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Development of 5-fluorouracil-loaded nano-sized liposomal formulation by two methods: Strategies to enhance encapsulation efficiency of hydrophilic drugs

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ABSTRACT: The number of studies conducted with liposomes to reduce side effects in systemic administration of chemotherapeutic agents is increasing day by day. One of these chemotherapeutic agents, 5-Fluorouracil (5-FU) is a good candidate for encapsulating into the liposomes; however, it has been difficult to obtain liposomal 5-FU with high encapsulation efficiency. The various factors such as preparation method (thin film hydration method and passive loading with small volume incubation method), drug amount (10 mg, 7.5 mg, and 5 mg), hydration volume (3.5 mL and 2 ml), and incubation volume (2 mL and 1 mL) were investigated to optimize the formulation of 5-FU encapsulated liposomes. Liposomes were characterized according to particle size, polydispersity index (PDI), zeta potential, and encapsulation efficiency (EE%). The *in vitro* release study was carried out using Franz diffusion cell. Based on the optimization of formulation, the average drug EE% and the mean particle size of 5-FU-loaded liposomes were found to be 25% and 188.6 nm. *In vitro* drug release of 5-FU-loaded liposomes (SVI-4) presented a biphasic release of 5-FU, and this behavior was in accordance with the first-order equation. According to the results, 5-FU can be effectively loaded into liposomes prepared by passive loading with small volume incubation method.

KEYWORDS: 5-Fluorouracil; liposomes; small volume incubation method; encapsulation efficiency.

1. INTRODUCTION

Chemotherapy is one of the most commonly applied approaches to treat many types of cancer, either alone or in combination with surgery and radiation therapy [1]. However, chemotherapeutic agents do not always provide great results in cancer treatment because they can also damage healthy cells and cause serious side effects. Therefore, there is a need for new drug delivery systems that can show high anticancer activity and minimize side effects.

Nano-sized drug delivery systems have many advantages in cancer treatment, including increasing circulation time, improving pharmacokinetic parameters, and reduced side effects. Liposomes are a widely investigated class of nanoparticles with an aqueous core enclosed in one or more phospholipid bilayers [2]. Several liposomal products that are approved for use in the treatment of different types of cancer are available on the market [3]. Although liposomes have many advantages such as biocompatibility and biodegradability [4]; it is hard to obtain high encapsulation efficiency (EE%) as the water-soluble drugs cause low affinity for the phospholipid [5]. The antineoplastic agent 5-fluorouracil (5-FU) is preferred in this study to use a model for water-soluble anticancer drug which has successful results for many types of cancer, such as colorectal, breast, liver and pancreatic tumor [6]. Like other hydrophilic drugs, 5-FU is difficult to retain in liposomes because of its water solubility [7].

The method used to produce liposomes should achieve a high EE% and narrow size distribution. For enhancing EE%, passive and active strategies are investigated. Proper selection of the liposome preparation method is critical for passive loading. There are many liposome preparation methods that have been reported to enhance hydrophilic drug encapsulation [8]. In this study, two different methods were investigated to obtain high EE%. Thin film hydration method (TFH) is the one of the simplest way to prepare liposomes [9] and because of this, it has been used in this work as a liposome preparation method. Also, small volume

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incubation method (SVI) [10] was developed and compared to TFH method. The aim of this study was to investigate the effect of the preparation method of hydrophilic drug-loaded liposomes on vesicle characteristic properties. Different factors, such as hydration volume for TFH method, drug amount and incubation volume for passive loading with SVI method were selected to further increase 5-FU loading in liposomes.

2. RESULTS AND DISCUSSIONS

2.1. Particle size, polydispersity index (PDI) and zeta potential

The physicochemical properties of the 5-FU-loaded liposomes prepared by TFH method were evaluated in terms of particle size, PDI, and zeta potential (Table 1). Particle size analysis showed no significant differences ($p>0.05$) in liposome size as a result of variations in the hydration volume. The average particle sizes were 194.7 nm and 194.5 nm for TFH-1 and TFH-2, respectively.

Table 1. The particle size, PDI, and zeta potential of liposomal formulations prepared by TFH method (mean ± SD, n = 3).

Formulation code	5-FU amount (mg)	Hydration volume (mL)	Particle size (nm)	PDI	Zeta potential (mV)
TFH-1	10	3.5	194.7 ± 8.4	0.198 ± 0.022	-12.7 ± 1.0
TFH-2	10	2	194.5 ± 4.3	0.135 ± 0.032	-14.3 ± 1.6

Similar results were obtained in liposomes prepared by SVI method (Table 2). Changes in the amount of active ingredient and incubation volume did not significantly affect the particle size ($p>0.05$). As a result, all the liposomes (both TFH and SVI method used) had average particle size less than 200 nm. All formulations may represent a convenient drug delivery system into tumors utilizing the enhanced permeability and retention (EPR) effect [11]. According to the EPR effect, liposomes of size below 200 nm can remain in circulation for a long time [12, 13]. The PDI indicates the homogeneity of particle. All formulations exhibited low PDI (<0.3), indicating a homogeneous dispersion. Another investigated parameter was the zeta potential, and all prepared liposomes displayed a negative zeta potential and were not influenced by formulation parameters, such as hydration volume, incubation volume, or amount of drug added to formulations.

Table 2. The particle size, PDI, and zeta potential of liposomal formulations prepared by SVI method (mean ± SD, n = 3).

Formulation code	5-FU amount (mg)	Incubation volume (mL)	Particle size (nm)	PDI	Zeta potential (mV)
SVI-1	10	2	184.6 ± 2.0	0.176 ± 0.010	-14.2 ± 1.2
SVI-2	7.5	2	191.3 ± 9.2	0.255 ± 0.005	-18,2 ± 0.2
SVI-3	5	2	189.3 ± 16.3	0.227 ± 0.011	-15.5 ± 0.3
SVI-4	5	1	188.6 ± 7.7	0.244 ± 0.034	-19.0 ± 0.6

2.2. Encapsulation efficiency (EE%)

An ideal nano-sized drug delivery system should encapsulate a sufficient amount of drug in order to obtain an effective treatment. As shown in Figure 1, hydration volume had a significant ($p < 0.05$) effect on the EE%. EE% was found to be more in TFH-2 (15.5%) as compared to TFH-1 (11.7%). The EE% increased with decreases in the hydration volume from 3.5 mL to 2 mL. This result can be explained by the high aqueous solubility of 5-FU. This hydrophilic drug can be easily dissolved in the external aqueous phase during liposome preparation. Because of the presence of relatively large amounts of external aqueous medium for TFH-1 (3.5 mL) compared with TFH-2 (2 mL), little 5-FU content can be encapsulated into TFH-1 [14]. The similar EE% result was also obtained by Glavas-Dodov et al. who prepared 5-FU-loaded liposomes using TFH method [15]. Also, it was observed that the whole thin lipid film layer was not hydrated with phosphate buffer solution (PBS) when lower volumes (<2 mL) of hydration was used. Because of this, much higher EE% cannot be obtained by TFH method.

Similar EE% results were obtained in TFH-2 and SVI-1 formulations. This may be due to the fact that both formulations have the same volume of external aqueous phase (2 mL) at the drug loading step. In

addition, similar EE% was obtained in SVI-1, SVI-2, and SVI-3 formulations including different amounts of 5-FU, but with the same incubation volume. These result was in agreement with the previous work done by Sabbagh et al. [16]. They reported that when 5-FU concentration increased, the encapsulation development could not be achieved when the lipid concentration remained the same. Therefore, 5 mg of 5-FU was chosen for the preparation of the formulations, and SVI-4 was prepared. In addition, the results indicated that if the incubation volume was 1 mL for SVI-1 and SVI-2, we could possibly achieve a much higher EE% compared to the 2 mL incubation volume. Since the water solubility of 5-FU is 12.2 mg/mL [17], the use of 5 mg of 5-FU in formulations gave us the chance to work at lower incubation volume such as 0.5 mL for further investigations. The highest EE% was achieved when the lowest incubation volume was used. The SVI-4 formulation had EE% of 25%. These results showed that since 5-FU does not strongly associate with the lipid bilayer [18], decreasing the incubation volume (provided that the same amount of hydrophilic drug is used) has a great effect on increasing EE%.

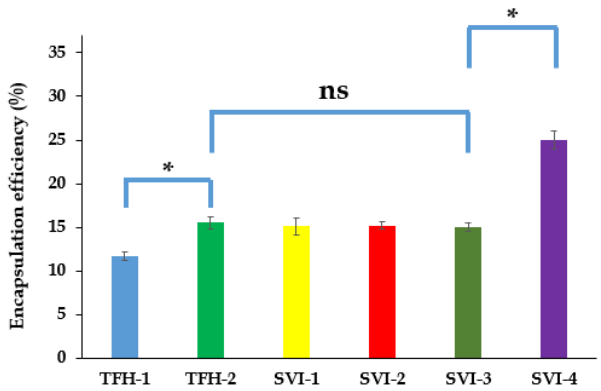

Figure 1. Comparison of EE% of liposomes prepared by different methods (mean ± SD, n = 3); ns: not significant; *: $p < 0.05$.

2.3. *In vitro* drug release study

In this study, the *in vitro* release of 5-FU from SVI-4 (optimum formulation with respect of EE%) was examined using Franz diffusion cell. The release rate of 5-FU from SVI-4 exhibited a biphasic mode: an initial fast rate of drug release followed by a slow rate of drug release. As shown in Figure 2, SVI-4 has 58.3% initial cumulative burst release in the first 2 h. The initial drug release could be attributed to the water solubility of active ingredient. 5-FU can leak through the lipid membrane rapidly since it is a hydrophilic drug [19]. Moreover, weakly bound or adsorbed drug molecules on the liposome surface [20] and the drug that leaked out of liposomes during the process of lyophilization [15] could be the reason for the burst release. After 4 h, 6 h and 12 h, 80.9%, 88.7%, and 94.3% of 5-FU was released from SVI-4, respectively. Also, the enclosed lipid shell could be the reason for the gradual repeated release of % 5-FU from SVI-4. The biphasic release pattern from 5-FU-loaded liposomes was also reported previously by AlQahtani et al. [21].

2.4. Drug release data modelling

Table 3 presents the adjusted coefficient of determination ($R^2_{adjusted}$) and the Korsmeyer-Peppas exponent (n) for SVI-4. Formulation SVI-4 showed a good fit to the first-order model with higher $R^2_{adjusted}$ value. The ''n value'' in case of Korsmeyer-Peppas model was used to predict the mechanism of 5-FU release from SVI-4. According to Korsmeyer-Peppas theory, when n is between 0.45 and 0.89, it characterizes non-Fickian release [22]. The fitted exponent was greater than 0.45 and lower than 0.89, which shows that diffusion and erosion have an important role in kinetic performance of the drug release [23]. Automatic reports from the DDSolver program are presented in Figure 3, and this data shows the predicted and observed *in vitro* release for 5-FU fitted for both calculated models.

3. CONCLUSION

In this study, various factors such as preparation method, drug amount, hydration volume, and incubation volume were investigated to optimize the formulation and preparation conditions of 5-FU encapsulated liposomes. In addition, this study demonstrates that the film hydration method is not very effective to increase hydrophilic drug EE%. Decreasing hydration volume and incubation volume increased

the EE% for both methods. Liposomes with high EE% of 5-FU and acceptable size for passive tumor targeting (EPR effect, particle size <200 nm) were obtained with the passive loading with SVI method. Further reduction of the incubation volume may increase the EE%. At this point, it may be necessary to reduce the amount of 5-FU used in formulations because of the limited water solubility of the drug. On the other hand, it could be useful that active encapsulation strategies help obtain higher 5-FU EE%. Adding materials such as cholesteryl hemisuccinate, which are more hydrophilic than cholesterol, to the formulation may also be beneficial to increase EE%.

Table 3. Release kinetic study of 5-FU from SVI-4.

Kinetic model	$R^2_{adjusted}$	n value
Zero-order	-1.870	-
First-order	0.986	-
Higuchi	0.302	-
Hixson–Crowell	0.360	-
Korsmeyer–Peppas	0.977	0.741

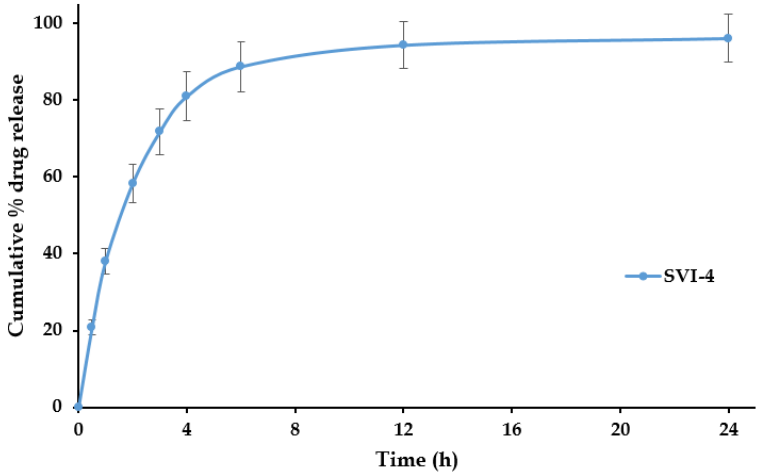

Figure 2. *In vitro* release of 5-FU from SVI-4 in PBS pH 7.4 at different time points. Triplicate data and values are expressed in mean ± SD (n=3).

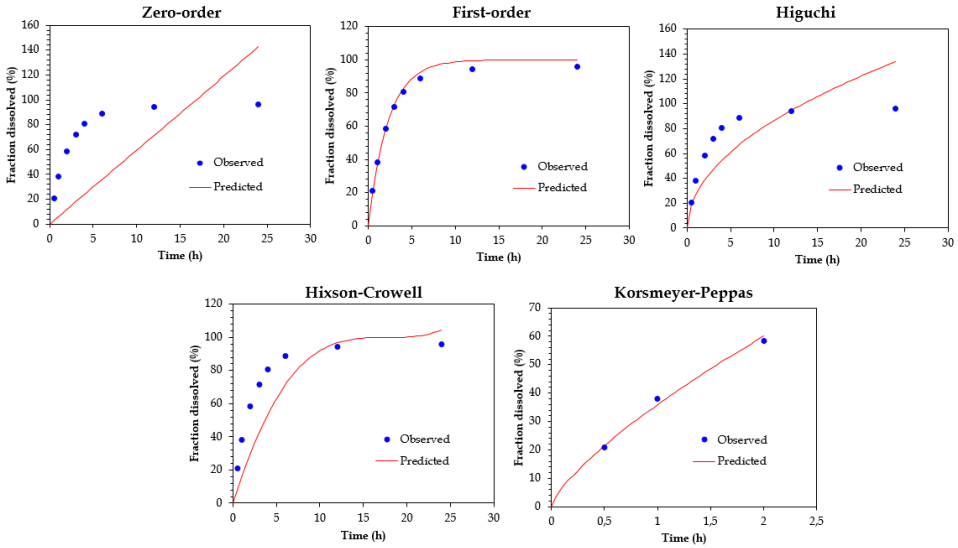

Figure 3. Fitting of 5-FU release from SVI-4. Automatically obtained from DDSolver.

4. MATERIALS AND METHODS

4.1. Materials

5-fluorouracil was purchased from Sigma-Aldrich (USA). L-α-phosphatidylcholine (PC) (Soy, 95%) and cholesterol (CHOL) were obtained from Avanti Polar Lipid Inc. (USA). Chloroform and acetonitrile (chromatographic grade) were purchased from Sigma-Aldrich (France and Israel, respectively). All other reagents and solvents were of analytical grade.

4.2. Preparation of 5-FU-loaded-liposomes

5-FU-loaded liposomes were prepared using two different methods (TFH and SVI method) as described below.

4.2.1. Thin film hydration (TFH) method

5-FU-loaded liposomes were prepared by using the TFH method as previously described [24, 25]. In the first step, 200 mg of PC and CHOL as lipids at a molar ratio of 8:2 in chloroform in a round-bottom flask. Then the flask was shaken. The film of lipid was obtained by evaporating the solvent under vacuum at 60 °C by using rotary evaporator (Rotavapor®, R-3, Buchi, Switzerland). The thin lipid film formed on the wall of flask was slowly hydrated with different volume of PBS pH 7.4 containing 5-FU (10 mg) at temperature of 60 °C for 1 h under magnetic stirrer. The obtained liposomes were sonicated using ultrasonic bath sonicator for 15 min. To increase homogeneity and to reduce particle size of liposomes, the formulations were extruded 10 times through 400 nm and 200 nm polycarbonate membrane filters using an extrusion device (Avanti Polar Lipids, Inc., USA), respectively. 5-FU-loaded liposomes were separated from unentrapped drug by centrifugation (Hitachi CS 150 GXL, Tokyo, Japan) at 70,000 rpm at 4 °C for 1 h. The formulation codes and characteristic properties of the prepared liposomes using TFH method are presented in Table 1.

4.2.2. Small volume incubation (SVI) method

In this method, firstly empty liposomes were prepared by TFH method using PBS (pH 7.4) as the hydration medium. In all formulations, the PC/CHOL molar ratio was 8:2, and total lipid amount was 200 mg. After the hydration of thin lipid film, the obtained liposomes were sonicated, extruded and ultra-centrifuged as described above. For active ingredient loading into the liposomes, 5-FU solutions were added and incubated to the empty liposomal pellets at 60 °C for 1 h under magnetic stirrer. The obtained 5-FU-loaded liposomes were centrifuged again for 1 h at 70,000 rpm at 4 °C to get rid of the untrapped 5-FU [26]. Formulation composition and physicochemical characterization of 5-FU-loaded liposomes using passive loading with SVI method are shown in Table 2. The general scheme for the preparation of liposomes is shown in Figure 4.

4.3. Lyophilization procedure

In this study, trehalose was chosen as the cryoprotectant. The obtained liposome pellets were resuspended in distilled water containing trehalose (10% w/w). The samples were frozen at −80 °C. The frozen samples were lyophilized at −55 °C for 40 h (Christ Alpha 1–2 LD plus, Germany) [27].

4.4. Particle size, polydispersity index (PDI) and zeta potential

The particle size and PDI of the 5-FU-loaded liposomes were determined by dynamic light scattering (DLS) technique [28], and the surface charge or zeta potential measurements were based on the electrophoretic mobility [29] of formulations using a Zetasizer Nano ZS (Malvern Instruments, UK). The results were represented as mean value ± SD (n = 3).

4.5. Determination encapsulation efficiency (EE%)

The amount of 5-FU in liposome formulations was evaluated by high performance liquid chromatography (HPLC). Briefly, 5 mg freeze-dried liposomes (n=3) were mixed with 5 mL of chloroform, and the mixture was sonicated in an ultrasonic bath for 1 min. Then 10 mL of PBS (pH 7.4) was added into this mixture; obtained final mixture was mixed by vortex mixing vigorously and stirred in an orbital shaker for 30 min [30]. Using a 0.22 µm membrane filter, the aqueous solution was filtrated and examined by HPLC at 265 nm. An Agilent 1220 LC HPLC system (Germany) was utilized to perform chromatographic analysis using a reverse phase C18 column (250 mm × 4.6 mm, 5 µm, Waters Xselect, Ireland). The mobile phase was consisted

of acetonitrile: water (10:90) mixture [31, 32], and the flow rate of mobile phase was adjusted at 1 mL/min. The percentage drug entrapped in the vesicles was then calculated using the following Eq. (1):

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

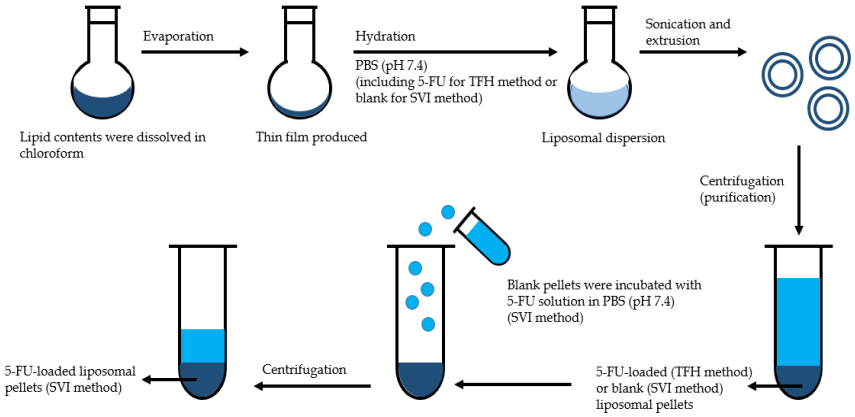

Figure 4. Schematic preparation of liposomes.

4.6. *In vitro* release study

The formulation with the highest EE% (SVI-4) was used in the release study. 5-FU release from SVI-4 was determined by dialysis method using Franz diffusion cell [33]. The diffusion membrane (MW: 12,000-14,000 dialysis membrane) was soaked in PBS (pH 7.4) before the study. 1 mL of freeze-dried liposome suspension in PBS pH 7.4 was placed in donor site and 2.5 mL PBS in receptor chamber. The whole diffusion cells were put in a thermostatic bath, which was maintained at 37 °C ± 0.5 °C. In predetermined time intervals, samples (2.5 mL) were collected from the receptor chamber, and substituted with an equal volume of fresh medium later. During all the experiment, a magnetic bar was stirring in each cell. The samples were analyzed by HPLC method as described above, and the experiments were carried out in triplicate (n=3).

4.7. Drug release data modelling

In vitro release data were analyzed by five most common kinetic models (which are zero order, first order, Higuchi, Hixson–Crowell and Korsmeyer–Peppas equations) to understand the 5-FU release profile, and the models were analysed using DDSolver software. The model with the highest adjusted coefficient of determination ($R^2_{adjusted}$) was considered to be the best fit for the kinetic release. With the aim of data modelling, all data were used expect for the Korsmeyer-Peppas model. To evaluate the drug release mechanism, release exponent "n" was calculated with the first 60% of drug release in Korsmeyer–Peppas model [34].

4.8. Statistical analysis

All the quantitative data were demonstrated as a mean ± standard deviation (SD). All data were analyzed statistically using a two-tailed unpaired t-test and one-way ANOVA followed by the Tukey's post-hoc test. Calculations were performed with the GraphPad Prism 5.0 program (GraphPad Software, Inc.).

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