Enhanced cytotoxic activity of beta carotene conjugated liposomes towards breast cancer cell line: comparative studies with cyclophosphamide

Article *in* Anti-Cancer Drugs · November 2021

DOI: 10.1097/CAD.0000000000001245

Nourhan S. Elkholy
Nawah Scientific Company

Gehan Safwat
University of Cambridge

Medhat W Shafaa
Helwan University

Enhanced cytotoxic activity of beta carotene conjugated liposomes towards breast cancer cell line: comparative studies with cyclophosphamide

Gina R. Mahrous[a], Nourhan S. Elkholy[b], Gehan Safwat[a] and Medhat W. Shafaa[b]

This work aims to evaluate cyclophosphamide (Cyclo) cytotoxic efficacy combined with liposomes in the presence or absence of beta carotene (beta) by detecting the effects of these compounds on the breast cancer cell line (MCF-7) DNA damage. The IC50 value for beta in cytotoxic assay with MCF-7 treated cells was 21.15 μg/ml, while with liposomal beta (LipoBeta) being 121 μg/ml. The free Cyclo IC50 value was 719.86 μg/ml, its liposomal form (LipoCyclo) was 172 μg/ml. The results indicated that in contrast with Cyclo and control values, all comet assay parameters for the LipoBeta were significantly increased ($P < 0.05$). In MCF-7 cells treated with beta, the findings show a higher intensity of comet tail than those treated with LipoBeta. The presence of several double-strand breaks suggests this high intensity relative to the head. The molecular combination between Cyclo and liposomes in the presence or absence of beta was characterized. Dynamic light scattering measurements confirmed the mono-dispersity of all samples. The incorporation of Cyclo or beta into liposomes exhibited a slight shift to higher temperature compared to the main peak of empty liposomes that exists at 101.5°C which creates a conformational disorder within the phospholipids. The FTIR study showed structural alterations in vesicles after liposome encapsulation. *Anti-Cancer Drugs* XXX: 000–000 Copyright © 2021 Wolters Kluwer Health, Inc. All rights reserved.

Anti-Cancer Drugs XXX, **XXX**:000–000

Keywords: beta carotene, cyclophosphamide, cytotoxicity, DSC, FTIR, liposomes

[a]Faculty of Biotechnology, October University for Modern Science and Arts, 6th October and [b]Medical Biophysics Division, Physics Department, Faculty of Science, Helwan University, Cairo, Egypt

Correspondence to Nourhan S. Elkholy, MSc, Medical Biophysics Division, Physics Department, Faculty of Science, Helwan University, Ain Helwan, P.O. 11795, Cairo, Egypt
Tel: +002 01061782583; e-mail: phy.nsayed@gmail.com

Received 21 July 2021 **Revised form accepted** 4 August 2021

Introduction

Cancer is characterized by the spread and uncontrolled growth of abnormal cells. It may result in death if the spread is not managed. In the USA, it is known to be the second most common cause of death, surpassed only by heart disease, and accounts for almost one in four deaths. Surgery, radiation, chemotherapy, hormonal therapy, immune therapy and targeted therapy are included in cancer treatments [1].

Managing and treating cancer cases involves usually using antineoplastic agents. These agents are toxic to rapidly proliferating cells and destroy neoplastic tissue as a result. However, they can also disrupt proliferating normal cells due to their low therapeutic index. Long-term use of antineoplastic agents is, therefore, a compromise with many destructive and unnecessary effects, and they are therefore a subject of growing concern [2]. Monitoring the mutagenic ability of anticancer agents may help reduce the immediate harmful effects on genetic materials and also to create another cancer in patients undergoing chemotherapy [3].

Breast cancer ranks second among women (after lung cancer) in the mortality rate. About 231 840 new cases of breast cancer and 40 730 deaths from breast cancer (40 290 women, 440 men) are reported worldwide in 2019. According to the WHO, it is estimated that 27 million new cases of breast cancer and 17.5 million deaths from breast cancer will occur annually by 2050 [4].

Cyclophosphamide (Cyclo), is also a well-known mutagen and clastogen [5]. It is an alkylating antineoplastic agent, creating the highly active carbonium ion that interacts with nucleic acids and proteins, which are extremely rich in electrons. In vivo, it also causes the development of free radicals or reactive oxygen species. Free radicals due to their high chemical reactivity induce cellular damage in several ways [3]. The most damaging effects of free radicals include damage to DNA, which can lead to a variety of pathological conditions, including cancer [6].

Beta-carotene is one of several naturally occurring compounds called carotenoids. Carotenoids are hydrophobic pigments prevalent in bacteria, plants and animal tissues [7,8]. There are about 50 of the 600 naturally occurring carotenoids present in the nutritional chain. As antioxidants and as prevention agents for cancer, cardiovascular, or skin disorders, dietary carotenoids play a significant role in vision [9].

 DOI: 10.1097/CAD.0000000000001245

Epidemiological studies have shown that the risk of cancer is inversely associated with green and yellow vegetables and the consumption of fruit. Since β-carotene is present in abundance in these vegetables and fruits, the cancer prevention agent has been studied as extensively as possible. The findings obtained in this study indicate that there is an anticarcinogenic activity of β-carotene distributed in our daily foods [8,9].

Natural treatment could be the best replacement for the treatment of cancer avoiding the various physical side effects caused by chemotherapy and radiation therapy. This treatment can effectively avoid the damaging of normal, healthy cells near cancer cells [10].

Liposomes are artificial membrane vesicles formed by bilayers of phospholipids. The similarity between the core of the liposomal and membrane bilayer makes liposomes a very useful method for investigating the importance of antineoplastic drug–membrane interactions [11,12].

An essential part of the liposome is made up of phospholipids, which are amphiphilic molecules (with a hydrophilic head and a hydrophobic tail) that form an integral part of the liposome. Once these phospholipids are dispersed in aqueous media, lipid molecules associate with noncovalent interactions form supramolecular structures as monolayers, micelles, bilayers or vesicles like structures called liposomes [13].

Distearoylphosphatidylcholine (DSPC), which is widely used in the delivery system of liposomal drugs, is used in vivo compartments to mimic the bulk lipids in eukaryotic and bacterial membranes. DSPC has many unusual physicochemical properties, such as a higher transition temperature, which in bilayers leads to decreased phospholipid tail movement. Thus, it has been increasingly used as the main bilayer portion in liposome formulation in recent years [14,15]. Literature also stated that DSPC liposomes as the main phospholipids are more stable than liposomes containing dipalmitoylphosphatidylcholine (DPPC) [16].

Several clinical trials have examined the use of carrier systems that can enhance the delivery specificity of therapeutic drugs; in particular, liposomes have been tested as carriers of several antineoplastic drugs, including cyclophosphamide and doxorubicin [3,17]. Animals have shown that anticancer drugs encapsulated into liposomes are much less toxic than unencapsulated anticancer drugs. Also, liposomes focus mostly on organs rich in reticuloendothelial cells when they are administered intravenously. Therefore, liposomal delivery of antineoplastic agents will improve some of their effectiveness by steering the drug away from healthy tissue or reducing the dose needed to achieve a cytotoxic effect on tumor cells [3].

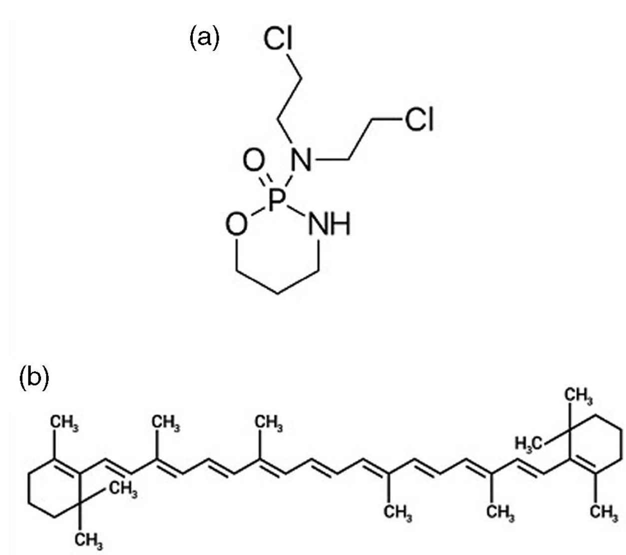
The objective of this study was to determine if free beta carotene or integrated into liposomes could inhibit human breast cancer MCF-7 cell proliferation compared to free cyclophosphamide and its conjugation with liposomes by detecting the possible effects of these compounds on the cell death of human breast carcinoma MCF-7 cell lines. The formulated beta carotene or cyclophosphamide / liposomal conjugate was characterized by analytical techniques to assess their size, size distribution, thermotropic changes and conformational changes along with in vitro potential cytotoxicity against MCF-7 (breast cancer cell line) in vitro.

Materials and methods

Chemicals

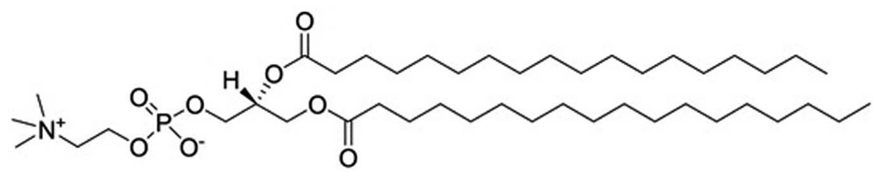
From Sigma (ST. Louis, Missouri, USA), beta-carotene was acquired. It was checked by HPLC for purity. The molecular weight of beta-carotene is 536.9. Cyclophosphamide with a molecular weight of 261.1, was purchased from Asta (Germany). The molecular structure of beta-carotene and cyclophosphamide are shown in Fig. 1. Ethanol alcohol was bought from DaeJung Chemicals (Seohaean-ro, Gyeonggi-do, Korea), with an absolute 99.9%. Distearoyl phosphatidylcholine (DSPC) was purchased from Sigma with a molecular weight of 790.161 in powder form and a purity of 99% (Fig. 2), Tris base (hydroxymethyl) in powder form, a molecular weight of 121.1 was purchased from CDH, New Delhi, India. Human tumor cell line MCF-7 was collected frozen in liquid nitrogen (−180°C) from the American Form Culture Array. The tumor cell lines were preserved in the National Institute of Cancer, Cairo, Egypt, by serial subculturing. Dimethylsulphoxide (DMSO), RPMI-1640 medium, sodium bicarbonate, Trypan blue: An isotonic

Fig. 1



The chemical structure of Cylophosphamide (a) and Beta-Carotene (b).

Fig. 2



Schematic chemical structure of DSPC. DSPC, distearoylphosphatidylcholine.

solution of 0.05 % trypan blue in normal saline, penicillin/streptomycin, trypsin, acetic acid, fetal bovine serum (FBS), sulphorhodamine-B (SRB), 0.4 % SRB dissolved in 1 % acetic acid, trichloroacetic acid (TCA), 100 % isopropanol were bought from Sigma Chemical Co. In distilled ultra-pure water, solutions were prepared. HPLC grade was used with all the other preparations used in this work.

Preparation of liposomes

DSPC:cyclophosphamide at a molar ratio of 7:2 were used to prepare neutral MLVs using the thin film hydration method of Bangham [18]. 10 mg of DSPC and 1 mg of cyclophosphamide in the absence or presence of 1.94 mg of beta-carotene at a molar ratio of 2:7 to DSPC were transferred to a 50 ml round bottom flask. Then 20 ml of ethanol (EtOH) were added, and the flask was shaken until all components were dissolved in the EtOH. The organic solvent was removed gradually using a rotary evaporator under a vacuum in a warm water bath (50°C) to produce a uniform thin film of lipid on the inner wall of the flask. In a water bath at 50°C for 15 min, the lipid film was hydrated with a Tris buffer (pH 7.4 at 37°C) to form multilamellar vesicles (MLV). At 50°C, the flask was shaken mechanically for 1 h. Then the flask was flushed through with a nitrogen stream and immediately stoppered. Empty liposome serves as control was prepared using the same process as mentioned above, using only DSPC mass aliquots previously used in the preparation.

Encapsulation efficiency measurement

By extracting untrapped cyclophosphamide by centrifugation at 11 000 rpm for 15 min, the encapsulation efficiency of cyclophosphamide into DSPC liposomes was achieved. Using a UV-Vis spectrophotometer (Jasco V-630, Germany), the unloaded cyclophosphamide in the supernatant was measured spectrophotometrically at a wavelength of 278 nm. At 278 nm (resonance absorption peaks of cyclophosphamide), the wavelength was adjusted. For each sample, the absorption of the supernatant was compared with the calibration curve for the absorption of different concentrations of cyclophosphamide.

The determination of the encapsulation efficiency of beta-carotene was performed by extraction according to Tan *et al.* [19]. 3 ml of ethyl acetate and aliquots of 1 ml of beta-carotene-loaded liposomes were combined strongly for 3 min at ambient temperature by vortexing to obtain the free amount of beta-carotene in suspension. The mixed sample was centrifuged for 5 min at 2000 rpm to extract the supernatant from the centrifuged sample. The procedure referred to above was replicated twice. Finally, the obtained supernatant was mixed in a tube and diluted with ethyl acetate. For beta carotene with ethyl acetate as blank, the free amount of carotenoid was spectrophotometrically quantized at 452 nm, respectively. Each experiment was conducted as a triplet.

Determination of entrapment efficiency percentage

The entrapment efficiency (EE%) was calculated through the following relationship [20]:

$$\mathrm{EE\%} = \frac{\text{Total drug imput}(mg) - \text{Drug in supernatant}(mg)}{\text{Total drug input}(mg)} \times 100$$

In vitro release studies

At pH 7.4 of 37°C, phosphate buffer saline (PBS) was prepared. In a shaking incubator at 50 rpm and 37°C, a certain volume of liposomal samples (5 ml) was combined with 15 ml of PBS. An aliquot of the mixture was removed at proper time intervals and replaced with the same volume of PBS. The collected samples were extracted and analyzed spectrophotometrically in the same way mentioned above. The amounts released were determined by subtracting the free quantity of the drug initially prepared. The rate of release was quantized as follows:

$$\text{Release rate} = \frac{\text{released amount of drug}}{\text{total amount of drug}} \times 100$$

Dynamic light scattering and zeta potential

The mean particle size, size distribution and zeta potential of freshly prepared empty liposomes and liposomes doped with either cyclophosphamide (LipoCyclo), beta carotene (LipoBeta) or cyclophosphamide combined with beta carotene (LipoCycloBeta) were calculated using a particle sizing method (Nanotrac Wave II, Microtrac, York, USA) for dynamic light scattering at 25 ° C in Tris buffer (pH 7.4). The experiment was carried out in a triplicate and all data are expressed as mean ± SD.

DSC measurements

To investigate the thermal behavior of lyophilized samples of empty liposomes and liposomes doped with either cyclophosphamide (LipoCyclo) or beta carotene (LipoBeta), or cyclophosphamide coupled with beta carotene (LipoCycloBeta), differential scanning calorimetry (DSC) (model DSC-50, Shimadzu, Kyoto, Japan) calibrated with indium was used. Analyses are conducted in standard aluminum pans on 5-mg samples sealed. The thermogram of each sample spans the temperature range of 25–200° C at a scanning rate of 3°C/min.

FTIR Spectroscopy

A Jasco FT / IR-4100 spectrometer (Tokyo, Japan) recorded FTIR spectra of lyophilized samples of empty liposomes and liposomes doped with either cyclophosphamide (LipoCyclo) or beta-carotene (LipoBeta) or cyclophosphamide combined with beta-carotene (LipoCycloBeta) deposited on KBr discs. The scanning was taken place at room temperature with a speed of 2 mm/s and a resolution of 4 cm^{-1} within the range of 400–4000 cm^{-1}.

In vitro cytotoxicity assay

In RPMI-1640 media, the breast cancer cell line (MCF-7) was cultivated, supplemented with decomplemented FBS (10 %, v/v), penicillin (100 IU/ml) and streptomycin (100 µg/ml) under 5 % CO_2 and 95 % air at 37°C in a humidified incubator.

Drug treatments were carried out in growth assays plates (96 well culture plates). For triplicate studies, drugs were applied to the culture medium at a determined final concentration. The SRB (Sulfo-RhodamineB-stain) assay was used to measure the level of cell viability and proliferation (cell numbers).

The potential cytotoxicity of free cyclophosphamide, beta carotene alone, CycloBeta, LipoCyclo, LipoBeta and LipoCycloBeta was tested separately using the method of Skehan *et al*. [21]. MCF-7cells were plated in 96-multi well plate (10^4 cells/well) for 24 h before treatment with the applied drugs to permit adhesion of the cell to the wall of the plate. With each dose, different concentrations of free cyclophosphamide, beta carotene alone, CycloBeta, LipoCyclo, LipoBeta, and LipoCycloBeta (5, 12.5, 25, 50, 100, 200, 400, 800, 1000 µg/ml) were applied to the triplicate cell monolayer wells (for further fertilization in the laminar flow cabinet). The free cyclophosphamide, beta carotene alone, CycloBeta, LipoCyclo, LipoBeta and LipoCycloBeta were incubated with monolayer cells separately for 48 h at 37°C and in an atmosphere of 5% CO_2. After 48 h, the cells were fixed, washed, and stained with sulforhodamine -B stain. The residual stain was cleaned with acetic acid and the attached stain with the Tris-EDTA buffer was retrieved. An ELISA reader (ELISA-TECAN-SUNRISE, Germany) measured the color intensity. The relationship between drug concentration and the percentage of cell viability (surviving fraction) is plotted to find the cell viability curve for the MCF-7 cancer cell line after treatment with free cyclophosphamide, beta carotene alone, CycloBeta, LipoCyclo, LipoBeta and LipoCycloBeta. The percentage of cell survival has been determined as follows:

Survival fraction = O.D. (treated cells)/O.D. (control cells).

IC50 values (the drug concentrations needed to produce 50 % cell growth inhibition). For each cell line, the experiment was replicated three times.

Single-cell gel electrophoresis (comet assay)

As a rapid, simple, visual and sensitive technique, comet assay is seen to evaluate DNA fragmentation typical for toxic DNA damage, including single and double-strand breaks, DNA adducts, cross-links and early stage of apoptosis. Comet assay was performed according to Singh *et al*. [22] with modifications according to Blasiak *et al*. [23].

Cell microgels were prepared as layers. The first layer of gel was made by applying 100 µl of normal melting point agarose (0.7%) onto precleaned microscope-charged slides and coverslipped gently. The coverslip was removed after the agarose solidified at 4ºC. Low melting-point agarose (0.5%) was prepared in 100 mmol/LPBS and kept at 37ºC. Mononuclear cells were mixed with the low melting-point agarose and 100 µl of the mixture was applied to the first gel layer. The slides were then covered with a coverslip and placed at 4ºC for solidification. After the second layer solidified, the coverslips were removed from the cell microgels. A final layer of low-melting agarose was added followed by coverslips, left to solidify for 10 min then the coverslips were removed. The slides were covered with 100 ml of fresh lysis buffer at pH 10 at 4ºC for 1 h. Buffer contains; 2.5 mol/l NaCl, 100 mmol/l EDTA, 1% sodium hydroxide, 10 mmol/l Tris, 1% Triton X-100, 10% DMSO. After draining, microgels slides were treated with DNA-unwinding solution (300 mmol/l NaOH, 1 mmol/l EDTA, pH 13) for 30 min at 4ºC, and placed directly into a horizontal gel electrophoresis chamber filled with DNA-unwinding solution. Gels were run with a constant current (300 mA at 4ºC) for 30 min. The microgels were neutralized with a 0.4 M Trisma base at pH 7.5 for 10 min after electrophoresis. The slides were stained with 20 µl ethidium bromide (10 µg/ml) [24].

Using a fluorescence microscope (IX70; Olympus, Tokyo, Japan) equipped with an excitation filter of 549 nm and a barrier filter of 590 nm, attached to a video camera (Olympus), the slides were examined at 400× magnification. As each cell had the appearance of a comet, with a brightly fluorescent head and a tail to one side formed by the DNA-containing strand breaks that were drawn away during electrophoresis, a damaged cell is visualized. Samples were analyzed by counting the damaged cell out of 100 cells per slide to calculate the percent of the damage [24].

Results and discussion

The entrapment efficiency percentage was found to be higher than 90% for all prepared liposomal suspensions when the drug was mixed with the lipid powder before dissolving it in ethanol.

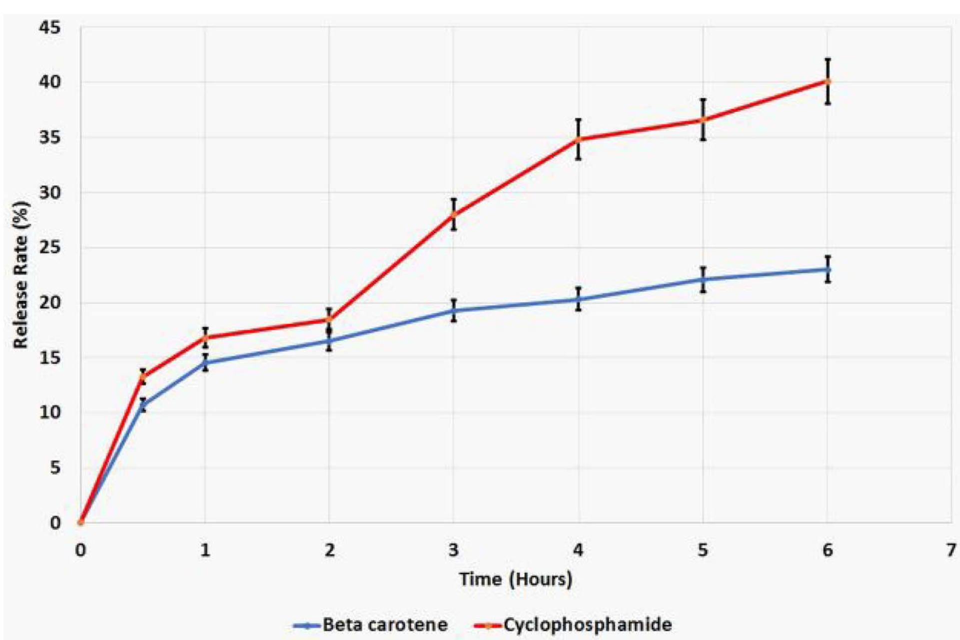
Some indications of drug delivery performance were estimated for the in vitro release of cyclophosphamide or beta-carotene from the DSPC liposomes. The leakage of cyclophosphamide or beta-carotene from liposomes was investigated by incubation in PBS at 37°C versus time in hours. Further analysis of Fig. 3 reveals that the initial step of continuous drug release is evident within the first half-hour for either cyclophosphamide or beta-carotene. The formulation of cyclophosphamide or beta-carotene is reasonably stable at a temperature of 37°C, releasing approximately 15–18 % over 2 h, followed by relatively rapid release rates over the next hours (23–40%). Beta-carotene doped with liposomes shows better stability of in vitro release (about 21%) than cyclophosphamide (about 34%) between the 3rd and 6th hours, manifested by a lower percentage of beta-carotene leakage rate which would lead to the higher beta-carotene retention. Additionally, the percentage of cyclophosphamide released was approximately 40 % over 6 h.

Beta-carotene vesicles show significantly improved stability of in vitro release ($P < 0.01$) and a lower rate of leakage manifested by higher drug retention than cyclophosphamide. The random distribution of nonpolar beta-carotene in the lipid bilayer without any desired orientation may explain these findings.

Beta-carotene vesicles show significantly improved stability of in vitro release ($P < 0.01$) and a lower rate of leakage manifested by higher drug retention than cyclophosphamide. These findings may be explained by the random distribution of the nonpolar beta-carotene in the lipid bilayer, without any desired orientation [25] resulting in higher stability of beta-carotene and a consequently lower rate of leakage. The results of the kinetic analysis revealed that the liposomal cyclophosphamide adopted a diffusion release order whereas beta carotene was released from liposomal form adopting first-order kinetics as summarized in Table 1.

Figure 4a shows the size distribution of a pure DSPC sample was concentrated around 230.8 ± 209.1 nm mean size diameter with 0.1711 PDI. Figure 4b displays an increase in mean size diameter of pure DSPC to 1417 ± 918 nm with 0.546 PDI upon the encapsulation of beta-carotene into DSPC and this probably could be attributed to that the beta-carotene molecule appears to be buried in a

Fig. 3



Release rates of cyclophosphamide and beta carotene from DSPC liposomes formulation. Data are expressed as mean ± SE. DSPC, distearoylphosphatidylcholine.

Table 1 **Summarized data obtained for the release kinetic analysis for liposomes encapsulated by cyclophosphamide and beta carotene.**

	Zero-order		1st order		Diffusion	
Drug	Release score	Correction coefficient	Release score	Correction coefficient	Release score	Correction coefficient
Cyclophosphamide	0.927	0.963	0.938	−0.969	0.979	0.990
Beta carotene	0.964	0.982	0.970	−0.985	0.957	0.979

Dynamic light scattering (DLS) is a technique used in the calculation of particle size. The index of polydispersity (PDI) efficiently accounts for colloidal suspension particle homogeneity. Values above 0.7 indicate that the sample has a very large size distribution and may not be stable for the DLS technique [26].

randomly distributed lipid bilayer without any desired orientation, resulting in increased motional freedom of the alkyl hydrocarbon chains, which may explain why the size is increased. The incorporation of cyclophosphamide into DSPC resulted in an increase in the calculated mean size diameter of blank liposomes to 385 ± 300 nm with 0.317 PDI. Such an increase could be attributed to the electrostatic repulsive force between the positive charge of the DSPC $N(CH_3)_3^+$ group and the positive charge of the NH group of cyclophosphamides where cyclophosphamide drug is mainly considered as a hydrophilic drug and could be entrapped in the aqueous core of the bilayer Fig. 4c. Figure 4d shows a significant increase in the calculated mean size of blank DSPC to 5340 ± 1726 nm with 0.272 PDI after incorporation of cyclophosphamide associated with beta carotene together at molar ratio (1:1) into DSPC liposomes. These results are consistent with the sum of DLS measurements obtained upon the entrapment of cyclophosphamide or beta carotene separately into DSPC liposomes. From the above results, beta carotene may increase the lipid bilayer size as well as cyclophosphamide increases the liposomal core size. These results are in strong alignment with the data observed by the studies of DSC and FTIR.

The possible stability of the colloidal system is indicated by the magnitude of the zeta potential. If the zeta potential increases, there will be an increased repulsion between particles, leading to a more stable dispersion of colloids. If all suspended particles have a strong zeta potential that is negative or positive, they seem to repel each other, and the particles are not likely to join together [27].

Following the findings of others, empty liposomes showed negative zeta potential (−6.58 mV) [28–31]. Cyclophosphamide or beta carotene-loaded liposomes had higher negative zeta potential (−19.2 mV and −7.51 mV, respectively) than blank liposomes due to the integration of both into the liposomal membranes.

Within liposomal membranes, the incorporation of cyclophosphamide (LipoCyclo) or beta carotene (LipoBeta) appears to increase the density of negative charge and hence made the zeta potential negative.

The liposomal formulation LipoCycloBeta in the presence of cyclophosphamide combined with beta carotene recorded the lowest zeta potential value (−2.31 mV) as the repulsion phenomenon between particles are smaller, thus leading to a less stable colloidal dispersion (Fig. 5). Table 2 summarizes the results of zeta potential measurements of different liposomal formulations.

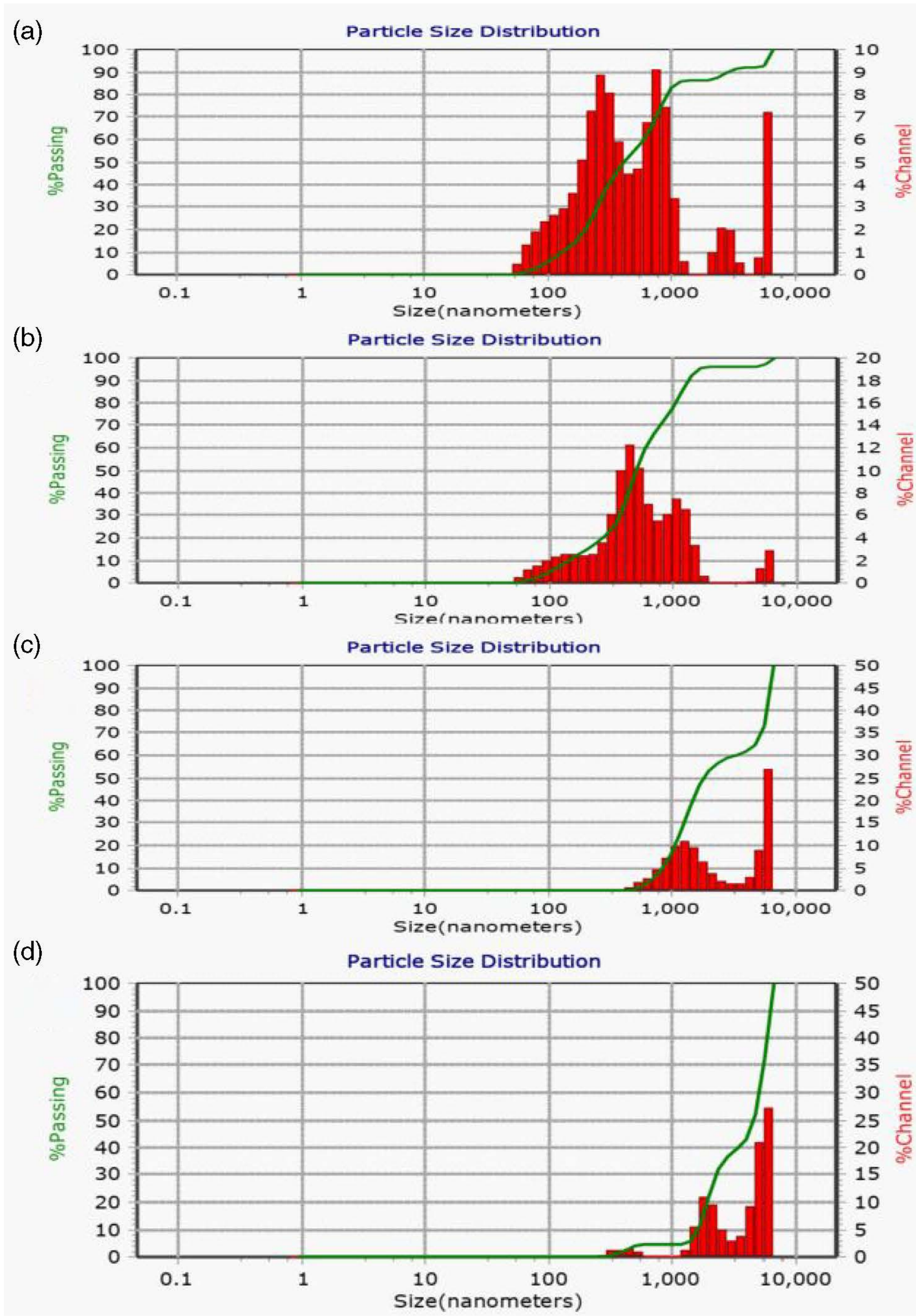
DSC characterization was used to analyze changes in phase transition temperature of the lipid bilayer due to altered interactions between the encapsulated drugs and liposomes [32,33].

As this phospholipid may mimic many characteristics of biological membranes, the DSPC vesicles were used as model membranes. When submitted to DSC analysis, pure DSPC vesicles upon dehydration showed a significant major endothermic peak (T_m) at 101.5°C (Fig. 6), in accordance with [34–37]. The pretransition temperature (T_p) was around 60°C for pure DSPC liposomes.

The presence of a compound in the DSPC membranes might influence the thermotropic parameters of the vesicle transition. Compared to the main endothermic peak (T_m) of empty DSPC present at 101.5°C, the incorporation of cyclophosphamide or beta carotene into DSPC liposomes showed a slight shift to a higher temperature at 104°C and 102.2°C, respectively, which suggests that cyclophosphamide or beta carotene had a major impact on the DSPC bilayers acyl chains creating a conformational disorder within the phospholipids and decrease the transition cooperatively of lipid acyl chains [38,39]. The increased temperature of the main endothermic peak (T_m) of empty DSPC indicated that the incorporation of cyclophosphamide or beta carotene is more favorable to the formation of acyl chains in a disordered and loose state. The pretransition temperature (T_p) peak for cyclophosphamide or beta carotene liposomes is disappeared, which revealed that either cyclophosphamide or beta carotene interacted with the polar head group of phospholipids. The incorporation of biomolecules into lipid bilayers could be a sensitive criterion for the disappearance of the pretransition [40].

Interestingly, the incorporation of cyclophosphamide combined with beta carotene into DSPC liposomes caused no noticeable shift to the main characteristic endothermic peak of pure DSPC existing at 101.5°C, its intensity was markedly depressed. The pretransition temperature was found to be shifted from 60−86.4°C for DSPC liposomes doped cyclophosphamide combined with beta carotene which revealed a membrane fluidizing effect. The insertion of the drug between the DSPC polar heads can favor the development of a less orderly liquid crystalline phase than the gel phase and slightly decrease the transition temperature of the gel-to-liquid

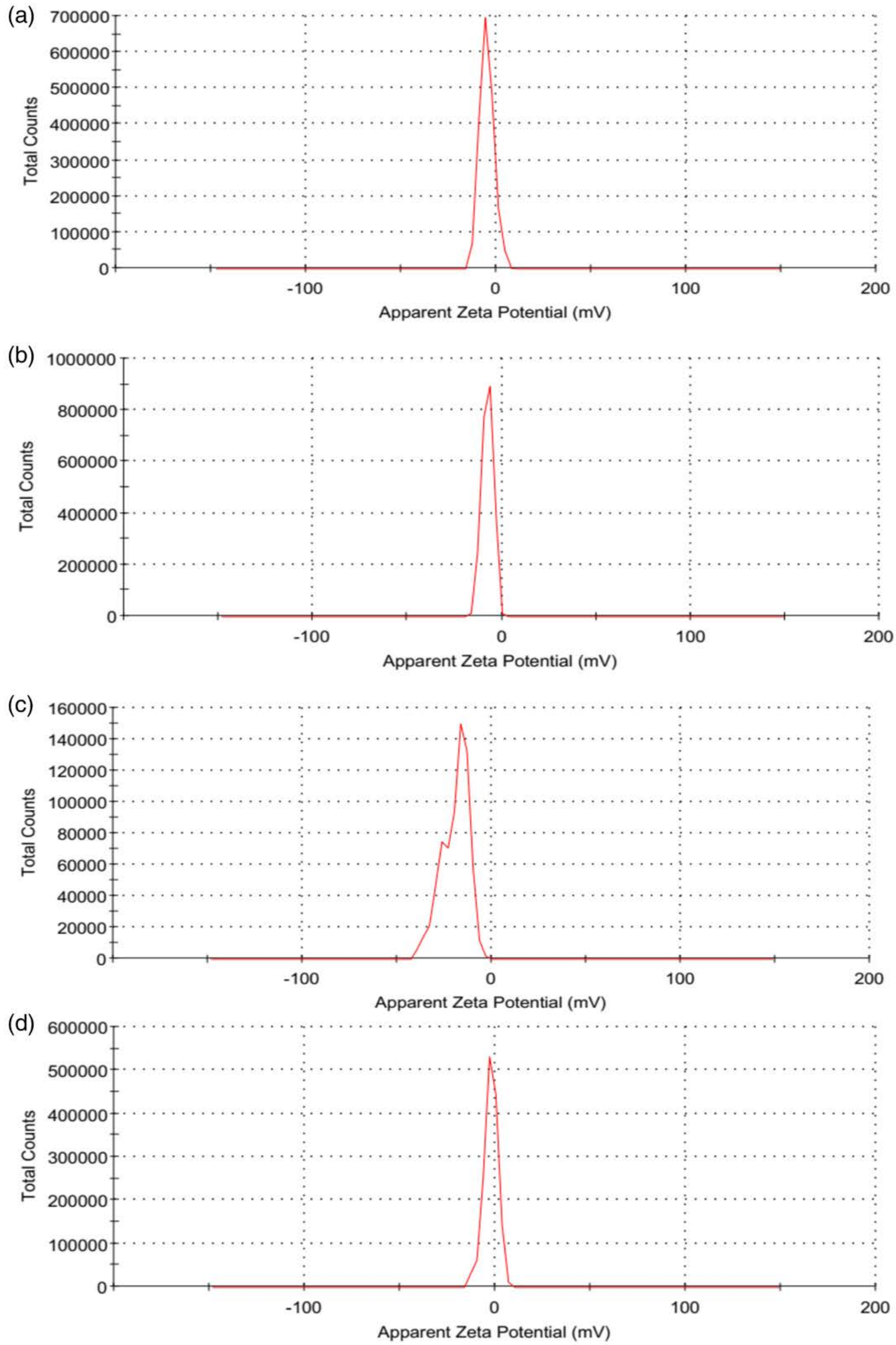
Fig. 4



Liposomes size distribution measured by dynamic light scattering (DLS) for (a) empty DSPC liposomal sample, (b) beta carotene-encapsulated liposomes, (c) cyclophosphamide-encapsulated liposomes and (d) cyclophosphamide combined with beta carotene into liposomes. DSPC, distearoylphosphatidylcholine.

crystal phase as observed by DSC [41]. For the DSPC/cyclophosphamide combined with a beta carotene sample, there was an appearance of an additional transition peak at nearly 128°C. The microheterogeneous distribution of cyclophosphamide in combination with beta carotene that interacts with DSPC can be presumed to the formation of lateral domains. Using DSC, it has been observed that DSPC and cyclophosphamide or beta carotene mixtures display a single peak, suggesting that they are miscible [42].

Fig. 5

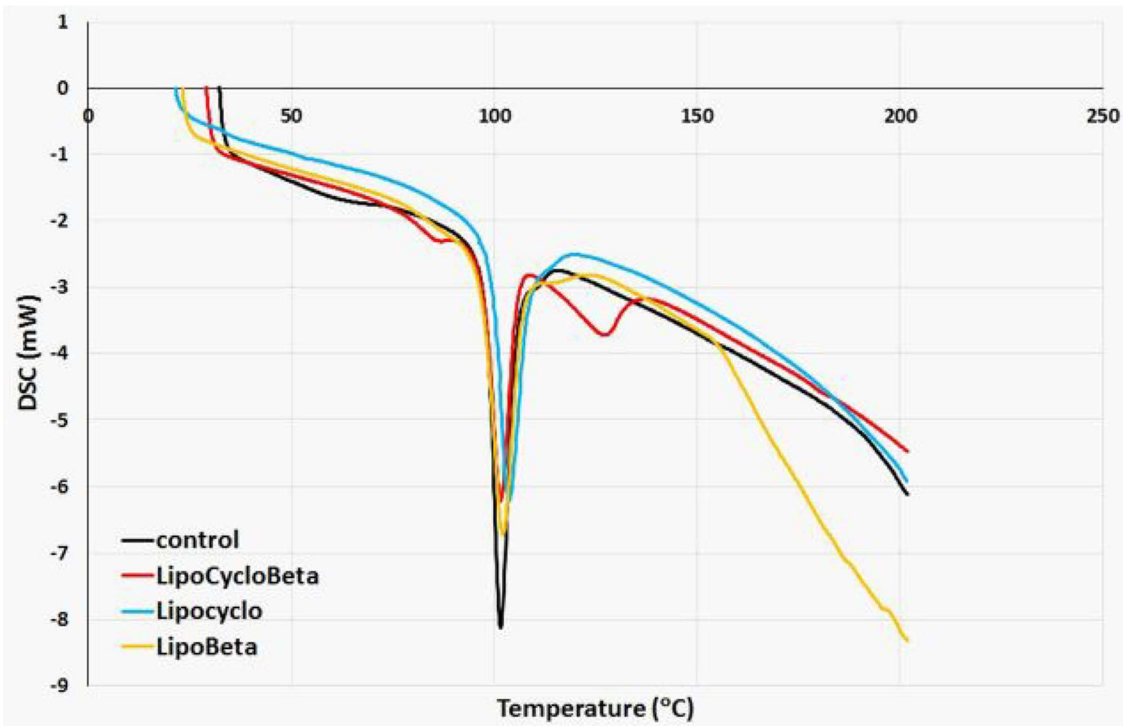


Zeta potential for (a) empty DSPC liposomal sample, (b) beta carotene-encapsulated liposomes, (c) cyclophosphamide-encapsulated liposomes and (d) cyclophosphamide combined with beta carotene into liposomes. DSPC, distearoylphosphatidylcholine.

Table 2 Summarized data obtained for the dynamic light scattering (DLS) and zeta potential for liposomes before and after encapsulation by beta carotene, cyclophosphamide or cyclophosphamide combined with beta carotene

Sample name	Mean diameter (nm) ± SD (nm)	PDI average	Mean zeta potential ± SD (nm)
Control	230.8 ± 209.1	0.1711	−6.58 ± 3.08
liposomal beta	385 ± 300	0.317	−7.51 ± 3.04
liposomal cyclo	1417 ± 918	0.546	−19.2 ± 4.27
liposomal cyclobeta	5340 ± 1726	0.272	−2.31 ± 3.69

Fig. 6



DSC diagrams of liposomes made of pure DSPC, liposomes doped with either cyclophosphamide or beta carotene or both. DSC, differential scanning calorimetry; DSPC, distearoylphosphatidylcholine.

Interestingly, its intensity was markedly depressed after the incorporation of cyclophosphamide combined with beta carotene into DSPC liposomes caused no noticeable shift to the main characteristic endothermic peak of pure DSPC existing at 101.5°C.

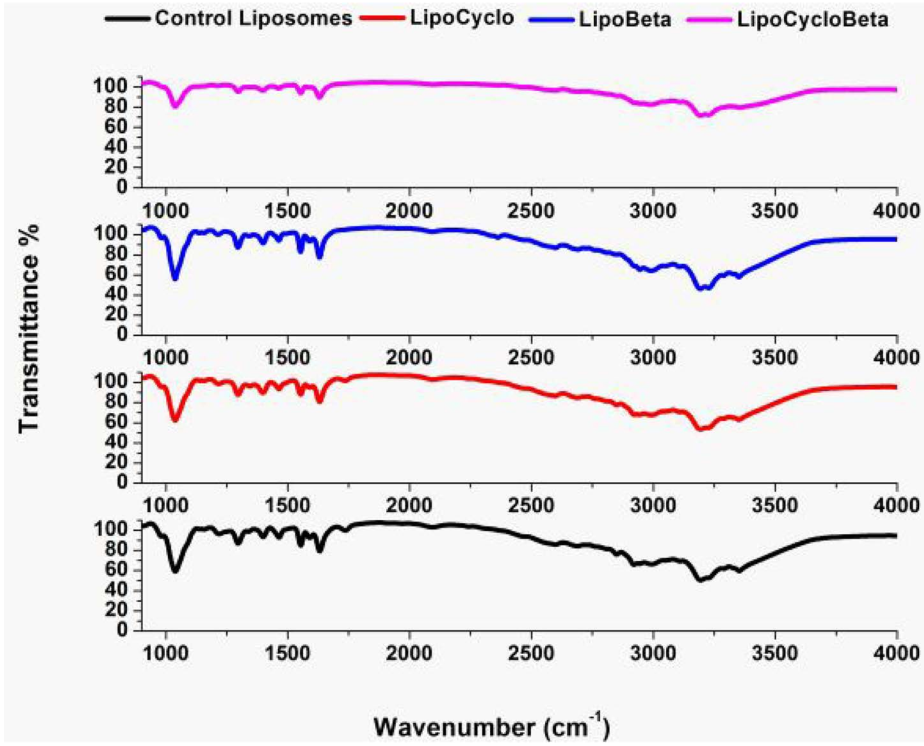
Some modifications observed in the current DSC work can be verified by FTIR, which has been used to detect any changes in the liposomal membrane structure by analyzing the wavenumber of various vibrational modes.

To analyze possible changes in the structure of DSPC, FTIR spectroscopy was used to analyze the wavenumber of different functional groups considering the acyl chains and the lipid molecule head group region in the presence or absence of foreign molecules.

Compared with cyclophosphamide or beta carotene or both/DSPC liposomal samples in the 4000–400 cm^{-1} region, FTIR spectra of empty lyophilized DSPC liposomes are presented (Fig. 7).

The DSPC liposome spectrum showed the highest absorption FTIR characteristic peaks [43]. Encapsulation of beta carotene or cyclophosphamide combined with beta carotene into the DSPC liposomes caused a change in the wavenumber of the symmetric CH_2 stretching bands in the acyl chain (Fig. 7), indicating that beta carotene or cyclophosphamide combined with beta carotene produce a conformational disorder within the phospholipid's acyl chains. In other words, they affected the order of the membrane. The peak at 2849.3 cm^{-1} for the pure DSPC is shifted towards a higher wavenumber 2851.2 cm^{-1} for cyclophosphamide associated with beta carotene liposomes. This could indicate an increase in the number of gauche conformers, suggesting an increase in bilayer disorder [44]. Interestingly, the signal intensity of cyclophosphamide in combination with beta carotene-loaded

Fig. 7



The full FTIR spectra of empty DSPC, liposomes doped with either cyclophosphamide, beta carotene, or both. DSPC, distearoylphosphatidylcholine.

liposomes has become less intensive. The peak at 2849.3 cm^{-1} for the pure DSPC is moved toward the lower wavenumber 2847.4 cm^{-1} after the introduction of beta carotene into DSPC, indicating a decrease in membrane fluidity and hence stabilization of the system in the gel phase. The CH_2 antisymmetric stretching band wavenumber shifted towards a higher wavenumber with the addition of cyclophosphamide combined with beta carotene (2923.6 cm^{-1}) or beta carotene alone (2920.7 cm^{-1}) in comparison to those of pure DSPC (2924.5 cm^{-1}) (Fig. 7).

The peaks of CH_2 symmetric and asymmetric stretching vibrations have been used as a sensitive alkyl chain ordering indicator. There are substantial changes in the CH_2 stretching band wavenumber, showing that beta carotene-associated cyclophosphamide increased the number of gauche conformers, suggesting an increase in bilayer conformational disorder [45,46].

The C = O stretching band is analyzed for the interaction between beta carotene or cyclophosphamide combined with beta carotene and the glycerol backbone near the head group of phospholipids in the interfacial region [47]. As seen from (Fig. 7), the wavenumber value of the C = O group at 1736. 6 cm^{-1} is disappeared for the liposomal samples containing beta carotene or cyclophosphamide combined with beta carotene, with evidence of hydrogen bonding formation. The degree of hydrogen-bond formation was regulated in the glycerol backbone region of the DSPC molecule by changes in the ester C = O stretching contours.

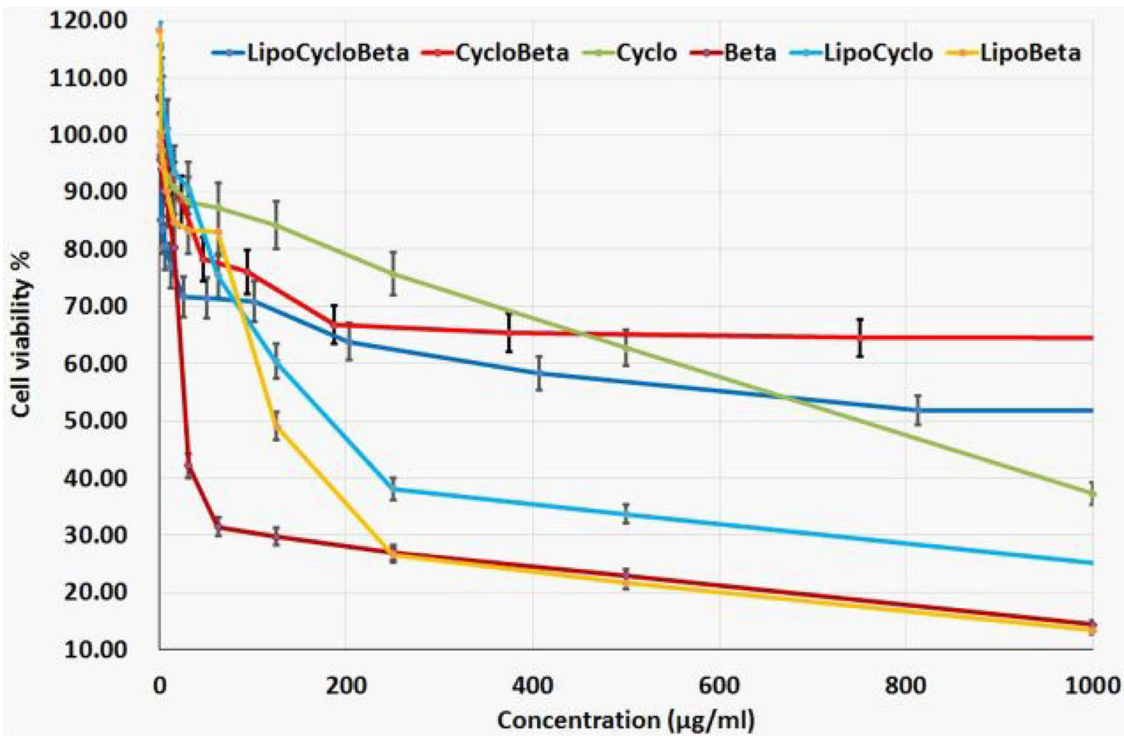
The C = O ester absorption bands are susceptible to changes in the polarity of their local environments and are affected by the bonding of hydrogen and other interactions. Therefore, any alteration in the spectrum of this region may be due to the interaction between cyclophosphamide or beta carotene and the polar/apolar interfacial region of the membrane [48].

The interaction between cyclophosphamide or beta carotene or a mixture of them and the head group of DSPC liposomes was studied through the PO_2^- antisymmetric stretching band, which is located at 1217.8 cm^{-1}. Figure 7 shows the PO_2^- antisymmetric stretching band for DSPC liposomes formulations in the absence and presence of cyclophosphamide or beta carotene or a mixture of them. As seen from Fig. 7, the wavenumber was shifted to lower values after the addition of cyclophosphamide (1216.8 cm^{-1}) or beta carotene (1213 cm^{-1}) or a mixture of them (1210.113 cm^{-1}) into DSPC liposomes. This meant that hydrogen bonding between the head group of the liposome and lutein or beta carotene was present. The decrease in the wavenumber value means that existing hydrogen bonds are strengthened, or new hydrogen bonds are formed between the components [44].

Table 3 The chemical shifts observed for cyclophosphamide or beta carotene or a mixture of them after the incorporation into DSPC liposomes

		Wavenumber (cm^{-1})			
Peak assignment	Wavenumber (cm^{-1})	Control	Cyclophosphamide	Beta Carotene	Cyclo mixed with beta
Symmetric stretching vibration of CH_2 in acyl chain	(2800–2855)	2849.3	2849.3	2847.3	2851.2
Antisymmetric stretching vibration of CH_2 in acyl chain	(2916–2921)	2920.66	2920. 66	2954.52	2923.55
Carbonyl stretching vibration C=O	(1730–1740)	1736.58	1736.58	–	–
CH_2 bending vibration	(1456–1470)	1463.7	1463.7	1463.7	1463.7
Aliphatic phosphates (P-O-C stretch	(920-1088)	1038.48	1038.48	1038.48	1038.48
Antisymmetric PO_2^- stretching vibrations	(1215–1260)	1217.82	1216.86	1213	1210.11
$(CH_3)_{3N}^+$ symmetric bending	1405	1399.10	1398.13	1399.10	1398.13
$(CH_3)_{3N}^+$ antisymmetric stretching	972	972.9	–	–	–

Fig. 8

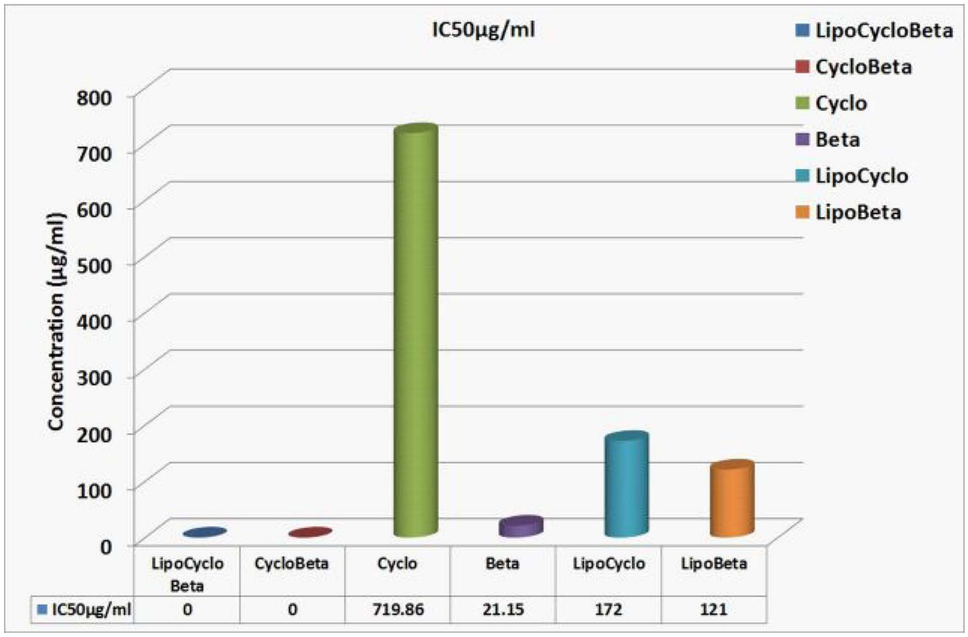


Cytotoxicity of Cyclophosphamide (Cyclo), Beta carotene (Beta), Cyclophosphamide with Beta carotene (CycloBeta) and separately their liposomal formulations (LipoCyclo, LipoBeta and LipoCycloBeta) against breast carcinoma (MCF-7) cell lines; incubated for 48 h with different concentrations (5, 12.5, 25, 50, 100, 200, 400, 800, 1000 µg/ml). The cell viability was determined using the SRB assay. The data represent mean ± standard error of triplicate experiments.

In the bulk hydrophobic interior of the bilayer, apolar carotenoid beta carotene is entirely buried and randomly distributed inside, without any preferred well-defined orientation, and retains a significant degree of mobility. The presence of beta carotene in PC liposomes leads to the enhancement of motional freedom of the lipid polar groups in the lipid–water interface and the liquid crystalline state of the fatty chains [25]. In the polar region of the lipid headgroups, xanthophylls or polar carotenoids are positioned almost perpendicular to the membrane plane and cross the double lipid layer anchoring their hydroxyl groups, while remaining immobilized within the membrane [49].

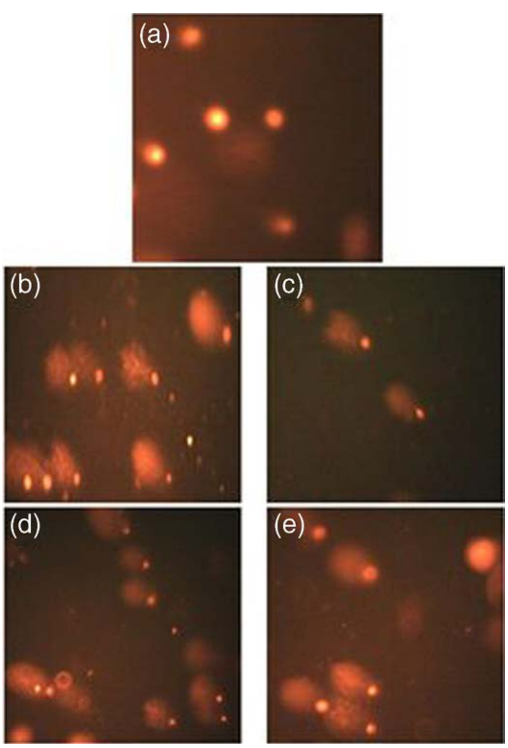
In addition, for all molecules studied in DSPC liposomal samples, the $N(CH_3)_3^+$ asymmetric stretching band at 972 cm^{-1} disappeared. Due to the electrostatic repulsive force between the DSPC $N(CH_3)_3^+$ and the NH group of cyclophosphamides, the presence of the $N(CH_3)_3^+$ group in the polar head of the DSPC may prevent the NH group of cyclophosphamides from approaching the PO_2^- group, thus weakening the interactions between the phospholipid PO_2^- group and the NH group of cyclophosphamides (Table 3). Nonpolar β-carotene, on the other side, is probably immersed deep in the lipid bilayer and in substantially weaker interaction with the phosphate group

Fig. 9



IC_{50} chart with significant values for beta, LipoBeta, LipoCyclo, Cyclo, LipoCyclobeta and CycloBeta against MCF7 cell lines by using SRB assay, 48 h posttreatment.

Fig. 10



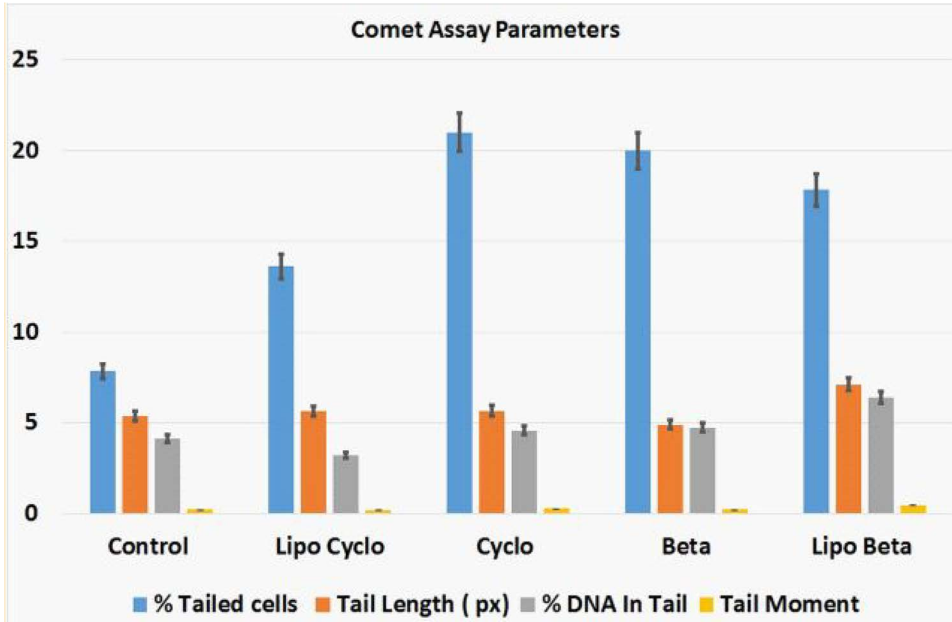
Comet assay images of MCF-7 cell line (evaluation of DNA damage induced by Beta (b), LipoBeta (c), Cyclo (d) and LipoCyclo (e) compared to control (a)

of phospholipids molecules. Table 3 shows the chemical shifts observed for cyclophosphamide or beta carotene or a mixture of them after the incorporation into DSPC liposomes.

The efficacy of the drug delivery system was investigated using cell viability assay (in vitro cytotoxicity SRB) at different drug concentrations of cyclophosphamide (Cyclo), beta carotene (beta), cyclophosphamide with beta carotene (CycloBeta) and separately their liposomal formulations (LipoCyclo, LipoBeta and LipoCycloBeta) against breast carcinoma (MCF-7) cell lines [21]. At zero concentration of each drug, untreated cells served as controls. Separately, the MCF-7 cancer cell lines were incubated with the same sequence of different drug concentrations, 5, 12.5, 25, 50, 100, 200, 400, 800, 1000 μg/ml, for 48 h, Fig. 8. After 48 h, the assay was terminated, and cell viability measurements were performed.

Free beta carotene (beta) demonstrated the highest rate of cytotoxicity against MCF-7 cell lines treated with the same sequence of different concentrations available to other drugs, according to cell viability measurements. At the highest beta carotene (beta) concentration (1000 μg/ml), MCF-7 treated cells displayed cell viability of about 15%, 48 h postincubation. While for beta-loaded liposomes (LipoBeta) treated cells, the cell viability was approximately the same 14% and for cyclophosphamide-loaded liposomes (LipoCyclo), the cell viability was approximately 34% compared to 38 % for free cyclophosphamide

Fig. 11



Indicates the comet assay parameters (percentage tailed cells, tail length, percentage tailed DNA, and tail moment) for the control and posttreatment groups, and the differences between the control and the posttreatment groups.

(Cyclo) at the same concentration (1000 μg/ml). The decrease in cell viability compared to free cyclophosphamide (Cyclo) may be due to the sustained release of cyclophosphamide from liposomes in cells treated with cyclophosphamide-loaded liposomes (LipoCyclo). So, as the concentration of the encapsulated drug increases, the quantity of the released drug also increases, and as a result, the viability of the cell decreases as the concentration of the encapsulated drug increases.

Beta-carotene prevented the development of many human colon adenocarcinoma cell lines, the induction of cell cycle arrest and apoptosis. Beta-carotene has been reported to be responsible for reducing cyclin A, a significant regulator of cell cycle progression [50]. In vitro studies support the assumption that for certain types of cancer, including prostate cancer [51]and melanoma [52], this carotenoid is either chemopreventive or chemotherapeutic.

Interestingly, cyclophosphamide combined with beta carotene (CycloBeta) or its liposomal forms (LipoCycloBeta) did not indicate a significant reduction in the cell viability when tested at the same concentration (1000 μg/ml) against MCF-7 cell lines. The cell viability was roughly 65 and 52% for CycloBeta and LipoCyclobeta, respectively. It seems that the combination of cyclophosphamide with beta carotene cannot act in synergism.

Cytotoxic activity among various drug formulations at higher concentrations displayed the order of beta > LipoBeta> LipoCyclo> Cyclo> LipoCyclobeta> CycloBeta according to Fig. 8.

At the lower concentration approximately at 31.25 μg/ml, MCF-7 treated cells with beta carotene (beta) displayed cell viability 42% relative to their liposomal form (LipoBeta) of about 83.33%, while 88.33 and 90.64% of the cell remained viable for Cyclo and its liposomal form (LipoCyclo), respectively Fig. 8.

The IC_{50} value for beta carotene (beta) in cytotoxic assay with MCF-7 treated cells has counted a minimum of 21.15 μg/ml, while LipoBeta treated cells for MCF-7 treated cells were counted as 121 μg/ml. IC_{50} was 719.86 μg/ml for MCF-7 treated cells with free cyclophosphamide and 172 μg/ml for liposomal type (LipoCyclo). This improved efficacy can be attributed to the liposolubilized state of the drug due to its entrapment within multiple lipoidal domains of vesicles. In addition, the phospholipid lamella can be incorporated with the cell membrane at the periphery of the vesicles, enabling the internalization of the vesicular contents. Based on the above results and depending on the cancer cells type, beta carotene (beta) showed the highest therapeutic efficacy against the MCF-7 cell line Fig. 9. It can be noticed that the IC50 of CycloBeta and its liposomal form (LipoCycloBeta) were not applicable in cytotoxic assay with MCF-7.

In another study, the potential of solid lipid nanoparticles (SLNs) as an effective delivery vehicle for beta-carotene has been confirmed. The carotenoid encapsulated inside the lipid layer of SLNs has demonstrated enhanced bioavailability and anticancer efficacy [53]. Work on the role of synthetic beta carotene, a natural food colorant, in the regulation of genotoxic damage caused by cyclophosphamide, a widely used chemotherapy drug and a well-known mutagen and clastogen, has been investigated. The results indicated the anticlastogenic activity of BC against cyclophosphamide providing significant protection against the genotoxicity of cyclophosphamide [54,55].

For all the formulations added to the MCF-7 cell line, the analysis of the comet assay photographs is given in Fig. 10. The intact DNA without migration was represented by the red round spot in the photograph, while the comet-shaped region adjacent to the nucleus represented breaks of DNA small enough to move in the gel, indicating the undamaged control cell. The DNA was tightly packed, and a typical nucleus was retained in its circular disposition. The photograph of the comet for the profile of nuclear DNA was altered with the presence of a fluorescence streak extending from the nucleus. Damaged DNA-containing cells looked like a comet with a bright head and tail.

In MCF-7 cells treated with beta alone, Fig. 10 shows a higher intensity of the comet tail than those treated with beta-loaded liposomes. The high intensity of the tail of the comet compared to the head means that there are a significant number of double-strand breaks. An increased percentage of mortality was observed for MCF-7 cells treated with Beta-loaded liposomes compared to those treated with plain beta carotene only. These results support the assumption that a nano-sized delivery system can reach cancer cells more effectively at a faster rate than its large-sized counterparts. On the other hand, compared to control cells and cells treated with LipoCyclo alone, MCF-7 cells treated with Cyclo exhibit highly significant ($P < 0.05$) DNA damage. As the tail length and density represent the amount of single-strand breaks in the DNA, a quantitative measure of the damaged DNA is given by the percentage of DNA in the tail. The increased mean tail moment is also indicative of injury to DNA.

Fig. 11 displays the comet assay parameters (percentage of tail cells, tail length, percentage of tail DNA and tail moment) for the MCF-7 control cell line and posttreatment with Beta, LipoBeta, Cycle and LipoCyclo separately, as opposed to the control group, and the variations between the control and posttreatment groups. The results showed that for the Beta-loaded liposomes group, all comet assay parameters were significantly increased ($P < 0.05$) relative to the control values.

Conclusion

The existing data sheds new light on a new treatment routine in which beta carotene or its liposomal form is replaced cyclophosphamide to raise its anticancer activity against the MCF-7cancer cell line. An indication was observed on the more impact of free beta carotene or its nanoliposomal form in destroying breast cancer cells. The study of nanoliposomal drug release patterns suggests that the release of drugs is relatively slow and as predicted, the use of liposomes as a drug delivery mechanism has a significant role to play in slowing drug release. This will boost the cyclophosphamide therapeutic index and is proof of concept in favor of the administration of liposomal co-encapsulated drugs. Natural treatment by beta carotene or its liposomal form could be the best replacement for the treatment of human breast cancer MCF-7 cells avoiding the various physical side effects caused by chemotherapies. This outcome provides a molecular basis for the production of natural compounds as new anticancer agents and will enable the dose of cytotoxic agents to be lowered, resulting in more specific and less toxic treatments for the treatment of human breast cancer. Future studies are suggested to test other natural combinations of liposomes with other therapies for the treatment of cancer.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

References

1 Tarver T. *Cancer facts & figures 2012.* American Cancer Society; 2012. 66p., pdf.
2 Arif K, Ejaj A, Maroof A, Azmat AK, Arun C, Fatima N, *et al*. Protective effect of liposomal formulation of tuftsin (a naturally occurring tetrapeptide) against cyclophosphamide-induced genotoxicity and oxidative stress in mice. *Indian J Biochem Biophys* 2009; **46**:45–52.
3 Abdella EM. Short-term comparative study of the cyclophosphamide genotoxicity administered free and liposome-encapsulated in mice. *Iran J Cancer Prev* 2012; **5**:51–60.
4 Talluri SV, Kuppusamy G, Karri VV, Tummala S, Madhunapantula SV. Lipid-based nanocarriers for breast cancer treatment - comprehensive review. *Drug Deliv* 2016; **23**:1291–1305.
5 Shokrzadeh M, Naghshvar F, Ahmadi A, Chabra A, Jeivad F. The potential ameliorative effects of melatonin against cyclophosphamide-induced DNA damage in murine bone marrow cells. *Eur Rev Med Pharmacol Sci* 2014; **18**:605–611.
6 Lobo V, Patil A, Phatak A, Chandra N. Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacogn Rev* 2010; **4**:118–126.
7 Milner JA. Molecular targets for bioactive food components. *J Nutr* 2004; **134**:2492S–2498S.
8 Kris-Etherton PM, Hecker KD, Bonanome A, Coval SM, Binkowski AE, Hilpert KF, *et al*. Bioactive compounds in food: their role in the prevention of cardiovascular disease and cancer. *Am J Med* 2002; **113**:71S–88S.
9 Fiedor J, Burda K. Potential role of carotenoids as antioxidants in human health and disease. *Nutrients* 2014; **6**:466–488.
10 Baskar R, Lee KA, Yeo R, Yeoh KW. Cancer and radiation therapy: current advances and future directions. *Int J Med Sci* 2012; **9**:193–199.
11 Lombardo D, Calandra P, Barreca D, Magazù S, Kiselev MA. Soft interaction in liposome nanocarriers for therapeutic drug delivery. *Nanomaterials (Basel)* 2016; **6**:E125.
12 Gao W, Hu CMJ, Fang RH, Zhang L. Liposome-like nanostructures for drug delivery. *J Mater Chem B* 2013; **1**:6569–6585.
13 Lasic DD. The mechanism of vesicle formation. *Biochem J* 1988; **256**:1–11.
14 Tardi PG, Gallagher RC, Johnstone S, Harasym N, Webb M, Bally MB, Mayer LD. Coencapsulation of irinotecan and floxuridine into low cholesterol-containing liposomes that coordinate drug release in vivo. *Biochim Biophys Acta* 2007; **1768**:678–687.

15 Dadashzadeh S, Vali AM, Rezaie M. The effect of PEG coating on *in vitro* cytotoxicity and *in vivo* disposition of topotecan loaded liposomes in rats. *Int J Pharm* 2008; **353**:251–259.
16 Rezler EM, Khan DR, Lauer-Fields J, Cudic M, Baronas-Lowell D, Fields GB. Targeted drug delivery utilizing protein-like molecular architecture. *J Am Chem Soc* 2007; **129**:4961–4972.
17 Din FU, Aman W, Ullah I, Qureshi OS, Mustapha O, Shafique S, Zeb A. Effective use of nanocarriers as drug delivery systems for the treatment of selected tumors. *Int J Nanomedicine* 2017; **12**:7291–7309.
18 Bangham AD, Hill MW, Miller NGA. Preparation and use of liposomes as models of biological membranes. *Methods in membrane biology.* Springer; 1974. pp. 1–68.
19 Tan C, Xue J, Lou X, Abbas S, Guan Y, Feng B, *et al*. Liposomes as delivery systems for carotenoids: comparative studies of loading ability, storage stability and *in vitro* release. *Food Funct* 2014; **5**:1232–1240.
20 Elbialy NS, Abdol-Azim BM, Shafaa MW, El Shazly LH, El Shazly AH, Khalil WA. Enhancement of the ocular therapeutic effect of prednisolone acetate by liposomal entrapment. *J Biomed Nanotechnol* 2013; **9**:2105–2116.
21 Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, *et al*. New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* 1990; **82**:1107–1112.
22 Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* 1988; **175**:184–191.
23 Blasiak J, Trzeciak A, Kowalik J. Curcumin damages DNA in human gastric mucosa cells and lymphocytes. *J Environ Pathol Toxicol Oncol* 1999; **18**:271–276.
24 Nandhakumar S, Parasuraman S, Shanmugam MM, Rao KR, Chand P, Bhat BV. Evaluation of DNA damage using single-cell gel electrophoresis (Comet Assay). *J Pharmacol Pharmacother* 2011; **2**:107–111.
25 Gabrielska J, Gruszecki WI. Zeaxanthin (dihydroxy-beta-carotene) but not beta-carotene rigidifies lipid membranes: a 1H-NMR study of carotenoid-egg phosphatidylcholine liposomes. *Biochim Biophys Acta* 1996; **1285**:167–174.
26 Kätzel U. Dynamic light scattering for the characterization of polydisperse fractal systems by the example of pyrogenic silica, 2007. *PhD Thesis Technische Universitat Dresden.* http://nbn-resolving.de/urn:nbn:de:swb:14-1197634640783-66357.
27 Paolino D, Fresta M, Sinha P, Ferrari M. Drug delivery systems. In: Webster JG, ed. *Encyclopedia of medical devices and instrumentation.* 2nd ed. Wiley; 2006. pp. 437–495.
28 Klein JW, Ware BR, Barclay G, Petty HR. Phospholipid dependence of calcium ion effects on electrophoretic mobilities of liposomes. *Chem Phys Lipids* 1987; **43**:13–23.
29 Law S, Lo W, Pai S, Teh G. The electrokinetic behavior of liposomes adsorbed with bovine serum albumin. *Int J Pharm* 1998; **43**:257–260.
30 Makino K, Yamada T, Kimura M, Oka T, Ohshima H, Kondo T. Temperature- and ionic strength-induced conformational changes in the lipid head group region of liposomes as suggested by zeta potential data. *Biophys Chem* 1991; **41**:175–183.
31 Plank L, Dahl CE, Ware BR. Effect of sterol incorporation on head group separation in liposomes. *Chem Phys Lipids* 1985; **36**:319–328.
32 Kolman I, Pippa N, Meristoudi A, Pispas S, Demetzos C. A dual-stimuli-responsive polymer into phospholipid membranes. *J Therm Anal Calorim* 2016; **123**:2257–2271.
33 Riske KA, Barroso RP, Vequi-Suplicy CC, Germano R, Henriques VB, Lamy MT. Lipid bilayer pre-transition as the beginning of the melting process. *Biochim Biophys Acta* 2009; **1788**:954–963.
34 Koynova R, Caffrey M. Phases and phase transitions of the phosphatidylcholines. *Biochim Biophys Acta* 1998; **1376**:91–145.
35 Spink CH. Differential scanning calorimetry. *Methods Cell Biol* 2008; **84**:115–141.
36 Shafaa MW, Sabra NM, Fouad RA. The extended ocular hypotensive effect of positive liposomal cholesterol bound timolol maleate in glaucomatous rabbits. *Biopharm Drug Dispos* 2011; **32**:507–517.
37 Shafaa MW, Elshazly AH, Dakrory AZ, Elsyed MR. Interaction of coenzyme Q10 with liposomes and its impact on suppression of selenite - induced experimental cataract. *Adv Pharm Bull* 2018; **8**:1–9.
38 Pedersen TB, Kaasgaard T, Jensen MØ, Frokjaer S, Mouritsen OG, Jørgensen K. Phase behavior and nanoscale structure of phospholipid membranes incorporated with acylated C14-peptides. *Biophys J* 2005; **89**:2494–2503.
39 Popova AV, Hincha DK. Effects of cholesterol on dry bilayers: interactions between phosphatidylcholine unsaturation and glycolipid or free sugar. *Biophys J* 2007; **93**:1204–1214.
40 Gruszecki WI, Strzałka K. Carotenoids as modulators of lipid membrane physical properties. *Biochim Biophys Acta* 2005; **1740**:108–115.
41 Fa N, Ronkart S, Schanck A, Deleu M, Gaigneaux A, Goormaghtigh E, Mingeot-Leclercq MP. Effect of the antibiotic azithromycin on thermotropic behavior of DOPC or DPPC bilayers. *Chem Phys Lipids* 2006; **144**:108–116.
42 Bafna SS, Sun T, Baird DG. The role of partial miscibility on the properties of blends of a polyetherimide and two liquid crystalline polymers. *Polymer* 1993; **34**:708–715.
43 Mady MM, Shafaa MW, Abbase ER, Fahium AH. Interaction of doxorubicin and dipalmitoylphosphatidylcholine liposomes. *Cell Biochem Biophys* 2012; **62**:481–486.
44 Severcan F, Sahin I, Kazanci N. Melatonin strongly interacts with zwitterionic model membranes–evidence from Fourier transform infrared spectroscopy and differential scanning calorimetry. *Biochim Biophys Acta* 2005; **1668**:215–222.
45 Mady MM, Fathy MM, Youssef T, Khalil WM. Biophysical characterization of gold nanoparticles-loaded liposomes. *Phys Med* 2012; **28**:288–295.
46 Kushwaha K, Saxena J, Tripathi BK, Agarwal MK. Detection of carotenoids in psychrotrophic bacteria by spectroscopic approach. *J Biosci Biotechnol* 2014; **3**:253–260.
47 Llansola-Portoles MJ, Pascal AA, Robert B. Electronic and vibrational properties of carotenoids: from *in vitro* to in vivo. *J R Soc Interface* 2017; **14**:20170504.
48 Hannon JP, Trammell GT, Blume M, Gibbs D. X-ray resonance exchange scattering. *Phys Rev Lett* 1988; **61**:1245–1248.
49 Grudzinski W, Nierzwicki L, Welc R, Reszczynska E, Luchowski R, Czub J, Gruszecki WI. Localization and orientation of xanthophylls in a lipid bilayer. *Sci Rep* 2017; **7**:9619.
50 Palozza P, Serini S, Maggiano N, Angelini M, Boninsegna A, Di Nicuolo F, *et al*. Induction of cell cycle arrest and apoptosis in human colon adenocarcinoma cell lines by beta-carotene through down-regulation of cyclin A and Bcl-2 family proteins. *Carcinogenesis* 2002; **23**:11–18.
51 Williams AW, Boileau TW, Zhou JR, Clinton SK, Erdman JW Jr. Beta-carotene modulates human prostate cancer cell growth and may undergo intracellular metabolism to retinol. *J Nutr* 2000; **130**:728–732.
52 Hazuka MB, Edwards-Prasad J, Newman F, Kinzie JJ, Prasad KN. Beta-carotene induces morphological differentiation and decreases adenylate cyclase activity in melanoma cells in culture. *J Am Coll Nutr* 1990; **9**:143–149.
53 Jain A, Sharma G, Thakur K, Raza K, Shivhare US, Ghoshal G, Katare OP. Beta-carotene-encapsulated solid lipid nanoparticles (BC-SLNs) as promising vehicle for cancer: an investigative assessment. *AAPS Pharmscitech* 2019; **20**:100.
54 Mukherjee A, Agarwal K, Aguilar MA, Sharma A. Anticlastogenic activity of β-carotene against cyclophosphamide in mice in vivo. *Mutat Res* 1991; **263**:41–46.
55 Salvadori DM, Ribeiro LR, Oliveira MD, Pereira CA, Beçak W. The protective effect of beta-carotene on genotoxicity induced by cyclophosphamide. *Mutat Res* 1992; **265**:237–244.