RESEARCH ARTICLE

Chlorambucil and Quantum Dots Co-Loaded Nanostructured Lipid Carrier for *In Vitro* Cytotoxicity and Imaging Evaluation

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ABSTRACT

Chlorambucil (CBL) is an efficient anticancer drug. It is a lipophilic agent with serious adverse effects. The objective of this study was to formulate a CBL-loaded nanostructured lipid carrier (NLC) and targeted the solid tumors using the emulsification-ultrasonication method. Folic acid was used as a targeting moiety for MCF-7 cells. Moreover, CdSe/ZnS quantum dots (QD) were utilized to be loaded inside the nanoparticles as an imaging probe. The nanocarrier's DLS results were 121.2 ± 3.2nm PS, 0.27 ± 0.05 PDI, and -41 ± 4 mV ZP with an excellent CBL entrapment efficiency (93.08 ± 1.83%). The *in vitro* cytotoxicity assay significantly affected the targeted formula relative to the control and non-targeted one. The cellular uptake study explained the excellent cellular internalization after 1-hour incubation and a significant result for the targeted formula compared to the non-targeted one. This formulation was effective for intracellular CBL delivery and enhancing its activity as an antitumor therapy.

Keywords: Chlorambucil, Confocal laser scanning microscopy, Folic acid-targeting, MCF-7 cells, Nanostructured lipid carrier, Quantum dots.

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INTRODUCTION

The unfavorable properties of the anticancer agents as a free drug molecule inside the human body, such as high toxicity, wide distribution, and poor specificity, were the main problems related to their administration. Moreover, the anticancer drugs are mostly hydrophobic. Hence, nanocarrier drug delivery systems like nanostructured lipid carriers (NLC) are designed to change these undesired properties.[1] For more selectivity and higher activity, the active targeting by grafting molecular moiety onto the surface of NLC was rationalized to be a promising approach to design the nanocarrier for tumor cells targeting.[2] Target affinity is adjusted by selecting the targeting agent corresponding to the tumor cells' surface overexpression receptors. In this project, folic acid was selected as a molecular probe model for chemotherapy drug targeting toward cancer cells overexpressed by folate receptors such as ovarian and breast tumors.[3] The percutaneous endoscopic gastrostomy (PEG)-conjugated lipid type could also participate in the formulation process. It is constricted from the lipid part, inserted into the hydrophobic lipid core and the PEG part, which extended into the external aqueous shell. PEGylation projections from the surface contribute to the stabilization and stealth effect of the NLC. This stealthiness character makes the nanoparticles unrecognized by the reticuloendothelial system (RES) and then elongates their circulation time.[4]

Quantum dots (QDs) were developed for several biomedical and pharmaceutical applications, especially for in vivo cell imaging, cancer cell detection, and drug delivery trafficking. Comparing to conventional dyes, QDs own many advantages, such as composition and size-dependent photoluminescence (PL) emission, wide range absorption spectrum, high photostability, narrow emission spectrum, and high level of brightness.[5] Theragnostic nanoplatform is a better approach for combining therapy and diagnosis strategies. QDs loaded inside

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the nanoparticles as a diagnostic and trafficking agent, and drug as a therapeutic agent can be incorporated simultaneously as a theranostic nanocarrier.[6]

In this study, CdSe/ZnS core-shell QDs were utilized as a diagnostic and imaging probe and chlorambucil (CBL) as an anticancer therapy, both incorporated inside the NLC as a theranostic approach.

MATERIALS AND METHODS

Materials

Quantum Dots (CdSe/ZnS) was purchased from Nano Research Elements, India. Distearoyl phosphatidyl ethanolamine (DSPE), PEG2000-DSPE, and folate-PEG2000-DSPE were purchased from Xi'an Ruixi Biological Technology Co., Ltd., China. Chlorambucil was purchased from Beijing Yibai Biotechnology Co., Ltd., China. Soybean oil and soy lecithin were purchased from Shaanxi Kang New Pharmaceutical Co., Ltd., China. Poloxamer F68 was purchased from Connect Chemicals GmbH, Germany. All other solvents and chemicals were of analytical reagents or HPLC grades.

Preparation of Co-loaded (QDs and CBL) NLC

The first formulations of nanostructured lipid carrier (NLC) were prepared by an emulsification-ultrasonication method using high-pressure homogenization, followed by high energy ultrasonication with some modifications.[7] Briefly, the organic phase was composed of distearoyl phosphatidyl ethanolamine (DSPE), soybean oil, DSPE-PEG2000, and lipophilic QDs (dissolved with hexane) with or without 2mg CBL and with or without folated lipid were mixed using suitable organic solvents. The aqueous phase consisted of de-ionized water, poloxamer F68, and soya lecithin. The two phases were separately heated by about 4°C above the lipid's melting point under a hot plate magnetic stirrer at 900 rpm. The aqueous phase was subsequently pipetted into the lipid phase while the organic solvent was evaporated almost completely. After the formation of a coarse emulsion with an Ultra-Turrax® digital Homogenizer (IKA T25 basic, IKA Werke GmbH, and Co., Germany) for 5 minutes at 12000 rpm, the latter was subjected to high energy ultrasonication (Misonix ultrasonic liquid processor S-4000, Hielscher, GmbH, Germany) for 10min (on-off 4-1 sec) to form the nanosized nanocarrier.

Two main types of NLC were prepared, targeted (FNLC) and non-targeted (BNLC) formulations. Both types are consisting of QDs and with or without CBL. The weight ratio of all lipid types used was equal to (13.5:6.5:0.8:0.8) for DSPE, soybean oil, PEG2000-DSPE, and folate-PEG2000-DSPE, respectively.

Characterization of Co-loaded NLC

Zeta Potential, Particle Size, and Polydispersity Index

Zeta potential (ZP), particle size (PS), and polydispersity index (PDI) were characterized by dynamic light scattering (DLS) using Zetasizer (Nano-ZS90; Malvern Instruments, Malvern, UK) at 90º scattering angle and 25°C. The formulation samples were diluted with de-ionized water according to the final concentration available. Each sample was analyzed in triplicate. After dilution with de-ionized water for ZP, the samples were placed in the electrophoretic cell and analyzed.

Drug Loading Capacity and Encapsulation Efficiency Determination

Drug loading capacity (LC) and entrapment efficiency (EE) were determined by an indirect method, using the ultrafiltration technique to measure the unencapsulated amount of the drug. Briefly, five milliliters of each formulation were poured into the upper chamber of a centrifuge tube (Amicon Ultra, MWCO 10KDa, Sigma-Aldrich, Germany) and centrifuged for 15 minutes at 4000 rpm and 4°C. The process was repeated for washing with de-ionized water at the same parameters of centrifugation. From the lower chamber of the Amicon tube, 50 µL was diluted with 5ml of ethanol and assayed spectrophotometrically at 258 nm wavelength.[8] The EE and LC were calculated according to the following equations:

$$EE=(W_t - W_f) / W_t \times 100$$

$$LC=(W_t - W_f)/ W_l \times 100$$

Where Wt is the total drug added, Wf is the free unentrapped drug, and Wl is the total lipid added.

Characterization of NLC Morphology

The NLC formulation's morphology in an aqueous media was examined by TEM (Philips EM208S, Netherland). A drop of the diluted sample was spread on a 200-mesh copper grid and negatively stained with 2% phosphotungstic acid (PTA) for 30 seconds. The grid was dried at room temperature, stored in a light protectant capsule, and then observed using TEM. TEM measurement under different magnifications.[9]

Cell Culture

The cell line of the human breast cancer, MCF-7, was purchased from the Iranian Biological Resource Center (IBRC®, Tehran, Iran) and cultured in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% of FBS (Fetal Bovine Serum) and 1% of penicillin/streptomycin under a humidified atmosphere with 5% CO_2 at 37°C. At 80% confluence, the cells were trypsinized, resuspended in complete medium (DMEM with FBS and antibiotics), and counted to evaluate the cell density. Cells were observed under a phase-contrast microscope (Olympus CKX41) to assess their overall appearance.

In vitro Cytotoxicity Assay

MCF-7 cells were seeded at the concentration of 2×10^4 cells/well into a 96-well plate. After 24 hours incubation, the cell culture medium was replaced by a culture medium containing 0, 1, 5, 10, 50, 100, 500, and 1000 µg/mL CBL. After 72 hours of incubation, the supernatants were removed. Subsequently, 50 µL of fresh DMEM was added to each well, followed by adding 50 µL of 5 mg/ml of 3-(4,5-dimethyl thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) and incubated for 3-4 hours at 37°C and 5% CO2 humidified atmosphere. Afterward, 150 µL DMSO was added to each well while shaking for 15 min; a microplate reader (Biotec, Tecan US, Inc.) was used to record the absorbance at 570 nm.[10] The viability of

the treatment groups was reported as the percentage of controls put on 100%. The mean and the standard error of the mean (SEM) of cell viability for each treatment were determined. The viability percentage calculations were performed by using the formula:

$$\% \text{ Viability} = (\text{Mean OD of sample}) / (\text{Mean OD of control}) \times 100$$

Where OD sample and OD control represent the absorbance determined for cells treated with formulations and for control, respectively.

The next step, the cytotoxicity of IC50 of free CBL solution and CBL loaded in lipid nanocarrier containing QDs with or without folic acid (FA) against MCF-7, was investigated using MTT assay. Control cells were cultured simultaneously in a time period (72 hours).

In vitro Cellular Uptake Study

For the confocal laser scanning microscopy (CLSM) study, MCF-7 cells were cultured on microscope slides in a 6-well plate (5×10^5 cells per well) for 24 hours until total adhesion was achieved. Then the cells were treated with targeted and non-targeted formulations. After 1 hour incubation at 37°C, the culture medium was removed, and the cells on the microscope slides were washed with ice-cold PBS three times. The cells were then fixed with paraformaldehyde solution 4% for 15–20 minutes, followed by cell nuclei staining with Hoechst 33258 (2 µg/mL) for another 5 to 10 minutes and washing three times with PBS. Finally, using a laser confocal microscope (Nikon Eclipse Ti, USA) captures and analyzes the fluorescent images of the cells.[11]

Statistical Analysis

All quantitative data were expressed as mean with ± standard deviation (SD), and the experiments were repeated three times at least. Every two quantitative values of interest were statistically analyzed to determine their difference was significant or not. The p-values of <0.05 and <0.01 were accepted as indicative of statistical difference and significant difference.[12]

RESULTS AND DISCUSSION

Preparation Method Evaluation

A method of emulsification-ultrasonication was applied to prepare the formulations of targeted and non-targeted NLC loaded and unloaded with CBL, to get a therapeutic, diagnostic, or theranostic nanocarrier. The representative image shown in Figure 1 indicates clear dispersion of the formulation. The outer appearance with transparency coordinate with the nanosized region. There is some difference in the clarity and transparency between targeted and non-targeted ones due to the folated-type lipid involvement in the targeted formulation.

Characterization of Co-loaded NLC

Mean particle size, polydispersity index, and zeta potential reports of the formulations encapsulated with QDs, surface grafted with folic acid or non-grafted, were illustrated in Figure 2. The mean particle sizes for BNLC and FNLC were 111.6 ± 2.3 nm and 121.2 ± 3.2nm, and PDI values were 0.23 ± 0.02 and 0.27 ± 0.05, respectively. The mean particle size for both formulations was within the acceptable range of the parenteral administration. It was recorded that the nanoparticles should not be very small or very large and the allowable limit is below 200 nm.[13] There was a low difference in PS between the two different formulations; it might be due to the difference in folate-type lipid addition to the targeted formulation. The low PDI values (below 0.3) indicate the uniform and narrow size distribution. Logically less than 0.5 can be used as a feasible dispersion of the nanoparticle formulation.[14]

Zeta potential is a potential surface function. The amplitude of the zeta potential exhibits the stability of the emulsified solution. Zeta potential greater than about ± 25mV is expected to be enough for colloidal stabilization.[15] The ZP for co-loaded

Figure 1: A: Representitive photographs of targeted (FNLC) and non-targeted (BNLC) formulations, in the right and left site, respectively; B: Representative photographs of targeted (FNLC) and non-targeted (BNLC) formulations, in the right and left site, respectively.

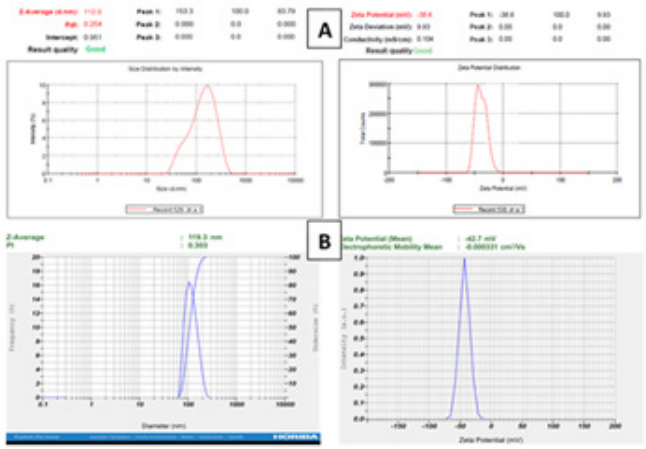


Figure 3: Mean size distribution and zeta potential of the BNLC (A) and FNLC (B) formulations.

NLC formulations was -37 ± 2mV and -41 ± 4mV for BNLC and FNLC, respectively. There is a difference in the amplitude of ZP between these two formulations, observing a higher absolute ZP value for the targeted one. This difference could be attributed to the terminal carboxyl group of the folic acid in FNLC formulation.[3] This value also confirms the outside projections of the folic acid. These terminal projections explain the difference in particle size, the magnitude of surface charge, and the right site for folic acid receptors affinity for cancer cells targeting.

The encapsulation efficiency and loading capacity were 91.73 ± 1.37% and 9.17 ± 0.13% for the BNLC formula, and 93.08 ± 1.83% and 9.30 ± 0.18% for FNLC, respectively. Solid lipid DSPE arranged with PEGylated one in a similar manner. At the same time, soybean oil which contains mono- and poly-unsaturated alpha-linoleic acid and linoleic acid, could create the " kink" like pattern between the solid lipid fatty acid chains, hence decrease lipid crystallinity, increase imperfection, and create voids permitting spaces for more drug incorporation.[16]

Characterization of NLC Morphology

Photographs of the transmission electron microscope (TEM) showed that the NLC has an ellipsoidal shape between sphere and rod shapes (Figure 3). This result is the same as what Zhang *et al.*[17] got in his research. As particle diameter was calculated using TEM and dynamic light scattering techniques, slight variations in particle size were observed. Particles seem smaller in size when measured by TEM comparing with the larger diameter revealed by DLS. In the DLS technique, NLCs are detected in an aqueous state, and lipid nanoparticles are extremely hydrated, and their diameters are hydrated and typically bigger than their actual sizes; hence, DLS measures a hydrodynamic diameter; in contrast, TEM has a drying step before measurement and measure the actual shape and size of the particles.[18]

In vivo Cytotoxicity Assay

A human breast cancer MCF-7 cell line was utilized in this study to simulate the activity of the prepared formulas on the cancer cells of breast cancer. Moreover, folic acid targeted formula FNLC effectiveness on MCF-7 cells could be evaluated, considering the overexpression of folic acid receptors on the surface of MCF-7 cells.[19] Chlorambucil solution at different concentrations (0, 1, 5, 10, 50, 100, 500, and 1000 µg/mL) were incubated for 72 hours in MCF-7 cells culture medium. An MTT reagent was then added, and cell viability was estimated to investigate the IC50 of CBL. Chlorambucil IC50 histogram was drawn using CBL concentration versus the percentage of cell viability (Figure 4), showing that the IC50 for CBL is 100 µg/mL.

Various formulations were studied in this assay to evaluate the effect of QD, CBL, and folic acid on the cell viability of MCF-7. The formulations of BNLC, CBL-BNLC, and CBL-FNLC were analyzed to study the effect of blanc formulation, anticancer activity of CBL, and folic acid targeting effects, respectively. Each formulation was prepared in a concentration equivalent to the IC50 of CBL. The proliferation of MCF-7 cells was significantly ($P < 0.001$) inhibited after treating with targeted or non-targeted theranostic lipid formulations (i.e., CBL-BNLC and CBL-FNLC). At the same time, there was a lower significant ($P < 0.05$) cytotoxicity property when using blanc formulation BNLC with control (see Figure 5).

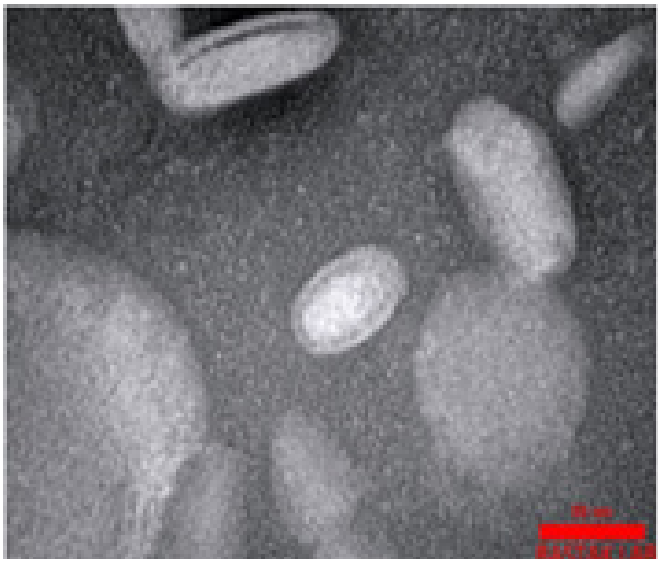


Figure 3: Morphology of NLC formulation observed by TEM at 50 nm scale bar.

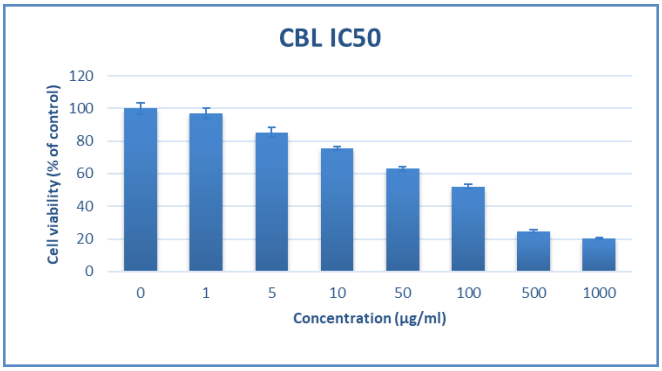


Figure 4: Chlorambucil IC50 histogram in MCF-7 cell line for 72 hours incubation at 37°C.

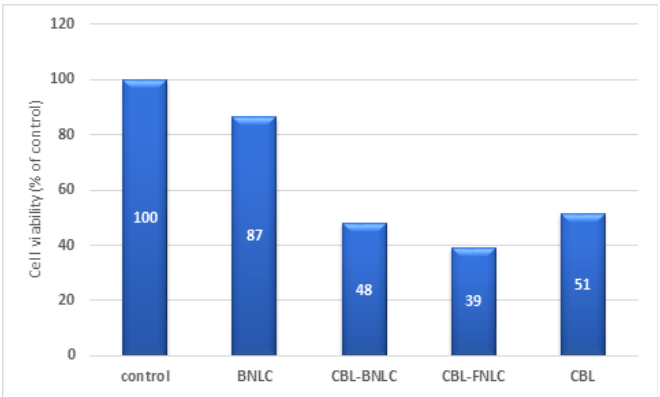
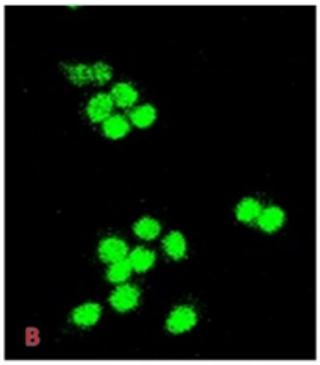
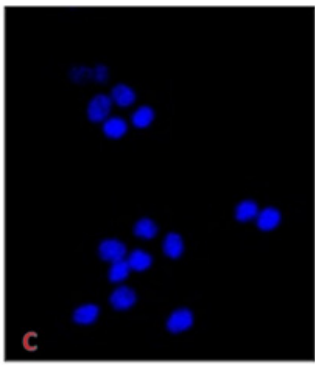
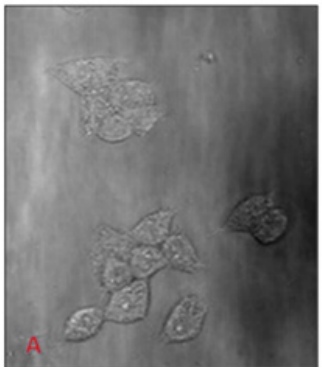


Figure 5: Cell viability of MCF-7 after 72hr incubation with different formulas, BNLC, CBL-BNLC, CBL-FNLC, and free CBL.







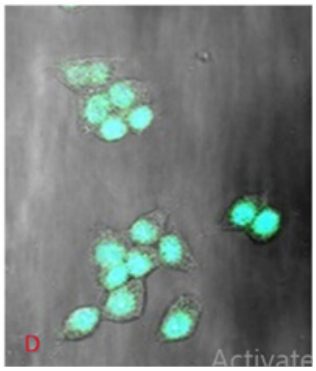
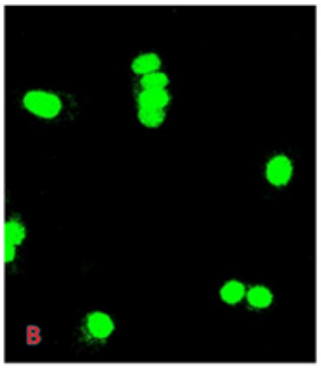
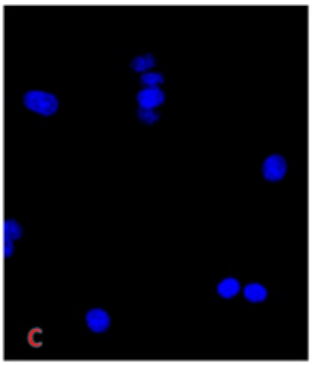
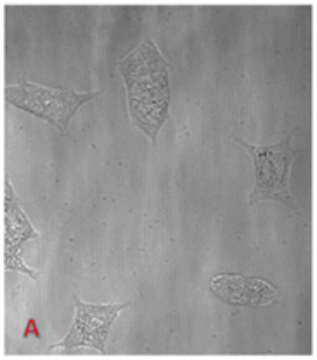


Figure 6: CLSM images of MCF-7 cell after 1hr incubation for targetd formulation. (A) is the bright field images, (B) is the blue fluorescence of the nucleus dyed by Hoechst333258, (C) is the green fluorescence of the FNLC, and (D) is the merge image.







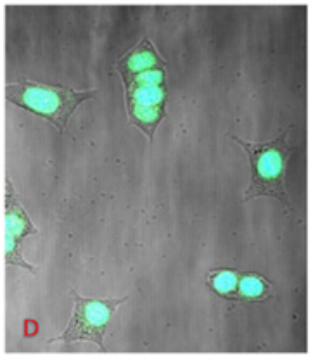


Figure 7: CLSM images of MCF-7 cell after 1hr incubation for non-targetd formulation. (A) is the bright field images, (B) is the blue fluorescence of the nucleus dyed by Hoechst333258, (C) is the green fluorescence of the BNLC, and (D) is the merge image.

The low cytotoxicity of the blanc formulation BNLC could be attributed to lecithin and non-ionic emulsifiers. The cytotoxicity effect might be due to particle adherence to the cell membrane, particle internalization, and degradation of the cell culture medium's products. On the other hand, although emulsifiers are considered toxic, it is safe to use lipid nanoparticles because living organisms are well tolerable to the lipids.[20] The high significant cell viability inhibition percentage related to the targeted formulation CBL-FNLC compared with non-targeted formulation CBL-BNLC was attributed to the folic acid ligand's targeting property toward MCF-7 cell due to it is overexpressed with folate receptors. Zhang *et al*. demonstrated that the *in vitro* experiments of folic acid-conjugated PEGylated nanoparticles have easily entered the breast cancer cells relative to non-targeted PEGylated nanoparticles or folic acid-conjugated nanoparticles directly bound to the surface without PEG linker.[21] Besides, it might be concluded that the cytotoxic activity of drug-loaded NLC, meaning CBL-BNLC and CBL-FNLC, was attributed to the therapeutic impact of the chemotherapy drug (CBL).

In vitro Cellular Uptake Study

MCF-7 cell line was utilized to investigate the QD-loaded nanoparticles' cellular internalization in targeted and non-targeted formulations. After 1hr incubation, there was higher QD fluorescence density for the targeted formula (FNLC), see Figure 6, comparing with the non-targeted one (BNLC), see Figure 7, which meaning higher cellular uptake. However, both formulas revealed a suitable uptake property. Hoechst 333258 is a DNA-specific fluorescence dye representing the nucleus site as a blue stain. Hence, the results not only prove that NCL formulas can enter the cells but also it can reach the nucleus.[22] Quantum dots emit a green fluorescence color according to their PL emission spectra. Hence, the green spots or dots in the slide field under CLSM scanning refer to the QD-loaded formulation. Cytoplasmic green dots in BNLC images were higher than those of the FNLC formula, meaning some BNLC formulations are still in the cytoplasm. These results were explained by the higher cellular uptake of the targeted formula and faster nucleus accumulation comparing with non-targeted one.

This data suggests that the folic acid targeted formula was integrated with the cell via the folic acid receptor, expressed on the surface of MCF-7, and internalized via the receptor-mediated endocytosis process. In contrast, the non-targeted formula was mostly entered inside the cell via the endocytosis method.[23] The higher cellular internalization related to folic acid targeted formulation was the same as that of Muthu *et al*. through the preparation of theranostic folic acid targeted liposome for co-delivery of quantum dots docetaxel.[24]

CONCLUSION

In this study, the co-loaded NLC was successfully formulated for parenteral use with an acceptable particle size limit, good physical stability, and high drug entrapment efficiency. There was significant inhibition of MCF-7 cancer cell proliferation. A high cell internalization confirms the high

ability for accessibility and cellular uptake for both formulations. Moreover, the pssssarticle surface grafting with folic acid improves its cytotoxicity ability and cellular internalization concerning the folic acid receptor overexpression. These results affirm that this formula could be the most efficient for breast cancer therapy.

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