due to the configuration of the PEG-lipid chains around the surface (Kowalska et al., 2021). Increasing the PEG-lipid ratio leads to the conversion of the PEG-lipid chains configuration from brush-like to mushroom configuration, which reduces the volume of the inner (Nicholas et al., 2000, Kowalska et al., 2021). Other parameters, such as lipid composition, phospholipid chain length, bilayer thickness, lamellarity of liposomes, and the manufacturing method, can mainly affect the encapsulation of hydrophilic drugs.

The manufacturing method of liposomes is an important parameter that must be addressed; one of the major reasons for the low EE% of hydrophilic drugs is the disadvantages of the manufacturing methods techniques. For example, the thin film hydration always shows a low efficacy in encapsulating hydrophilic drugs due to the condition of the procedure that the volume of the hydration medium is always significantly higher than the volume entrapped as the aqueous core within the liposomes (Eloy et al., 2014). Similar drawbacks were noticed with the extrusion method due to the size reduction step that highly reduces the EE% (Eloy et al., 2014). The MF show a superior effect in raising the passive EE% of hydrophilic drugs compared to other traditional methods. For example, the highest concentration of carboplatin

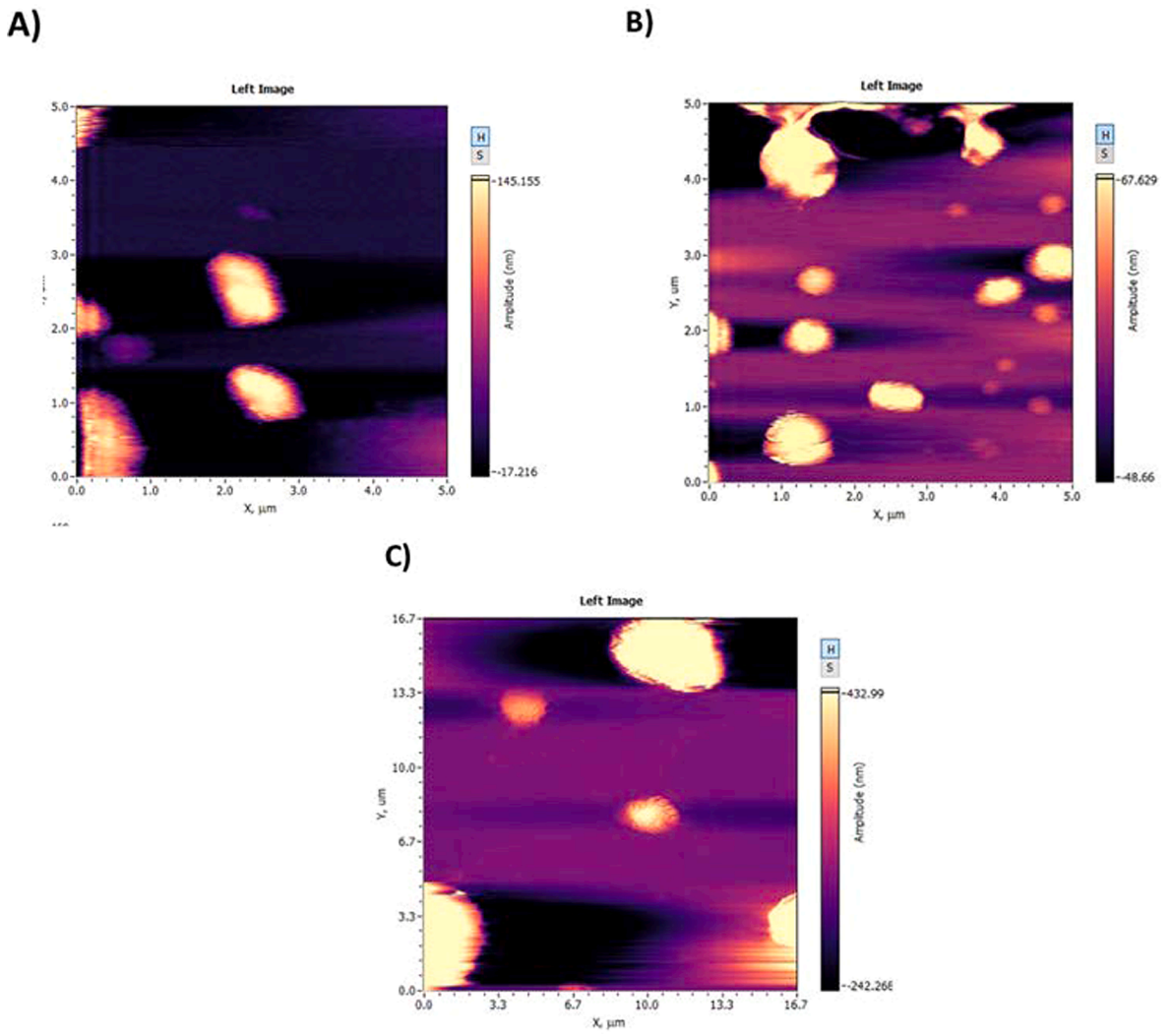

Fig. 8. AFM images for (A) conventional carboplatin-loaded NLs, (B) 2:2:2 PEGylated carboplatin NLs, (C) 2:0.9:0.1 PEGylated carboplatin NLs.

encapsulated in liposomes composed of DPPC: Chol: PEG2000 utilising thin film hydration method was 0.32 mg per 1 mg of lipid (Shi et al., 2018). Compared to the result of this work, the calculated concentration of encapsulated carboplatin was 0.49 mg per 1 mg of lipid for the composition of PEGylated liposomes. Alavizadeh et al., used the ethanol injection method followed by extrusion to manufacture cisplatin-loaded PEGylated liposomes. Cisplatin and carboplatin have the same backbone of chemotherapy regimens and are analogous in properties and hydrophilicity. The result showed that the EE% of the PEGylated liposomes created with DPPC, cholesterol, and DSPE-PEG2000 was 17.5 ± 0.56 % (Alavizadeh et al., 2014).

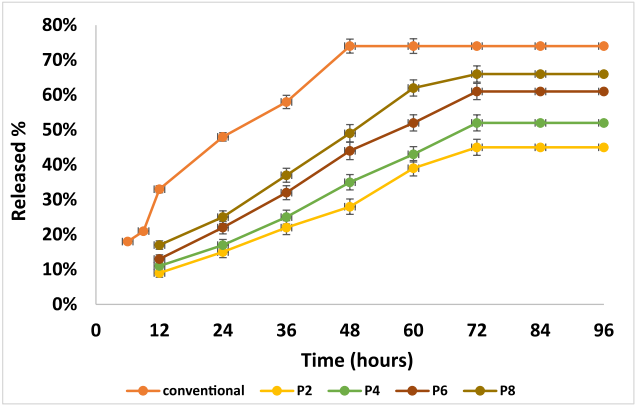

Fig. 9. Drug release profile of conventional and PEGylated carboplatin-loaded Conventional NLs.

3.6. *In vitro drug release*

The release profile shows a variation in the release rate and % of the released drug between the conventional and PEGylated liposomes. The conventional liposomes represent a faster drug release and a higher % of released drug (Fig. 9); the average of released drug % reached 74 % compared to 54 % correlated to different ratios of PEGylated liposomes. The release of carboplatin starts to be detectable after 6 h and increases gradually to reach a steady state at 48. The release profile of the PEGylated NLs represents a more delayed and sustained release compared to the conventional NLs; the release starts after 12 h and reaches a steady- state after 72 h (Fig. 9). The delayed release of the PEGylated NLs is mainly due to the PEG coating on the surface; the PEG chains on the liposome surface slow down the release of the encapsulated molecule sustainably (Panwar et al., 2010). Incorporating the PEG lipid provides an advantage of the delayed release; the delayed release evades the premature release of the drug and inhibits the collateral toxicity of the healthy tissues (Adepu and Ramakrishna, 2021). The different ratios of incorporated PEG show a variation in the release profile; the lower PEG concentration in P6 and P8 formulations offers

earlier release, a faster release rate, and a higher % of drug release compared to P2 and P4. The release of carboplatin starts to be detectable after 12 h for both formulations, but the average % of the released drug at 12 h was 15 % for P6 and P8 and 10 % for formulations P2 and P4. The higher ratio of PEG- lipid in the component of formulations P2 and P4, slows the release of the drug due to the higher rigidity of the liposomes. The studies show that increasing the PEG-lipid concentration increases liposome rigidity, one of the main parameters affecting drug release (Aghaei et al., 2021, Jaafar-Maalej et al., 2010). Multiple studies report a decline in drug release after incorporating PEG lipids (Dadashzadeh et al., 2008, Sivadasan et al., 2022, Ju et al., 1995). The higher drug concentration of each formulation shows a slight increase in the % of the drug released. However, based on the statistical analysis, the change in the drug concentration has not demonstrated a significant difference in the % of drug released ($P > 0.05$).

4. Conclusions & future work

The conducted experiments, analyse the impact of modifying the carboplatin-loaded NLs with PEGylated lipids on the characteristics of NLs as a DDS for a hydrophilic drug. The study investigates the effect of liposome PEGylation from multiple sides, including the liposome diameter, PDI, ζ-potential, stability, EE%, and drug release profile. The comparison between the PEGylated and conventional NLs represents a decrease in the diameter of the liposome after incorporating PEG-lipid. The homogeneity of the conventional NLs was better as the average of the PDI values was lower. The role of the MF system is evident in controlling the liposome diameter and PDI by offering high-quality mixing and advanced controlling of manufacturing parameters such as the FRR and TFR. The results highlighted the impact of increasing the FRR on decreasing the liposomes' diameter and PDI. The ζ-potential of the PEGylated NLs was more anionic due to the slightly negative charge of DSPE-PEG2000. The result of the EE% showed a higher EE% of the carboplatin in the conventional NLs as the larger size allows them to encapsulate a higher amount of carboplatin in the core of the liposomes. The microfluidic system represents another positive impact on increasing the passive EE% of the hydrophilic drugs compared to the other traditional methods. The release profile for both formulations showed a more delayed release of carboplatin from the PEGylated NLs. The release rate from the PEGylated NLs was lower than the conventional, which resulted in a more sustained release of the carboplatin-loaded PEGylated liposomes. The PEGylated NLs were superior in some areas, such as the controlled size, enhanced stability, and sustained release, and the conventional NLs were superior in offering higher EE% and more homogeneous formulations. The determination between the conventional and PEGylated NLs as the optimum DDS of hydrophilic drugs depends mainly on the primary goal of the DDS; for example, immediate or delayed release is needed. Since the PEGylated liposomal formulations represent promising formulation characteristics, additional optimisations can be conducted to increase their EE% of hydrophilic drugs, such as changing the type or molecular weight of PEGylated lipids and extending the microfluidic ship mixing area to prolong the exposure time between the aqueous and lipid phase, which can facilitate the intake of higher quantities of the liquid phase containing the dissolved drug into the liposome core. Overall, both formulations offer a high-quality DDS by achieving the optimum size, PDI, and stability.

Various additional examinations can be conducted to enhance and bolster the optimised NL characterisation. For instance, employing the CryoTEM microscope to visualise the internal structures of conventional and PEGylated NL can offer a more accurate characterisation and obtain higher-resolution images that can help in identify differences in particle size, shape, and distribution between the formulations. Further, the promising characteristics of the resultant formulations allow them to qualify for in vitro cytotoxic studies to investigate any discrepancies in their cytotoxic effect. In conjunction with biological in vivo investigation, a comprehensive assessment of the formulation's effectiveness, safety, and potential toxicity can be conducted. Over and above that, the surface of the optimised PEGylated NLs can be modified with targeting ligands, such as antibodies, peptides, or aptamers, to develop actively targeting DDS that offer precise delivery to specific tissues or cells.

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CRediT authorship contribution statement

Eman Jaradat: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Adam Meziane:** Writing – review & editing, Validation, Supervision, Resources, Methodology. **Dimitrios A. Lamprou:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary material

Supplementary Materials: Figure S1: Stability study of the conventional carboplatin liposomes at TFR 1 ml/ min and 1:4 FRR; Figure S2: Stability study of P6 and P8 formulations at TFR 1 ml/min and 1:4 FRR; Figure S3: Stability studies of P2 and P4 formulations, at TFR 1 ml/min and 1:4 FRR; Figure S4: FTIR spectra of the carboplatin loaded liposomes; A) conventional liposomes and B) PEGylated liposomes; Figure S5: EE% of conventional and PEGylated liposomes at 0.5 and 1 mg/ml of carboplatin. Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2024.124077.

References

Adepu, S., Ramakrishna, S., 2021. Controlled drug delivery systems: current status and future directions. Molecules, 26.

Aghaei, H., Solaimany Nazar, A.R., Varshosaz, J., 2021. Double flow focusing microfluidic-assisted based preparation of methotrexate–loaded liposomal nanoparticles: encapsulation efficacy, drug release and stability. Colloids Surfaces A: Physicochem. Eng. Aspects, 614, 126166.

Aisha, A.F., Majid, A.M., Ismail, Z., 2014. Preparation and characterization of nano liposomes of Orthosiphon stamineus ethanolic extract in soybean phospholipids. BMC Biotech. 14, 23.

Alavizadeh, S.H., Badiee, A., Golmohammadzadeh, S., Jaafari, M.R., 2014. The influence of phospholipid on the physicochemical properties and anti-tumor efficacy of liposomes encapsulating cisplatin in mice bearing C26 colon carcinoma. Int. J. Pharm. 473, 326–333.

Azhar, F., Shahbazpour, E., Olad, A., 2017. pH sensitive and controlled release system based on cellulose nanofibers-poly vinyl alcohol hydrogels for cisplatin delivery. Fibers Polym. 18, 416–423.

Bernal-Chávez, S.A., Romero-Montero, A., Hernández-Parra, H., Peña-Corona, S.I., Del Prado-Audelo, M.L., Alcalá-Alcalá, S., Cortés, H., Kiyekbayeva, L., Sharifi-Rad, J., Leyva-Gómez, G., 2023. Enhancing chemical and physical stability of pharmaceuticals using freeze-thaw method: challenges and opportunities for process optimization through quality by design approach. J. Biol. Eng. 17, 35.

Briuglia, M.-L., Rotella, C., McFarlane, A., Lamprou, D.A., 2015. Influence of cholesterol on liposome stability and on in vitro drug release. Drug Deliv. Transl. Res. 5, 231–242.

Cao, Y., Dong, X., Chen, X., 2022. Polymer-modified liposomes for drug delivery: from fundamentals to applications. Pharmaceutics 14, 778.

Chatterley, A.S., Laity, P., Holland, C., Weidner, T., Woutersen, S., Giubertoni, G., 2022. Broadband multidimensional spectroscopy identifies the amide II vibrations in silkworm films. Molecules 27.

Chaudhury, A., Das, S., Bunte, R.M., Chiu, G.N., 2012a. Potent therapeutic activity of folate receptor-targeted liposomal carboplatin in the localized treatment of

intraperitoneally grown human ovarian tumor xenograft. Int. J. Nanomed. 7, 739–751.
Chaudhury, A., Das, S., Lee, R.F., Tan, K.-B., Ng, W.-K., Tan, R.B., Chiu, G.N., 2012b. Lyophilization of cholesterol-free PEGylated liposomes and its impact on drug loading by passive equilibration. Int. J. Pharm. 430, 167–175.
Cheung, C.C.L., Al-Jamal, W.T., 2019. Sterically stabilized liposomes production using staggered herringbone micromixer: effect of lipid composition and PEG-lipid content. Int. J. Pharm. 566, 687–696.
Dadashzadeh, S., Vali, A.M., Rezaie, M., 2008. The effect of PEG coating on in vitro cytotoxicity and in vivo disposition of topotecan loaded liposomes in rats. Int. J. Pharm. 353, 251–259.
Duong, T.T., Isomäki, A., Paaver, U., Laidmäe, I., Tõnisoo, A., Yen, T.T.H., Kogermann, K., Raal, A., Heinämäki, J., Pham, T.-M.-H., 2021. Nanoformulation and evaluation of oral berberine-loaded liposomes. Molecules 26, 2591.
Ebrahimifar, M., Nili-Ahmadabadi, A., Akbarzadeh, A., Shahemabadi, H.E., Hasanzadegan, M., Moradi-Sardareh, H., Madadizadeh, H., Rezaee-Diyan, J., 2017. Preparation, characterization and cytotoxic effects of pegylated nanoliposomal containing carboplatin on ovarian cancer cell lines. Indian J. Clin. Biochem. 32, 230–234.
Eloy, J. O., Claro De Souza, M., Petrilli, R., Barcellos, J. P. A., Lee, R.J., Marchetti, J.M., 2014. Liposomes as carriers of hydrophilic small molecule drugs: Strategies to enhance encapsulation and delivery. Colloids Surfaces B: Biointerfaces, 123, 345–363.
Garbuzenko, O., Barenholz, Y., Priev, A., 2005. Effect of grafted PEG on liposome size and on compressibility and packing of lipid bilayer. Chem. Phys. Lipids 135, 117–129.
Glavas-Dodov, M., Fredro-Kumbaradzi, E., Goracinova, K., Simonoska, M., Calis, S., Trajkovic-Jolevska, S., Hincal, A., 2005. The effects of lyophilization on the stability of liposomes containing 5-FU. Int. J. Pharm. 291, 79–86.
Gonzalez Gomez, A. Hosseinidoust, Z., 2020. Liposomes for antibiotic encapsulation and delivery. ACS Infect. Diseases, 6, 896-908.
Guimarães, D., Cavaco-Paulo, A., Nogueira, E., 2021. Design of liposomes as drug delivery system for therapeutic applications. Int. J. Pharm. 601, 120571.
Herath, N.I., Berthault, N., Thierry, S., Jdey, W., Lienafa, M.-C., Bono, F., Noguiez-Hellin, P., Sun, J.-S., Dutreix, M., 2019. Preclinical studies comparing efficacy and toxicity of DNA repair inhibitors, olaparib, and AsiDNA, in the treatment of carboplatin-resistant tumors. Front. Oncol. 9.
Iliescu, I., Andronescu, E., Ghitulica, C., Berger, D., Ficai, A., 2011. Montmorillonite-alginate nanocomposite beads as drug carrier for oral administration of carboplatin-preparation and characterization. UPB Sci. Bull., Series B: Chem. Mater. Sci. 73, 3–16.
Immordino, M.L., Dosio, F., Cattel, L., 2006. Stealth liposomes: review of the basic science, rationale, and clinical applications, existing and potential. Int. J. Nanomed. 1, 297.
Jaafar-Maalej, C., Diab, R., Andrieu, V., Elaissari, A., Fessi, H., 2010. Ethanol injection method for hydrophilic and lipophilic drug-loaded liposome preparation. J. Liposome Res. 20, 228–243.
Jaradat, E., Weaver, E., Meziane, A., Lamprou, D.A., 2021. Microfluidics technology for the design and formulation of nanomedicines. Nanomaterials 11, 3440.
Jaradat, E., Weaver, E., Meziane, A., Lamprou, D.A., 2022. Microfluidic paclitaxel-loaded lipid nanoparticle formulations for chemotherapy. Int. J. Pharm. 628, 122320.
Jaradat, E., Weaver, E., Meziane, A., Lamprou, D.A., 2023. Synthesis and characterization of paclitaxel-loaded PEGylated liposomes by the microfluidics method. Mol. Pharm. 20, 6184–6196.
Joshi, S., Hussain, M.T., Roces, C.B., Anderluzzi, G., Kastner, E., Salmaso, S., Kirby, D.J., Perrie, Y., 2016. Microfluidics based manufacture of liposomes simultaneously entrapping hydrophilic and lipophilic drugs. Int. J. Pharm. 514, 160–168.
Ju, R.T., Nixon, P.R., Patel, M.V., Tong, D.M., 1995. Drug release from hydrophilic matrices. 2. A mathematical model based on the polymer disentanglement concentration and the diffusion layer. J. Pharm. Sci. 84, 1464–1477.
Kępczyński, M., Nawalany, K., Kumorek, M., Kobierska, A., Jachimska, B., Nowakowska, M., 2008. Which physical and structural factors of liposome carriers control their drug-loading efficiency? Chem. Phys. Lipids 155, 7–15.
Kowalska, M., Broniatowski, M., Mach, M., Płachta, Ł., Wydro, P., 2021. The effect of the polyethylene glycol chain length of a lipopolymer (DSPE-PEGn) on the properties of DPPC monolayers and bilayers. J. Mol. Liq. 335, 116529.
Le-Deygen, I.M., Safronova, A.S., Kolmogorov, I.M., Skuredina, A.A., Kudryashova, E.V., 2022. The influence of lipid matrix composition on the microenvironment of levofloxacin in liposomal forms. Russ. J. Bioorg. Chem. 48, 710–719.
Leung, S.S.Y., Morales, S., Britton, W., Kutter, E., Chan, H.-K., 2018. Microfluidic-assisted bacteriophage encapsulation into liposomes. Int. J. Pharm. 545, 176–182.
Lin, W., Kampf, N., Goldberg, R., Driver, M.J., Klein, J., 2019. Poly-phosphocholinated liposomes form stable superlubrication vectors. Langmuir 35, 6048–6054.
Maherani, B., Wattraint, O., 2017. Liposomal structure: a comparative study on light scattering and chromatography techniques. J. Dispers. Sci. Technol. 38, 1633–1639.
Manojlovic, V., Winkler, K., Bunjes, V., Neub, A., Schubert, R., Bugarski, B., Leneweit, G., 2008. Membrane interactions of ternary phospholipid/cholesterol bilayers and encapsulation efficiencies of a RIP II protein. Colloids Surf. B Biointerfaces 64, 284–296.
Masaki, S., Nakano, Y., Ichiyoshi, K., Kawamoto, K., Takeda, A., Ohnuki, T., Hochella, M., Utsunomiya, S., 2017. Adsorption of extracellular polymeric substances derived from S. cerevisiae to ceria nanoparticles and the effects on their colloidal stability. Environments 4, 48.
Milla, P., Dosio, F., Cattel, L., 2012. PEGylation of proteins and liposomes: a powerful and flexible strategy to improve the drug delivery. Curr. Drug Metab. 13, 105–119.
Morini, M.A., Sierra, M.B., Pedroni, V.I., Alarcon, L.M., Appignanesi, G.A., Disalvo, E.A., 2015. Influence of temperature, anions and size distribution on the zeta potential of DMPC, DPPC and DMPE lipid vesicles. Colloids Surf. B Biointerfaces 131, 54–58.
Najlah, M., Said Suliman, A., Tolaymat, I., Kurusamy, S., Kannappan, V., Elhissi, A.M.A., Wang, W., 2019. Development of injectable PEGylated liposome encapsulating disulfiram for colorectal cancer treatment. Pharmaceutics, 11, 610.
Nakamoto, K., 2008. Applications in inorganic chemistry. infrared and raman spectra of inorganic and coordination compounds.
Nele, V., Holme, M.N., Kauscher, U., Thomas, M.R., Doutch, J.J., Stevens, M.M., 2019. Effect of formulation method, lipid composition, and PEGylation on vesicle lamellarity: a small-angle neutron scattering study. Langmuir 35, 6064–6074.
Nguyen, D.H., Bach, L.G., Nguyen Tran, D.-H., Cao, V.D., Nguyen, T.N.Q., Le, T.T.H., Tran, T.T., Thi, T.T.H., 2019. Partial surface modification of low generation polyamidoamine dendrimers: gaining insight into their potential for improved carboplatin delivery. *Biomolecules,* 9, 214.
Nicholas, A.R., Scott, M.J., Kennedy, N.I., Jones, M.N., 2000. Effect of grafted polyethylene glycol (PEG) on the size, encapsulation efficiency and permeability of vesicles. Biochimica et Biophysica Acta (BBA) - Biomembranes 1463, 167–178.
Nosova, A.S., Koloskova, O.O., Nikonova, A.A., Simonova, V.A., Smirnov, V.V., Kudlay, D., Khaitov, M.R., 2019. Diversity of PEGylation methods of liposomes and their influence on RNA delivery. MedChemComm 10, 369–377.
Nsairat, H., Khater, D., Sayed, U., Odeh, F., Al Bawab, A., Alshaer, W., 2022. Liposomes: structure, composition, types, and clinical applications. Heliyon, 8, e09394.
Panwar, P., Pandey, B., Lakhera, P., Singh, K., 2010. Preparation, characterization, and in vitro release study of albendazole-encapsulated nanosize liposomes. Int. J. Nanomed. 101–108.
Petrilli, R., O Eloy, J., Fv Lopez, R., J Lee, R. 2017. Cetuximab immunoliposomes enhance delivery of 5-FU to skin squamous carcinoma cells. Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents), 17, 301-308.
Pourmadadi, M., Eshaghi, M.M., Shaghaghi, M., Das, S.S., Arshad, R., Ghotekar, S., Rahdar, A., Manicum, A.-L.-E., Pandey, S., 2023. Nano-scale drug delivery systems for carboplatin: a comprehensive review. OpenNano 13, 100175.
Rahim, M.A., Jan, N., Khan, S., Shah, H., Madni, A., Khan, A., Jabar, A., Khan, S., Elhissi, A., Hussain, Z., 2021. Recent advancements in stimuli responsive drug delivery platforms for active and passive cancer targeting. Cancers 13, 670.
Rozenberg, M., Shoham, G., Reva, I., Fausto, R., 2004. Low temperature FTIR spectroscopy and hydrogen bonding in cytosine polycrystals. Spectrochim. Acta A Mol. Biomol. Spectrosc. 60, 463–470.
Sedighi, M., Sieber, S., Rahimi, F., Shahbazi, M.A., Rezayan, A.H., Huwyler, J., Witzigmann, D., 2019. Rapid optimization of liposome characteristics using a combined microfluidics and design-of-experiment approach. Drug Deliv. Transl. Res. 9, 404–413.
Shenoy, V., Gude, R., Rayasa, M., 2011. Investigations on paclitaxel loaded HSPC based conventional and PEGylated liposomes: In vitro release and cytotoxic studies. Asian J. Pharm. Sci. 6, 1–7.
Shi, M., Anantha, M., Wehbe, M., Bally, M.B., Fortin, D., Roy, L.O., Charest, G., Richer, M., Paquette, B., Sanche, L., 2018. Liposomal formulations of carboplatin injected by convection-enhanced delivery increases the median survival time of F98 glioma bearing rats. J Nanobiotechnology 16, 77.
Sivadasan, D., Sultan, M.H., Madkhali, O.A., Alsabei, S.H., Alessa, A.A., 2022. Stealth liposomes (PEGylated) containing an anticancer drug camptothecin: in vitro characterization and in vivo pharmacokinetic and tissue distribution study. Molecules, 27.
Sriwongsitanont, S., Ueno, M., 2004. Effect of a PEG lipid (DSPE-PEG2000) and freeze-thawing process on phospholipid vesicle size and lamellarity. Colloid Polym. Sci. 282, 753–760.
Trang Le, N.T., Nguyen, C.K., Nguyen, D.H., 2020. Carboplatin delivery system based on poly(ethylene glycol) methyl ether–cholesterol modified soy lecithin liposomes. Adv. Nat. Sci.: Nanosci. Nanotechnol., 11, 045016.
Tsermentseli, S.K., Kontogiannopoulos, K.N., Papageorgiou, V.P., Assimopoulou, A.N., 2018. Comparative study of PEGylated and conventional liposomes as carriers for Shikonin. Fluids 3, 36.
U.S, F. 2018. Liposome drug products; chemistry, manufacturing, and controls; human pharmacokinetics and bioavailability; labeling documentation. Guidance for Industry; April 2018 Pharmaceutical Quality/CMC.; U.S. .
Villasmil-Sánchez, S., Rabasco, A.M., González-Rodríguez, M.L., 2013. Thermal and 31P-NMR studies to elucidate sumatriptan succinate entrapment behavior in phosphatidylcholine/cholesterol liposomes. Comparative 31P-NMR analysis on negatively and positively-charged liposomes. Colloids Surf. B Biointerfaces 105, 14–23.
Weaver, E., O'Connor, E., Cole, D.K., Hooker, A., Uddin, S., Lamprou, D.A., 2022. Microfluidic-mediated self-assembly of phospholipids for the delivery of biologic molecules. Int. J. Pharm. 611, 121347.
Xu, X., Khan, M.A., Burgess, D.J., 2012. Predicting hydrophilic drug encapsulation inside unilamellar liposomes. Int. J. Pharm. 423, 410–418.
Yamamoto, T., Yokoyama, M., Opanasopit, P., Hayama, A., Kawano, K., Maitani, Y., 2007. What are determining factors for stable drug incorporation into polymeric

micelle carriers? Consideration on physical and chemical characters of the micelle inner core. J. Control. Release 123, 11–18.
Yang, X., Salado, D., Porcel, E., González, C., Savina, F., Dragoe, D., Remita, H., Lacombe, S., 2020. A facile one-pot synthesis of versatile PEGylated platinum nanoflowers and their application in radiation therapy. Int. J. Mol. Sci. 21, 1619.
Zamboni, W.C., Gervais, A.C., Egorin, M.J., Schellens, J.H., Zuhowski, E.G., Pluim, D., Joseph, E., Hamburger, D.R., Working, P.K., Colbern, G., 2004. Systemic and tumor disposition of platinum after administration of cisplatin or STEALTH liposomal-cisplatin formulations (SPI-077 and SPI-077 B103) in a preclinical tumor model of melanoma. Can. Chemother. Pharmacol. 53, 329–336.
Zhang, J., Huang, C., Huang, H., 2014. Antitumor and antimetastasis effects of carboplatin liposomes with polyethylene glycol-2000 on SGC-7901 gastric cell-bearing nude mice. Oncol. Lett. 8, 2209–2214.
Zhen, S., Li, X., 2020. Liposomal delivery of CRISPR/Cas9. Can. Gene Ther. 27, 515–527.