Research Article

Development and Evaluation of Artesunate-Loaded Chitosan-Coated Lipid Nanocapsule as a Potential Drug Delivery System Against Breast Cancer

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Abstract. Artesunate (ART)—a well-known hydrophobic anti-malarial agent was incorporated in a polymer-lipid hybrid nanocolloidal system for anti-cancer therapeutic. The lipid negatively charged nanoemulsion was formulated by modified hot homogenization method then covered with positively charged chitosan via electrostatic interaction to obtain chitosan-coated lipid nanocapsule (ART-CLN). Physical properties of the system were characterized in terms of size, charge, morphology, drug loading capacity, and physical state. In addition, anti-cancer activities were confirmed by conducting MTT assay for ART and ART-CLN on different cancer cell lines. Obtained ART-CLN after coating chitosan revealed positive charge (13.2 ± 0.87 mV), small particle size (160.9 ± 3.5 nm), and spherical shape. High drug entrapment efficiency ($95.49 \pm 1.13\%$) and sustained release pattern were observed. Moreover, the good cellular uptake was recorded by flow cytometry as well as confocal image. Finally, ART-CLN exhibited stronger anti-cancer activity than free ART on breast cancer cell lines (MCF-7, MDA-MB-231). These results suggested that by loading ART into lipid core of polymer-lipid hybrid carrier, the activity and physical stability of ART can be significantly increased for cancer chemotherapy.

KEY WORDS: anti-cancer; artesunate; breast cancer; chitosan; lipid nanoparticles.

INTRODUCTION

Artesunate (ART), a derivative of artemisinin (the active principle of *Artemisia annua*; sweet wormwood), initially used in the anti-malarial combination therapy against multidrug-resistant *falciparum* and *vivax* malaria was later discovered by the National Cancer Institute (NCI, USA) to possess potent anti-proliferative and anti-angiogenic activity against cancer, especially against breast cancer, leukemia, and colon cancer (1). Though it has been well established that oxidative stress-reactive oxygen species (ROS) is the major cause of anti-neoplastic action of ART, the full mechanism of action is still being studied. In combinational chemotherapy, ART has been reported to offer no cross resistance and no overlapping toxicities with other anti-cancer agents. Indeed, with excellent safety profile, ART has been shown to enhance potency and reduce dose of other more toxic anti-cancer agents, with little to no additional side effects (2). However, ART has poor aqueous solubility and is easily degraded in

acidic condition, which translates to low oral bioavailability (~40%) (3,4). In addition, the drug has very short half-life (5,6). In order to overcome these drawbacks, some groups tried to incorporate ART into lipid system like liposome (7) or solid lipid microparticles (8). However, the efficacy of plain drug as well as drug-loaded formulation on anti-cancer model has not yet been studied.

In this work, a polymer-lipid hybrid nanocolloidal system was formulated by solvent-free procedure. The oily liquid core of oleoyl polyoxyglyceride Labrafil-containing drug was enveloped by a membrane made of lecithin and low molecular weight chitosan. The anionic nanoemulsion prepared by high-shear emulsification method was coated with cationic polymer chitosan to provide outer rigid hydrophilic polymer layer. This system is very versatile with tunable particle size and narrow size distribution. Chitosan is a hydrophilic linear copolymer composed of random units of β -1-4-*N*-acetyl-D-glucosamine and D-glucosamine and is derived by *N*-deacetylation of chitin (9,10). It exhibits polycationic nature at pH below its pK_a (~6.5) owing to the presence of primary amines (11). Thus, at pH lower than 6, it gets protonated, develops positive charge, interacts with lecithin electrostatically, and helps to form the outer envelope of the nanocapsule. The protonation of chitosan in acidic extracellular matrix of solid tumor helps nanoparticles to adhere to tumor cell membrane glycocalyx via electrostatic interaction (12). This characteristic is also responsible for its mucoadhesive property. Moreover, chitosan is known to confer stealth properties to nanoparticles, thereby

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evading recognition and uptake by mononuclear phagocytic system and increasing their blood circulation half-life. Besides, chitosan has been reported to act on tumor cells independently and induce apoptosis by interfering with cell metabolism (13).

The aim of this study is to improve physicochemical properties and anti-neoplastic activity on cancer cell lines of artesunate by loading into chitosan-coated lipid nanocapsules (CLN). The physicochemical properties of artesunate-loaded CLN (ART-CLN) characterized were particle size, zeta potential, drug entrapment efficiency, and *in vitro* drug release behavior. The cellular uptake efficacy of ART-CLN in human breast cancer cells (MCF-7 and MDA-MB-231) were evaluated by flow cytometry and confocal laser scanning microscopy (CLSM) analyses. The *in vitro* toxicity as well as cell apoptosis was further studied to confirm the potential of ART-CLN for delivering ART in cancer therapy.

MATERIALS AND METHODS

Materials

Artesunate (ART) was provided by Sao Kim Pharma (Hanoi, Vietnam). Lecithin (Phospholipids GmbH, Ludwigshafen, Germany) is a combination of vegetable phospholipids. Polysorbate 80 (Tween 80), a non-ionic surfactant, was purchased from Duksan Chemical Co (Ansan, South Korea). Chitosan (low molecular weight, 50–190 kDa), a cationic polysaccharide, and Thiazolyl Blue Tetrazolium Blue (MTT) were purchased from Sigma-Aldrich Chemical Co. Ltd, Sigma (St. Louis, MO, USA). Labrafil® M1944CS (Labrafil) was purchased from Gattefosse (St-Priest, France). Palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-sn-glycero-3-phosphocholine (NBD-PC) was provided by Avanti Polar Lipid, Inc. (Alabama, USA), whereas 2,5'-Bi-1H-benzimidazole, 2'-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-23491-52-3 (Hoechst 33324), and LysoTracker Red were supplied by Thermo Fisher Scientific Inc. (Waltham, MA, USA). Deionized water was used and was freshly prepared using a Milli Q-water purification system (Millipore, MA, USA) whenever required. All other chemicals were of analytical grade and were used without further purification.

Preparation of Blank Chitosan-Coated Lipid Nanoparticles

Chitosan-coated lipid nanocapsules were prepared by hot homogenization method. Briefly, the lipid phase was prepared by melting the mixture of lecithin, Labrafil, and Tween 80 (weight ratio, 6:3:1) at 50°C accompanied with vigorous stirring. Five milliliters of distilled water was slowly added to the lipid phase with continued stirring (1200 rpm) for 2 min to form coarse emulsion. The obtained emulsion was sonicated by a probe sonicator (Vibracell VCX130, Sonics, USA) at 100 W for 3 min. Then, the obtained nanoemulsion was chilled in ice for 30 min. Blank nanocapsules containing different lecithin/chitosan ratios were fabricated by slowly adding the nanoemulsion to 1% (w/v) chitosan aqueous solution. The formulation was continuously stirred for 1 h at room temperature. Finally, the CLN was washed three times with water and stored at 4°C until further analysis.

Preparation of Artesunate-Loaded CLNs

ART-CLNs were prepared by dissolving different amounts of drug (2.5, 5, 7.5, 10 mg) in the lipid phase under stirring to form a homogeneous solution. Then the drug-loaded nanocapsules were processed using the same procedure as the blank nanocapsule preparation. All components are described clearly in Table I.

Lyophilization

Lyophilization of CLN dispersion was carried out with mannitol as a cryoprotectant using freeze dryer (FDA5518, IIShin, South Korea). The dispersion was pre-frozen (−80°C) for 12 h and subsequently lyophilized at a temperature of −25°C for 24 h followed by a secondary drying phase of 12 h at 20°C.

Physicochemical Characterization

Particle Size and Zeta Potential

Particle size and polydispersity index (PDI) of nanocapsules were measured by the dynamic light scattering (DLS) method using Zetasizer Nano 90 (Malvern Instruments Ltd, Worcestershire, UK) at a fixed angle of 90°. The zeta potential was analyzed using the same device, with cuvettes primarily designed for zeta potential measurement. Samples were diluted ten times before measurements. All experiments were performed three times and expressed as the mean ± standard deviation (SD).

Transmission Electron Microscopy Analysis

The morphology of the particles was observed by using transmission electron microscopy analysis (TEM H7600, Hitachi, Tokyo, Japan). A single drop of nanocapsule sample, stained with phosphotungstic acid (2%, w/v) for contrast enhancement was placed on a copper grid coated with a carbon film. The grid was dried for 10 min under an infrared lamp prior to the analysis.

Table I. Composition of Artesunate-Loaded Chitosan-Coated Lipid Nanoparticles

Formulation	Labrafil (mg)	Lecithin (mg)	Tween 80 (mg)	Chitosan (mg)	ART (mg)
1	300	50	150	0	0
2	300	50	150	0.5	0
3	300	50	150	1.25	0
4	300	50	150	2.5	0
5	300	50	150	3.75	0
6	300	50	150	5	0
7	300	50	150	2.5	2.5
8	300	50	150	2.5	5
9	300	50	150	2.5	7.5
10	300	50	150	2.5	10

X-ray Diffraction

Diffraction patterns were obtained using a PANalytical X'Pert pro X-ray diffractometer system (PANalytical, Almelo, The Netherlands). The X-ray generator was operated at 40 kV and 30 mA. The characteristic K α copper line with a wavelength of 1.54 Å was generated. The range of the diffraction patterns was between 10° and 50° 2-theta.

Fourier Transform Infrared Spectroscopy

The interaction between drug and the other components was confirmed by Fourier transform infrared spectroscopy using a Thermo Scientific Nicolet Nexus 670 Fourier Transform Infrared Spectroscopy (FT-IR) Spectrometer and Smart iTR with a diamond window (Thermo Fisher Scientific Inc., Waltham, MA). The spectra were employed at 550–4000 cm⁻¹ and obtained at a resolution of 4 cm⁻¹ and at 32 scans per sample. Background scanning and correction were performed before each measurement, and all measurements were performed thrice at room temperature.

Encapsulation Efficiency and Drug Loading

The drug encapsulation efficiency and loading capacity were determined using indirect method by calculating the free drug in the filtrate. Firstly, 1 mL of ART-CLN suspension was centrifuged in ultrafiltration tube (MWCO, 10 kDa; Amicon Ultra, Millipore Co., USA) at 4500 rpm for 15 min (5,14). The amount of free drug in filtrated solution was analyzed using HPLC system (Model L2100, Hitachi, Japan) with C18 analytic column (Inertsil ODS3, GL Sciences Inc., Tokyo, Japan). The mobile phase was the mixture of acetonitrile-phosphate buffer solution (pH 3.0) at a ratio of 55:45 (v/v). Flow rate was set at 1.0 mL/min and 50 µL injection volume. Encapsulation efficiency (EE) and drug loading capacity (LC) were calculated by the following equations:

$$EE(\%) = (W_{\text{initial drug}} - W_{\text{unbound drug}}) / W_{\text{initial drug}} \times 100$$

$$LC(\%) = (W_{\text{initial drug}} - W_{\text{unbound drug}}) / W_{\text{CLN}} \times 100$$

In Vitro Drug Release Study

In vitro release study of ART-CLN was performed within the 48 h of preparation. Two milliliters of ART-CLN suspension was charged into a dialysis bag (MWCO, 3.5 kDa) and immersed in 30 mL of phosphate buffer solution (PBS; pH 7.0) as release media and subsequently put into a 50-mL Falcon tube. Then the tube was placed in an orbital water bath shaker (HST-205 SW, Hanbaek ST Co., South Korea) at 100 rpm and 37°C temperature. At specific time points, 0.5 mL of samples were taken and the same volume of fresh media was added. The quantity of ART-CLN was analyzed by HPLC

system mentioned above. The ART release data were kinetically evaluated by (8):

$$\begin{aligned} \text{Zero - order : } C &= K t \\ \text{First-order : } C &= [1 - \exp(-Kt)] \times 100 \\ \text{Higuchi : } C &= K t^{0.5} \\ \text{Korsmeyer-Peppas models : } C &= K t^n \end{aligned}$$

where C is ART concentration (percent) at time t , K is kinetic constant, and n is release exponent.

In Vitro Cytotoxicity of ART-Loaded CLN

The human breast adenocarcinoma MCF-7 and MDA-MB-231 cells were originally obtained from the Korean Cell Line Bank (Seoul, South Korea). All cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 containing 10% fetal bovine serum (FBS), 100 U/mL penicillin G sodium, and 100 µg/mL streptomycin sulfate and incubated at 37°C in 5% CO₂ atmosphere. All the cell threats such as fungi, bacteria, mycoplasma, etc. were eliminated before carrying out the experiments.

Cellular cytotoxicity was assessed by MTT assay with breast cancer cell line (MCF-7 and MDA-MB-231). Sample cells were plated in 96-well plates at 1×10⁴ cells per well density for 24 h. Then the cells were treated with blank nanocapsules, free ART, and ART-CLN nanocapsules in various concentrations (1, 10, 25, 50, 100 µg/µL) for 24 h. MTT solution (100 L of 1.25 mg/mL MTT in supplemented RPMI medium) was added to the cultures and incubated at 37°C. After 4 h, 100 µL of dimethyl sulfoxide was added to each well to dissolve purple formazan. The absorbance of individual well was analyzed at 570 nm using a microplate reader. Cell viability was determined based on the following equation:

$$\text{Cell viability}(\%) = \frac{OD_{570}(\text{sample}) - OD_{570}(\text{blank})}{OD_{570}(\text{control}) - OD_{570}(\text{blank})} \times 100$$

Flow Cytometry Study

In cellular uptake measurement, cells were seeded in 6-well plates with a density of 2×10⁵ cells per well in 2.0 mL RPMI and incubated for 24 h, and then treated with NBD-PC-CLN at concentration of 2 µg/mL for 30 min. Cells without NBD-PC treatment were used as the control. The harvested cells were suspended in PBS and analyzed by flow cytometer. All studies using flow cytometry method were carried out by fluorescence-activated cell sorting (FACS) Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) in all cell lines.

Confocal Laser Scanning Microscopy

The cells were seeded on coverslip in 12-well plates with a density of 1×10⁵ cells per well in 2 mL of RPMI medium and cultured for 24 h. For the cellular uptake studies, NBD-PC-labeled CLNs in media was replaced for old media. After 30 min incubation, the cells were continuously stained with Lyso Tracker Red at concentration of 1 µg/mL for 10 min then the cells were washed and fixed with 4% formaldehyde. On

the other hand, nuclear morphologies of cell lines under different treatments were observed by the Hoechst 33342 staining method; 10^5 cells per well were seeded onto a 12-well plate, with coverslips over each well and incubated for 24 h. The cells were then treated with medium containing ART or ART-CLNs at ART concentrations of 25 $\mu\text{g/mL}$. The control group was treated with drug-free culture medium. After incubation for 24 h, the wells were rinsed three times with cold PBS and then fixed with 200 μL of 4% paraformaldehyde for 15 min. The cells were further washed three times with 500 μL of PBS and stained with 200 μL of Hoechst 33342 (10 $\mu\text{g/mL}$) for 8 min. The cellular and intracellular localization of NBD-PC-CLNs and nuclei morphology were observed using a laser scanning confocal microscope Leica TCS SP2 (Leica Co., Wetzlar, Germany).

Stability Study

The stability of ART-CLNs was evaluated by storing the formulations in different refrigerated conditions: (1) in dispersed and (2) lyophilized forms. After 30 days of storage, the following physical properties of ART-CLNs were characterized: particle size, size distribution, zeta potential, drug loading capacity, and entrapment efficiency.

RESULTS AND DISCUSSION

Preparation of ART-CLNs

ART-CLN (Scheme 1) was fabricated by modified hot homogenization method. ART, a hydrophobic drug was solubilized in a melted solution containing (a) Labrafil, an oleoyl polyoxyglyceride derived from apricot kernel oil, (b) a

lipophilic surfactant lecithin, and (c) non-ionic surfactant Tween 80, under magnetic stirring. This solution was mixed with water to form coarse emulsion which was subjected to high shear via probe sonicator to obtain nanoemulsions. Lecithin is a negatively charged food-grade emulsifier that can produce small oil droplets during homogenization and sonication (15). According to our scheme, lecithin molecules get anchored on the oil–water interface of the oil nanodroplets, with hydrophobic tail in the oil phase and hydrophilic and negatively charged phosphate head group in the water phase, thereby conferring surface negative charge to the nanoemulsions. Chitosan dissolved in 1% acetic acid gets protonated and develops a positive charge, which then electrostatically adsorbs onto the nanoemulsion forming a rigid layer. This rigid layer also provides protection for ART which tends to degrade in an acidic environment. In this study, Tween 80 was used along with lecithin to produce a more stable nanoemulsion, as suggested by our previous studies (14,16). All components used in this work are approved by the FDA for oral, topical, and parenteral administration. In order to obtain the highest loading capacity, the lipid was selected based on the solubility study of ART in various lipids. Labrafil showed the best performance among the oil tested in terms of solubility of ART (data not shown).

The effects of different lecithin/chitosan ratios on CLN size and size distribution were evaluated to find the optimum ratios. As shown in Fig. 1a, particle size, PDI, and zeta

Scheme 1. Illustration of artesunate-loaded chitosan-coated lipid nanocapsules

Fig. 1. Effect of **a** lecithin/chitosan ratio and **b** drug concentration on formulation parameters: particle size, polydispersity index (PDI), and zeta potential (ZP). Data are expressed as mean $\pm$ SD ($n=3$)

Table II. Drug Entrapment Efficiency and Loading Capacity of Artesunate-Loaded Chitosan-Coated Lipid Nanoparticles

Formulation	Entrapment efficiency (%)	Loading capacity (%)
7	93.00±1.69	0.47±0.01
8	95.49±1.13	0.95±0.01
9	92.93±1.47	1.39±0.02
10	88.59±3.56	1.77±0.07

Data are expressed as mean±SD (*n*=3)

potential were measured to confirm the formation of a chitosan coating layer on the particles' surface. When the lecithin/chitosan ratio decreased at a constant lecithin amount, the particle size increased from 60.5±2.3 to 96.7±2.6 nm and the zeta potential reversed from highly negative to a positive territory confirming that lipid core was coated by a chitosan layer. Chitosan, along with its own bioactivity and natural positive charge also provides potential for enhanced bioadhesion to negatively charged cell membrane glycolyx via electrostatic attraction (17). However, strongly positive nanoparticles are quickly adsorbed by serum proteins onto their surface that "tag" them for removal by the mononuclear phagocyte system (MPS) inside the liver and spleen (18) so the minor positive charge on the nanocapsule surface was deemed more suitable for further studies and a formulation containing a lecithin/chitosan ratio of 20/1 was selected as an optimum formulation for ART loading.

Figure 1b indicates that when drug concentration increased, there was concomitant increase in the particle size. However, ART being non-ionic, the zeta potential remained almost the same. On the other hand, by increasing the amount of ART added to the preparation, the encapsulation efficiency decreased from 93.00±1.69% to 88.59±3.56% (Table II).

Fig. 2. TEM image of ART-CLN

Based on the particle size as well as loading efficacy, formulation 8 containing a lecithin/chitosan ratio of 20/1 and 5 mg ART was selected for further studies.

Characterization of ART-CLNs

Transmission electron micrographs of ART-CLN are shown in Fig. 2. They show that the nanocapsules are spherical and poly-dispersed, with sizes in range of 160 nm which is consistent with the DLS data. The structure of particle shows a compact lipid core surrounded by a contrasted layer of chitosan which confirms the concept of this study.

The inner structures of the lipid nanoparticles were studied by X-ray diffraction (19). The X-ray diffraction patterns of the lyophilized blank CLN and ART-CLN powders as well as ART are displayed in Fig. 3a. Mannitol was used as a cryoprotectant which demonstrated the low intensity peaks in X-ray diffraction pattern of blank CLN and ART-CLN. The

Fig. 3. a X-ray diffraction patterns of free ART, blank CLN, and ART-CLN. b FT-IR spectra of free ART, chitosan, and ART-CLN

Fig. 4. *In vitro* drug release (data are expressed as mean $\pm$ SD, $n=3$)

diffraction curve of pure ART is different from that of the blank CLN and ART-CLN. The diffractogram of ART exhibits sharp peaks at 2θ equals 12.2° , 12.9° , 14.2° , 15.4° , 18.5° , 19.8° , and 21.9° , corresponding to the crystalline nature of ART (6). However, those peaks disappeared on diffractogram of ART-CLN, indicating that the drug was in an amorphous form or molecularly dispersed form (20,21).

FT-IR studies were performed to detect the possible interactions between the ART and other components in the ART-CLNs. Figure 3b shows the IR spectra of ART and chitosan and their formulations. Pure ART displays a peak characteristic to the O–H bending vibration at 3272.3 cm^{-1} and a band with a main strong peak at 1753.3 cm^{-1} indicative of the C=O stretch of the ester group. The similar pattern of ART-CLN spectra especially at the important wavelength points suggests that there was no chemical change between the drug and other components and ART structure was maintained (5,6).

In Vitro Drug Release

In vitro drug release studies were carried out at 37°C in pH 7.0 media. ART-CLN showed a biphasic release profile with a sustained release characteristic after a burst release at the initial state ($\sim 35\%$ after 6 h) (Fig. 4). The burst release phenomenon demonstrated that some ART molecules were adsorbed onto the particle surface, while the sustained release characteristics suggested the diffusion of ART from the core of the lipid matrix (22). The

physicochemical nature of ART and its interaction with the lipid nanoparticles may be the reason for this biphasic release pattern. ART release from CLNs was kinetically studied using zero-order, first-order, Higuchi, and Korsmeyer–Peppas models. Table III lists the model constants and their corresponding correlation coefficients. According to their correlation coefficients, the best model was found to be Korsmeyer–Peppas and the worst model was the first order. On another hand, the n value is 0.400 (<0.5), indicating that the release phenomenon is mainly governed by Fickian diffusion mechanism (8).

In Vitro Cellular Uptake

The internalization of the drug as well as carrier into the cells is the pre-requisite for its pharmacological effect. To evaluate the cellular uptake of the ART-CLN, MCF-7 and MDA-MB-231 cells were incubated with NBD-PC-labeled CLN nanoparticles at 37°C , and then studied by CLSM and FACS. Figure 5 clearly shows that after 30 min of incubation, the uptake of CLN significantly shifted to the right side compared with the control (free treated), which indicates that the CLN nanoparticles penetrated into the cells. In addition, as shown in Fig. 6, the internalization of NBD-PC-labeled CLN nanoparticles in cells can be clearly observed. The cells exhibit red color in the cytoplasm region with a diffused distribution, suggesting that CLN nanoparticles (stained green) are mainly distributed uniformly in the entire cell cytoplasm and not in the nucleus. Chitosan may play some important roles to enhance intracellular uptake. The electrostatic interaction between positive-charged polymer and the negative-charged cell membrane urges the penetration of particles into the cell. On another hand, some studies noticed the good non-ionic interaction between chitosan backbone and the cell surface proteins (23).

Cytotoxicity Studies of the ART-CLN

Cytotoxicity is one of the major concerns when developing drug delivery systems for anti-cancer drug. The viability of MCF-7 and MDA-MB-231 cells in the presence of various concentrations (1 to $100\text{ }\mu\text{g/mL}$ ART concentration accordingly) of blank CLN, free ART, and ART-CLN are displayed in Fig. 7. Blank CLN induced insignificant cell growth inhibition (cell viability of more than 80%) at each concentration and time interval tested. According to ISO 10993-5, the blank CLN is safe for biomedical application (14). However, at a higher concentration or longer incubation time, some proportions of cells were killed which could be the effect of chitosan (24). In both cell lines, free ART and ART-CLNs showed a clear dose- and time-dependent effect. In case of MCF-7 cell

Table III. Correlation Coefficient and Constants of Release Kinetics of Artesunate from Chitosan-Coated Lipid Nanocapsules

	Kinetic model					
	Zero-order	First-order	Higuchi	Weibull	Hixson–Crowell	Korsmeyer–Peppas
R^2	0.7826	0.5751	0.9274	0.7761	0.6516	0.9475
$K\text{ (h}^{-1}\text{)}$	1.7402	0.0624	10.728	0.3999	0.0617	16.184

Fig. 5. Quantitative uptake of analysis by flow cytometry of CLN on **a** MCF-7 cells and **b** MDA-MB-231 cells. Control (*purple*) and treated CLN (*orange*)

line, at concentration of 10 $\mu\text{g/mL}$ of ART, the cell viability after 24 h of incubation is 77.6% whereas after 48 h of incubation is 54.6%. The phenomenon is similar in case of MDA-MB-231, and they are consistent with previous studies (25).

Furthermore, ART-CLN induced cell growth inhibition statistically when compared with the free ART ($p < 0.05$). These results demonstrate that ART not only maintained the biological activity but also augmented it after encapsulation into

Fig. 6. Intracellular uptake of CLN into MCF-7 and MDA-MB-231 cells by confocal laser scan microscope images. CLN containing NBD-PC (*green*) and LysoTracker Red (*red*) staining lysosome were used for this experiment

Fig. 7. *In vitro* cytotoxicity of blank CLNs, free ART, and ART-CLNs after 24- and 48-h exposures in MCF-7 and MDA-MB-231 cells ($p < 0.05$). Data are expressed as mean $\pm$ SD ($n=8$)

CLN. This might be contributed by the enhancement of fluidity of the cell membrane induced by chitosan polymer; as a result, more ART-CLNs could be easily taken up by the cells (13). Also, chitosan has a pK_a of around 6–6.5 and is, therefore, readily soluble in the intra- and intercellular acidic environment of solid tumor which might lead to a faster drug release (12).

Nuclei Morphology Studies

It has been reported that ART can induce DNA damage and cell death via the apoptotic pathway (26). In order to evaluate the effect of drug on nuclei, the nuclei morphology study was carried out by CLSM. As shown in Fig. 8, the nuclei of the control MCF-7 and MDA-MB-231 cells were homogeneously stained. However, chromatin condensation and apoptotic body formation could be clearly observed in the cells treated with ART and ART-CLN, which are typical apoptotic features in MCF-7 and MDA-MB-231 cells. The ART-CLN induced more cell apoptosis than free ART. Similar features were also reported in another cell line by some groups (27,28). All the above results also support the MTT assay results.

Stability Study

Physical stability of ART-CLN in the dispersed and lyophilized forms was assessed by examining the changes in physical properties. As shown in Fig. 9 and Table IV, after a month, the particle size and PDI increased in both forms. In contrast, drug entrapment efficiency and loading capacity decreased as compared with the original formulations. However, the zeta potential remained almost the same indicating the stability of ART-CLNs structure. In addition, between the two conditions, the lyophilized samples were more stable than the dispersed one. Although there are such changes in the formulations, the selected formulation (F8) with the favorable size and loading capacity is still in range of acceptance for further trials.

CONCLUSIONS

In conclusion, it can be stated that chitosan-coated lipid nanocapsule can be a promising carrier for the delivery of artesunate for chemotherapy. ART-CLN had nanometric size and narrow size distribution and showed core-shell morphology with enhanced solubility and

Fig. 8. Nucleus apoptosis assay after 24-h treatment at the ART concentration of 10 µg/mL on MCF-7 and MDA-MB-231 cells by confocal laser scan microscope images

stability properties. In addition, the sustained release pattern can provide the sufficient drug concentration for a safe and effective therapy. ART-CLN provided a fast and efficient uptake of the encapsulated drug into tumor cells.

Consequently, the efficacy of carriers is better than free drug in terms of cytotoxicity. According to these results, chitosan-coated lipid nanoparticles represent a promising vehicle for hydrophobic drug delivery.

Fig. 9. Physical properties of ART-CLNs after 30 days of storage in different conditions. Data are expressed as mean±SD (n=3)

Table IV. Drug Entrapment Efficiency (EE) and Loading Capacity (LC) of Artesunate Loaded Chitosan-Coated Lipid Nanocapsules After 30 Days of Storage

Formulation	Dispersed formulation		Lyophilized formulation	
	EE (%)	LC (%)	EE (%)	LC (%)
7	79.35±6.03	0.40±0.03	91.75±0.46	0.46±0.01
8	77.15±1.52	0.77±0.02	92.71±0.65	0.93±0.01
9	78.29±1.84	1.17±0.03	89.62±2.87	1.34±0.04
10	74.24±1.85	1.48±0.04	88.71±0.86	1.77±0.02

Data are expressed as mean±SD (n=3)

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Conflict of Interest The authors report no conflicts of interest.

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