Formulation and Characterization of Tamoxifen loaded stealth Liposomes for Breast Cancer

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Abstract
The present study deals with the formulation and in-vitro characterization of tamoxifen loaded stealth liposomes. Passive targeting by stealth liposomes, once combined with efficient intracellular delivery, may be a very useful strategy to improve the antitumor efficacy for the anticancer agents. Stealth liposomes were prepared by using Cholesterol, DMPC, DSPC, and Polyethylene Glycol 4000 (PEG 4000) in order to achieve prolonged circulation time and sustained release. The prepared liposomes were evaluated for size, shape, profile, degree of drug entrapment, and in-vitro release efficiency. The effect of various formulation and drug release was investigated.

Key words:
Tamoxifen, DMPC, DSPC, Polyethylene Glycol, Stealth.

1) INTRODUCTION
Breast cancer is the most commonly diagnosed form of cancer and the second leading cause of cancer death in women [1]. Between one out of eight and one out of ten women will develop breast cancer during her lifetime [2]. Approximately two thirds of all breast cancers are estrogen receptor positive and are treated with adjuvant hormonal therapy [3].

Tamoxifen is the most widely used in hormonal treatment for all stages of breast cancer. It acts as an anti-estrogen by binding to the estrogen receptor [4]. Tamoxifen was approved by the Food and Drug Administration in 1977 for the treatment of women with advanced breast cancer and several years later for adjuvant treatment of primary breast
Tamoxifen has a relatively low toxicity and is less harmful than most chemotherapeutics. The major adverse effects of tamoxifen include higher incidence of endometrial cancer, liver cancer, thromboembolic disorders and development of drug resistance. These unwanted effects of tamoxifen, as well as various barriers to the effective administration of the drugs to tumor demands targeted delivery to the site of tumor and enhanced uptake by the tumor cells.

One of the major problems facing cancer therapy is achieving the required therapeutic concentration of the drug at the tumor site for desired period of time. It would, therefore, be desirable to develop targeted drug delivery system that can passively target cancerous cells. The greatest breakthrough leading to more general targeted antitumor therapy was the discovery of the EPR effect. The endothelial wall of all healthy human blood vessels is encapsulated by endothelial cells that are bound together by tight junctions. These tight junctions stop any large particles in the blood from leaking out of the vessel. Tumor vessels do not contain the same level of seal between cells and are diagnostically leaky. This ability is known as Enhanced Permeability and Retention effect (EPR effect). Passive targeting exploits the characterizing features of tumor biology that allow nanocarriers to accumulate in the tumor by the EPR effect.

The EPR effect was first reported by Matsumura and Maeda in 1986. Their investigations showed that most solid tumors have blood vessels with defective architecture and usually produce extensive amounts of various vascular permeability factors. Most solid tumors therefore exhibit enhanced vascular permeability, which will ensure a sufficient supply of nutrients and oxygen to tumor tissues for rapid growth. The EPR effect considers this unique anatomical-pathophysiological nature of tumor blood vessels that facilitates transport of macromolecules into tumor tissues. In contrast, this EPR effect-driven drug delivery does not occur in normal tissues. This unique EPR effect in solid tumors is considered to be a landmark principle in tumor targeting chemotherapy and is a promising paradigm for the development of anticancer targeted drug delivery system. Thus EPR effect has now become the “Gold standard” in anticancer drug design.

A liposome is a spherical vesicle with a membrane composed of a phospholipid and cholesterol bilayer used to deliver drug. Liposomes have been extensively investigated as targeting carriers for a variety of anticancer agents. The drug–loaded liposomes are usually injected intravenously for systemic application. However, there is usually rapid clearance of them from the circulation by the reticuloendothelial system (RES). To avoid this disadvantage, “Stealth technology” or called sterically stabilized liposomes has been developed.

Polyethylene glycol (STEALTH polymer) coating reduces mononuclear phagocyte system (MPS) uptake and provides long plasma residence time and plasma stability. Anticancer drug are limited in their use due to their systemic toxicity, poor stability and short biological half life. Hence, the use of liposomes offers a great way to delivering drug at a higher efficacy and lower toxicity. Liposomes are concentric bilayered vesicle in which an aqueous volume is entirely enclosed by a membranous lipid bilayer, mainly composed of natural or synthetic phospholipids. Liposomes are microscopic vesicles ranges from 20nm up to several µm composed of one or several concentric membranes, each 4nm in thickness. Saturated and unsaturated fatty acids found in lipids used to form liposomes. The main disadvantage of liposome is, following I.V administration they primarily come in contact with macrophages residing in liver and spleen (the mononuclear phagocyte system or MPS). Once internalized by macrophages, the lipid membrane of the liposome was digested by intracellular enzymes and the drug released. This combination of instability and MPS uptake restricts the opportunity for “true”
targeting\textsuperscript{[17]}. This can be overcome by incorporation of hydrophilic polymers (Polyethylene Glycol-4000). Surface modification with PEG has been shown to increase the circulation persistence of liposomes by decreasing recognition and uptake by reticuloendothelial system. PEG incorporation greatly increases the circulation time of liposomes and allows for greater delivery to tumors\textsuperscript{[13]}.

2) **MATERIALS AND METHODS**

2.1 Materials

Tamoxifen citrate was obtained as gift sample from Ar-Ex Laboratories, Ahmedabad. DMPC and DSPC were generous gifts from Lipoid GmbH, Frigenstrasse 4, D-67065 Ludwigshafen, Germany. Cholesterol was purchased from Sigma-Aldrich, Mumbai. Polyethylene glycol-4000 was purchased from Qualigens fine chemicals (Glaxo Smithkline). Methanol and chloroform was obtained from Merck Ltd, Mumbai. All other chemicals were high purity grade and obtained from commercial source.

2.2 Formulation of Tamoxifen Stealth Liposomes

Stealth Liposomes were prepared by the lipid thin-film hydration method. Accurately weighed quantity of cholesterol and lipid (DMPC &/or DSPC) was dissolved in chloroform: methanol (1:1, 5ml v/v) and transferred into a suitable round bottom flask. The flask was then connected to a Buchi rotary evaporator (Flawil, Switzerland) and rotated at 100 rpm for 20 minutes in a thermostatically controlled water bath at 37°C. The flask was rotated at 1.5 cm above the water bath. Vacuum was applied to the flask to evaporate organic solvents and form a homogeneous lipid film on the wall of a round bottom flask. To the thin dry lipid film formed, tamoxifen citrate, polyethylene glycol 4000 and polyoxyethylene lauryl ether were dissolved in 10 ml of distilled water was added and the flask was rotated again at same speed and temperature as before but without vacuum for 30 minutes for lipid film removal and dispersion. The liposomal suspension so formed was then transferred to a suitable glass container and sonicated for 30 minutes using a probe sonicator in an ice bath for heat dissipation. The sonicated dispersion was then allowed to stand undisturbed for about 2 hours at room temperature to form unilamellar liposomes. Each batch was prepared three times and stored in refrigerator.

2.3 Shape and surface morphology by SEM

The shape and surface morphology of stealth liposomes were visualized using scanning electron microscopy (JEOL, Japan). The stealth liposomes were first dried under vacuum. Stealth liposomes then were glued to aluminum sample holders and gold coated under argon atmosphere. The coated stealth liposomes were finally characterized for surface morphology under suitable magnification\textsuperscript{[18]}.

2.4 Vesicle size analysis

Liposome mean diameter and particle size distribution were determined by laser scattering light using Malvern Laser Analyzer Instrument. In order to analyze particle size, drug loaded liposomes were dispersed in deionized water, vortexed for 10 min and sonicated for 5 min before sampling.

2.5 Lamillarity by TEM

5µl undiluted tamoxifen stealth liposome dispersion was placed on a copper grid covered by a holey carbon film (Quantifoil R 1.2/1.3, pore size 1.2µm, 400 mesh, Quantifoil Micro Tools, D-Jena). Excess of sample was blotted automatically for 3s between 2 strips of filter paper and the grid was rapidly plunged into liquid ethane (cooled to about −180 °C with liquid nitrogen) in a cryo box. After blotting excess ethane on the grid with filter paper, the frozen specimen was transferred with a cryo-transfer unit (Gatan 626-DH) into the pre-cooled cryo-electron microscope (Cryo-TEM, Philips CM 120) operated at 120 kV and viewed under low dose conditions\textsuperscript{[19]}. The images were recorded with a 1k CCD Camera (FastScan F114, TVIPS, Gauting, Germany).

2.6 Zeta potential

The electrophoretic mobility and zeta potential were measured using a zeta potentiometer.
(Zeta Meter 3+, USA). To determine the zeta potential, liposome sample was diluted with KCl (0.1 M) and placed in the electrophoretic cell where an electric field of 15.2 V/cm was applied. Each sample was analyzed in triplicate.

2.7 Encapsulation Efficiency

The concentration of tamoxifen in the produced stealth liposomes were determined by HPLC method. A reversed phase C8 Column [(250 x 4.6 mm) 5µ] & guard column (4.0 x 3.0 mm, 5µ) was used. The mobile phase consisted of a mixture of methanol, water (90:10, v/v) and Triethylamine 0.1%; delivered at a flow rate of 1 ml/min with a pump. 1 ml of chloroform was added to 1 ml aliquots of the extruded suspension in tubes and mixed, followed by sonication for 2 min. The mixture was kept under a nitrogen stream. After complete chloroform evaporation, solution was centrifuged for 5 min at 5000 rpm, after which the clear solution was filtered through 0.22 µ nylon membrane (Millipore). The filtered solution was used for the analysis of tamoxifen citrate. The cumulative percentage drug release was calculated to establish the drug release profile of the tamoxifen loaded stealth liposomes.

2.8 In vitro drug release study

In vitro release studies of tamoxifen loaded stealth liposomes were carried out at 37±2°C in pH 7.4 phosphate buffer saline for a period of 10 days using an apparatus, which was indigenously designed and fabricated to conduct in vitro release studies. Liposomes equivalent to 10 mg of tamoxifen citrate was placed in a dialysis tube and was tightly sealed. Then the tube was immersed in 200 ml of release medium, ie., PBS (pH 7.4). While stirring the release medium using the magnetic stirrer at 300rpm, samples were taken at predetermined time intervals from the release medium. At periodic intervals, initially at 24 hours and then followed by every 2 days, 5 ml of the release medium was withdrawn and 5 ml of fresh release medium was replaced to provide the necessary sink condition. Samples were analyzed by HPLC for tamoxifen citrate content by solvent extraction method, ie. 5 ml of chloroform was added to withdrawn samples to which 5 ml of methanol: water (90:10 v/v) was added and the mixture was vortexed vigorously. The mixture was kept under a nitrogen stream. After complete evaporation of chloroform, solution was centrifuged for 5 min at 5000 rpm, after which the clear solution was filtered through 0.22 µ nylon membrane (Millipore). The filtered solution was used for the analysis of tamoxifen citrate. The cumulative percentage drug release was calculated to establish the drug release profile of the tamoxifen loaded stealth liposomes.

3) RESULTS AND DISCUSSION

To investigate the possible morphological changes of tamoxifen stealth liposomes on loading process, samples of tamoxifen stealth liposomes were observed under the scanning electron microscope (SEM). Scanning electron photomicrographs of all the formulations are taken, Formulation (F3 & F6) result is shown in Fig. 1a & 1b. Magnification of 7,500- 20,000 X was used while taking these photographs. Average particle size of Tamoxifen stealth liposomes was 482 ± 0.013 nm, 488 ± 0.081 nm, 431 ± 0.093 nm, 492 ± 0.078 nm, 592 ± 0.073nm, 600 ± 0.121nm, 526 ± 0.11nm, 598 ± 0.273nm, 892 ± 0.285 and 887 ± 0.336 nm for formulations F1 to F10 respectively. Particles of all formulations were in nanosize having smooth surface. The results of particle size data are shown in Table 1 for formulation F-1 to F-10.

Tamoxifen loaded PEGylated phospholipids in the formulations led to the formation of more colloidal structures (mixed micelles) with a disc-like shape (Fig. 2a & 2b). Often, these disc-like micelles appeared deformed (see e.g. marked structure in Fig.
The Figures represented that the liposomes are multilamellar (many layers) and disc shaped.

The possible effects of surface charge may affect the in vivo life span of the natural drug delivery system. The surface charge on the particle produced a difference in the electric potential in mV between the surface of each particle and bulk of the suspending liquid. That difference is called as zeta potential. It is easily measured because the charge of the potential will move as the suspension is placed between the two electrode that have D.C. voltage across them and the velocity will be proportional to the zeta potential of the particle. The technical term for this is electrophoresis. The electric charge present on the liposomes was evaluated by measuring the zeta potential by the zeta meter. Zeta potential of all formulated liposomes was in the range of –37.2 to –41.2 mV which indicates good stability. The result for zeta potential is shown in Table 1.

The entrapment efficiency in all the batches of tamoxifen stealth liposomes was studied. The results for entrapment efficiency are shown in Table 2. The maximum entrapment of tamoxifen was found in F-2 and F-7.

All the formulations of prepared tamoxifen stealth liposomes were subjected to in vitro release studies. Cumulative percentage drug released for F-1 to F-10 are shown in Fig. 3a & 3b. It was observed that the drug release showed a biphasic release with initial burst effect. The mechanism for the burst release can be attributed to the drug adsorbed on the liposomes or due to leakage of drug from tamoxifen stealth liposomes. The Peppas model is widely used when the release mechanism is not well known or when more than one type of release phenomenon could be involved. To characterize different release mechanisms 'n' value could be used. At the initial stage (24 hrs), DMPC based tamoxifen stealth liposomes showed burst effect related to the drug entrapped near the surface of the liposomes. At the later stage (after 24 hrs), the drug release was slower and release rate is determined by the diffusion/erosion of lipids. This initial burst drug release was later followed by more sustained/controlled pattern of drug release for up to 10 days. The percentage cumulative drug release at the end of 10 days period for F-1 to F-4 formulations was 22.765 ± 0.885, 17.342 ± 0.287, 13.682 ± 0.749 and 11.347 ± 0.995 respectively.

In case of DSPC based tamoxifen stealth liposomes, the burst effect was more when compared with DMPC based tamoxifen stealth liposomes; such initial burst was probably due to the high permeability of DSPC based tamoxifen stealth liposomes. Another possible reason is that the release of loosely bound drug which was present on the surface of DSPC based tamoxifen stealth liposomes. This loosely bound drug would be released by a mechanism of diffusion through the aqueous pores on the surface of the liposomes. The percentage cumulative drug release at the end of 10 days period for F-5 to F-8 formulations was 29.289 ± 1.98, 21.225 ± 1.028, 14.899 ± 0.879 and 17.448 ± 2.55 respectively.

In case of DSPC & DMPC based tamoxifen stealth liposomes, the vesicle size increased due to the lipid mixture, but in-vitro release was more or less the same. The percentage cumulative drug release at the end of 10 days period for F-9 & F-10 formulations was 14.899 ± 0.879 and 22.337 ± 2.15 respectively.

4) CONCLUSION

Liposomes are novel form of drug delivery. They offer a great way of delivering drugs at a higher efficacy and lower toxicity. However they have their limitations and as far as drug delivery concerns, there seems to be an emphasis on the use of sterically stabilized liposomes. In the present study, stealth liposomes were formulated and evaluated to explore the advantages of stealth liposomes over conventional liposomes. The formulations were characterized and evaluated for in vitro studies of tamoxifen loaded stealth liposomes, got higher entrapment efficiency and showed prolonged drug
release *in-vitro* due to the presence of stealthing effect.

5) **ACKNOWLEDGEMENTS**

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![Fig. 1a: Scanning Electron Photomicrograph of tamoxifen loaded stealth liposomes formulation F-3](image1)

![Fig. 2a: TEM image of tamoxifen loaded stealth liposomes formulation F-3](image2)

![Fig. 1a: Scanning Electron Photomicrograph of tamoxifen loaded stealth liposomes formulation F-6](image3)

![Fig. 2b: TEM image of tamoxifen loaded stealth liposomes formulation F-6](image4)

![Graph showing percentage cumulative drug release over time](image5)
Fig. 3a & 3b: Comparative in vitro release of tamoxifen loaded stealth liposomes according to zero order kinetics. Data represents the mean ± S.E. (n=3).

Table 1. Physicochemical characteristics of tamoxifen loaded stealth liposomes

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Particle Size (nm)</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>482 ± 0.013</td>
<td>-38 ± 2</td>
</tr>
<tr>
<td>F2</td>
<td>488 ± 0.081</td>
<td>-41 ± 1</td>
</tr>
<tr>
<td>F3</td>
<td>431 ± 0.934</td>
<td>-49 ± 3</td>
</tr>
<tr>
<td>F4</td>
<td>492 ± 0.078</td>
<td>-41 ± 2</td>
</tr>
<tr>
<td>F5</td>
<td>592 ± 0.073</td>
<td>-37 ± 2</td>
</tr>
<tr>
<td>F6</td>
<td>600 ± 0.121</td>
<td>-41 ± 2</td>
</tr>
<tr>
<td>F7</td>
<td>526 ± 0.11</td>
<td>-39 ± 2</td>
</tr>
<tr>
<td>F8</td>
<td>598 ± 0.273</td>
<td>-37 ± 3</td>
</tr>
<tr>
<td>F9</td>
<td>892 ± 0.285</td>
<td>-38 ± 3</td>
</tr>
<tr>
<td>F10</td>
<td>887 ± 0.336</td>
<td>-40 ± 2</td>
</tr>
</tbody>
</table>

Table 2: The encapsulation efficiency of the tamoxifen loaded stealth liposomes

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Intraliposomal tamoxifen concentration (mg/ml)</th>
<th>Encapsulation efficiency (%) after complete dialysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before dialysis</td>
<td>After dialysis</td>
</tr>
<tr>
<td>F-1</td>
<td>8.7 mg/ml ± 1.74</td>
<td>2.2 mg/ml ± 1.74</td>
</tr>
<tr>
<td>F-2</td>
<td>6.9 mg/ml ± 1.74</td>
<td>2.5 mg/ml ± 1.74</td>
</tr>
<tr>
<td>F-3</td>
<td>4.5 mg/ml ± 1.74</td>
<td>2.0 mg/ml ± 1.74</td>
</tr>
<tr>
<td>F-4</td>
<td>9.2 mg/ml ± 1.74</td>
<td>1.6 mg/ml ± 1.74</td>
</tr>
<tr>
<td>F-5</td>
<td>10.2 mg/ml ± 1.74</td>
<td>2.1 mg/ml ± 1.74</td>
</tr>
<tr>
<td>F-6</td>
<td>9.3 mg/ml ± 1.74</td>
<td>2.1 mg/ml ± 1.74</td>
</tr>
<tr>
<td>F-7</td>
<td>6.7 mg/ml ± 1.74</td>
<td>2.3 mg/ml ± 1.74</td>
</tr>
<tr>
<td>F-8</td>
<td>9.1 mg/ml ± 1.74</td>
<td>1.7 mg/ml ± 1.74</td>
</tr>
<tr>
<td>F-9</td>
<td>5.7 mg/ml ± 1.74</td>
<td>1.2 mg/ml ± 1.74</td>
</tr>
<tr>
<td>F-10</td>
<td>9.7 mg/ml ± 1.74</td>
<td>1.8 mg/ml ± 1.74</td>
</tr>
</tbody>
</table>

6) REFERENCE


