Endocytic pathways of optimized resveratrol cubosomes capturing into human hepatoma cells

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ABSTRACT

Resveratrol (RSV) is a natural polyphenolic compound with high affinity to hepatocytes. It has numerous benefits as anticancer, antioxidant, immunomodulatory and cardioprotective actions. Nevertheless, RSV therapeutic applications are hindered by its low solubility, light sensitivity and extensive first-pass metabolism. Cubosomes are collooidally stable dispersed liquid crystalline nanoparticles. The incorporation of RSV into cubosomes could overcome some of its physicochemical limitations. A Design-Expert® software was applied to optimize cubosomes in terms of particle size and encapsulation efficiency (EE%). The used model proved its suitability in predicting optimum cubosomal size. The prepared cubosomes showed an enhanced HepG2 cytotoxicity except at particle size of ~20 nm. Different endocytic pathways mechanisms as macropinocytosis, clathrin-mediated endocytosis and caveolae-mediated endocytosis were identified in the cellular uptake of RSV cubosomes depending on particle size. Caveolae-mediated transport was shown to have a significant effect on RSV cubosomes internalization efficiency and cytotoxicity.

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1. Introduction

The successful ability of nanocarriers’ applications in cancer therapy necessitates efficient cellular internalization. Hence, understanding nanosystem-cell interactions and the mechanism of intracellular trafficking is crucial [1]. In general, endocytic cellular uptake is governed by two types of competitive energy that affect the rate and amount of nanoparticles internalization. The first one is the binding energy between ligands and receptors. This energy denotes the interaction and the diffusion between receptors and their ligands. The second force is the thermodynamic driving force. This force represents the amount of free energy essential to internalize nanocarriers inside the cells [2]. Obviously, paracellular and transcellular transport are the two main pathways for nanocarriers’ uptake [3]. Paracellular transport is a passive diffusion mechanism via the intercellular spaces and tight junctions. Previous studies reported that negatively charged particles have no influence on disturbing the tight junctions [4]. Consequently, negatively charged nanosystems could be implemented in transcellular endocytic pathway only. Transcellular endocytosis includes macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis or caveolae- and clathrin-independent endocytosis [5]. These different endocytic pathways differ in the size it could internalize and the fate of the penetrated nanocarrier [6]. Generally, the relevant applications of nanocarriers are closely related to their size, shape, and surface properties which with the membrane dynamics would affect the cellular uptake [7]. Particle size could be considered one of the major factors that affect cellular uptake mechanism and biodistribution of nanocarriers [8]. Nanocarriers with size less than 200 nm have superior extravasation and accumulation into tumors by the enhanced permeability and retention effect [9]. Nanosystems rigidity seems also to influence the cell entry pathway; rigid nanoparticles are more susceptible to phagosomes formation, macropinocytosis is more involved in soft nanocarriers uptake, clathrin-mediated mechanism for stiff nanoparticles, and finally multiples mechanisms are implicated for nanosystems with intermediate elasticity [10].

Among various nanocarrier systems, cubosomes have gained a great interest either in drug and/or vaccine delivery [11]. Cubosomes are collooidally dispersed nanostructured liquid crystalline nanoparticles characterized by a three-dimensional cubic inner structure [12]. They are lipid bilayer-based particulates built
on self-assembled systems disseminated in an aqueous environment. Cubosomes are composed of biodegradable and biocompatible lipids as glycerol monooleate (monoolein), mixtures of soy phosphatidylcholine and glycerol diolate which can be dispersed in water by the use of stabilizers and/or surfactants as TPGS (D-alpha-tocopheryl poly(ethylene glycol) 1000 succinate), Tween 80 or Pluronics. By virtue of their structure, they offer more appealing characteristics than liposomes such high stability and mechanical rigidity [13].

Resveratrol (RSV) is a polyphenolic compound naturally exists in grapes and peanuts which exhibits antioxidant, immunomodulatory and cardioprotective actions. RSV is supposed to constrain different tumorigenesis stages; initiation, promotion and progression [14]. Previous reports had demonstrated the high affinity of RSV for hepatocytes [15,16]. Unfortunately, RSV suffers from low aqueous solubility, extensive first pass metabolism, and isomerization to the inactive cis-isomer by light exposure [17]. Drug encapsulation in nanocarriers could avert these challenges where the bioavailability will depend on the nanosystem rather than physicochemical properties of the drug [3]. Due to its immense pharmaceutical uses, RSV had been extensively studied in various nanoformulations. Previous studies had demonstrated the incorporation of RSV in different nanocarriers as polymeric nanoparticles, micelles, liposomes and solid lipid nanoparticles. These nanocarriers afford numerous benefits as improved aqueous solubility, controlled drug release, improved stability and bioavailability as well as enhanced cytotoxicity [18–20].

Hence, the aim of this study was the tuning of RSV cubosomes characters to improve RSV cellular uptake and cytotoxicity against HepG2 human hepatoma cells. This study also focused on tracking the different endocytic pathways that RSV cubosomes could be captured into cells.

2. Materials and methods

2.1. Materials

Resveratrol (RSV) was obtained from Xi’an Sonwu Biotech Co., Ltd., China; glycerol monooleate (GMO), poloxamer 407 (P407), RPMI-1640 medium, heat-inactivated fetal bovine serum (FBS), penicillin, streptomycin, L-glutamine, sulforhodamine B (SRB), Tris buffer, trypsin-EDTA, amiloride, chlorpromazine, nystatin and glacial acetic acid from Sigma–Aldrich Company, St. Louis U.S.A; acetonitrile, ethanol and methanol (HPLC grade) from Riedel-de HaenGmbh, Germany.

2.2. Experimental design

A three-factor, each at three-level, Box-Behnken design was exploited to statistically optimize the variables for the preparation of RSV nanocubic vesicles using Design Expert® software (Version 9.0.6.2, Stat-Ease Inc. Minneapolis, MN, USA). The influence of three independent variables namely: glycerol monooleate/poloxamer 407 (GMO/P407) ratio (X1), homogenization speed (X2) and homogenization time (X3) on the dependent variables, particle size and entrapment efficiency (EE%) were assessed. The influence of different factors on the characters of RSV cubosomes was investigated to obtain RSV with particle size range of 20–100 nm and maximum EE%. All experiments were performed in triplicate to validate the final predicted results compared to the experiments [21]. The optimized formulations were selected depending on the calculated desirability and were then prepared in triplicate to check the validity of the evolved statistical model before being subjected to further investigations. The different levels of the independent factors used in the experiment design and the dependent responses were clarified in Table 1.

2.3. Preparation of RSV cubosomes

Briefly, GMO and P407 (5%w/w) were molten in a water bath at 70 °C. RSV was accurately weighed and added to the molten mixture and vortexed to ensure thorough mixing. The mixture was added gradually into deionized water (95%/w/v) at 60 °C [22]. The obtained dispersion was subjected to different homogenization speed and time using high-speed homogenizer (Unidrive X1000D Homogenizer, CAT Scientific, USA). The concentration of RSV in the prepared cubosomes was kept 1%/w/v for all formulations. The prepared nanocubic vesicles were allowed to cool gradually to room temperature and kept in screw-capped amber glass vials for further investigations.

2.4. Characterization of the prepared RSV cubosomes

2.4.1. Particle size and zeta potential measurements

Particle size, polydispersity index (PDI) and zeta potential of the prepared RSV cubosomes were measured by photon correlation spectroscopy using Zetasizer (Malvern Instruments Ltd., UK). The measurements were determined at 25 °C using an angle of 90°.

2.4.2. Determination of entrapment efficiency

The entrapment efficiency percent (EE%) of RSV in the prepared cubosomes was determined by centrifugation at 2400g for 3h at 4 °C using Ultra Centrifuge (Jouan, France). The pellets were dissolved with ethanol and RSV content was determined spectrophotometrically at 306 nm (UV–vis double beam spectrophotometer Shimadzu 2450, Japan). An empty cubosomes were used as a blank. EE% was calculated according to the following equation [23]:

\[ EE = \frac{\text{amount of entrapped RSV}}{\text{Total amount of RSV added}} \times 100 \]  

(1)

2.4.3. Transmission electron microscopy (TEM)

The morphology of the optimized RSV cubosome was examined using TEM (JEOL, JEM-HR-2100, Japan). The cubosome was dried on a carbon coated copper 300-mesh grid and was stained with 1% phosphotungstic acid before the examination.

2.4.4. In vitro RSV release study

The in vitro release of RSV from the optimized cubosomes was assessed, in the dark, using dialysis method [24]. An accurately weighed RSV cubosomes (0.5 g) were placed into presoaked...
Table 2
The observed responses in three-factor each at three-level Box-Behnken design for the formulation of RSV loaded cubosomes.

<table>
<thead>
<tr>
<th>Run</th>
<th>X1: GMO: P407 ratio</th>
<th>X2: Homogenization Speed (rpm)</th>
<th>X3: Homogenization Time (min)</th>
<th>Y1: Particle size (nm)</th>
<th>Y2: Entrapment Efficiency (%)</th>
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dialysis bag (Spectra/Por® HDI cut off MWCO12–14 kD, flat width 10 mm and diameter 6.5 mm, USA). The dialysis bag was attached to the shaft of the USP dissolution testing apparatus type I (Hanson Research Corporation, USA) containing 150 mL PBS (pH 7.4). The shaft was rotated at 50 rpm ± 0.1 at 37 °C ± 0.5 and the experiment was conducted for 30 h. At predetermined time intervals, an aliquot of 1 mL was withdrawn and replaced with the same volume of fresh dissolution medium. Samples were diluted with ethanol in a ratio of 1:1 (v:v) and quantified for drug content by spectrophotometric analysis at 306 nm [24]. An empty cubosomes were used as a blank.

2.4.5. Stability studies

2.4.5.1. Thermal stability. Thermal stability of the optimized RSV cubosomes was conducted in dark at 4 and 25 °C for 28 days. At day 7, 14, 21 and 28 samples were visually inspected and entrapment efficiency, particle size, zeta potential were determined [25].

2.4.5.2. Freeze-They stability. The optimized RSV cubosomes were subjected to three repeated cycles of freezing at −80 °C for two days followed by storage at 25 °C for another two days. Samples then were analyzed in terms of entrapment efficiency, particle size and zeta potential [25].

2.4.6. In vitro cytotoxicity assessment of the optimized RSV cubosomes

In vitro antitumor activities of the both the empty and optimized RSV nanocubics vesicles as well as RSV solution in PBS (pH 7.4) were assessed on human hepatoma HepG2 cell line. The cells were grown as monolayers in RPMI-1640 medium containing 100 units/mL of penicillin, 100 μg/mL of streptomycin and 1% L-glutamine. The medium was supplemented with 10% heat-inactivated FBS at 37 °C under a humidified atmosphere containing 5% CO2 (Thermo Scientific Forma™, Germany). The grown cells were seeded in 96-well microtitre plates at a concentration of 104 well and incubated for 24 h at 37 °C with 5% CO2. Afterward, cells were incubated with serial concentrations of cubosomes in RSV range of 0.78–25 μg/mL at 37 °C for 24 h. The cytotoxicity was measured using sulforhodamine B (SRB) assay. Briefly, cells were fixed with 10% w/v trichloroacetic acid for 1 h at 4 °C then stained with 0.4% w/v SRB for 15 min. The formed dye complex was solubilized with 10 mM unbuffered Tris-HCl. The optical density (OD) of each well was assessed spectrophotometrically at 540 nm using a plate reader (ChroMate-4300, FL, USA). The absorbance of control untreated cells was considered as 100% proliferation. The 50% inhibitory concentration (IC50) was assessed graphically [26].

2.4.7. Evaluation of transport across HepG2

The cellular uptake of nanoparticles is an energy-dependent process highly affected by time and concentration. Therefore, the effect of the optimized cubosomes in different RSV concentrations and incubation time on the intracellular uptake efficiency would be evaluated as follow:

2.4.7.1. Effect of time. The exponentially grown HepG2 cells were incubated with 100 μL (containing 20 μg/mL RSV) of different cubosomes for 0.5, 1, 2 or 4 h. Cells were washed three times with ice-cold PBS to remove un吞 cubosomes. Cells were trypsinized and lysed using 0.1% sodium dodecyl sulfate, 0.1 M sodium hydroxide and 2% sodium carbonate [27]. The cell homogenate was centrifuged at 1530g for 10 min at 4 °C. The obtained suspension was mixed with an equal volume of acetonitrile and centrifuged at 860g for 30 min. The clear supernatant was used for drug quantification. RSV concentration was assayed using HPLC (Agilent 1100, Germany) equipped with G 1311 A quaternary pump and UV detector (WWD-G1314 A). A reverse phase column (ACE 5-C18, 250 × 4.6 mm, 5 μm) was used at 25 °C. RSV was eluted with a mobile phase consisted of methanol: water (containing 0.05% acetic acid) in ratio 52:48 (v:v) at a flow rate 1 mL/min. The UV detector was set at 310 nm [24].

2.4.7.2. Effect of concentration. The seeded HepG2 cells were incubated with 100 μL of different concentrations of the prepared cubosomes (1, 5, 10 and 20 μg/mL RSV) for 4 h [1]. Cells were washed three times with ice-cold, trypsinized and lysed by the same procedures as mentioned above. The RSV concentration was assayed using HPLC as previously described.

2.4.7.3. Effect of energy variation. The grown HepG2 cells were incubated at 4 °C for 2 h [3]. Subsequently, cells were incubated with 100 μL of the prepared RSV cubosomes (containing 20 μg/mL RSV) for 4 h. Cells were treated as previously mentioned to determine RSV cellular concentration.

2.4.8. Tracking pathway of cellular uptake

The influence of different endocytic inhibitors on the cellular uptake was assessed. Briefly, the seeded HepG2 cells were incubated separately with chlorpromazine hydrochloride (5 μg/mL) as an inhibitor of clathrin-mediated endocytosis, nystatin
(50 μg/mL) as caveolae-mediated endocytosis inhibitor and amiloride (13.3 μg/mL) as macropinocytosis inhibitor for 1 h at 37 °C [1].) Afterward, cells were treated with 100 μL of different RSV cubosomes (containing 20 μg/mL RSV) for 4 h and RSV cellular concentration was determined as previously described.

2.5. Statistical analysis

Results were expressed as the mean of three replicates ± standard deviation (SD). For comparing different parameters between groups, one-way analysis of variance (ANOVA) followed by Tukey HSD test was conducted using SPSS 18 (Chicago, U.S.A.). The differences were statistically significant at a probability level (p) less than 0.05.

3. Results and discussion

For the response surface methodology using Box-Behnken design experiment, a total of 17 runs were implemented to investigate the influence of GMO: P407 ratio, homogenization speed and time on RSV cubosomes particle size and EE%. Suitable statistical models for RSV cubosomes optimization were chosen based on the adjusted R², predicted R² and the lowest predicted residual sum of squares (PRESS) [28]. Table 2 shows the 17 experiment runs with three independent variables each at three levels and the obtained particle size and EE% as responses. The 2FI and quadratic models were selected as the statistical models describe the effect of different RSV cubosomes formulation on particle size and EE% respectively since they had the least PRESS values (Table 3).

3.1. Effect of formulation variables on the particle size (Y1) of the prepared RSV cubosomes

Nanocarrier size is one of the most factors affecting cellular uptake and biodistribution. The mean particle size of the prepared RSV cubosomes ranged from 22 ± 1.26 to 195 ± 2.99 nm (Table 2). The polydispersity index PDI of all formulations was <0.2 which indicated the narrow homogenous particle size distribution of the prepared RSV cubosomes [29]. Fig. 1 illustrates the response surface for the influence of different formulation variables on particle size. Equation (2) shows the effect of the different variable on RSV cubosomes particle size:

\[
\text{Particle size} = +110.00 + 3.67 \times X1 - 57.25 \times X2 - 29.00 \times X3 - 32.83 \times X1^2 - 14.83 \times X2 \times X3
\]

Where X1 is GMO: P407 ratio, X2 is homogenization speed and X3 is homogenization time.

The ANOVA results revealed the insignificant effect of GMO: P407 ratio on particle size in the tested concentrations (p > 0.05). Furthermore, the negative sign of X2 and X3 coefficients indicated a significant inverse relation between homogenization speed and time and particle size (p < 0.05). High homogenization speed and time could amplify mechanical and hydraulic shear that break cubic gel structure into nanocubic vesicles with smaller particle size [30,31].

3.2. Effect of formulation variables on the EE% (Y2) of the prepared RSV cubosomes

The influence of different formulation variables on EE% could be depicted from Table 2 and Fig. 2 according to the following equation:

\[
\text{Entrapment efficiency} = +90.24 - 0.80 \times X1 - 0.62 \times X2 + 2.26 \times X3 + 0.75 \times X1 \times X2 + 0.64 \times X1 \times X3 + 1.43 \times X1^2 + 0.91 \times X2^2 + 2.85 \times X3^2
\]

Where X1 is GMO: P407 ratio, X2 is homogenization speed and X3 is homogenization time.

Negative signs of both GMO: P407 and homogenization speed indicate an antagonistic effect on EE%. At high GMO: P407 ratio, the surfactant amount is not sufficient to solubilize high amount of RSV into the lipid vesicles [32]. Moreover, increasing homogenization speed decreases the contact between RSV and GMO. On the contrary, a direct relation between homogenization time and EE% could be observed. As by increasing contact time between drug and cubosome components, EE% could be improved.

3.3. Optimization of RSV cubosomes

To verify the developed models, five formulations, selected according to high desirability, were prepared as a checkpoint. Table 4 shows the composition, predicted and experimental responses. The linear correlation plots between experimental and predicted values for both responses showed high R² (0.998 and 0.979 for particle size and EE% respectively). Hence, the model was suitable for studying and predicting the most suitable conditions for the fabrication of RSV negatively charged cubosomes of size range 20–100 nm. Thus 5 formulations were subjected for further characterization.

3.4. Zeta potential of the optimized RSV cubosomes

The selected RSV cubosomes had zeta potential values ranged from −17.32 ± 2.71 to −32.12 ± 1.41 mV (Table 4). Where the cubosomes with the highest GMO ratio (highest fatty acid content) having the highest charge. The negative charge was possibly imparted by the free fatty acids present in the lipid [33].

3.5. Transmission electron microscope

The TEM image of F2 is depicted in Fig. 3A. Scattered nanocubic vesicles with a diameter range of 40–50 nm in agreement with the droplets size results determined by DLS. Spherical globules appearing in the field might be molten cubosomes under the high-energy beam of the TEM [30]. Moreover, P407 that did not incorporate into the cubosomal matrix could participate in the formation of mixed micelles [23].

3.6. In vitro RSV release

Fig. 3B illustrates release profile of RSV from different cubosomes over 30 h. By inspecting release profiles, an obvious effect of GMO: P407 ratio could be discernible. Cubosomes are considered as a biphasic burst release delivery system with

<table>
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<th>Table 3</th>
<th>Results of regression analysis for responses Y1 (particle size) and Y2 (EE%).</th>
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<td>Response</td>
<td>Model</td>
</tr>
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<td>Particle size</td>
<td>2FI</td>
</tr>
<tr>
<td>EE%</td>
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</tbody>
</table>
diffusion release mechanism from the cubic-phase matrix [34].

The prepared formulae showed a biphasic release pattern with an initial burst release for 6h. This might be attributed to the inadequately adsorbed RSV on the cubicosome surface. The second sustained phase which prolonged for 30h could be due to the architecture of cubicosome matrix that assists as a rate controlling membrane, hampered RSV release [34]. Furthermore, Nanocubic vesicles may exist in different types according to P407 concentrations. At low P407 concentration, cubic particles have Pn3m (cD) forms where most of the P407 stick to the cubicosome surface. On the contrary, P407 incorporated in the cubic bulk matrix by increasing its concentration and cubicosomes exists in Im3m (cP) patterns. It is to be noticed that cubicosomes with cD structure have superior drug release efficiency than cP cubicosomes [35]. Moreover, at the same GMO: P407 ratio, the increase in particle size decrease particles surface area in contact with dissolution medium and therefore caused a restriction in dissolution efficiency. Concerning the kinetics of RSV release from the optimized cubicosomes, the highest R² was >0.99 obtained from diffusion model indicating a controlled diffusion release mechanism. The release of RSV occurred via multiple consecutive steps where the drug is dissolved within the matrix of the cubicosomes then RSV diffuses the system via lipid/water interface [36].

3.7 Stability studies of RSV cubicosomes

All the prepared RSV cubicosomes reserved its original appearance with no signs of separation or precipitation. No significant

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**Table 4**

<table>
<thead>
<tr>
<th>Formula code</th>
<th>GMO: P407 ratio</th>
<th>Homogenization speed (rpm)</th>
<th>Homogenization time (min)</th>
<th>Particle size (nm)</th>
<th>EE%</th>
<th>Zeta potential (mV) ± SD</th>
<th>IC50 (µg/mL)</th>
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Fig. 3. TEM micrograph of RSV cubosome (A), in vitro resveratrol release from different cubosomes (B). Data are the mean of three determinations ± SD.

Fig. 4. The effect of freeze-thaw cycles and storage at: 4 °C (A) and 25 °C (B) on entrapment efficiency, 4 °C (C) and 25 °C (D) on particle size, 4 °C (E) and 25 °C (F) on zeta potential. Results are the mean of three ± SD.
difference in entrapment efficiency, particle size or zeta potential could be observed after storage at either 4 or 25 °C up to 28 days (Fig. 4). Moreover, RSV cubosomes exhibited no significant change in entrapment efficiency, particle size or zeta potential after freeze-thaw stability study as evidence by Fig. 4.

3.8. In vitro cytotoxicity of the optimized RSV cubosomes

Fig. 5A illustrates high cell viabilities more than 80% with all the tested empty cubosomes indicating that plain formulations had no cytotoxicity [37]. The deterioration in HepG2 human hepatoma cells viability incubated with both RSV cubosomes and solution demonstrated a rapid decline in viability of cells in a concentration-dependent manner (Fig. 5B) [38]. It was obvious that the calculated IC50 were also particle size dependent, with the superior antitumor activity of all RSV cubosomes over solution (IC50 14.5 µg/mL) except in formulation F3 with the size ≈ 28.61 ± 1.51 nm Table 4. Moreover, cubosomes with a particle size of 45–50 nm showed superior cytotoxic activity over other formulations. Therefore, the particle size could affect the in vitro cytotoxicity of RSV cubosomes.

3.9. Factors affecting RSV cubosomes internalization efficiency

Cellular recognition of the fabricated nanocarriers has been reported to be mainly size and surface chemistry dependent. It was previously mentioned that the cellular uptake of negative nanocarriers occurs mainly via endocytosis [4]. In addition, the rate of endocytic uptake of nanoparticles with size less than 200 nm is strongly size dependent. Accordingly, the prepared RSV cubosomes internalization will be evaluated in terms of time, concentration and energy.

In the present study, the cellular uptake of RSV cubosomes by HepG2 was found to be increased after 4 h in a size dependent manner. Longer incubation time was accompanied by better nanocarriers’ uptake (Fig. 6A) [39,40]. Similarly, the increase in concentration from 1 to 20 µg/mL also went along with the increase in cellular uptake by 12–17 fold especially in the case of F2 and F5 (Fig. 6B). Finally, incubation of HepG2 cells at 4 °C significantly (p < 0.05) decreased the cellular concentration of RSV (Fig. 6C). At low temperature, enzymes activity could be deteriorated and the efficiency of energy production was altered hence inadequacy of cellular energy for uptake. Accordingly, the nanocarriers’ cellular uptake was an active energy-dependent process. The energy was produced by the mitochondrial aerobic oxidation and glycolysis in the cytoplasm [41].

3.10. Tracking pathway of cellular uptake

Cellular internalization is assumed to occur by the formation of a protein coat vesicle on the cytoplasmic outer side of the cell membrane [42]. Amiloride is a macropinosis inhibitor as it inhibits Na+/H+ exchange in the plasmatic membrane essential for macropinosis [43]. When added to the cells it inhibited the uptake of F1 (97.65 nm) significantly by 27% (p < 0.05). Chlorpromazine hydrochloride hinders of clathrin-mediated endocytosis by promoting clathrin agglutination in late endosomes obstructs the endocytosis sag [44]. Uptake of the formulations F2, F3, F4 and F5 (28–76 nm) had significantly declined by 20–30% in the presence of chlorpromazine hydrochloride (p < 0.05) (Fig. 6D). Finally, nystatin constrains caveolea-mediated endocytosis by inhibiting caveolea-mediated endocytosis due to cholesterol segregation in the plasma membrane hinders sag vesicles formation [43]. Accordingly, the treatment of cells with nystatin prior to incubation with different RSV cubosomes resulted in a significant diminish in RSV uptake in cubosomes F2, F4 and F5 (p < 0.05) (Fig. 6D). It is to notice that these three cubosomes had a particle size in the range 45–76 nm.

Cellular uptake mechanism was clear to be a particle size dependent process. Cellular penetration of F1 with a high particle size (97.65 ± 4.50 nm) was mainly followed micropinosis only, endocytosis was abolished for particles with size more than 80 nm [2], F3 with the least evaluated size (≈28 nm), was infiltrated into cells by clathrin-mediated endocytosis mechanism only and had the least efficacy. Particles with size less than 40 nm are unable to produce sufficient free energy to enwrap to the cellular membrane surface which may avert endocytosis [2]. Finally, formulations F2, F4 and F5 with size range 45–76 nm were internalized by both clathrin- and caveolea-mediated endocytosis. The dual uptake pathways contributed with cubosomes in the size range 45–76 nm could explain their superior cytotoxicity. In addition, formulations F2 and F5 with 45–50 nm in diameter showed lower IC50 than F4 (76 nm). These results are in good agreement with previous results which showed that nanocarriers with size ≈ 45 nm had the highest cellular internalization rate [9].

Correlating the cellular uptake with the trafficking results revealed a poor cell penetration in case of F1 and F3 cubosomes where only one cellular uptake mechanism, either macropinosis or clathrin-mediated endocytosis, was involved in the internalization process. In macropinosis process, actin which is the most abundant cellular protein initiates the folding of membrane projections with the plasma membrane and constructing macropinosomes. Unfortunately, macropinosomes vesicles could route the captured nanocarriers either to lysosomes or
Moreover, mediated by 80 parameters. Consequently, caveolae both allocate drugs into nanocarriers, which could facilitate the internalization of endocytosis inhibitors. Therefore, nanocarriers are susceptible to lysosomal degradation after their internalization by the aforementioned mechanisms.

However, F2, F4 and F5 cubosomes, which were internalized by both clathrin- and caveolae-mediated endocytosis, have got improved cellular uptake results. It was previously reported that caveolae are membrane curvature with diameter range from 60 to 80 nm with 10–50 nm neck protrusion structure [46]. Therefore, the efficiency of nanocarriers’ uptake with a particle size smaller than caveolae diameter is superior over larger particles [47]. Albeit caveolae have the capability to expand their neck to uptake larger nanocarriers, the internalization efficiency is diminutive [47]. Moreover, caveolae dissociate from the plasma membrane and allocate their content to caveosomes during the caveolae-mediated endocytosis. Caveosomes circumvents lysosomes and consequently protect its nanocargo from lysosomal degradation [48,49]. Consequently, the fabrication of nanosystems that can deliver drugs into the cells by exploiting caveolae-mediated endocytosis could have tremendous potential in drug delivery.

4. Conclusion

Concisely, cubosomes provide numerous benefits as ease of preparation, low cost, no need for organic solvent and improve RSV solubility. It could be inferred that tailoring the different process parameters could optimize RSV cubosomes to improve its efficacy. The proposed cubosomes were able to control in vitro RSV release for 30 h. Efficient cytotoxicity and cellular uptake of RSV cubosomes were attained with a particle size range 45–76 nm which was additionally internalized by caveolae-mediated endocytosis. Caveolae were found to improve RSV cubosomes internalization efficiency and cytotoxicity as it could avert lysosomal attack. The internalization of RSV with caveolae-mediated endocytosis could help in achieving higher cellular concentration and achieve better chemotherapeutic outcomes. Understanding the cellular internalization mechanism is a crucial phase in the optimization of cellular drug targeting strategies. A finding which should be dealt with in depth.

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References


