Original Article

Niosomal Formulation for Co-Administration of Hydrophobic Anticancer Drugs into MCF-7 Cancer Cells

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Abstract

Introduction: Designing and developing drug delivery systems has received tremendous attention during the last decade. The treatment of cancer cells is a complicated process due to the existence of different biological pathways. Therefore, the co-delivery of different drugs could have a synergic effect on the treatment process.

Materials and Methods: In this study, different types of span (20, 60, 80) and cholesterol were utilized to formulate tamoxifen/curcumin co-loaded niosomes as a drug carrier system for breast cancer chemotherapy. Niosome characterization was performed through a set of instrument analysis techniques including scanning electron microscopy (SEM) and dynamic light scattering. Release behavior was studied by dialysis method at (pH = 5, 7.4). The stability was monitored during two months storage at two temperatures (4 and 25 °C). Cytotoxicity activity of the best niosomal formulation were assessed on MCF-7 cells, using MTT assay.

Results: The optimal niosomal formulation with span 80 and lipid-to-drug molar ratio of 20 was selected, with maximum encapsulation of both drugs and minimum size. Drug release behavior at physiological pH (7.4) (with significant drug release under acidic conditions (pH = 5) and storage stability of up to 2 weeks with little change in drug efficacy and measurement makes it a proper candidate for breast cancer treatment.

Conclusion: Finally, the results of this study showed the importance of creating highly biocompatible formulations, allowing the simultaneous transfer of two drugs with controlled release to cancer cells which could improve the chemotherapy process with the synergistic effect of the two drugs.

Keywords: Niosome, Tamoxifen, Curcumin, Breast cancer, MCF-7

1. Introduction

Nano-carrier-based DDSs (drug delivery systems) work efficiently on malignant sites in chemotherapy. Encapsulating chemotherapy drugs using a nanoscale device is the best way to reduce the side effects and improve the availability of drugs for cancer. Nanoparticles have some advantages over the free drug, including improved drug delivery and release and improved drug stability in biological environments [1]. One of the most widely used nanoparticles are niosomes. Self-aggregated non-ionic amphiphiles in aqueous media form two-layered structures called niosomes[2]. Niosomes have unique structures and can encapsulate hydrophilic and hydrophobic materials[3].
Cancer has long been considered an incurable disease, and its prevention, diagnosis, and treatment are among serious and perennial challenges in medicine [1]. Each of the cancer treatment approaches have a number of undesirable side effects that are dangerous to the health of the patient. In contrast, recent advances in nanotechnology have dramatically reduced these side effects and raised hopes for effective and safe treatments [4]. Breast cancer is a heterogeneous disease and is the most common malignancy in women [5]. It is estimated that about every 18 seconds a case of breast cancer is diagnosed and has a high mortality rate. Different classes of therapeutic agents and methods are used to treat breast cancer. Chemotherapy is one of the therapeutic methods that can be more effective if two or more drugs are used concurrently [6].

Curcumin (Cur) is a water and ether insoluble polyphenol and has antioxidant properties at acidic and neutral pHs. Its mechanism of action involves the inhibition of several cellular signaling pathways at multiple stages, with the effect on cellular enzymes such as cyclooxygenase and glutathione-s transferase, and the effect on angiogenesis and cell adhesion to each other which makes it suitable for chemical treatment [7]. Tamoxifen (Tmx) is also widely used as a non-steroidal estrogen receptor antagonist and adjuvant drug for the treatment of breast cancer [8]. This study aimed to investigate the role of nano-carriers in drug delivery to breast cancer cells and evaluate the synergistic effect of Tmx and Cur in the treatment process.

2. Materials and Methods

2.1 Reagents

Cholesterol, Span80, Span60, Span20, Sodium dodecyl sulfate (SDS) and Dialysis membrane (MWCO 12kDa) were bought from Sigma Aldrich (USA). Tmx and Cur drugs were provided by Iran Hormone and Exir Nano Sina companies (Iran) respectively. Chloroform, Amicon (Ultra-15-Membrane, MWCO 30kDa) and Dimethyl sulfoxide (DMSO) were purchased from Merck (Germany). Medium RPMI-1640, DMEM (Dulbecco’s Modified Eagle Medium), Trypsin-EDTA, Trypan blue, Fetal Bovine Serum (FBS), Phosphate-buffered saline (PBS), MTT (dimethylthiazol-2-yl)-2,5, and Penicillin / Streptomycin (PS)100 X were taken from Gibco, (USA). HEK-293 and MCF-7 cell lines were obtained from Pasteur Cell Bank (Iran).

2.2 Niosome Preparation

Loading of Tmx and Cur in the niosomes was performed by thin-layer hydration method; the details of this method is presented in previous work [9]. Briefly, cholesterol, surfactants, and drugs were dissolved in chloroform and chloroform evaporation was performed using a rotary evaporator (60 °C, 30 min). Then, PBS was used to hydrate dry thin films at 60 °C (30 min). In order to co-load Tmx / Cur in the niosomes with uniform size distribution, the sample was sonicated for 7 min. (Table 1).
Table 1. Composition of niosomal formulations.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Formulation</th>
<th>Type of Surfactant</th>
<th>Lipid *Drug (mol ratio)</th>
<th>Drug concentration (mg/ml)</th>
<th>Sonication time (min)</th>
<th>Surfactant: Cholesterol (molar ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tmx/Cur (TC)</td>
<td>TC1</td>
<td>Span20</td>
<td>10</td>
<td>1-1</td>
<td>7</td>
<td>2:1</td>
</tr>
<tr>
<td></td>
<td>TC2</td>
<td>Span60</td>
<td>10</td>
<td>1-1</td>
<td>7</td>
<td>2:1</td>
</tr>
<tr>
<td></td>
<td>TC3</td>
<td>Span80</td>
<td>10</td>
<td>1-1</td>
<td>7</td>
<td>2:1</td>
</tr>
<tr>
<td></td>
<td>TC4</td>
<td>Span20</td>
<td>20</td>
<td>1-1</td>
<td>7</td>
<td>2:1</td>
</tr>
<tr>
<td></td>
<td>TC5</td>
<td>Span60</td>
<td>10</td>
<td>1-1</td>
<td>7</td>
<td>2:1</td>
</tr>
<tr>
<td></td>
<td>TC6</td>
<td>Span80</td>
<td>20</td>
<td>1-1</td>
<td>7</td>
<td>2:1</td>
</tr>
</tbody>
</table>

* Lipid is the total amount of cholesterol and surfactant.

2.3 Morphology
To investigate the morphology of the optimum formulation, SEM was used (NOVA NANOSM 450 FEI, USA).

2.4 Size and Polydispersity of Index Measurements
Malvern Zeta Sizer (Malvern Instrument, UK) was used to distribute the size and polydispersity index based on dynamic light scattering.

2.5 Determination of Encapsulation Efficiency (EE)
The niosomes were ultra-filtered for 20 min at 4000xg, utilizing an Amicon. Throughout filtration, free drugs passed through the filter membrane and the drug-loaded niosomes remained in the top chamber. Drug concentration at a wavelength of maximum absorbance peak of each drug molecule was analyzed by UV visible spectroscopy (JASCO, V-530, Japan) (420 nm and 236 nm for Cur and Tmx) and drugs concentration was evaluated according to its standard curve. Finally, the encapsulation efficiency was computed using the following equation:

Encapsulation Efficiency (%) = [(A – B)/A] * 100

In this equation, A represents the amount of initial drug trapped into the niosomal formulations and B is the amount of free drug released from the membrane.

2.6 In Vitro Drugs Release Kinetic Study
The in vitro Tmx/ Cur release from niosomes was studied through the following method. Briefly, 2 ml of each sample was added to a dialysis bag. The dialysis bag containing each sample was put in PBS-SDS (0.5%, w/v) solution (pH = 5, 7.4) and stirred at 37 °C (50rpm). Then Aliquots were then taken at specified intervals and replaced with fresh medium. Different kinetic models were utilized to investigate and analyze the release profile.

2.7 Niosome Stability studies
By measuring the average size, PDI and EE of the niosomes while storing the samples at 25 °C and 4 °C for two months, the stability of the samples was evaluated.

2.8 In vitro Cytotoxicity Assay (MTT)
Cytotoxicity of samples in both HEK-293 cell lines and MCF-7 cell lines using MTT assay was investigated. For this purpose, the cells were cultured and seeded into 96-well plates (10^4 cells/well) utilizing RPMI-1640 as a medium that containing 1% PS (1%) and FBS, 10%, and then incubated under 5% CO_2 atmosphere (T=37°C, 24 h). Subsequently, different concentrations of the sample were added to the cell lines and incubated for 72 h. Then, each well medium was replaced by MTT (20 μL, 5 mg/mL) and was incubated for 4h in a 5% CO_2 atmosphere (T=37°C). The supernatant was eliminated and 100 μL of isopropanol was added to dissolve the formazan crystals generated by the living cells. Finally, MTT absorbance was
measured at 570 nm, using an ELISA Reader (Organon Teknika, Oss, Netherlands.

2.9 Statistical analysis
In this study, all data were reported as Mean ± SD, and were analyzed and plotted by GraphPad Prism, version 8 (GraphPad Software, San Diego, CA). Statistical analysis was carried out by performing analysis of variances (ANOVA) test as followed by post hoc Tukey. The significance level was considered less than 5 percent to assess the differences.

3. Results
Characterization of Niosomal Tmx/Cur Formulations

Table 2. Vesicle size, PDI, and EE % of different niosomal formulations. Data are represented as mean ± SD, n = 3.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Formulation</th>
<th>Vesicle Size (nm)</th>
<th>PDI</th>
<th>EE (%)</th>
<th>Tmx</th>
<th>Cur</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tmx/Cur (TC)</td>
<td>TC₁</td>
<td>319.15±6.58</td>
<td>0.223±0.033</td>
<td>90.5476±2.7531</td>
<td>86.2213±1.9238</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TC₂</td>
<td>190.20±8.63</td>
<td>0.169±0.009</td>
<td>94.2103±1.0278</td>
<td>90.2741±0.7894</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TC₃</td>
<td>172.70±3.96</td>
<td>0.189±0.028</td>
<td>95.2278±0.6342</td>
<td>93.7823±1.8852</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TC₄</td>
<td>367.00±14.57</td>
<td>0.218±0.039</td>
<td>92.2996±1.5021</td>
<td>90.0250±2.0763</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TC₅</td>
<td>286.55±2.47</td>
<td>0.208±0.030</td>
<td>96.7421±1.1139</td>
<td>93.1279±1.4895</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TC₆</td>
<td>159.45±10.39</td>
<td>0.192±0.001</td>
<td>98.3674±1.1930</td>
<td>96.3941±2.3383</td>
<td></td>
</tr>
</tbody>
</table>

Morphological Characterization of Optimized Niosomes
Figure 1 shows the optimal niosomal formulation that confirms uniform spherical morphology and a smooth surface with an average length of 80nm without accumulation of niosomes.

Drugs Release Study
The study results of the Tmx/Cur release profile from the optimum formulation for 0.5-72 h, at 37 ° C (pH=7.4 and 5) show that the cumulative diffusion profile is
biphasic and decreased for the nano-drugs formulation compared to the free drugs [10]. In general, the release data were examined with different kinetic models and were found to follow the Korsmeyer-Peppas model and the drug release was controlled by diffusion and erosion mechanism (Table 3).

![Tamoxifen](image1.png) ![Curcumin](image2.png)

**Figure 2.** Evaluation of the effect of pH on the release of Tmx/Cur loaded in the niosomes by dialysis method

<table>
<thead>
<tr>
<th>Release model</th>
<th>Tmx (pH 7.4): R²=0.7105</th>
<th>Cur (pH 7.4): R²=0.9150</th>
</tr>
</thead>
<tbody>
<tr>
<td>First-Order</td>
<td>Tmx+ Cur (pH 7.4): R²=0.6453</td>
<td>Tmx+ Cur (pH 7.4): R²=0.9484</td>
</tr>
<tr>
<td></td>
<td>TC₆ (pH 5): R²=0.7672</td>
<td>TC₆ (pH 5): R²=0.6895</td>
</tr>
<tr>
<td></td>
<td>TC₆ (pH 7.4): R²=0.5608</td>
<td>TC₆ (pH 7.4): R²=0.7453</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Tmx (pH 7.4): R²=0.8507</th>
<th>Cur (pH 7.4): R²=0.9688</th>
</tr>
</thead>
<tbody>
<tr>
<td>Higuchi</td>
<td>Tmx+ Cur (pH 7.4): R²=0.8081</td>
<td>Tmx+ Cur (pH 7.4): R²=0.9877</td>
</tr>
<tr>
<td></td>
<td>TC₆ (pH 5): R²=0.8028</td>
<td>TC₆ (pH 5): R²=0.9579</td>
</tr>
<tr>
<td></td>
<td>TC₆ (pH 7.4): R²=0.7288</td>
<td>TC₆ (pH 7.4): R²=0.9109</td>
</tr>
</tbody>
</table>

|                                      | Tmx (pH 7.4): R²=0.8474, n=0.4950 | Cur (pH 7.4): R²=0.9240, n=0.6113 |
| Korsmeyer-Peppas                    | Tmx+ Cur (pH 7.4): R²=0.8913, n=0.4135 | Tmx+ Cur (pH 7.4): R²=0.8700, n=0.5980 |
|                                      | TC₆ (pH 5): R²=0.8942, n=0.5286 | TC₆ (pH 5): R²=0.9737, n=0.4316 |
|                                      | TC₆ (pH 7.4): R²=0.7856, n=0.4794 | TC₆ (pH 7.4): R²=0.9394, n=0.5791 |

**Table 3.** Different models of kinetic and their parameters for Tmx/Cur release from niosomal formulation at different pH

**Physical stability study**

As shown in Figures 3A and 3B, the size and PDI of the niosomal formulation containing Tmx/Cur increased with increasing storage temperature and storage time, whereas the EE (Figure 3C) was less sensitive to these parameters. The size variations were significant on days 14, 30 and 60 (p-value <0.05, p-value <0.001 and p-value <0.01 respectively) and the PDI for TC₆ formulation was significant only on day 60 (p-value<0.05). In addition, for the EE, there was a significant difference between the two temperatures studied, only for Cur at 30 and 60 days (Figure 3C).
Figure 3. The effect of time and temperature of storage on the average size (A), PDI (B), and EE (C) of Tmx/Cur loaded niosomal formulation, (* p-value <0.05, ** p-value <0.01, and *** p-value <0.001).

In Vitro Cell Viability

Cytotoxicity was evaluated by MTT assay for niosome, Tmx, Cur, Tmx+ Cur and Tmx/ Cur loaded to niosomes (Nano (Tmx+ Cur)) on HEK-293 cell lines and MCF-7 cell lines. As can be seen in Figure 4 B, the encapsulation of drugs by niosomes significantly improved drug biocompatibility(p-value<0.05), which may be due to the low rate of release of drug molecules from the niosomal formulation into the physiological environment. This is consistent with the release data at this pH too. The results showed (Figure 4A) that the treatment of breast cancer cells (MCF-7) with niosomes loaded with Tmx/ Cur had a significantly higher inhibitory effect than the free drug solution (p-value<0.05) which can be attributed to the acidic environment of the cancer cells and the release of drugs from the niosomes (Figure 2). The synergy of Tmx and Cur also results in a stronger inhibitory effect (p-value<0.05), with the simultaneous loading of the two drugs in the niosomal formulation enhancing their inhibitory effects on the cancer cells tested (Table 4 for IC50 values).

Figure 4. The effect of samples on the viability of A) MCF-7 cells. B) The effect of Tmx + Cur and Nano (Tmx+ Cur) on the viability of HEK-293 cells by MTT assay (* p-value <0.05, ** p-value <0.01, and *** p-value <0.001).

Table 4. The calculated IC50 values for different samples against MCF-7 cell line using MTT assay

<table>
<thead>
<tr>
<th>IC50(µg/ml)</th>
<th>Niosome</th>
<th>Tmx</th>
<th>Cur</th>
<th>Tmx+ Cur</th>
<th>Nano (Tmx+ Cur)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>565.35±2.58</td>
<td>41.98±2.33</td>
<td>63.12±7.31</td>
<td>36.16±1.99</td>
<td>20.68±1.25</td>
</tr>
</tbody>
</table>

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4. Discussion

In the present study, effect of some parameters such as surfactant type, and lipid to drug ratio on size, PDI and entrapment efficiency of niosomal formulations were assessed. The results showed that the size of niosomal formulations synthesized with span 20 and 60 using lipid to drug 10 ratio was significantly smaller than formulations with lipid/drug ratio of 20. Also, in the case of niosomal formulations with span 80, nanoparticles prepared with lipid to drug ratio of 20 showed smaller size than lipid to drug ratio of 10. This process can be attributed to the hydrophobic chain length of the Span structure, which increases from Span 20 to Span 80 and the greater hydrophobic-hydrophobic interaction between encapsulated Tmx/Cur, cholesterol and the surfactant hydrophobic chain [11]. Also, according to the results, the encapsulation efficiency of Tmx and Cur is reliant on the size of the niosome and can be the result of hydrophobic-lipophilic balance (HLB) which is the ratio of hydrophobic to the hydrophilic portion of surfactant and depends on the type and amount of the surfactant [11, 12].

Results of the drug release from niosomal formulation showed that the cumulative diffusion profile is biphasic and decreased for the nano-drugs formulation compared to the free drugs [10]. The first stage of rapid release may be due to the desorption of drugs from the outer surface of the niosomes, and the lower rate of release often relates to drug release through the two layers [13]. Significant release of both drugs can be seen by decreasing the pH of the environment[14]. The reason for this trend may be due to swelling and breakage of niosomes in acidic situations, which is a common behavior in niosomes. PH-Dependent release of drugs is often suitable for the treatment of cancers because the environment around the cancer cells is acidic while it is not so in healthy cells. The drug release was controlled by diffusion and erosion mechanism. N values calculated at each pH (0.43 <n <0.85) indicate anomalous transport mechanism for drug release[15].

Based on the data obtained from stability test, the niosomal formulations can be maintained for at least two weeks with minimal change in size and content of the drug; however, for the samples stored at 4 °C compared to 25 °C are lower and slower, it might be due to less mobility of bilayer at 4°C [16]. However, experiments typically show an increase in the size of vesicles during storage due to their fusion [17] or aggregation [18].

The IC50 content of the nanocarrier containing 20.68 μg/ml decreased significantly compared to the combination of the two drugs (36.16 μg/ml) and the drug solution (41.98 and 63.12 μg/ml for Tmx and Cur, respectively) (P-value <0.05). In conclusion, it appears that encapsulation of Tmx/Cur by niosomes (TCn) increases anti-proliferative activity[19]. Also, IC50 of niosome (about 570 μg/ml) indicates that the niosomes not only have a low toxicity to cancer cells but at high concentrations, they do not have toxic effects on the MCF-7 cell line [20, 21].

In a 2015 study by Roghayeh Abbasali Porkhir et al., apoptosis induced by Tmx and Tmx loaded on solid lipid nanoparticles (SLN) was determined on MCF-7 and MDA-MB231 breast cancer cells and the results showed that Tmx loaded with SLN had a cytotoxic effect on MCF-7 and MDA-MB231 cells compared to free Tmx[22].

5. Conclusion

Optimal formulations of niosomes containing two drugs were prepared by varying the surfactant type (span 20, 60 and span 80) and lipid-to-drug ratio and finally selected according to the size and encapsulation efficiency (span 80 and lipid to drug molar ratio of 10). Optimum formulations had a controlled release at physiological pH while burst release at acidic pH was observed. This property helps niosomal carriers to be more effective in the
acidic environment of breast cancer cells than normal cells. Also, the while niosomes carrying Tmx and Cur have good biocompatibility with HEK-293 normal cells, they show significant toxicity in MCF-7 breast cancer cells. Finally, the data from this study may provide one possible way to optimally treat breast cancer.

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Conflict of interest
The authors declare no conflict of interest.

References