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ORIGINAL ARTICLE

Influence of Formulation Composition on the Characteristic Properties of 5-fluorouracil-loaded Liposomes

Tahir Emre YALÇIN*, Ceren YETGİN

Gazi University Faculty of Pharmacy, Department of Pharmaceutical Technology, Ankara, Türkiye

ABSTRACT

Objectives: Variations in the types and quantities of excipients used to prepare liposomes can affect the physicochemical properties of liposome formulations. This study aimed to provide information about the design and fabrication of 5-fluorouracil (5-FU)-loaded liposome formulations using different lipid and cholesterol (CHOL) derivatives.

Materials and Methods: Passive loading *via* a small-volume incubation method was used to prepare liposomes. The particle size, polydispersity index, zeta potential, and encapsulation efficiency (EE%) of the formulations were determined. The release studies of the formulations were conducted using a Franz diffusion cell at 37 ˚C. In this study, a high-pressure liquid chromatography device was used to measure the amount of 5-FU.

Results: The mean particle sizes of all formulations were between 134 and 166 nm, and they had a negative charge on their surface. Increasing the cholesteryl hemisuccinate content reduced the size of the liposomes. Additionally, all formulations exhibited a low polydispersity index (0.3). The EE% of all formulations exceeded 30%. The *in vitro* release of 5-FU from liposome formulations followed the Korsemeyer-Peppas model.

Conclusion: Modifying the lipid and CHOL content in the formulations, as indicated by the experimental results, can change the characteristic properties of liposomes. The use of soybean phosphatidylcholine and cholesteryl hemisuccinate appears to be a promising combination for the preparation of hydrophilic drug-loaded liposome formulations.

Keywords: 5-fluorouracil, liposomes, cholesteryl hemisuccinate, small-volume incubation method, drug release data modeling

INTRODUCTION

Cancer is a global public health issue and ranks second in deaths caused by diseases.[1] Despite the advancement of novel therapeutic approaches for cancer, chemotherapy continues to be a primary treatment strategy. Nevertheless, the clinical use of chemotherapeutic agents faces limitations due to their toxicity and insufficient specificity. 5-Fluorouracil (5-FU) is a type of chemotherapy drug that is used to treat various solid tumors, such as colon, breast, and liver cancers. While 5-FU is commonly employed in treating cancer, its short half-life (~10-20 min)[2] and minimal affinity to tumor cells constrain the therapeutic potency of the drug.[3] Because of these limitations, a significant amount of 5-FU is needed to boost therapeutic efficiency, thereby increasing drug toxicity.[4] Thus, to address these issues, new technologies, such as nanocarrier drug delivery systems, have been introduced.

Nanocarriers are highly advantageous in cancer treatment owing to passive targeting because they exhibit minimal side effects. The term "nanocarrier" includes nanosized drug carrier systems such as nanoparticles, nanoemulsions, nanosuspensions, liposomes, niosomes, dendrimers, transferosomes, and polymeric micelles.[5-7] Based on the number of studies conducted and the number of commercial products available, liposomes are important nanocarrier systems.[8,9]

*Correspondence: tahiremreyalcin@gmail.com, ORCID-ID: orcid.org/0000-0001-5834-2335
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Liposomes are spherical vesicles consisting of a lipid bilayer structure that can encapsulate various drugs and molecules.[10] These drug delivery systems are biocompatible, biodegradable, and flexible, and their nano-size enables passive targeted drug delivery for cancer treatment. Some commercial liposomal products used for cancer treatment are Marqibo®, Mepact®, DepoCyt®, and Doxil®.[11]

Lipid and cholesterol (CHOL) derivatives can significantly influence the characteristic properties of liposomes. These components can affect the particle size (PS), zeta potential (ZP), encapsulation efficiency (EE%), drug release profiles, and other attributes of formulations. This study aimed to characterize liposome formulations prepared with different types of lipid and CHOL, loaded with 5-FU, and to evaluate the impact of excipients on the characteristic properties of the formulations.

MATERIALS AND METHODS

Materials

5-FU, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), dialysis membrane (MW: 12,000-14,000 Da), and CHOL were acquired from Sigma-Aldrich (USA). Soybean L-α-phosphatidylcholine (SPC, 95%) and cholesteryl hemisuccinate (CHEMS) from Avanti Polar Lipid Inc. (USA). All other reagents and solvents were of analytical grade.

Methods

Preparation of phosphate buffer solution (PBS) (pH 7.4)

PBS (pH 7.4) was prepared according to the methods described in the U.S. Pharmacopeia (the second supplement, USP 35-NF 30). The steps for preparing PBS are as follows: Transferred KH_2PO_4 solution (0.2 M, 250 mL) into a 1 L volumetric flask, NaOH solution (0.2 M, 195.5 mL) was added, the volume was diluted to 1 L with purified water, and the mixture was mixed well.

Preparation of 5-FU-loaded liposomes

Fourier transform infrared analyses from previous studies have indicated the compatibility of the excipients used in this research with each other and with the active substance, 5-FU.[12-14] Therefore, these excipients were selected as suitable candidates for the preparation of a 5-FU-loaded liposome formulation. The passive loading with small volume incubation (SVI) method was used to prepare liposomes.[15] In this method, empty liposomal pellets devoid of active substances are initially obtained using the thin-film hydration technique. The thin-film hydration technique, also known as the Bangham method, is the most common fabrication technology for liposomes. The SVI method is a passive drug-loading approach that relies on drug diffusion from a solution, creating a substantial concentration gradient across the liposomal membrane to facilitate efficient drug influx into the liposomes.[16] In this method, the derivatives of phospholipid and CHOL are first solubilized in chloroform in a round bottom flask at the amounts shown in Table 1 and then shaken. The organic solvent in the mixture was removed using an evaporator (Rotavapor® R-3, Büchi, Switzerland) at 60 °C, resulting in the formation of a thin film layer on the flask's wall. The obtained films were slowly hydrated with blank PBS under a magnetic stirrer at 60 °C for 1 h. The liposomes were subjected to sonication using an ultrasonic bath sonicator (Bandelin Sonorex Digitec, Bandelin electronic GmbH & Co, Germany). Furthermore, to reduce PS and improve homogeneity, all formulations were gradually extruded through 400- and 200-nm polycarbonate membranes (10 times each). To obtain empty liposomal pellets, the liposomal suspensions were centrifuged at 70,000 rpm for 1 h using a centrifuge (Hitachi CS 150 GXL, Tokyo, Japan). Subsequently, 5-FU solutions (5 mg 5-FU in 0.5 mL PBS) were added to the empty liposomal pellets and mixed thoroughly by gentle pipetting up and down several times. The resuspended formulations were transferred in 2 mL Eppendorf tubes and incubated at 60 °C for 1 h under magnetic stirring using a 5 mm x 2 mm magnetic stir bar. The resulting 5-FU-loaded liposomes were then centrifuged at 70,000 rpm for 1 h to remove any unencapsulated 5-FU.[17]

Lyophilization procedure

The acquired liposomal pellets were resuspended in purified water containing trehalose as a cryoprotectant. After freezing the samples at 80 °C, they were rapidly transferred to a freeze dryer (Christ Alpha 1-2 LD plus, Germany). The samples were freeze-dried inside the device at 55 °C for 40 h.[18] The lyophilized powder was collected and stored at 5 ± 3 °C for further experiments.

Characterization of 5-FU-loaded liposomes

PS, polydispersity index (PDI), and ZP

The PS, PDI, and ZP values of the formulations were measured using a Zetasizer Nano ZS (Malvern Instruments, UK).[19,20] Before each measurement, the lyophilized formulations were redispersed in purified water (n= 3).

EE%

Drug EE% was determined using the direct method.[21,22] A certain amount of lyophilized liposomes were ruptured by adding chloroform. Then, 10 mL of PBS was added to the mixture to extract 5-FU into the aqueous phase. The organic phase was mixed with the aqueous phase. The PBS solution was analyzed using an Agilent 1220 LC high-pressure liquid chromatography (HPLC) system (Germany) to determine 5-FU in the samples. Chromatographic separation was performed using a Waters Xselect reverse phase C18 column (5 µm, 250 mm×4.6 mm *i.d*),

Table 1. Content and codes of formulations

Formulation code	SPC (mg)	DOPE (mg)	CHOL (mg)	CHEMS (mg)
F1	140	-	45	15
F2	140	-	15	45
F3	133	7	60	-
F4	112	28	60	-

SPC: Soybean L-α-phosphatidylcholine, DOPE: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, CHOL: Cholesterol, CHEMS: Cholesteryl hemisuccinate

isocratic conditions (90% acetonitrile and 10% purified water) with 1 mL/min flow rate and detected at 265 nm.[23] The EE% was then calculated using the following Equation (1):

$$EE\% = \frac{\text{(Amount of the drug in liposomes)}}{\text{(Amount of the drug added to liposomes)}} \times 100 \quad (1)$$

In vitro release study

To determine the *in vitro* release rate of 5-FU from the formulations, Franz diffusion cells were used. Franz diffusion cells were purchased from Çalışkan Cam (Ankara, Türkiye). Prior to the release experiment, the diffusion membrane was soaked in PBS. The study was carried out under sink conditions, in which the release media (PBS) were able to dissolve at least three times the amount of 5-FU in the samples. A volume of 1 mL of the liposome suspension in PBS was added to the donor chamber, whereas a volume of 2.5 mL of PBS was added to the receptor chamber as the release media. The diffusion cell was then placed in a thermostatic bath maintained at 37 °C. At predefined intervals, all release media in the receptor chamber were withdrawn and an equal volume of PBS was added. Throughout the experiment, a magnetic bar was used to stir the contents of each cell. The samples were then analyzed using the HPLC method, as previously described, with all measurements performed three times.

Drug release data modeling

Drug release data were assessed using kinetic models, which included zero-order, first-order, Higuchi, Hixson-Crowell, and Korsmeyer-Peppas, using the DDSolver add-in in Excel. The model with the highest adjusted coefficient of determination (R^2 adjusted) was chosen as the most appropriate model for describing the release kinetics. In the context of data modeling, all data were used, except for the Korsmeyer-Peppas model. The release exponent "n" was determined using the initial 60% drug release within the Korsmeyer-Peppas model.[24]

Statistical analysis

The data were expressed as mean values with standard deviations. Statistical analysis was performed using a One-Way analysis of variance followed by Tukey's posthoc test, using the GraphPad Prism 5.0 software (GraphPad Software, Inc.). Significance was established at $p \leq 0.05$.

RESULTS

PS, PDI, and ZP

Liposomes prepared using different lipids (SPC and DOPE) and CHOL derivatives (CHOL and CHEMS) were evaluated in terms of PS, PDI, and ZP (Table 2). The mean PSs of the F1 (CHOL 45 mg: CHEMS 15 mg) and F2 (CHOL 15 mg: CHEMS 45 mg) formulations were 138 and 134 nm, respectively. The ZP values of the F1 and F2 formulations were 27.3 and 32.4 mV, respectively. Furthermore, no statistically significant difference was observed in terms of mean PS in formulations containing DOPE (F3 formulation: 166 nm and F4 formulation: 162 nm) ($p > 0.05$).

EE%

The 5-FU encapsulation efficiencies ranged between 30.8% and 35.8% for the formulations, as shown in Table 2. There is no significant difference was observed between F3 and F4 formulations containing DOPE according to EE% ($p > 0.05$).

In vitro release studies

The *in vitro* drug release profiles of all formulations are depicted in Figure 1. The way in which 5-FU was released from formulations was observed to have consisted of two distinct stages: an initial rapid release of approximately 50% of the drug within the first 2 h, followed by a gradual and slower release for all formulations.

Drug release data modeling

Various models were used to determine the kinetics of the formulations. Based on the values of the R^2 adjusted, the model that best describes 5-FU release from liposomes was the Korsmeyer-Peppas model (highest R^2 adjusted) for all formulations (Table 3).

DISCUSSION

Pre-formulation studies conducted in this study and previous studies in the literature indicate that the lyophilization process generally increases the PS of liposomes due to the fusion/aggregation of vesicles.[25] To enhance the stability of liposomes,[26] all formulations prepared in this study were

Table 2. PS, PDI, ZP, and EE% of liposomal formulations (n= 3)

Formulation code	PS (nm)	PDI	ZP (mV)	EE%
F1	138 ± 2	0.130 ± 0.025	-27.3 ± 1.2	32.9 ± 0.7
F2	134 ± 4	0.194 ± 0.010	-32.4 ± 2.1	35.8 ± 1.8
F3	166 ± 2	0.197 ± 0.009	-31.5 ± 0.9	30.8 ± 0.6
F4	162 ± 4	0.247 ± 0.010	-21.0 ± 0.2	32.3 ± 1.8

PS: Particle size, PDI: Dispersity Polydispersity index, ZP: Zeta potential, EE: Encapsulation efficiency

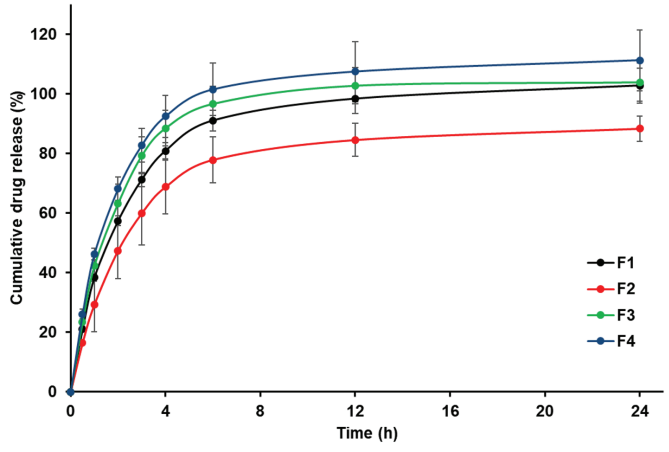

Figure 1. *In vitro* release profile of 5-FU from formulations

Table 3. Results of model fitting of the formulations

Model	Parameter	F1	F2	F3	F4
Zero-order	R^2 adjusted	-0.4365	-0.3180	-0.6475	-0.6658
First-order	R^2 adjusted	0.9822	0.8055	0.9971	0.9829
Higuchi	R^2 adjusted	0.6867	0.7210	0.5921	0.5968
Hixson-Crowell	R^2 adjusted	0.6692	0.6543	0.5986	0.5738
Korsmeyer-Peppas	R^2 adjusted	0.9913	0.9954	1	1
	n	0.724	0.724	0.857	0.824

lyophilized and characterized. The results indicate that an increase in the amount of CHEMS within the formulation leads to a slight reduction in PS. Similar results were reported by Kulig et al.[27] An increase in the CHEMS content may have resulted in an increase in net negative ZP, which could have led to the production of smaller particles. CHEMS has a negative charge due to the carboxylic acid structure in its composition,[28] which increases the net negative ZP value of the formulations. An increase in ZP may have prevented the formulation from aggregation.[29] The narrow PS distribution in colloidal dispersions indicates their suitability and quality. For this purpose, the PDI must be less than 0.5.[30] All formulations exhibited low PDI (< 0.3) and the PDI values indicate that all formulations have a homogeneous PS distribution.

EE% mainly depended on the compound solubility in the lipids or CHOL materials. The EE% findings are higher than those of some previously published 5-FU-loaded liposome formulations,[31] whereas the results are similar to or lower than those of other formulations.[32] As evident from this comparison, the EE% value of 5-FU in the formulations varied depending on the quantity and type of lipids and CHOL present in the liposome. Higher EE% was achieved using higher CHEMS (F2). This phenomenon is probably due to the presence of CHEMS on the surface of the liposomes. CHEMS has a relatively higher water solubility than CHOL,[27] and this property may make it a suitable candidate for binding highly water-soluble active substances such as 5-FU.

Because 5-FU is a hydrophilic drug (saturation solubility in distilled water and pH 7.4 PBS has been reported as 13.56 mg/mL and 16.76 mg/mL, respectively)[33], it can rapidly permeate the lipid membrane, potentially leading to the initial release of the drug. The continuous release of the drug could potentially affect the degradation rate of the liposome's structure.[34] The formulations containing DOPE (F3 and F4) were observed to have a higher *in vitro* release percentage at all time points than the formulations without DOPE (F1 and F2). This phenomenon may have been caused by the conical shape of DOPE and its disruption of the bilayer structure upon incorporation into the formulation.[35] In the F2 formulation with a higher amount of CHEMS (45 mg), the 5-FU release occurred more slowly than in the F1 formulation with a lower amount of CHEMS (15 mg). This could be attributed to the fact that CHEMS enhances membrane stability more effectively than CHOL.[36]

The Korsmeyer-Peppas model was the best model for explaining the release of 5-FU from all formulations. Previous publications on 5-FU-loaded different nanocarriers have also reported that the Korsmeyer-Peppas model best describes the release of 5-FU from these carriers.[37,38] The value of n could be used as an approximation to describe the mechanism of drug release. If n is less than 0.5, drug diffusion occurs within the polymer matrix following Fickian diffusion. If n lies within the range of 0.5 to 1, it indicates a non-Fickian diffusion mechanism, suggesting a combination of both diffusion and matrix erosion. If n > 1, the drug-release mechanism follows supercase II diffusion.[39] In the present study, n values were obtained in the range of 0.724-0.857, indicating a non-Fickian diffusion process. This drug-release mechanism is commonly observed in most drug delivery systems that incorporate liposomes.[40]

CONCLUSION

The SVI method was effective in preparing liposomes containing 5-FU with liposomes having a PS in the nanometer range, displaying a negative ZP and high drug EE% of > 30%. The experiments revealed that liposome properties, such as PS, EE%, and drug release, were influenced by the amounts of DOPE and CHEMS used in the formulations. The mathematical models used to analyze drug release kinetics indicated that the n values were within the range of 0.5-1. This strongly suggests that the drug-release mechanism follows a non-Fickian diffusion process. Using SPC, DOPE, CHOL, and CHEMS together in the formulation may be useful for obtaining the optimal PS, ZP, EE%, *in vitro* release profile, and stability, although further studies are needed to evaluate the anticancer activity.

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Ethics

Ethics Committee Approval: Not required.

Informed Consent: Not required.

Authorship Contributions

Concept: T.E.Y., Design: T.E.Y., Data Collection or Processing: T.E.Y., C.Y., Analysis or Interpretation: T.E.Y., C.Y., Literature Search: T.E.Y, C.Y., Writing: T.E.Y, C.Y.

Conflict of Interest: The authors have no conflicts of interest to declare.

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