Maximizing the Therapeutic Efficacy of Imatinib Mesylate—Loaded Niosomes on Human Colon Adenocarcinoma Using Box-Behnken Design

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Abstract

This research purposed to formulate an optimized imatinib mesylate (IM)–loaded niosomes to improve its chemotherapeutic efficacy. The influence of 3 formulation factors on niosomal vesicular size (Y1), zeta potential (Y2), entrapment capacity percentage (Y3), the percentage of initial drug release after 2 h (Y4), and the percentage of cumulative drug release after 24 h (Y5) were studied and optimized using Box-Behnken design. Optimum desirability was specified and the optimized formula was prepared, stability tested, morphologically examined, checked for vesicular bilayer formation and evaluated for its in vitro cytotoxicity on 3 different cancer cell lines namely MCF-7, HCT-116, and HepG-2 in addition to 1 normal cell line to ensure its selectivity against cancer cells. The actual responses of the optimized IM formulation were 425.36 nm, −62.4 mV, 82.96%, 18.93%, and 89.45% for Y1, Y2, Y3, Y4, and Y5, respectively. The optimized IM-loaded niosomes confirmed the spherical vesicular shape imaged by both light and electron microscopes and further proven by differential scanning calorimetry. Moreover, the optimized formula exhibited improved stability on storage at 4 ± 2 °C and superior efficacy on MCF7, HCT-116, and HepG2 as IC50 values were 6.7, 16.4, and 7.3 folds less than those of free drug, respectively. Interestingly, IC50 of the optimized formula against normal cell line was ranged from 3 to 11 folds higher than in different cancer cells indicating a higher selectivity of the optimized formula to cancer cells. In conclusion, the incorporation of IM in niosomes enhanced its efficacy and selectivity toward cancer cells, presenting a promising tool to fight cancer using this approach.

Keywords:
imatinib mesylate
novesicles
cancer cell lines
in vitro cytotoxicity
optimization

Introduction

Several techniques were developed and well recognized for defeating cancer. One of the most rapidly progressive disciplines is nanomedicine and nanotechnology that proved successful returns and mechanisms for fighting cancer.1 Many benefits were recorded from applying nanomedicine over the conventional medicine in either cancer diagnosis or therapy with a very good prognosis results such as metabolism reduction, better pharmacokinetic profile, and improved selectivity on a particular tissue of interest.

Another recent and interesting advantage is nanoparticles (NPs) role in cancerous autophagy pathway interruption. Autophagy is substantially involved in many different mechanisms of cancer treatments and even cancer progress. Nanocarriers (NCs) application is considered an exploited tool in cancer remediation.2,3 From nanotechnology, NCs have been assembled from inorganic and organic materials to boost remedies efficacy, decrease their adverse events, and reinforce their therapeutic effectiveness. NCs could deliver drugs as adsorbed on or attached to their surfaces or encapsulated within their cavities.5 NCs used for medicinal purposes are synthesized from biocompatible materials which apart them a minimum toxicity and also offer high characteristic uptake adequacy in the affected cells rather than healthy.

Conflicts of interest: All authors declare that there are no conflicts of interest.

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IM was purchased from Shaanxi Pioneer Biotech Co. (Xi'an, China). Chloroform for HPLC was obtained from Honeywell Riedel-de Haën (Seelze, Germany). Absolute ethyl alcohol and sodium chloride 0.9% for injection were provided from El-Nasr Pharmaceutical Chemicals Co. (Abuzaabal, Cairo, Egypt). Sorbitan oleate (Span 20), sorbitan monopalmitate (Span 40), and Sorbitan monostearate (Span 60) were dissolved completely in 100-mL pear-shaped phial of Büchi-M/HB-140, (Flawil, St. Gallen, Switzerland) rotary evaporator, and rotated, 100 rpm, on a 58°C–60°C water bath (temperature above the transition temperature of cholesterol-surfactant mixture). For the thin film to be established on the wall of the flask, the chloroform was removed under vacuum. The thin film was then hydrated with 10-mL PBS (pH 5.5) containing 5-mg IM.

### Table 1

<table>
<thead>
<tr>
<th>Independent Variables (Factors)</th>
<th>Levels</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>X1: HLB of surfactant</td>
<td>Low</td>
<td>4.7</td>
</tr>
<tr>
<td>X2: cholesterol conc.</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>X3: DCP conc.</td>
<td>0.05</td>
<td>0.075</td>
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</table>

<table>
<thead>
<tr>
<th>Dependent Variables (Responses)</th>
<th>Units</th>
<th>Goal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y1: mean vesicle size</td>
<td>nm</td>
<td>Minimize</td>
</tr>
<tr>
<td>Y2: zeta potential</td>
<td>mV</td>
<td>Maximize</td>
</tr>
<tr>
<td>Y3: entrapment efficiency</td>
<td>%</td>
<td>Maximize</td>
</tr>
<tr>
<td>Y4: initial release</td>
<td>%</td>
<td>Maximize</td>
</tr>
<tr>
<td>Y5: cumulative release</td>
<td>%</td>
<td>Maximize</td>
</tr>
</tbody>
</table>
This step was implemented with 7 glass beads entity (4-mm diameter) for assuring the niosomal film complete hydration. It is important to mention that surfactant concentration used in each formula was constant (1 molar ratio) to make the value of HLB as the only independent variable concerning the surfactant (X1). Formulations compositions are presented in Table 2.

**Separation and Washing of Niosomal Dispersions**

The free drug isolation from the entrapped IM was carried out by cooling centrifugation at 4°C temperature and at 5200 × g force using large-capacity table-top refrigerated centrifuge (Union 32R, Hanil Science Ind. Ltd., Incheon, Korea). The niosomal vesicles were then washed twice with 30-mL PBS (pH 5.5), and volume was completed to 100 mL. By using a spectrophotometric assay with a Shimadzu DSC-50 differential scanning calorimeter (Shimadzu Corporation, Kyoto, Japan) was used for recording all thermo-graphs of BBD-optimized formula were taken after the sample negatively stained. The dye (1% potassium phosphotungstate) was freshly prepared by dissolving sufficiently in distilled water. One drop of the niosomal formulation was placed on the grid and mixed with 1 drop of the dye, left to dry, and then examined by the (Zeiss EM 10; Oberkochen, Germany) electron microscope.

**EE% = \( \frac{\text{IM Amount entrapped in niosomes}}{\text{Total IM added amount}} \times 100 \) (1)

**Characterization of IM-Loaded Niosomes**

**Determination of Zeta Potential and Vesicle Size**. The zeta potential and distribution by intensity of vesicle size for each formula was measured using dynamic light scattering (DLS) based on laser diffraction at room temperature (DLS Zetasizer Nano ZPS Instrument; Malvern, Worcestershire, UK). For each sample, 3 replicates were possessed. As a size uniformity mensuration, the polydispersity index (PDI) was specified.

**IM Niosomal In Vitro Release Study**. The in vitro release profiles were implemented as a simulation assessment of the predicted attitude of IM niosomal formulations under the in vivo hydrodynamic stress conditions. The experiment was conducted in triplicate. Each preparation was separated, washed twice, and the IM entrapped amount was quantified. The amount of retained drug at initial time was considered as the total amount of drug. Then, the pellet of each formulation was dispersed in 10-mL PBS. The rotary shaker (model GLF 3203; Hilab, Düsseldorf, Germany) was used to carry out the experiment. The device rate was adjusted to 150 strokes/min with temperature at 37 ± 0.5°C. A sample of 1-mL niosomal dispersion was taken at different time intervals, namely 1, 2, 3, 4, 5, 6, 8, 12, 24, and 48 h. Samples were separated and washed twice. The amount of IM released was determined at each time interval, and the amount of IM retained was then calculated at each time interval for all formulations.

**Kinetic Treatment of the Release Data**. IM release profiles from niosomes can be mathematically represented to get acquaintance about the release mechanism. Data procured from release study were conformed to various kinetic models predominantly used to illustrate drug release pattern from the prepared niosomes which are as follows: zero order, first order, second order, Higuchi, and Korsmeyer-Peppas models.

**Prediction, Elaboration, and Evaluation of the Optimized Formulation**

The optimized IM-loaded niosomes formulation was predicted after analysis of data and multiple response optimization using the software. This predicted formulation was produced and evaluated for mean vesicular size, zeta potential, EE%, and the initial release percentage of IM after 2 h and the cumulative release percentage of IM after 24 h. Each measurement was conducted in triplicate. In addition, the optimized formulation was subjected to morphological examination using both light microscope and electron microscope. In addition, the interaction between the vesicle-forming ingredients and the drug was checked by differential scanning calorimetry.

**Light Microscopy**. The morphological aspects were evaluated by using light microscopy. The optimized formula was examined under (Leica DM300, Wetzlar, Germany) optical microscope and photographed at 400× magnification power by means of a digital camera (HTC MS).

**Electron Microscopy**. Transmission electron microscope micrographs of BBD-optimized formula were taken after the sample negatively stained. The dye (1% potassium phosphotungstate) was freshly prepared by dissolving sufficient amount in distilled water. One drop of the niosomal formulation was placed on the grid and mixed with 1 drop of the dye, left to dry, and then examined by the (Zeiss EM 10; Oberkochen, Germany) electron microscope.

**Scanning electron microscope (SEM)**. Examination of BBD-optimized formula was performed after drying 1 drop of the sample and consigned using adhesive double-faced tapes on aluminum stubs and gold coated using sputter coater and photographed by (model Quanta 250 FEG, FEI, Eindhoven, Netherlands) SEM.

**Differential Scanning Calorimetry**. To confirm the drug and cholesterol interaction with phospholipid-forming vesicles, DSC was used. DSC was carried out for the dehydrated vesicles of optimized formula. The niosomal formulation was prepared, separated, washed twice, and dehydrated by freeze dryer, and the dehydrated vesicles were used for this thermal analysis. DSC thermograms of the individual components of the niosomes, namely; cholesterol, Span 40, DCP, and IM were also investigated. The Shimadzu DSC-50 differential scanning calorimeter (Shimadzu Corporation, Kyoto, Japan) was used for recording all thermograms. With nitrogen purging (100 mL/min), a 5°C/min heating rate was used over a 20°C–250°C temperature range. For the niosomal preparation, a weight equivalent to 2-mg Span 40

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Table 2

<table>
<thead>
<tr>
<th>Run</th>
<th>Factors</th>
<th>Responses</th>
<th>X1 (Value)</th>
<th>X2 (Molar Ratio)</th>
<th>X3 (Molar Ratio)</th>
<th>Y1 (nm)</th>
<th>Y2 (mV)</th>
<th>Y3 (%)</th>
<th>Y4 (%)</th>
<th>Y5 (%)</th>
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<tbody>
<tr>
<td>F1</td>
<td>6.65</td>
<td>1.5</td>
<td>0.1</td>
<td>520.24</td>
<td>–560</td>
<td>83.9</td>
<td>13.6</td>
<td>88.6</td>
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<tr>
<td>F2</td>
<td>4.7</td>
<td>1.5</td>
<td>0.075</td>
<td>573.15</td>
<td>–43.0</td>
<td>84.3</td>
<td>92.3</td>
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<tr>
<td>F3</td>
<td>8.6</td>
<td>1.0</td>
<td>0.05</td>
<td>567.98</td>
<td>–54.0</td>
<td>84.1</td>
<td>17.3</td>
<td>92.5</td>
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<td>F4</td>
<td>6.65</td>
<td>0.5</td>
<td>0.05</td>
<td>462.59</td>
<td>–58.0</td>
<td>78.6</td>
<td>18.0</td>
<td>94.1</td>
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<tr>
<td>F5</td>
<td>8.6</td>
<td>0.5</td>
<td>0.075</td>
<td>567.37</td>
<td>–53.0</td>
<td>85.04</td>
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<td>92.1</td>
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<tr>
<td>F6</td>
<td>4.7</td>
<td>1.0</td>
<td>0.1</td>
<td>408.64</td>
<td>–62.0</td>
<td>79.6</td>
<td>11.3</td>
<td>89.6</td>
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<tr>
<td>F7</td>
<td>8.6</td>
<td>1.5</td>
<td>0.075</td>
<td>574.92</td>
<td>–49.0</td>
<td>85.9</td>
<td>13.2</td>
<td>86.8</td>
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<td>F8</td>
<td>4.7</td>
<td>1.0</td>
<td>0.05</td>
<td>419.66</td>
<td>–51.0</td>
<td>77.4</td>
<td>15.3</td>
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<td>F9</td>
<td>8.6</td>
<td>1.0</td>
<td>0.1</td>
<td>565.8</td>
<td>–64.0</td>
<td>85.1</td>
<td>14.5</td>
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<td>F10</td>
<td>6.65</td>
<td>0.5</td>
<td>0.1</td>
<td>499.73</td>
<td>–62.0</td>
<td>79.8</td>
<td>18.95</td>
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<td>F11</td>
<td>6.65</td>
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<td>0.05</td>
<td>516.61</td>
<td>–53.0</td>
<td>81.3</td>
<td>12.3</td>
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<td>F12</td>
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<td>0.5</td>
<td>0.075</td>
<td>391.18</td>
<td>–59.0</td>
<td>71.4</td>
<td>18.90</td>
<td>94.3</td>
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<td>F13</td>
<td>6.65</td>
<td>1.0</td>
<td>0.075</td>
<td>437.21</td>
<td>–63.0</td>
<td>81.4</td>
<td>17.3</td>
<td>90.8</td>
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<tr>
<td>F14</td>
<td>6.65</td>
<td>1.0</td>
<td>0.075</td>
<td>449.82</td>
<td>–61.0</td>
<td>80.7</td>
<td>16.8</td>
<td>93.1</td>
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<tr>
<td>F15</td>
<td>6.65</td>
<td>1.0</td>
<td>0.075</td>
<td>435.46</td>
<td>–64.0</td>
<td>82.9</td>
<td>14.9</td>
<td>92.7</td>
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</table>

* Responses were measured in triplicate when SD values did not exceed 5% of the stated values.
was used. For each individual component, an accurate weight of 2 mg was used in the investigation. All weights were measured and examined as sealed with pinholes into an aluminum pan. An empty aluminum pan was used as a reference.\textsuperscript{57} Computer reports of the DSC thermograms were provided.\textsuperscript{58-60}

**Effect of the Storage on the Optimized IM-Loaded Niosomes Stability**

Stability study on the optimized formula of IM-loaded niosomes was conducted for 3 months at the refrigerator temperature (4 ± 2°C). Stability parameters for the prepared formulation were defined in terms of the percentage of drug entrapment during the study period, change in the vesicle size, PDI, and zeta potential,\textsuperscript{46} as well as the morphological changes observed by light microscope.\textsuperscript{59} The optimized IM-loaded niosomes was contained in 30-mL clear glass vials, well-sealed, and then stored at 4 ± 2°C temperature.\textsuperscript{41,58,61-64} At specified time intervals (30, 60, and 90 days), samples were collected to specify the residual amount of the vesicular retained drug.\textsuperscript{65,66} The separation technique of the leaked out drug during the storage period was done by dilution of an aliquot of the formulation with PBS and centrifugation. Besides, the change in vesicle size, PDI, and zeta potential and light microscopic examination were also observed. Leaching of IM from optimized formulation was studied and also determined according to the most suitable kinetic order. By knowing K, it was possible to calculate the shelf life (t\textsubscript{90%}). The leaching rate constant (K) was determined by using both zero- and first-order kinetic model.\textsuperscript{46} The leaching rate constant (K) was determined according to the most suitable kinetic order. By knowing K, it was possible to calculate the shelf life (t\textsubscript{90%}), the time after which the dosage forms lost 10% of their initial drug content and which the dosage forms would remain complying with the official requirements of drug content.

**In Vitro Cytotoxicity Study of the Optimized Formulation**

In this experiment, the cytotoxicities of both the optimized formulation of IM-loaded niosomes and the pure IM were studied and compared against different cancer cell lines as well as normal cell line. The concentration that induces 50% growth inhibition (IC\textsubscript{50}) was calculated in accordance to the most suitable kinetic order. By knowing K, it was possible to calculate the shelf life (t\textsubscript{90%}), the time after which the dosage forms lost 10% of their initial drug content and which the dosage forms would remain complying with the official requirements of drug content.

**Cell Lines Chosen for the Study**

Three different cancer cell lines namely, human hepatocellular carcinoma (HepG2), human colon adenocarcinoma (HCT-116), and human breast adenocarcinoma (MCF-7) in addition to baby hamster kidney (BHK) cells were originally purchased from American type Culture collection (ATCC, Manassas, VA) and were allowed to metabolize the dye into colored-insoluble formazan crystals for 2 h. The remaining MTT solution was discarded from the wells, and the formazan crystals were dissolved in 200 μL of 5% MTT solution/well (Sigma-Aldrich) and were allowed to metabolize the dye into colored-insoluble formazan crystals for 2 h. The remaining MTT solution was discarded from the wells, and the formazan crystals were dissolved in 200 μL/well acidified isopropanol for 30 min, covered with aluminum foil and with continuous shaking using a MaxQ 2000 plate shaker (Thermo Fisher Scientific Inc., Kalamazoo, MI) at room temperature. Using a Stat Fax\textsuperscript{R} 4200 plate reader (Awareness Technology, Inc., Palm City, FL), absorbance was measured at 570 nm.\textsuperscript{66} Cell viability was expressed as percentage of control and the concentration that induces 50% maximum inhibition of cell proliferation (IC\textsubscript{50}) were determined using version 5 GraphPad Prism software (GraphPad software Inc., La Jolla, CA).\textsuperscript{43}

**Red Blood Cell Hemolysis Assay**

The effect of the optimized formula on the integrity of RBCs was examined as an indicator of its toxicity. To assess the hemolytic activity of the optimized IM-loaded niosomes, blood sample was drawn from an anonymous human donor, directly into K2-EDTA-coated vacutainer tubes to prevent coagulation. The blood sample was then centrifuged at 500 g for 5 min to remove the plasma and to ensure that there is no hemolysis. Plasma then was aspirated gently via a micropipettor and discarded into biohazard waste. This step was repeated 3 times after addition of PBS with gentle mix for washing purposes. The blood sample then was diluted 1:50 in PBS, and 150 μL of diluted erythrocytes was transferred to each well in 96-well plate and incubated with the desired concentration of IM-loaded niosomes, PBS as negative control or triton X 20% as positive control for 1 h at 37°C. The plate then was centrifuged for 5 min at 500 × g to pellet intact erythrocytes and the supernatant was transferred to a new flat bottom 96-well plate, and the absorbance was measured at 450 nm using Stat Fax\textsuperscript{R} 4200 plate reader (Awareness Technology, Inc.). To calculate the percentage of hemolysis, triton X absorbance was considered as 100% hemolysis and all values were normalized to it. The data were represented as mean ± standard error of the mean for 3 independent experiments.\textsuperscript{70}

**Statistical Analysis**

Data are presented as the mean ± standard error of the mean. Statistical analysis was performed using GraphPad Instat software version 3 (ISI® software).

**Results and Discussion**

**Preliminary Study**

**Effect on the Entrapment Efficiency of IM in Niosomes**

The preliminary study revealed that the negatively charged niosomes exhibits higher percentage of the entrapped IM than those of the neutral and the positively charged niosomes at the investigated ratios. These results can be explained first by the theory of the attraction forces between the negative charge inducer (DPC) and the cationic drug (IM) which make the negative charged niosomes with higher entrapment efficiency than the neutral one. On the contrary, the positively charged niosomes exhibited the lowest entrapment efficiency due to the repulsion forces between SA and the drug. This postulation was in good agreement with the previously reported finding that investigated the effect of charged
Effects on the In Vitro Release Profile of Imatinib Mesylate From Niosomes

It was revealed that all IM niosomal formulations released approximately 100% of their drug content after 48 h, whereas the release of IM from niosomes with molar ratio (1:1:0.1) of Span 60: cholesterol: DCP in the first 24 h was slower than the other molar ratios of negatively charged niosomes and the neutral niosomes with Span 60. The efficient stabilization of the niosomal membrane structure may be imputed to the addition of DCP with cholesterol presence, which in turn decreases the release of drug and increases its retention in niosomes. The negatively charged niosomes released IM with lower degree than its neutral niosomes analogues. This can be referred to the better stability of negatively charged niosomes compared with neutral ones. Besides, the increase of cholesterol concentration to certain limit has a great role in stabilization of the niosomes and in prevention of leakage which leads to a slower release rate.

Optimization of IM-Loaded Niosomes

Based on the raised results from the preliminary study, 15 formulations of IM-loaded niosomes were prepared by taking the chosen factors values (X1, X2, and X3) at varied levels as displayed in Tables 1 and 2. A BBD of 3-factor, 3-level paradigm was used to investigate, optimize, and assess main interaction and quadratic effects of the chosen variables on the 5 responses stated for the design. Three surfactants with varied HLB values, namely Span 60, Span 40, and Span 20 with HLB values 4.7, 6.7, and 8.6, respectively. Cholesterol concentration (molar ratio) was used as X2 with 3 different ratios (0.5, 1, and 1.5). And for X3, concentration of DCP was used with 3 different molar ratios (0.05, 0.075, and 0.1). An essential variation of the observed datum for size of vesicles, zeta potential, the efficiency of entrapment, and the initial and the cumulative drug release percentage were accomplished, concerning the different factor levels (Table 2). Besides, kinetic treatment results of the release profiles of the drug (IM) from the niosomal formulations were also presented in Table 3.

Quantitative Factors Effects Estimation

From the previously mentioned software, polynomial equations were generated which represented the mathematical linkages between the observed responses and the factors and tested for their significance by ANOVA. In Table 4, the predestined effects of factors and the correlating p values from ANOVA on the 5 responses were shown. The factor effectiveness will be regarded significant when the effect does not equal zero and its p value is less than 0.05. It should be noted that a negative sign will be assigned for the antagonistic factor effect, whereas a synergistic effect is denoted by a positive sign. Based on the obtained results, the HLB of surfactant (X1) possessed a considerable synergistic effect on the niosomal mean vesicle size (Y1) and the drug entrapment efficiency in niosomes (Y2) with 0.002 and 0.001 p values, respectively. Cholesterol concentration (X3) was found to have a significant synergistic effect on Y1 and Y3 with 0.0232 and 0.0035 p values, respectively. Furthermore, X0 possessed a respectable antagonistic influence on zeta potential (Y2), initial release percentage of IM after 2 h (Y4), and cumulative release percentage after 24 h (Y5) with 0.0131, 0.0034, and 0.001 p values, respectively. In addition, zeta potential of the prepared niosomes (Y2) has been influenced significantly by DCP concentration (X3) and the quadratic term of X1 with 0.0193 and 0.0142 p values, respectively. In addition, the quadratic term of X2 was noted to have a significant synergistic effect on niosomal mean vesicular size (Y1) and to have a significant antagonistic effect on zeta potential (Y2) with 0.025 and 0.0102 p values, respectively. In addition, it was found that the interaction term (X1X2) experienced a significant synergistic effect on Y1 and, in contrast, a significant antagonistic effect on Y3 with 0.0295 and 0.0077 p values, respectively.

Effects on the Vesicle Size and Zeta Potential (Y1 and Y2). Vesicles size, PDI, and zeta potential are important parameters for evaluation of niosomes stability and efficiency which influence the biopharmaceutical feature of niosomes as NCS. The prepared niosomes vesicular sizes were ranged from 391.18 nm for F12 to 574.92 nm for F7 (Table 2). In addition, IM-loaded niosomes rendered a PDI values ranged from 0.126 to 0.498, evidencing a homogeneous population with comparatively narrow vesicle size distribution which was agreed with the earlier published work. F12 was observed to have the smallest vesicle size, the formulation that have low level of HLB value of surfactant (4.7) and lower level of cholesterol (0.5) with 0.075 molar ratio of charge-inducing agent. Although the largest vesicle size acquired in F7 that have higher level of HLB value of surfactant (8.6) and higher level of cholesterol (1.5) with 0.075 molar ratio concentration of charge-inducing agent. Consequently, as displayed in the Pareto chart and response surface plot of Y1 (Figs. 1 and 2), mean vesicular size affected mainly by both surfactant HLB values (X1) and cholesterol concentration (X3). A direct connection was observed between surfactant HLB (X1) and niosomal mean vesicle size (Y1). At the same level of X2 and X3 in F6 and F9, the increment in HLB of surfactant from 4.7 to 8.6 was along with vesicle size increase from 408.64 to 565.8 nm (Table 2). In addition, the decrease of surfactant HLB from 8.6 to 4.7 in F5 and F12, respectively, was associated with the decrease of mean vesicle sizes from 567.37 to 391.18 nm at the same level of other factors X2 and X3 which is in conformity with the prior work. In addition, vesicle size decreased from 573.15 nm in F2 to 391.18 nm in F12 as cholesterol concentration (X3) decreased from 1.5 to 0.5 at the same level of X1 and X3.

In addition, zeta potential ranged from −43.0 mV for F2 to −64.0 mV for F9 and F15 (Table 2) which indicates that all formulations were of good stability, which was compatible with many other earlier studies. Physical stability of niosomes is influenced by particle aggregation due to the niosomal surface charge alteration as aforementioned in the literature for other carriers systems. Particularly, NCS with high negatively zeta potential values will possess good stability with minimal aggregation. Therefore, colloidal suspensions should render zeta potential not less than −30 mV, which ensures enough electrostatic repulsion between dispersed NCS. To achieve our goal in maximizing zeta potential of the prepared niosomal formulations and avoid the misuse of the data by the software, the results were applied in numerical values neglecting the negative sign of the zeta potential charge. It was found that cholesterol concentration (X3) significantly affects zeta potential of the prepared formulation with negative trend as displayed in the Pareto chart and response surface plot of Y2 (Figs. 1 and 2). As X2 decreased from 1.5 to 0.5 molar ratio, Y3 increased from −56 mV in F1 to −62 mV in F10, noting that the other factors levels are the same. Besides, if X2 increased from 0.5 to 1.5 molar ratio, zeta potential (Y2) decreased from −58 mV in F4...
Effects on the Entrapment Efficiency ($Y_3$). Table 2 showed the observed values of the entrapment efficiency for BBD formulations. It was revealed that the prepared vesicles entrapment efficiency varied from 71.4% to 85.9% for F12 and F7, respectively. Outcomes showed that the rise in surfactant HLB leads to augmentation of IM capacity of entrapment in the niosomes. IM entrapment percentage was ameliorated with surfactant of higher HLB compared to other grades which agreed with what reported in previous work.\textsuperscript{53} In addition, entrapment efficiency percentage improved with the increase in cholesterol and DCP concentration. The effect of DCP is not a part of but correlated to the type of nonionic surfactant and amount of cholesterol used. Raising DCP amount above certain limit will hinder the niosomal vesicles formation. However, the increase of cholesterol amount resulted in a more membrane rigidity and hence increased entrapment efficiency percentage and leads to less-leaky vesicles formation. Thus, the addition of cholesterol reduced the fluidity of niosomes and imparts rigidity to vesicles. It prevents the transition from gel to liquid phase resulting in less-leaky niosomes.\textsuperscript{11,49,56}

It was observed that both HLB of surfactant ($X_1$) and cholesterol concentration affect significantly the entrapment efficiency percentage of the formulations with positive trend as displayed in Pareto chart and response surface plot of $Y_3$ (Figs. 1 and 2). The increase in the value of surfactant HLB from 4.7 to 8.6 was accompanied with increase in entrapment efficiency percentage from 84.3% in F2 to 85.9% in F7 at the same level of $X_2$ and $X_3$. In addition, the decrease of $X_1$ from 8.6 to 4.7, without changing the other factors, would yield a decrease in $Y_3$ from 84.1% in F3 to 77.4% in F8 as well. In addition, at the same level of $X_1$ and $X_3$, as the cholesterol concentration ($X_2$) decreased, the entrapment efficiency percentage decreased from 84.3% in F2 to 71.4% in F12. Moreover, the entrapment efficiency percentage increased from 78.6% ($F_4$) to 81.3% ($F_11$) as $X_2$ increased from 0.5 to 1.5 molar ratio concentrations.

Effects on the Initial and Cumulative Release Percentage ($X_4$ and $X_5$). Data obtained from the release profiles evidences (Table 2 and Fig. 3) that every formulation has released at least 80% of their drug content at the end of 24 h. The initial release percentages were varied from 9.2% for F2 to 18.95% for F10, whereas cumulative release percentages were ranged from 83.6% for F2 to 94.3% for F12. Cholesterol concentration ($X_2$) was the only factor that affect significantly on both $Y_4$ and $Y_5$ as displayed in Pareto charts and response surface plot of $Y_4$ and $Y_5$ (Figs. 1 and 2). There was an inverse relationship between $X_2$ and both $Y_4$ and $Y_5$. As $X_2$ decreased from 1.5 to 0.5 molar ratio at the same levels of the other factors, $Y_4$ and $Y_5$ increased from 13.6% in F1 to 18.95% in F10 and

---

### Table 3

<table>
<thead>
<tr>
<th>Run</th>
<th>$r$ Value</th>
<th>Zero Order</th>
<th>First Order</th>
<th>Second Order</th>
<th>Higuchi Model</th>
<th>Korsmeyer-Peppas Model, n Value$^a$</th>
<th>Release Rate Constant ($h^{-1}$)$^b$</th>
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<td>0.94095</td>
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$^a$ Non-Fickian release; anomalous, the release is controlled by a combination of diffusion and polymer relaxation.

$^b$ Release rate (K) of first-order model.

---

### Table 4

<table>
<thead>
<tr>
<th>Factors</th>
<th>$Y_1$</th>
<th>$Y_2$</th>
<th>$Y_3$</th>
<th>$Y_4$</th>
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<td>$X_1X_5$</td>
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<td>0.6784</td>
<td>-0.8767</td>
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$X_1$ is the HLB of surfactant (value); $X_2$ is the charge-inducing agent DCP molar ratio concentration; $X_3$ is the charge-inducing agent DCP molar ratio concentration; $X_1X_2$, $X_1X_3$, and $X_1X_4$ are the interaction terms between the factors; $X_1^2$, $X_2^2$, and $X_3^2$ are the quadratic terms of the factors; $Y_1$ is the mean vesicle size (nm); $Y_2$ is the zeta potential; $Y_3$ is the entrapment efficiency percentage; $Y_4$ is the percentage of IM initial release after 2 h; and $Y_5$ is the percentage of IM cumulative release after 24 h.

$^a$ Significant effect of factors on individual responses.
Figure 1. Standardized Pareto charts showing the effects of the investigated factors on all responses (Y1-Y5).

Figure 2. Estimated response surface plots for the effects of the investigated factors on all responses (Y1-Y5).
from 88.6% in F1 to 93.8% in F10, respectively. In addition, the increase in Y4 and Y5 from 9.2% in F2 to 18.9% in F12 and from 83.6% in F2 to 94.3% in F12, respectively, was along with the decrease of X2 from 1.5 to 0.5 molar ratio concentration at the same levels of other factors. The most reliable explanation for these results was owing to the cholesterol concentrations used in formulation. As cholesterol concentration increased, drug efflux from niosomes was significantly lowered, which is in compatibility with its stabilizing ability of niosomal membrane.\(^{58,83,86}\) Table 3 displayed results of the mathematical modeling of release mechanism of the prepared niosomes. The results indicated that the IM release from niosomal formulations followed first-order release which in a good agreement with the previous studies.\(^{83,87,88}\) In addition, kinetic treatment revealed that (n) values for the same formulations were confirmed a non-Fickian diffusion mechanism for the release of IM from the prepared niosomes which indicates that the release is controlled by a combination of diffusion and polymer relaxation or erosion.\(^{89,90}\)

**Mathematical Modeling and Statistical Analysis of the Experimental Data**

Values for the mean vesicle size of niosomes (Y1), zeta potential (Y2), entrapment efficiency (Y3), initial release percentage of IM after 2 h (Y4), and cumulative release percentage of IM after 24 h (Y5) were investigated, and mathematical model for each response was generated. For each response variable, multiple linear regression analysis results derived by the best-fit method are shown in Equations 2-6.

### Mean vesicle size (Y1)

\[
Y1 = 571.399 - 61.566X_1 + 33.296X_2 - 2232.72X_3 + 10.066X_1^2 - 44.723X_1X_2 + 45.333X_1X_3 + 190.195X_2^2 - 670.2X_2X_3 + 18262.0X_3^2
\]

### Zeta potential (Y2)

\[
Y2 = -9.078 + 17.157X_1 + 21.955X_2 + 34.103X_3 - 1.468X_1^2 + 3.077X_1X_2 - 5.128X_1X_3 - 24.333X_2^2 - 20.0X_2X_3 + 1066.67X_3^2
\]

### Entrapment efficiency (Y3)

\[
Y3 = 39.188 + 4.183X_1 + 26.196X_2 + 153.123X_3 + 0.085X_1^2 - 3.087X_1X_2 - 6.154X_1X_3 - 1.313X_2^2 + 28.0X_2X_3 - 701.333X_3^2
\]

### Initial release (Y4)

\[
Y4 = 12.053 + 3.293X_1 - 13.275X_2 + 57.827X_3 - 0.315X_1^2 + 1.026X_1X_2 + 6.154X_1X_3 - 0.342X_2^2 + 7.0X_2X_3 - 856.667X_3^2
\]

### Cumulative release (Y5)

\[
Y5 = 94.62 + 3.578X_1 - 3.308X_2 - 239.744X_3 - 0.401X_1^2 + 1.385X_1X_2 + 7.179X_1X_3 - 5.9X_2^2 - 8.0X_2X_3 + 1160.0X_3^2
\]

**Preparation of the Optimized IM-Loaded Niosomes**

Multiple response optimization helps to get an optimized IM-loaded niosomes formulation that attains our requirement in obtaining minimal vesicular size values, maximal zeta potential, entrapment capacity, and release after 2 h (initial) and release after 24 h (cumulative) values. The optimized final experimental parameters were deliberated and analyzed to compromise among different responses to attain an integration of factor levels which amplify the desirability function. The combination of factor levels which boost the desirability function over the study aim specified that X1, X2, and X3 optimum levels are 6.67, 0.7, and 0.06 molar concentrations, respectively. An optimized IM-loaded niosomes, with the aforementioned inspected responses optimum levels, was formulated and evaluated as described earlier. The observed values for Y1, Y2, Y3, Y4, and Y5 were found to be 425.36 nm, \(-62.4 \text{ mV}\), 82.96%, 18.93%, and 89.45%, respectively, whereas these responses predicted values were 437.311 nm, \(-61.37 \text{ mV}\), 79.7%, 18.45%, and 93.99%, respectively.

**Evaluation of the Optimized IM-Loaded Niosomes**

**Light and Electron Microscopy**

Vesicles formation and its morphological manifestations were visualized using photo microscopy. Figure 4 showed the morphology of the optimized formulation which demonstrated the oval and regular rounded vesicles. The magnifications used for transmission electron microscope were (×15000, 200 kV), whereas the magnifications used for SEM were ranging from \(\times20000\) to \(\times30000\), at an acceleration voltage of 20 kV. The photomicrographs showed generally the outline of the vesicles and, to some extent, the core of the vesicles. The electron micrographs of the optimized formulation demonstrated the oval and regular rounded vesicles at a range of 300-450 nm in diameter.
Differential Scanning Calorimetry

The thermograms (Fig. 5) revealed sharp melting endothermic peaks for niosomal individual components indicating their crystallinity. A definite endothermic peak representing gel-liquid transition temperature of Span 40 used for the preparation of the optimized niosomal formulation. An endothermic peak was noted in Span 40 thermal analysis at its melting point 50.57°C, whereas cholesterol and dicetyl phosphate showed also endothermic peaks at 147.76°C and 76.73°C, respectively. In addition, pure IM showed endothermic peaks at 97.55°C and 210.51°C. As observed in DSC thermograms of the optimized formulation, it showed that a decrease in the value of Span transition temperature as well as a distinct peak broadening of niosomal formulation, compared with Span in its pure form. Where the inclusion of cholesterol in niosomal preparation did not shift the transition temperature of Span to higher temperatures. In addition, DSC thermograms showed disappearance of the characteristic endothermal peak of IM at 210°C which may be owing to considerable interaction among IM and the bilayer components of niosomes which can explain the enhanced entrapment of IM into these formulations.30,60,91 All these findings confirm interactions among the components forming the niosomal bilayers that enhance entrapment of IM into this formulation.68

Effect of Storage on the Physical Stability of the Optimized IM-Loaded Niosomes

The optimized IM-loaded niosomes stability study was proceeded to investigate changes in vesicular size, its PDI, zeta potential, and percentage of IM retained in vesicles (as a suspension) during storage at temperature 4 ± 2°C for 3 months. It was found that the optimized formulation after 3 months possessed an increment in vesicular size along with its PDI with the decrease of zeta potential values which is an expected finding as a consequence of increasing the size according to Stoke’s law theory. The increase in size from 425.36 to 459.45 nm with minimal increase of PDI from 0.201 to 0.305 led to increase tendency for aggregation and settling of particles and so, by default, also led to decrease in zeta potential value.62 Although the change in zeta potential from −62.4 to −49.0 mV may lead to instability of vesicles, zeta value was high enough to render the suitable electrostatic repulsion that prevents the aggregation. These results were in accordance with previous stability studies on niosomes.44,85,93 Fortunately, it was noticed that there was no appreciable change in the drug-retained amount in niosomes (95.68%) of its initial amount at the end of 3 months. This finding is mostly due to the storage at low temperature that leads to low fluidity of the niosomal bilayers which decrease the tendency of drug leakage.10,59 Morphological changes examined by light microscope were observed in Figure 4 and revealed rounded shape of vesicles that remained unaffected with least aggregation. Finally, the t90% of optimized IM-loaded niosomes formulation was calculated to be 11.4 months which arises another proof of its high stability.

In Vitro Cytotoxicity Study of the Optimized IM-Loaded Niosomes

The results of MTT cell proliferation assay were presented in Figure 6 to study the cytotoxicity of the optimized IM-loaded niosomes on the viability of different cancer cell lines compared with pure IM by determination of their IC50. Results revealed that...
IC50 values of the optimized formulation in MCF-7, HCT-116, and HepG2 were 18 μg/mL, 6.5 μg/mL, and 22 μg/mL, respectively, whereas the IC50 values of pure IM in PBS pH 5.5 were 122 μg/mL, 107 μg/mL, and 161.5 μg/mL for the 3 cancer cell lines, respectively. Thus IM in niosomal formulation was superiorly more cytotoxic and effective than the pure form of drug. It was clear that the highest effect of free and niosomal IM was on HCT-116. This could be explained by the fact that IM has a higher efficacy against colorectal and colon cancer cells.21 On the contrary, there is a little effect on either breast cancer or hepatocellular cancer cell lines with serious side effects on the liver.17-19 Interestingly, while IC50 of free drug was almost the same on normal BHK cells, as a model of normal cells, the optimized formulation has higher IC50 (3, 4, and 11 folds) compared to MCF7, HCT-116, and HepG2 cancer cell lines; respectively. This indicates that the optimized formulation has higher selectivity profile and works preferentially against cancer cells. This may decrease the side effects and enhance the toxicity profile of IM. The IC50s were calculated using GraphPad Prism software and the means of 3 independent experiments were compared using student t-test for unpaired data using GraphPad Instat software version 3. A significance signs were placed on the Figure 6. The mean IC50s of free drug and IM-loaded niosomes were extremely significant at a level of probability ranging from 0.003 in HepG2 cells, 0.001 in MCF-7, and 0.0001 at HCT116. As a substantial and popular property of NCs, it can be concluded that the incorporation of IM in niosomal formulation significantly improved its interaction with the cancer cells. This type of cell-NPs interaction was generally accompanied with internalization through rapid nonspecific phagocytosis.68 In fact, NCs in general could propose a variety of benefits over free drugs, as improved absorption selectivity into a targeted tissue and protection from degradation. Interestingly, the intracellular nanomaterials may be selectively compartmentalized on autophagic sequestration. Autophagy is strongly involved in cancer therapies affirmative outcomes, and nanomaterials can efficiently interrupt the autophagic pathway.2 These results were in accordance with earlier studies on nanoparticles in general,45,57,64,90 and on niosomes in particular.63,68,94 This approach ensures the improvement in the efficacy of chemotherapeutic drugs and decreases their therapeutic dose that in turn minimizes their harmful effects and increases their safety margin.

**Hemolytic Activity of the Optimized IM-Loaded Niosomes**

Considering the percentage of hemolysis occurred by triton X as 100% hemolysis, the optimized formulation of IM-loaded niosomes has a little hemolysis effect on RBCs compared with those treated with Triton X-100 with the same concentration at the same temperature and pH. The lysis percent were 3.7 ± 0.2, 13 ± 0.05, 17.3 ± 1.5, 21 ± 2.2, 25 ± 5, and 39.2 ± 2.4 for 0, 6.25, 12.5, 25, 50, and 100 μg/mL, respectively, to be increased with increasing the concentration of the formulation.

**Conclusion**

NC system has potentially revolutionized cancer therapy and diagnosis. In this attempt, we have developed an optimized niosomes for their drug delivery potential using IM (anticancer drug) as model bioactive. Developed formulation was characterized and assessed for its vesicular size, zeta potential, entrapment efficiency, in vitro drug release profile, physical stability, and cytotoxicity

![Figure 6. Cytotoxicity profile of the optimized formula of IM-loaded niosomes compared to the free IM against MCF-7, HCT-116, HepG2, and BHK cell lines.](image-url)
(anticancer benefit). From the outcomes of our studies, it can be concluded that in general, the optimized IM-loaded niosomes showed minimum vesicular size, high negative surface charge, maximum entrapment efficiency, and sustained release profile. In addition, the results revealed that IM niosomal formulation exhibited superior cytotoxic efficacy compared to the pure form of the drug when examined against colon cancer cell line (HCT-116), breast cancer cell line (MCF-7), or hepatocellular carcinoma (HepG2). Moreover, the optimized formula showed a preferential cytotoxicity against cancer cells compared to normal cells and has little effect on the RBCs hemolysis. Full toxicological screening, detailed mechanisms of action, as well as in vivo therapeutic efficacy warrant further investigations which will be the forefront of our laboratory future directions.

References
