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Original Research Article

Preparation and optimization of glyceryl monooleate-low molecular weight chitosan nanoparticles for delivery of morpholinopyrrolizine derivative

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Abstract

Purpose: *To develop and optimize glyceryl monooleate–low-molecular-weight chitosan (GMO-LMWC) nanoparticles loaded with morpholinopyrrolizine derivative (NPM).*
Methods: *Molecular mechanics was used to determine the main driving force for the complexation between glyceryl monooleate (GMO) and chitosan. Nanoparticles were fabricated using a modified film-rehydration method. Optimization was carried out using a statistical design approach. The effects of formulation factors (concentrations of GMO, LMWC and Tween 80) on zeta potential and particle size were investigated using a 2^3 factorial design.*
Results: *A steady increase in binding energy was observed when chitosan length was increased from 22 to 142 Å, and thereafter it remained almost constant. The examined independent variables had significant effects on particle size and zeta potential. The particle size of the nanoparticles varied from 265 to 1270 nm while zeta potential was in the range of 3 – 12 mV. The optimized preparation showed a significantly low half-maximal inhibitory concentration (IC50) when compared to a free anticancer agent in the hepatocellular carcinoma (HePG-2) cell line.*
Conclusion: *A nanoparticulate system composed of GMO and LMWC is a potential nanocarrier for delivery of morpholinopyrrolizine derivative.*

Keywords: *Glyceryl monooleate, Chitosan, Pyrrolizine, Nanoparticles, Anticancer*

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INTRODUCTION

Several nanosystems such as nanoemulsions, polymeric nanoparticles, dendrimers, and lipid-based nanocarriers have been investigated as carriers of chemotherapeutics [1-3]. In this work, the nanotechnology approach was utilized to deliver a new chemotherapeutic agent, a pyrrolizine derivative [4]. This derivative had shown a promising anticancer activity on both HCT116 (colon) and HEPG-2 (liver) cancer cell lines [5]. It was shown that this compound had 88.4 % anti-epidermal growth factor receptor (EGFR) activity. The EGFR was over-expressed in many solid tumors and therefore monoclonal

antibodies and chemotherapeutic agents were synthesized to target these receptors [5].

The major shortcoming of morpholinopyrrolizine derivative (NPM) is its poor water solubility. Nano-carriers improve the bioavailability of many hydrophobic anticancer agents [6,7]. Their high surface area per unit volume improves solubility, dissolution and absorption of bioactive pharmaceutical ingredients. Further enhancement in bioavailability could be achieved by formulation of low solubility substances with lipids, which allows them to access the systemic circulation via the lymphatic route [7].

A nano-system composed of glycerylmonooleate (GMO) as a lipoidal material and chitosan as a hydrophilic polymer were chosen to encapsulate NPM. Gycerylmonooleate (GMO) forms a cubic phase in contact with an aqueous medium and has been investigated as an anticancer agent delivery system [8]. However, these nanocarriers are unstable at physiological pH [9]. Chitosan is a natural cationic polyamine polymer. It represents an advantageous excipient in drug delivery due to its biocompatibility, biodegradability, permeation enhancing effect and mucoadhesive properties [10]. The major drawbacks of chitosan are insolubility in alkaline medium and poor incorporation of hydrophobic drugs [11]. Therefore, in this study, the combination of GMO and chitosan were used to overcome their limitations and combine the advantages of each of them. Other researchers have prepared GMO–chitosan nanoparticles using the multiple emulsion method [12]. In this study, another method for fabrication of GMO-LMWC nanoparticles has been described. The objective of this work was to prepare GMO-LMWC nanostructures loaded with chemotherapeutic agent. The nature of the interaction between chitosan and GMO was studied at the molecular level to choose an optimum chitosan molecular weight for preparation of the nanoparticles.

EXPERIMENTAL

Materials

High molecular weight chitosan was purchased from Xiamen Xing, China, (molecular weight > 250 kilodaltons (KDa) and degree of deacetylation (DDA) of 95 %). Glycerylmonooleate (GMO) and Polysorbate 80 (Tween 80®) were obtained from Sigma-Aldrich (Lyon, France). The reagents: RPMI-1640 medium, MTT and DMSO were obtained from Sigma Chemical Co. (St. Louis, USA). Fetal bovine serum was purchased from GIBCO (UK). The hepatocellular carcinoma (HePG-2) human tumor cell line was purchased from ATCC via Holding Company for Biological Products and Vaccines (VACSERA), Cairo, Egypt.

Preparation of the pyrrolizine derivative

The synthesized anticancer active pyrrolizine derivative was prepared as reported previously [4]. The structure of the morpholinopyrrolizine derivative is depicted in Figure 1.

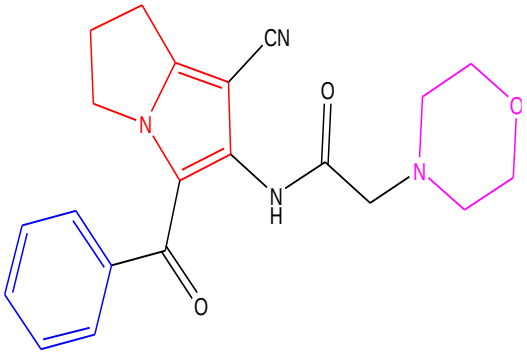

Figure 1: N-(3-Benzoyl-1-cyano-6,7-dihydro-5H-pyrrolizin-2-yl)-2-morpholin-4-yl-acetamide

Depolymerization of chitosan

Low-molecular-weight chitosan was prepared from high-molecular-weight chitosan using the method mentioned previously [13].

Computational calculation studies

Computations were performed with Hyperchem® (release 8.05) using the MM+ (atomic charges) force field implemented in Hyperchem. Partial atomic charges were obtained by performing Austin Method 1 (AM1) semi-empirical calculations. Energy minimizations were obtained using the conjugate gradient algorithm (0.01 kcal/mol/Å gradient). All the calculations were performed in a vacuum. The three-dimensional structures of the CS monomer and GMO (Figure 2) were built up with natural bond angles as defined in the Hyperchem software. Initially, the structures were minimized with MM+ (atomic charges) method, and then optimized at the HF-*ab initio* level using the 3-21G* basis set. This protocol has been used successfully in a previous publication to optimize the molecular structure of different compounds [14]. Polymer chains of different lengths were considered to estimate the influence of molecular weight (Mwt) on the interaction with GMO. The molecular weights of single polymeric chains were of 0.9, 1.8, 2.7, 3.6, 5.4, 9, 13.5, 18 and 36 kDa (calculated based on the molecular weight of the glucosamine monomer (~180 Da). All chitosan

amino groups were considered as completely deacetylated.

GMO-CS complex formation

GMO molecules were manually positioned close to (and in front of) the amino groups of the CS copolymer (1:1 ratio) and allowed to freely optimize, leading to a GMO-CS complex that was further optimized using the MM+ (atomic charge) force field (0.1 gradient). The optimized structure of GMO-CS is depicted in Figure 2.

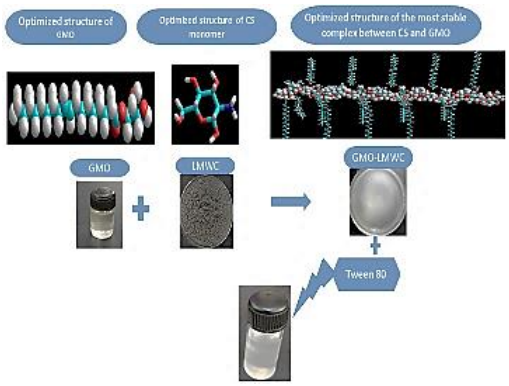

Figure 2: Images of LMWC, GMO-LMWC films and final preparation and molecular mechanics optimized structures of GMO, LMWC and GMO-LMWC

Preparation of GMO-LMWC nanostructure carriers

One milligram of morpholinopyrrolizine derivative was dissolved in 2 mL of (1:1 v/v) mixture of ethanol/chloroform. This solution was added to the melted GMO (at 40 °C) and mixed for 5 min. The LMWC was dissolved in water and then 2 mL of the solution was added slowly to the anticancer agent–GMO mixture. The mixture was stirred for 15 min using a magnetic stirrer, poured into a Petri dish and then left at room temperature for 48 h under forced air. A film was obtained after complete evaporation of the liquid. This film was hydrated with 10 mL of Tween 80® solution. The pH of the preparations was adjusted to 6.5. The obtained dispersion was homogenized for 10 min and used for further studies.

The final concentrations of chitosan and Tween 80® and the amount of GMO used for fabrication of 2^3 preparations are shown in Table 1.

Optimization of the preparation using statistical design

A factorial design (2^3) was used to screen the most important parameters in the formulation of the GMO-LMWC nanocarriers. Processing factors were kept constant throughout the experiment. Three formulation variables were studied (the amount of GMO, initial concentrations of Tween 80® and LMWC) at two levels. The independent factors together with their levels are depicted in Table 1 A and B. The dependent variables were particle size and zeta potential. Eight formulas were prepared and replicated to determine the experimental error. Sixteen experiments were conducted randomly as suggested by the software (Design-Expert® DX7 Stat-Ease, Inc. 2021 East Hennepin Ave, Suet 480 Minneapolis, MN 55413, Trial Version.). Table 1 and Table 2 show the composition of each preparation together with the order of run of the experiments. The main effects and interactions were calculated. Analysis of variance (ANOVA) was used to differentiate the important factors from those that were not. The optimum preparation was searched for using the same software.

Table 1: Composition of the 16 experiments of 2^3 factorial design

Factor	Name	Units	Low level	High level
A	GMO	Mg	40	100
B	LMWC	%	2	4
C	Tween 80	%	2	4

Determination of particle size and zeta potential

The particle size and zeta potential were analyzed using Zetasizer Nano ZS (Malvern Instruments, UK) at 25 °C. Samples were diluted (1 mL from sample + 9 mL water) prior to measurements. The average of three independent measurements was calculated.

Cytotoxicity

The MTT assay protocol was used to compare the *in vitro* cytotoxicity of empty nanoparticles, NPM-loaded nanoparticles and free anticancer agent as stated previously [14]. Briefly, 1 x 10^4 cells/mL were seeded in 96 wells for 48 h at 37 ^oC under 5 % CO_2. Blank nanoparticles, NPM-loaded nanoparticles (directly diluted with serum free medium), or free anticancer agent (dissolved in DMSO) were added to the cells at different serially diluted concentrations (6.25 – 100 µg/mL) and incubated for 24 h. Then, cells were washed and treated with MTT dye (5 mg/mL) and left for 4 h. After dissolving the purple formazan using DMSO, a plate reader (EXL 800, USA) was used to measure its absorbance at 570 nm. The anticancer agent concentration needed to inhibit the growth of 50 % of cells (IC_{50}) was calculated [15].

Table 2: Responses of the 16 experiments of 2^3 factorial design

Run order	Experiment Code	Factor A (mg)	Factor B (%)	Factor C (%)	Zeta Potential (mv)	Particle Size (nm)
1	1	40	2	2	7.73	415
13	BC	40	4	4	8.62	853
9	C	40	2	4	5.77	265
7	AB	100	4	2	5.44	1605
5	B	40	4	2	11.2	519
12	AC	100	2	4	3.33	505
11	AC	100	2	4	3.7	502
10	C	40	2	4	5.16	279
15	ABC	100	4	4	5.62	1262
8	AB	100	4	2	5.61	1609
14	BC	40	4	4	8.69	856
2	1	40	2	2	7.6	420
3	A	100	2	2	3.47	494
4	A	100	2	2	3.93	502
6	B	40	4	2	12.2	523
16	ABC	100	4	4	5.71	1270

Statistical analysis

Design-Expert® software was utilized to study the effect of formulation variables on quality attributes of nanoparticles and optimize the preparation. In addition, the cytotoxicity of the encapsulated anticancer agent was compared to a free anticancer agent and the IC_{50} values were calculated using MTT method. One-way ANOVA was performed with Excel 2013 software (Microsoft Corporation, USA).

RESULTS

GMO-CS interactions

The number of glucosamine units of polymers built with Hyperchem® is illustrated in Table 2. The binding energies between GMO and CS were evaluated using Eq 1.

$E_{binding} = E_{total} – (E_{CS} + E_{GMO})$ …… (1)

where E_{total} represents the energy of the GMO-CS complex, and ECS and E_{GMO} are the energies of chitosan and GMO, respectively. However, the average binding energies were estimated by dividing the evaluated binding energy ($E_{binding}$) by the number of glucosamine units as shown in Table 3. The primary result from Table 3 was that the higher the negative values of $E_{binding}$, the more thermodynamically favorable was the pathway of CS-GMO complex formation.

Statistical optimization of GMO-LMWC nanocarriers: *Model adequacy*

Analysis was performed without transformation as suggested by the Box-Cox plot for both responses (Design-Expert® DX7 software). The importance factors and the interactions were seen in the Pareto chart and half-normal plot and then verified using ANOVA analysis. As illustrated in Table 3, $R^2 > 0.99$ indicating linearity of the model. The “Predicted R-Square" was in reasonable agreement with the "Adjusted R-Square" (difference less than 0.2). Adequate precision measures the signal to noise ratio. The obtained ratios indicated an adequate signal. In addition, most of the residuals were fitted on the straight line, which supports the assumption of normality. These data suggest the model was adequate [16]. The final equations generated by the software for zeta potential and particle size are as shown in Eq 2 and 3.

Table 3: Binding energies of chitosan composed of different numbers of glucosamine units

Number of glucosamine unit	$E_{binding}$ kCal/mol	MM (kDa)	CS length (Å)
5	-10.2	0.9	22.2
10	-18.57	1.8	45.03
15	-21.18	2.7	75.6
20	-22.75	3.6	97.43
30	-24.8	5.4	141.7
50	-24.97	9	228
75	-25.09	13.5	337.4
100	-25.12	18	451.2
200	-25.13	36	898.5

Zeta potential = 6.42438 – (1.94687 x GMO) + (1.40562 x LMWC) – (0.599375 x Tween 80) – (0.400625 x GMO x LMWC) + (0.711875 x GMO x Tween 80) – (0.070625 x LMWC x Tween 80) + (0.140625 x GMO x LMWC x Tween 80) …… (2)

Particle size = 742.438 + (226.188 x GMO) + (319.688 x LMWC) – (18.4375 x Tween 80) +

(148.188 x GMO x LMWC) (65.4375 x GMO x Tween 80) + (16.5625 x LMWC x Tween 80) – (103.188 x GMO x LMWC x Tween 80) ……. (3)

Effect of independent variables on zeta potential

Zeta potential values varied from 3.33 to 12.2 mv (Table 1). All factors investigated had a significant effect on zeta potential. The most important factor that affect zeta potential was the amount of GMO (Factor A) followed by the percentage of LMWC (Factor B). The main effects and ANOVA analysis are displayed in Table 3. There were also significant AC and AB interactions.

Effect of independent variables on particle size

Particle size of the investigated preparations ranged from 265 to 1605 nm (Table 1). The particle size of nanoparticles was affected by the three studied independent variables. Two level interactions were also important (Table 4). The most important factor was LMWC, followed by GMO. When a low level of Tween 80 was combined with LMWC, the particle size increased only slightly as the percentage of LMWC was increased (Figure 3 A). In contrast, the presence of LMWC and a high level of Tween 80 led to a steady increase in particle size as illustrated in Figure 3 B.

Table 4: Analysis of variance results (main effects and *P*-values) for zeta potential and particle size responses

Source	Zeta potential main effects	*P*-value Prob > F
Model		< 0.0001
A-GMO	-3.77	< 0.0001
B-LMWC	2.8	< 0.0001
C-Tween 80	-1.3225	< 0.0001
AB	-0.8125	0.0017
AC	1.3	< 0.0001
BC	-0.13	0.5000
Residual	0.2925	

Optimization of the nanocarrier system

The optimum preparation was searched for using the Design Expert® software. The criteria for selection was to maximize the zeta potential and minimize the particle size. As the amount of Tween 80 increased, the number of solutions decreased as shown in overlay plots (Figure 4). Therefore, the percentage of Tween 80 was set at the lower level. The software generated 18 solutions; three of them with a desirability greater than 0.6. A preparation composed of 40 mg GMO, 4 % LMWC and 2 % Tween 80 was selected for further studies. This optimum preparation had a zeta potential of 11.7 mv and a particle size equal to 521 nm. The optimum formula was prepared, and its zeta potential and particle size were comparable to the software solution as shown in Figure 5.

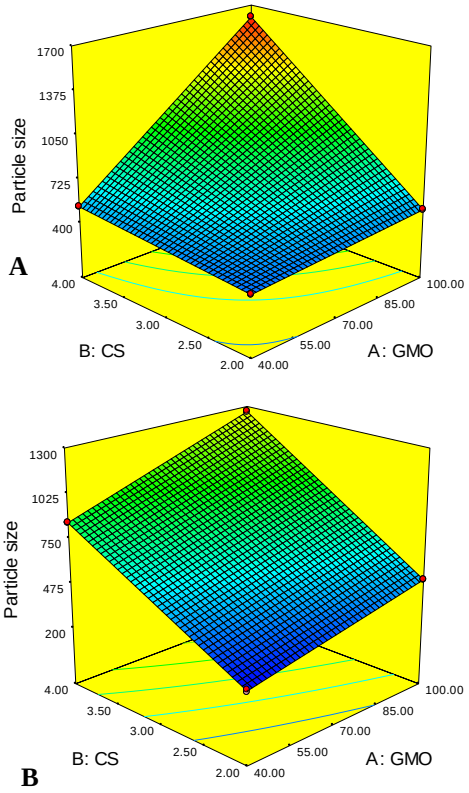

Figure 3: Response surface plots of the effect of Tween 80 on particle size of GMO-LMWC nanoparticles. (A) Low level of Tween 80 (2 %) (B) High level of Tween 80 (4 %)

Cytotoxicity of GMO-LMWC nanocarriers

The responses increased as the dose increased from 6.25 to 100 µg/mL as depicted in Figure 6. Cell viabilities were significantly lower ($p < 0.05$) for NPM-loaded nanoparticles compared to free drug at 6.25 – 25 µg/mL (Figure 6). The IC_{50} values for the free anticancer agent and the NPM-loaded nanoparticles were 13.19 and 9.62 nM/mL, respectively.

The percentage viabilities of blank nanoparticles were above 90 %, indicating that the cytotoxicity of the NMP-loaded nanoparticles was not due to their surface charge or their composition.

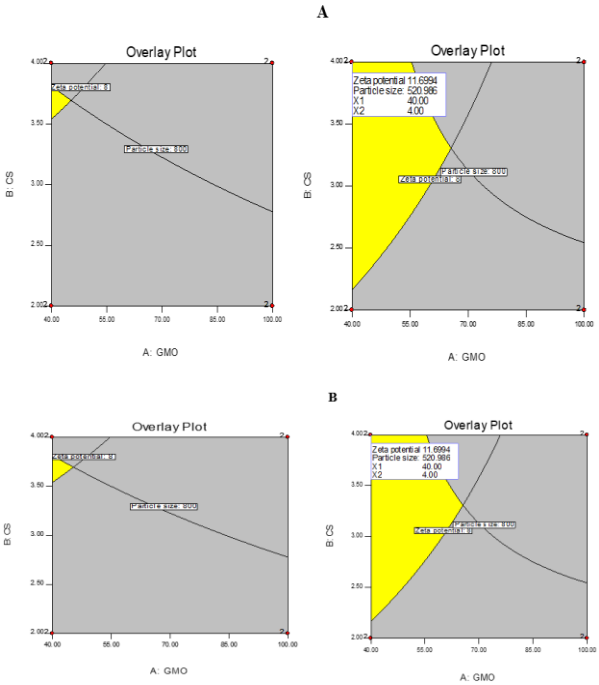

Figure 4: Overlay plots for optimum solutions obtained by the software at two levels of Tween 80. (A) Low level of Tween 80, (B) High level of Tween 80. Best solution: zeta potential = 11.5 mv, particle size = 521 nm obtained when amount of GMO = 40 mg and concentrations of LMWC and Tween 80 were 4 and 2 % w/v, respectively

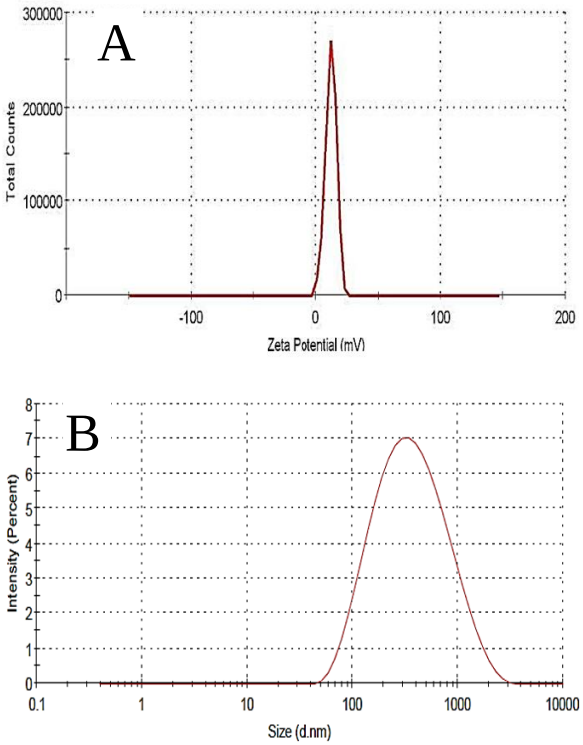

Figure 5: Zeta potential and particle size of the optimum preparation. A: zeta potential; B: Particle size distribution by intensity

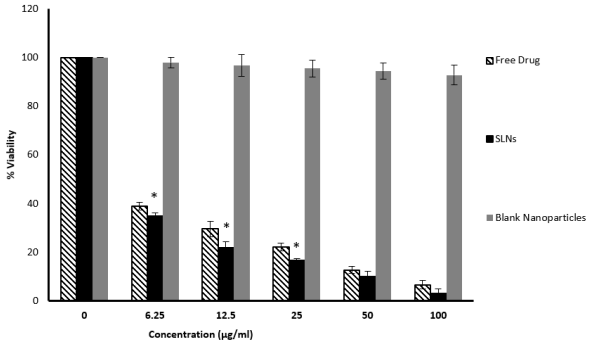

Figure 6: Cytotoxic effect of blank nanoparticles, free and SLNs-loaded chemotherapeutic agent in HePG2 cell-lines. Results are expressed as mean ± SD, *p < 0.05

DISCUSSION

Nanocarriers based on modification of GMO nanostructures by LMWC were prepared. In the optimized complex structure chitosan chains surrounded GMO molecules. The lipid core allowed encapsulation of the hydrophobic anticancer agent, while the chitosan shell might provide intimate contact between the nanoparticles and the mucosal membranes and consequently enhanced permeability and retention at the tumor site.

To choose an optimum chitosan (CS) molecular weight, molecular mechanics was explored to gain understanding of the nature of the GMO-CS interaction process at the molecular level. The analyses of the evaluated binding energies revealed a well-defined correlation between the CS binding capacity and its length. The average concerted binding energies indicated that the GMO-CS binding energy increases with increasing CS length (22 Å (0.9 kDa) to about 142 Å (5.4 kDa)), after which it leveled off. In summary, the net negative binding energy in these complexes was substantial and disclosed the possible formation of a 1:1 molecular complex between CS and GMO. The main driving force for intermolecular attractions was van der Waals and columbic interactions. This did not rule out other interactions, such as hydrogen bonding between the glyceride group of GMO and chitosan or electrostatic attractions between positively charged chitosan and negatively charged GMO. Low molecular weight chitosan (M. wt 3.57 ± 0.52 KDa) was prepared and used for production of Nano systems.

Nanocarriers were prepared by the modified film rehydration method to maximize the interactions between GMO and LMWC and to remove organic solvents, which was used to solubilize

NPM. A previous study demonstrated formation of a nanocomposite film of chitosan and rosehip seed oil emulsion [17]. The emulsion/casting method was used for production of GMO-LMWC film. Low molecular weight chitosan has good emulsifying properties compared to the high molecular weight candidate [17]. Glycerylmonooleate has a hydrophilic-lipophilic balance (HLB) of 3.8 and it has been used as a lipophilic emulsifying agent in many applications. A continuous film was produced from an emulsion obtained by mixing GMO and LMWC, whereas LMWC film showed signs of cracking. This is because chitosan polymer exists in a glassy state at room temperature; its glass transition temperature (Tg) value ranges between 140 and 150 ^{o}C as reported elsewhere [18]. In contrast, the GMO-LMWC emulsion formed smooth film which was attributed to increased flexibility of the polymer by GMO. The film was rehydrated using aqueous solution of Tween 80 and homogenized to produce the nanoparticles.

Statistical design was carried out to identify the effect of formulation variable on two important nanocarriers' parameters which are zeta potential and particle size. As the concentration of chitosan increases from a low to a high level, there was a concomitant increase in zeta potential as evidenced by the positive sign of its main effect. This is to be expected since LMWC possesses a positive charge due to ionization of its amino groups. The matrix lipid used also affects the value of the zeta potential. Glycerylmonooleate (GMO) is a neutral lipid, and it was expected that it would have no influence on zeta potential. However, it had a significant negative effect (−3.77), which was ascribed to ionization of fatty acids released from the hydrolysis of GMO [19]. Tween 80 is a non-ionic surfactant, i.e., it bears no charge. However, it had a negative effect on zeta potential, i.e., it reduced the zeta potential of the nanoparticles. At low and high levels of Tween 80, the maximum zeta potentials obtained were 11.8 and 8.5 mv, respectively. This effect might be attributed to the adsorption of the surfactant on the surface of the nanoparticles, resulting in shielding of their charge. The nanoparticles could be stabilized by both electrostatic and steric stabilization due to presence of charge and adsorption of the non-ionic surfactant, Tween 80.

The main effect of LMWC was the increase of particle size (the sign of the effect is positive) as it goes from a low to a high level. This result agrees well with another study where liposome sizes increased with the increase of chitosan concentration [20]. The increment in size of the colloidal particles was attributed to the presence of the chitosan layer on their surfaces and to aggregation of the nanovesicles because of screening of liposome surface charge. Glycerylmonooleate also increased the particle size of the carriers. In contrary, the Tween 80 main effect had a negative sign, which means that it reduced the size of the particles as its level was increased. This is to be expected since it decreased the interfacial tension between the lipid and the aqueous phase and stabilized the system. However, its effect was not so large compared to the effect of LMWC and GMO. It seems that the Tween 80 modest reduction effect was overwhelmed by LMWC tendency to increase the particle size.

A hepatocellular carcinoma (HePG-2) human tumor cell line was used in this study to compare cell viability between the compound as a raw material and its formulated form. Loading the anticancer agent in nanoparticles increased the *in vitro* cytotoxicity significantly The nanosize and mucoadhesive properties of chitosan might be the reason for this increment in activity. However, the increase in activity was modest, which could be attributed to the size of the nanoparticles (> 500 nm). It has been demonstrated that the cellular uptake of nanoparticles is affected by particle size, where it was greater for particle sizes of 100 – 200 nm, when compared to particles greater than 500 nm [20,21]. In this study, processing variables were not investigated; a future study will optimize these variables to obtain the optimum particle size.

CONCLUSION

Nanostructures composed of GMO and LMWC were prepared using a modified film rehydration method. The binding energy between CS and GMO is substantial and indicates the possible formation of a 1:1 molecular complex. All formulation variables investigated using 2^3 factorial design had a significant effect on particle size and zeta potential. The optimized preparation has a 1.4-fold lower IC_{50} than the free drugs in HePG-2 cell line. Thus, nanoparticles prepared from LMWC and GMO are a potential carrier for the delivery of morpholinopyrrolizine derivative for cancer therapy.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Amani Elsayed designed the study, methodology, and wrote the original manuscript. Amani Belal synthesized the anticancer agent and revised the manuscript. Khadoun Al-Sou'od performed computational study and revised the manuscript.

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