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Influence of Formulation Composition on Characteristic Properties of 5-Fluorouracil-Loaded Liposomes

Influence of Formulation Composition on Liposomes

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ABSTRACT
Objectives: Variations in the types and quantities of excipients used to prepare liposomes can affect the physicochemical properties of the formulation. This study aims to provide information about the design and fabrication of 5- Fluorouracil (5-FU)-loaded liposome formulations using different lipid and cholesterol derivatives.
Materials and Methods: Passive loading with the small volume incubation method was used for the preparation of liposomes. Particle size, polydispersity index, zeta potential, and encapsulation efficiency (EE%) of formulations were determined. The release studies of the formulations were conducted using a Franz diffusion cell at 37 °C. In the study, a high-pressure liquid chromatography (HPLC) device was used to measure the amount of 5-FU.
Results: All formulations' mean sizes were between 134 to 166 nm, and they had a negative charge on their surface. Increasing the cholesteryl hemisuccinate content resulted in a reduction in the size of liposomes. Additionally, all formulations had a low polydispersity index value, which was less than 0.3. All formulations have an EE% of more than 30%. Also, in vitro release of 5-FU from liposome formulations followed the Korsemeyer-Peppas model.
Conclusion: Modifying the lipid and cholesterol content in formulations, as indicated by the experimental results, can change the characteristic properties of liposomes. The use of soybean phosphatidylcholine and cholesteyl hemisuccinate appears as 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 modelling

INTRODUCTION

Cancer is a global public health issue and ranks second in deaths attributed to diseases.[1] In spite of the advancement of novel therapeutic approaches for cancer, chemotherapy continues to be seen as a primary strategy in cancer treatment. 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 utilized to combat various types of solid tumors such as colon, breast, and liver cancer. 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 this limitation, a significant amount of 5-FU is needed to boost therapeutic efficiency, thereby causing increased drug toxicity.[4] Thus, to deal with these issues, new technologies have been introduced, such as nanocarrier drug delivery systems.
Nanocarriers are highly advantageous in cancer treatment, thanks to passive targeting, as they exhibit minimal side effects. The term nanocarrier includes nano-sized 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, it can be observed that liposomes are one of the important nanocarrier systems.[8,9]
Liposomes are spherical vesicles and consist of a lipid bilayer structure that can encapsulate a variety of drugs and molecule. [10] These drug delivery systems are biocompatible, biodegradable and flexible, and their nano-size

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enables passive targeted drug delivery for cancer treatment. Some of the commercial liposomal products used in cancer treatment are Marqibo®, Mepact®, DepoCyt®, and Doxil®.[11]

Lipid and cholesterol 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. The objective of this study is to characterize liposome formulations developed using different types of lipids and cholesterol, loaded with 5-FU, and to investigate the impact of excipients on the formulation.

MATERIALS AND METHODS

Materials

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

Methods

Preparation of phosphate buffer solution pH 7.4

Phosphate buffer solution (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 the PBS are as follows: Transferred KH_2PO_4 solution (0.2 M, 250 mL) into a 1 L volumetric flask, added NaOH solution (0.2 M, 195.5 mL), diluted volume to 1 L with distilled water, and mixed well.

Preparation of 5-FU-loaded-liposomes

The Fourier transform infrared (FTIR) 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, initially, empty liposomal pellets devoid of active substance are obtained using the thin-film hydration technique. The thin-film hydration technique, also known as Bangham method, is the most common fabrication technology for liposomes. The SVI method is a passive drug loading approach that relies on the diffusion of the drug from a solution, creating a substantial concentration gradient across the liposomal membrane to facilitate the efficient influx of the drug into the liposomes.[16] In this method, firstly, derivatives of phospholipid and cholesterol are solubilized in chloroform in a rounded 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 and resulted in the formation of a thin film layer on the flask's wall. The obtained film was slowly hydrated with blank PBS-under a magnetic stirrer at 60 °C for 1 h. The liposomes obtained were subjected to sonication using an ultrasonic bath sonicator (Bandelin Sonorex Digitec, Bandelin electronic GmbH & Co, Germany). Furthermore, in order to reduce PS and improve homogeneity, all formulations were extruded gradually 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 gently 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 5 mm x 2 mm magnetic stir bar. The resulting 5-FU-loaded liposomes were then subjected to centrifugation at 70,000 rpm for 1 h to remove any unencapsulated 5-FU.[17]

Lyophilization procedure

The acquired liposomal pellets were resuspended in distilled water that contained 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

Particle size (PS), polydispersity index (PDI) and zeta potential (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 with distilled water (n=3).

Encapsulation efficiency (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 the 5-FU into the aqueous phase. The organic phase was mixed with the aqueous phase. The PBS solution was analyzed by Agilent 1220 LC HPLC system (Germany) to determine 5-FU in the samples. The chromatographic separation was performed using Waters Xselect reverse phase C18 column (5 µm, 250 mm × 4.6 mm i.d), isocratic conditions (90% acetonitrile and 10% water) with 1 mL/min flow rate and detected at 265 nm.[23] The EE% was then calculated using the following Eq.(1):

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

In vitro release study

To determine the in vitro release rate of 5-FU from formulations, Franz diffusion cells were used. Franz diffusion cells were purchased from Çalışkan Cam (Ankara, Turkey). The diffusion membrane was soaked in PBS to ensure complete swelling of the membrane. The study was carried out under sink conditions, where the release media (PBS) used was able to dissolve at least 3 times the amount of 5-FU that is in the samples. A volume of 1 mL of liposome suspension in PBS was added to the donor part, while 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 that was maintained at a temperature of 37 °C. At predefined time intervals, all release medium 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 outlined, with all measurements three times.

Drug release data modelling

The 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 one for describing the release kinetics. In the context of data modeling, all data were employed, except for the Korsmeyer-Peppas model. The release exponent "n" was determined using the initial 60% of drug release within the Korsmeyer-Peppas model.[24]

Statistical analysis

The data were expressed as the mean value along with the standard deviation (SD). Statistical analysis was carried out through a one-way ANOVA 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 AND DISCUSSIONS

Particle size, polydispersity index and zeta potential

Pre-formulation studies conducted in this research and previous research 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 lyophilized and characterized. Liposomes prepared using different lipids (SPC and DOPE) and cholesterol derivate (CHOL) and CHEMS) were evaluated in terms of PS, PDI, and ZP (Table 2). The results indicate that an increase in the amount of CHEMS within the formulation leads to a slight reduction in PS.

The mean PS of the F1 (CHOL 45 mg:CHEMS 15 mg) and F2 (CHOL 15 mg:CHEMS 45 mg) formulations were 138 nm and 134 nm, respectively. Similar results were found by Kulig et al.[27] An increase in the amount of CHEMS may have resulted in an increase in the net negative ZP, which could have led to the production of smaller particles. The ZP of the F1 and F2 formulations were -27.3 mV and -32.4 mV, respectively. CHEMS has a negative charge due to the carboxylic acid structure in its composition[28], and this increases the net negative ZP value of the formulations. An increase in ZP may have prevented the formulation from aggregation.[29]

Also, no statistically significant difference was observed in terms of mean PS in formulations containing DOPE (F3 formulation:166 nm and F4 formulation:162 nm) ($p>0.05$). The narrow PS distribution in colloidal dispersions indicates the suitability and quality of the dispersion. For this, 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.

Encapsulation efficiency (EE%)

EE% depends mainly on the compound solubility in the lipids or cholesterol materials. 5-FU encapsulation efficiencies ranged between 30.8% and 35.8% for formulations as shown in Table 2. These results are higher than some previously published 5-FU-loaded liposome formulations [31], while the results are similar to or lower than others [32]. As evident from this comparison, the EE% value of 5-FU in the formulations varies depending on the quantity and type of lipids and cholesterol that constitute the liposome. Higher EE% in liposomes is achieved by using higher amounts of 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 cholesterol[27], and this property may make it a suitable candidate for the binding of highly water-soluble active substances such as 5-FU. Also, no significant difference was observed between F3 and F4 formulations containing DOPE according to EE% ($p>0.05$).

In vitro release studies

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. Because it is a hydrophilic drug (saturation solubility in distilled water and pH 7.4 phosphate buffer solution has been reported as 13.56 mg/mL and 16.76 mg/mL, respectively)[33], 5-FU can rapidly permeate the lipid membrane, potentially leading to the initial release of the drug. The continuous release of the

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drug could potentially impact the degradation rate of the liposome's structure.[34] The formulations containing DOPE (F3 and F4) were observed to have higher in vitro release percentage at all-time points compared to 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), 5-FU release occurred more slowly compared to the F1 formulation with a lower amount of CHEMS (15 mg). This may result from the fact that CHEMS may enhance membrane stability more than CHOL.[36]

Drug release data modelling

Various models were investigated for the determination of the kinetics of the formulations. Based on the values of the R^2 adjusted, the model that best describes the 5-FU release from liposomes is the Korsmeyer-Peppas model (highest R^2 adjusted) for all formulations (Table 3). Previous publications on 5-FU-loaded different nanocarriers have also mentioned that the Korsmeyer-Peppas model best describes the release of 5-FU from these carriers[37,38].

If the n value is less than 0.5, drug diffusion occurs within the polymer matrix following Fickian diffusion. If n is 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 value is greater than 1, the drug release mechanism follows super Case-II diffusion[39]. In the present study, n values were obtained in the range of 0.724 to 0.857, indicating a non-Fickian type of diffusion process. This drug release mechanism is commonly observed in the majority of drug delivery systems that incorporate liposomes [40].

CONCLUSION

The SVI method was effective in preparing liposomes that contained 5-FU with the liposomes having a PS in the nanometer range, displaying a negative ZP and a high drug EE% of over 30%. The experiments revealed that liposome properties, such as PS, EE%, and drug release could be influenced by the amount 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 to 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 to obtain 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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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	-

Table 2. The 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

Table 3. Results of kinetic model fitting of 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

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