Supplementary material

1 Materials and methods

1.1 Physical characterization of niosomal vesicles

To determine the physical stability of lysine-mediated niosomal nanoparticles loaded with Cur during storage, the change in particle size distribution, zeta potential, polydispersity index (PDI), and the remaining amount of the drug in vesicle, was assessed over 15, 30, and 60 days [1,2], using a dynamic light scattering method (Brookhaven Instruments, Holtsville, NY, USA). The surface morphology of niosomes and the internal structure of the lysine-mediated niosomal nanoparticles were characterized using a scanning electron microscope (SEM, model KYKY-EM3200-30 kV, China) and atomic field microscope (AFM), respectively. For this purpose, an amount of the nano-niosome solution was placed on the mesh copper grid 400. Then, the copper grid was located in an evacuated desiccator for evaporating the solvent. The measurements were made in triplicate and then their mean values were assessed. The functional group characterization of the prepared samples was evaluated using fourier transforms infrared (FTIR) spectrometer (Model 8300, Shimadzu Corporation, Tokyo, Japan) for blank niosome, niosomal nanoparticles loaded with Cur, and lysine-mediated niosomal nanoparticles loaded with Cur. For preparation, the samples were lyophilized as a dry powder, and mixed with potassium bromide (KBr). Then, the samples were placed in a hydraulic press, to form the pellets. The FTIR spectrum was scanned in the wavelength range of 400–4,000 cm^{-1} [3].

1.2 *In vitro* drug release study

The *in vitro* release of Cur from niosomes, was monitored using a dialysis bag (MW = 12 kDa) against PBS (containing 2% Tween-20 to imitate a physiological environment) for 72 h at physiological pH (pH 7.4) and acidic environment (pH 5.5) [4]. First, the nanoniosome-loaded Cur samples were suspended in a dialysis tube, and the release of drug was evaluated in 10 mL of PBS with continuous stirring. Then, 2 mL of the sample was collected from the incubation medium at precise time intervals, and was immediately substituted with an equal volume of fresh PBS. A UV-Vis spectrometer was used to determine the quantity of released Cur at 429 nm.

1.3 Cell culture, cytotoxicity analysis and cellular uptake of nanoniosomes-loaded Cur

A270s and A270cp-1 human ovarian cancer cells purchased from Pasteur Institute of Iran, were cultured in DMEM/F12 Ham's combination (InoClon, Iran) complemented with 21 mg mL^{-1} of penicillin/streptomycin (Gibco, USA), 10% fetal bovine serum (FBS; Gibco, USA), and GlutaMAX™-I (100X, Gibco, USA). The cytotoxicity of optimal formula was determined by MTT assay (Sigma, USA). Briefly, A270s and A270cp-1 cells were seeded in 96-well plates at 10,000 cells per well [5,6]. After attachment for 24 h, the cells were treated with 200 µL fresh medium containing serial dilutions of various niosome/drug formulations including unloaded niosome, niosomal Cur, and modified niosome by lysine. Then, 20 µL MTT solution (5 mg mL^{-1} in PBS) was added to each well after 14, 48, and 72 h, prior to 3 h incubation at 37°C. Subsequently, 180 µL DMSO was added to each well that the dissolving formazan crystals formed. The absorbance was recorded by EPOCH Microplate Spectrophotometer (synergy HTX, BioTek, USA) at 570 nm. 2×10^5 A270cp-1 and A270s cells were seeded per well in six wells for 24 h. The cells were then incubated for 3 h with free Cur, unloaded niosome, and lysine-modified niosomal Cur, rinsed 3 times with cold PBS, fixed with a 4% paraformaldehyde solution (Sigma, USA), and stained with DAPI (0.125 µg mL^{-1}, Thermo Fisher Scientific, USA) before visualization with fluorescence microscope (BX61, Olympus, Japan). A fluorescent label (Dil) was used to detect and track the modified niosomal NPs [7].

1.4 Cell apoptosis assay

Apoptosis was measured by an annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double

staining (Sigma-Aldrich, USA). Apoptosis was induced by treating the cells with free Cur and the modified nanocarrier administered in aqueous solution or in nano-niosome formulations at an IC50 concentration for each drug. The cells without any drug treatment, were the control. An annexin V-FITC/PI double staining assay was carried out to detect cell apoptosis stimulated either by free Cur or Cur loaded in niosomes, when administered in an aqueous solution and nanoniosome formulation. Moreover, propodeum iodide (PI) was a fluorescent intercalating element that could be used as a DNA stain within flow-cytometry. PI was not able to pass the membrane of apoptotic and live cells. Nevertheless, it marked dead cells and thus was useful for differentiating necrotic, apoptotic, dead and healthy cells. Data was represented as scatter plots based on percentage of detected cells in 4 quadrants. Q1 quadrant (FITC–/PI+) represented the secondary necrotic cells, Q2 quadrant (FITC+/PI+) represented the late apoptotic cells, Q3 quadrant (FITC+/PI–) represented the early apoptotic cells and finally the Q4 quadrant (FITC–/PI-) represented the living cells.

1.5 *In vivo* experiments

Thirty-five female BALB/c mice of 20–25 g and 6–8 weeks old were bought from Pasteur Institute and maintained in a germ-free environment. The animal experiments complied with NIH and IACUC guidelines. Each mouse was inoculated subcutaneously into the right flank with 5×10^6 4T1 cells. After the inoculated tumor volume reached nearly 100 mm^3, they were randomized into seven groups (five animals in each group). The tail vein of the animals was chosen to inject free curcumin, niosomal curcumin (DOTAP), niosomal curcumin modified by lysin. Normal saline was injected into the control group. The injections were performed on the 0, 3rd, 6th, 9th, and 12th days. The dose of the free drug was 2.5 mg/kg and that of the niosomal drugs was 10 µg per mouse. The body weight and the tumor volume were measured every three days. The following equation was employed to calculate the tumor volume:

$$V(\mathrm{mm}^3) = \frac{1}{2} \times LW^2,$$

where W and L are the lengths in short and long directions of the tumor, respectively. We sacrificed all mice on the 21st day. The tumor inhibition rate was calculated by the following formula:

$$\text{Tumor inhibition rate (TIR, \%)} = \left[\frac{(W_c - W_t)}{W_c}\right] \times 100$$

where W_c represents the average weight of the tumor in the controls and W_t stands for the average weight of tumors in the treated group.

1.6 Statistical analysis

Statistical data was investigated *via* GraphPad Prism 6 software and stated as mean ± SD. A student *t*-test was utilized when the two independent groups were compared. A multiple comparison was performed using an ANOVA test. $P < 0.05$ was considered as statistically significant.

References

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