Formulation, Characterization, and Evaluation of Doxorubicin-loaded Cubosome as a Cytotoxic Potentiator against HCT-116 Colorectal Cancer Cells

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ABSTRACT

Background: Colorectal cancer (CRC) has grown to be the world’s fourth-biggest cause of cancer-related mortality. It is critical to identify more effective treatment options for it. Objectives: Doxorubicin a potent antineoplastic agent is widely used but has severe adverse effects. Therefore, our objective is to use cubosome as an efficient drug delivery system to reduce the off-target effects and increase the cytotoxic effects in CRC cells. Materials and Methods: Pluronic F127-Based Cubosomes were prepared by low energy stirring method and loaded with doxorubicin. Physicochemical characteristics were studied using X-ray diffraction, dynamic light scattering, microscopic techniques, FTIR UV spectrophotometer, and entrapment efficiency. \textit{In vitro} activity of doxorubicin-loaded cubosomes was studied on HCT-116 cells. Results: The prepared cubosomes have the required nano-size and the FTIR results showed the presence of both doxorubicin and Pluronic F-127. The entrapment efficiency of doxorubicin was high too. DOX-CBs have a better cytotoxic effect and induced ROS and reduced NO levels in HCT-116 cells. Conclusion: The obtained results show that doxorubicin-loaded cubosomes can be used to increase the cytotoxic effect in CRC cells and can be used with other chemotherapeutic agents.

Keywords: Doxorubicin, HCT-116 cells, Pluronic F127, \textit{in vitro} activity, Cubosomes, Entrapment efficiency.

INTRODUCTION

Colorectal cancer (CRC) results in approximately 800,000 deaths each year with more than one million cases newly diagnosed each year.\textsuperscript{1,2} In spite of the progress made in the treatment of CRC, mortality remains high and several CRC patients suffer from recurrence and metastasis.\textsuperscript{3} Major treatment options include chemotherapy and surgery.

Doxorubicin (DOX) is an antineoplastic agent commonly used in the treatment of several cancers. It intercalates with the DNA and inhibits a key enzyme, topoisomerase II resulting in the death of cancer cells.\textsuperscript{4,5} Doxorubicin and other chemotherapeutic agents such as mitomycin, and cisplatin are usually the first-line treatment and are also given after surgery to kill residual cancer cells and obstruct the proliferation of CRC cells.\textsuperscript{6-8} Although doxorubicin is very effective, its therapeutic ability is hindered by systemic toxicity specifically DOX-induced cardiomyopathy and nephrotoxicity.\textsuperscript{8,9} Therefore, methods to increase the efficacy and uptake of DOX in malignant cells and decrease the toxicity associated with noncancerous cells are required.

Drug delivery approaches using Nano-systems are often preferred because of their possibility to improve drug efficacy.\textsuperscript{10} Cubosomes are lyotropic liquid crystalline (LLC) nanoparticles that are...
thermodynamically stable and provide a possibility of sustained drug release.\textsuperscript{11} Since cubosomes viscosity is low and they mostly are in suspension, it makes it easier to deliver drugs to targeted sites.\textsuperscript{12,13} As cubosomes have been proven to be an efficient drug delivery system and have been utilized to deliver anticancer medications,\textsuperscript{14,15} we formulated a DOX-loaded cubosome nanoparticle (DOX-CB) stabilized by Pluronic F127 aimed to enhance the therapeutic window and maximize the anticancer effect of DOX. The formulated DOX-CB was characterized using UV-Spectroscopy, FTIR, XRD, DLS, TEM, and FESEM with EDAX, and DOX-CB was used on CRC cell line - HCT 116 and tested for cytotoxicity, Nitric oxide, and Reactive oxygen species. Morphometric analysis was also carried out to check the effect of formulated cubosomes.

MATERIALS AND METHODS

Pluronic F127-Based Cubosomes preparation

Pluronic-F127 cubosomes and the weight ratio of ethanol were prepared by the low-energy stirring method reported by Alexandridis \textit{et al}.\textsuperscript{16} with slight modifications. 30–70\% wt/wt pluronic-F127 was mixed with ethanol to make the final volume of 100\% wt/wt. The solution was stirred continuously until a homogeneous mixture was obtained. This mixture was left at room temperature for 72 hr to equilibrate, after that the mixture was centrifuged for 30 min at 15,000 rpm at a temperature of –15°C. Finally, the cubosome NPs were obtained.

Formulation of Doxorubicin loaded Pluronic-F127 cubosomes

Doxorubicin-loaded Pluronic-F127 cubosomes were prepared by the protocol reported by Esposito \textit{et al}. and Yakaew \textit{et al}.,\textsuperscript{17,18} with minimal modifications. 5\% wt/wt of Doxorubicin loaded 30–70\% wt/wt Pluronic F127 was weighed and blended with the ethanol (1:100\% wt/wt). The mixture was stirred continuously until a homogeneous mixture was obtained and allowed to reach equilibration for 72 hr to develop the cubic phases fully. Next, the reaction mixture was centrifuged at 15,000 rpm for 15 min at a –15°C. Finally, DOX-loaded cubosome NPs were obtained.

Characterization of Formulated Doxorubicin Cubosome (DOX-CB)

X-ray Diffraction

DOX-CBs were characterized by an X-ray diffractometer (model: X’PERT PRO PANalytical). The diffraction patterns were recorded in the range of 25°–80° for the Doxorubicin-loaded (pluronic-F127)-based cubosomes, where the monochromatic wavelength of 1.54 Å was used. The spectrum was taken at a pressure using an ultra-high vacuum with Al Kz excitation at 250 W. The average crystal size of DOX-CBs was calculated using the Debye-Scherrer equation:

\[ D = \left( \frac{0.941 \lambda}{\beta \cos \theta} \right). \]

Particle Size Measurement using Dynamic Light Scattering (DLS)

Dynamic Light Scattering (DLS) Nano Particle Sizer was, used for the particle size analysis. DOX-CB was diluted 25-fold and more so that multiple scattering in the cuvette can be prevented. The measurements were performed using the Malvern Panalytical instrument at 25°C.

Field Emission SEM with EDAX

FESEM was performed on the DOX-CBs using a SUPRA: CARL ZEISS 55 microscope operating at 30 kV. The microscope was equipped with a charge-coupled device (CCD) camera. The samples were prepared by using 1mg of Cubosome sample loaded with 1.2 nm gold particle separation on a carbon tape using a low vacuum. Energy-dispersive X-ray spectroscopy was done using an EDAX (model: ULTRA 55) with SUPRA: CARL ZEISS 55 Field Emission scanning electron microscope operated at 30 kV. Dry powdered samples were attached to the substrate using double-sided carbon tape and mounted onto the sample holder.

Transmission Electron Microscope

Morphological analysis of DOX-CB was performed by TEM (FEI Tecnai G2 20 S-TWIN model, United States). A drop of cubosome suspension was placed on the copper grid and a filter paper was used to remove excess fluid. 1\% sodium phosphotungstate solution was used to stain the sample. After the stained sample was dried, it was analyzed using TEM.

Fourier Transformer Infrared Spectroscopy

The physical state of DOX-CB was identified by using FTIR (Perkin-Elmer spectrometer). The sample was compressed, and potassium bromide discs were prepared. IR spectra of DOX-CB were screened in the region 450–4000 cm\textsuperscript{-1}.

Optical Density Measurements and UV- spectroscopy

The absorption spectra of Doxorubicin-loaded (pluronic-F127)-based cubosomes were studied in the
range between 200 and 800 nm by the Jasco UV-730 spectrophotometer.

**Entrapment Efficiency**

To entrapment efficiency of DOX-CBs, the methods previously reported by Cytryniak et al. and Kazi et al. with minimal modifications. Free DOX from DOX-CBs were obtained by centrifugation for 30 min at 12,000 rpm. The supernatant was diluted using PBS (pH 7.4) and free DOX concentration was calculated using a UV-Vis spectrophotometer at 485 nm. The entrapment/encapsulation efficiency (EE) was calculated using the following formula:

$$EE = \frac{C_{(DOX\ in\ CB)} - C_{(Free\ DOX)}}{C_{(DOX\ in\ CB)}} \times 100$$

Where $C_{(DOX-CB)}$ is the total concentration of doxorubicin in DOX-CBs and $C_{(Free\ DOX)}$ is the DOX concentration obtained in the supernatant after centrifugation.

**Cell Line**

Human colorectal cancer cell line HCT 116 was cultured in cultured using DMEM with 10% heat-inactivated FBS and 1% Penicillin-Streptomycin. Cells were incubated in 5% CO$_2$ environment and once they reached 80-90% confluency, it was passaged. Cells cultured at 3rd - 6th passages were mostly used for the study.

**Morphological Analysis**

To assess and visualize the morphological changes of cells because of blank cubosomes, DOX, and DOX-CBs. HCT 116 cells with a seed density of $2.5 \times 10^5$/well were grown in a 6-well plate at 37°C for a day. Different concentrations of blank cubosomes, DOX, and DOX-CBs were added to the cells and after 24 hr of incubation, the morphological changes were observed using a phase-contrast inverted microscope.

**Cytotoxicity Assay**

MTT assay was performed by the method reported by Sylvester et al. to assess the viability of cells. Yellow tetrazole dye is reduced by viable cells to form an insoluble purple formazan product. The absorbance of the reduced product is read at 595 nm. This assay was performed to assess the effects of blank cubosomes, DOX, and DOX-CBs on HCT 116 colorectal cancer cell viability. HCT 116 cells were seeded in a 96-well plate with a seed density of 12,000 cells/well. Blank cubosomes, DOX, and DOX-CBs were dissolved in distilled water separately and subsequently added to the wells at 10 different concentrations (1-10 μg/ml). After 48 hr of incubation of HCT 116 cells, the culture media was discarded and 10 μl MTT solution (0.5 mg/mL) was added to each well. The cells were incubated again for 4 hr in the dark at 37°C. After the 4-hr incubation, the MTT solution was discarded and 1% DMSO (0.1 ml) was used to dissolve the MTT metabolite, Formazan crystal. The plates were maintained at room temperature for 10 min and using a microplate reader the absorbance was measured at 490 nm and 630 nm. The viability was expressed in %. The cellular viability of the cells in the control group was considered to be 100%. The rate of inhibition was calculated by the equation:

$$Inhibition\ % = 1 - \frac{Absorbance\ at\ 490\ nm - Absorbance\ at\ 630\ nm\ (treated)}{Absorbance\ at\ 490\ nm - Absorbance\ at\ 630\ nm\ (control)} \times 100$$

**Reactive Oxygen Species Measurement**

Colorimetric Nitro blue tetrazolium reduction assay performed by Choi et al. was followed with minimal modifications. About 80,000 HCT 116 cells were seeded in a 96-well plate and incubated at 28°C for 24 hr. Once they reached 80-90% confluency blank DOX-CBs were added at various concentrations and further incubated for 48 hr, the media was discarded and given a wash with PBS twice. 100 µl of 0.1% NBT was added to the wells in the dark and was incubated at 28°C for an hour then washed with methanol. After the wash, 120 µl of KOH (2M) and 120 µl of DMSO were added to the well and the absorbance was determined at 620 nm.

**Nitric Oxide Measurement**

To estimate nitric oxide measurement, the modified experimental method of Wahyuni et al. was employed. Griess reagent A (2% sulfanilamide) and Griess reagent B (0.2% N-(1-Naphthyl) ethylenediamine) were prepared using 5% H$_3$PO$_4$. The Griess reagents (A&B) were mixed with an equal volume of the media in the 96 wells. This mixture was mixed by shaking the 96-well plate and incubated in dark for 5 min. Once the color is developed the absorbance was read at 540 nm.

**RESULTS**

**Formulation and Characterizations of DOX-CBs**

Cubosomes with different ratios of Pluronic-F127 were prepared with different concentrations to reach the desired criteria. The crystal size detection revealed that formulated DOX-CBs were in the nano-size range. The average size of DOX-CBs was 27.42 nm (Figure 1). The DLS spectra of synthesized doxorubicin-loaded cubosome NPs measured 133.20 nm (Figure 2). Moreover, because the Doxorubicin-loaded cubosome NPs were...
surrounded by an aqueous medium, the DLS particle size resulted in significant changes in the size of cubosomes relative to the XRD and TEM measurements known as the hydrodynamic size.

DOX-CBs were also analyzed in FESEM, and the results are illustrated in Figure 3a. DOX-CBs have a spherical structure with uniform grain boundaries and an average particle size of 35.54 nm, which is relatively similar to the XRD results. Again, the EDAX spectrum was used to determine the chemical composition of doxorubicin-loaded cubosome NPs. As shown in Figure 3b, the chemical elements present in the doxorubicin-loaded cubosome NPs were Cl, C, O, and N molecules.

The surface topography of DOX-CBs was investigated by a TEM image, which is shown in Figure 4. TEM images indicate that the DOX-CBs exhibit a spherical structure (core Pluronic F-127 and shell doxorubicin formation). Furthermore, the results show that the spherical formation (Pluronic F-127 is a cubic structure) is covered with doxorubicin (it is marked with a TEM image). These results indicate that the Pluronic F-127 phases are due to both the steric effects and the intermolecular hydrogen bonds between the doxorubicin-loaded cubosome matrixes.

**UV- spectroscopy and FTIR**

DOX-CBs were dispersed into water by the ultrasonication method employed using the UV-visible spectrum. As determined by quasi-elastic light scattering (QELS), the absorbance edge of the cubosome NPs was 317 nm.23 The UV spectrum of the hydrated lipid and doxorubicin absorption peak is at 279 nm.24 This is illustrated in Figure 5.

The FTIR spectrum of doxorubicin-loaded cubosome NPs is shown in Figure 6. These results confirmed that the Doxorubicin and Pluronic-F127 (cubosome) various functional groups were doxorubicin-loaded cubosome NPs. The typical doxorubicin characteristics peaks were observed at 3453 cm⁻¹ for N–H stretch and O–H stretch, 1747 cm⁻¹ for C=O stretch (carbonyl group), and 1655 cm⁻¹ for C=C ring stretch, and 845 cm⁻¹ for C=H bending vibration. The cubosome (Pluronic-F127) signals associated with the functional groups present in these polymer peaks were found at 2971 cm⁻¹ for asymmetric and 2890 cm⁻¹ for symmetric stretching, 1339 cm⁻¹ for O–H bend, 1290 and 1247 cm⁻¹ for C–O–C stretches, 1111 cm⁻¹ for C–O–C stretching, respectively.
Entrapment Efficiency

The total concentration of DOX encapsulated in the cubosomes compared to the free DOX revealed an encapsulation efficiency of 87.61%.

Cytotoxicity and Morphological Analysis

The cytotoxic activity of empty cubosome, DOX, and DOX-CBs on CRC cells was established by MTT assay. Different doses of DOX and DOX-CBs (1-10 μg/ml) were added to the cells. Higher doses showed higher cytotoxicity. The IC₅₀ value of DOX-CBs was 6 mg/ml whereas, for free DOX, it was 9 mg/ml indicating that DOX-CBs have a better cytotoxic effect than DOX itself as shown in Figure 7. The morphological analysis obtained corroborates the aforementioned results. The exposure of HCT-116 cells to DOX-CBs provokes morphological changes that include membrane blebbing and morphological shape distortion (Figure 8).

ROS Estimation

High levels of ROS can lead to cellular death activation. ROS levels were measured after DOX-CBs were added to HCT-116 cells and incubated for a day. Based on the IC₅₀ values obtained, ROS was estimated for 3 different concentrations of DOX-CBs. A significant elevation in the ROS levels was seen in the highest concentration (8 μg/ml) of DOX-CBs (191.41 ± 0.43) compared to the cells in the control group (100%). The ROS levels for 4 and 6 μg/ml of DOX-CBs were 107.92 ± 1.29 and 121.06 ± 0.45% respectively as illustrated in Figure 9. This experiment was carried out in triplicates and the values are expressed in mean ± SD.
The nitric oxide levels in the HCT-116 cells were measured for 3 different concentrations of DOX-CBs. A dose-dependent suppression was seen when the HCT-116 cells were supplemented with DOX-CBs compared to the control group (22.49 ± 1.18). The mean values of NO levels were 17.08 ± 1.17, 15.20 ± 0.88, and 12.49 ± 0.58 μMol for doses 4, 6, and 8 μg/ml as depicted in Figure 10. This experiment was carried in triplicates and the values are expressed in mean ± SD.

DISCUSSION

Doxorubicin has been a widely used chemotherapeutic drug against several malignant neoplasias. Through the decades, DOX has proven to slow disease progression. The adverse effects of DOX usage are severe. So, the development of various drug delivery methods can counteract the problem by decreasing the off-target toxicity and increasing the efficacy of DOX in cancer cells are needed. Different systems of DOX drug delivery has been developed in the past decade to overcome the adverse effects. The major advantage offered by cubosome-based drug delivery is the controlled and targeted release of drugs. Because of its advantages, it has been widely used for delivering many cancer drugs. The current research work aimed to develop a cubosome based delivery system. Pluronic F127-based cubosomes were developed. Pluronic F127 is a surfactant polyl used as a stabilizer.

The DOX-loaded cubosomes were characterized. The formulated cubosomes had a larger surface area that has various advantages such as enhancing drug concentration, higher drug payload, and avoiding any leakage of drugs before it reaches the intended site of action. The FTIR spectrum results confirmed that doxorubicin has successfully interacted with cubosome (Pluronic F127) of the doxorubicin-loaded cubosome surface matrix. These interactions are due to the electrostatic interaction between the doxorubicin with Pluronic-F127 molecules. The characteristic curve for doxorubicin was observed at 3453 cm⁻¹ which was similar to the results obtained in previous works of literature, and the FTIR spectra obtained for Pluronic-F127 were relatively similar to those obtained in other literature. Despite the potential of nanocarriers in therapeutics, their applications are restricted by their interactions with the mononuclear phagocyte system (MPS) which leads to quick clearance. This issue can be counteracted if the size is within 200 nm, and the formulated DOX-CBs fall under that category. DOX Entrapment efficiency in DOX-CBs was high 87.61%. This attributes to the nature of the drug and its solubility in the lipid bilayers. The results are in accordance with previous studies. The cytotoxicity of DOX, DOX-CBs, and empty cubosomes was tested on HCT-116 cells. The cytotoxic activity of empty cubosomes was very minimal and it was only observed with higher doses. So, empty cubosomes are biocompatible at selected doses. The IC₅₀ values of DOX-CBs were higher than empty DOX suggesting that DOX-CB has enhanced therapeutic potential than DOX. This is significant as it indicates the intensification of cytotoxic action of DOX even at very low concentrations whilst decreasing the risk of undesired effect off-target. The morphological analysis corroborates the cytotoxicity effects of DOX-CBs. The epithelial morphology of HCT-116 cells is clearly visible in the control group but as we increase the dose the membrane is disrupted, and the adherence is lost.
Reactive Oxygen Species play contradictory roles in cancer and its progression. ROS can increase the expression of protumorigenic signals, and aid in the survival, proliferation, and adaption of cancer cells in a hypoxic environment. Contrariwise, ROS has the ability to promote signals from antitumorigenic and promote oxidative stress that leads to the senescence and death of cancer cells. ROS generation was high at an 8 μg/ml concentration of DOX-CBs. This result proves that DOX-CBs can act as a chemotherapeutic agent and also be a supplement to existing colon cancer therapies that target ROS induction that directly promotes the death of CRC cells.

Nitric oxide is a free radical and water-soluble gas. Just like reactive oxygen species, the role of nitric acid in cancer is controversial. It can act as a tumoricidal molecule as well as a protumorigenic molecule, but it is understood that this dichotomous role is based on multiple factors such as the location of the tumor, the concentration of nitric oxide, and the timing. The results obtained show treating CRC cells with DOX-CBs suppressed NO levels. NO also upregulates matric metalloproteases and the expression of angiogenic factors that promotes angiogenesis in cancer cells. Previous studies on colorectal cancer cells treated with doxorubicin have shown a reduction in NO levels. The exact mechanism of how DOX suppress NO production is not known and this can be further analyzed.

CONCLUSION

In summary, the current work evaluated the cytotoxic effect of cubosomes loaded with doxorubicin and free doxorubicin. The results showed cubosomes loaded with doxorubicin have better antiproliferative activity than free doxorubicin on the HCT-116 colorectal cancer cell line in vitro. The study also showed the ability of DOX-CBs to elevate ROS and reduced NO levels leading to suppression of CRC cells proliferation. Although the exact mechanism is not known, this study opens the field for further investigation of DOX-CBs as a potent anticancer drug delivery system.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Almoshari, et al.: Doxorubicin-loaded Cubosomes Promotes Better Cytotoxicity than Free Doxorubicin


Colorectal cancer (CRC) is the third leading cause of cancer death globally. Doxorubicin is a potent anticancer drug and it is widely used to treat various types of cancer including colorectal cancer. The remedial effects of doxorubicin are obstructed by its adverse effects. Drug delivery approaches such as cubosomes can reduce off-target toxicity and increase the cytotoxicity in the tumour cells. Therefore we formulated a doxorubicin-loaded cubosome and tested its effects compared to free doxorubicin on HCT-116 cells. Doxorubicin-loaded cubosome had an increased cytotoxic effect compared to free doxorubicin. An increase in reactive oxygen species was also seen. Increased ROS can directly promote the death of CRC cells. A reduction in Nitric Oxidase levels was also seen. Our findings pave the way for further research into DOX-CBs as a strong anti-cancer agent.

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