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Original article

Synthesis and cytotoxicity study of magnetoliposomes loaded doxorubicin

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Abstract:

The limitation of clinical use of doxorubicin is its side effects especially a cumulative dose-dependent cardiotoxicity. To overcome this, doxorubicin can be targeted to accumulate on the site of action and limit dispersion in healthy tissue. Magnetic liposomes or magnetoliposomes (MLs) are enabling targeted delivery of doxorubicin to a specific area exposed in a magnetic field. The objective of this study was synthesis and cytotoxicity study of magnetoliposomes loaded doxorubicin (MLs-Dox). MLs-Dox were prepared from phosphatidylcholine and cholesterol by a modified version of the evaporation and sonication method. The average size was 45.1 ± 7.5 nm with narrow uniform size distribution and its magnetization was 670 emu/g. Furthermore, the percentage of doxorubicin encapsulation is 81.6 ± 4.9. We found that the cytotoxicity of MLs-Dox was increased according to the strength and applied time of magnetic field. The results of this study suggested that MLs-Dox should be able to archive the good magnetic carriers for targeting doxorubicin delivery.

Keywords: Cytotoxicity; Doxorubicin; Liposomes; Magnetoliposomes; SPIONs

Introduction

Antineoplastic agent of the anthrax-23-cycline called doxorubicin is frequently used with a combination of other anticancer drugs or methods to treat many cancers such as ovarian cancers, breast cancers, or others [1]. The limitation of clinical use of doxorubicin is its side effects especially a cumulative dose-dependent cardiotoxicity [2]. To overcome this, doxorubicin can be targeted to accumulate on the site of action and limit dispersion in healthy tissue. Such a minimization has already been proposed by using liposomes roughly 40 years [3]. Liposomes have been proven to be a unique tool for studying both structural and dynamic aspects of natural membranes [4-5] and give biocompatibility, biodegradability and low immunogenicity [6].

Although the progress of using liposomes as drug delivery has been archived, liposomal drug vehicles have not led to significant improvement in the clinical outcome [7-8] because of the non-specific distribution of liposomes and fast elimination from blood circulation and uptake by the reticulo-endothelial system (RES) in the liver and spleen. There are many investigations to address this problem [8-10]. The most successful development was so called long circulating liposomes [9-10] This performed by modified the surface of liposomes by using antibody [11-13], folate [14-16], transferring [17-19], cancer cell homing peptide [20-22], arginine-glycine-aspartic acid (RGD) [23-25] or others. Besides the surface modification chemistry, physiochemical methods represent another approaching way to control the delivery and targeting process for liposomal drugs. Liposomes based on these physicochemical elements could respond to a stimulus with the micro-environment of tumor sites and release the loaded drug by a second internal or external stimulus such as temperature [26], light [77], pH [28], or magnetic field [29].

Magnetic liposomes or magnetoliposomes (MLs) are liposomes which incorporate either magnetic materials in the aqueous core or magnetized polymers within the lipid bilayer structure and enable targeted delivery of drugs to a specific area exposed in a magnetic field. This technique has been shown and well developed.

Magnetic liposomes for the magnetic drug delivery system are essentially based on superparamagnetic iron oxide nanoparticles (SPIONs) proven to be biocompatibility [30]. One important question concerning the efficiency of MLs for targeting anticancer drug delivery is the applied magnetic field and incubation time. In this work, we prepared magnetoliposomes loaded doxorubicin (MLs-Dox) and determined the factors of magnetic field on cytotoxicity of MLs-Dox in Hela cells by using MTT test.

Materials and Methods

Preparation of SPIONs

To synthesize the SPIONs, 2 M Fe^{2+} and 1 M Fe^{3+} chloride solution were prepared by dissolving $FeCl_2.4H_2O$ (Sigma-Aldrich) and $FeCl_3.6H_2O$ in degassed 0.5 M HCl solution and filtered by 0.45 μm filter disc, respectively. Five mL of Fe^{2+} and 5 mL of Fe^{3+} were mixed together and stirred under nitrogen environment (760 mmHg). Fifty mL of 0.5 M degassed NaOH were preheated to 80°C for 10 min. The mixture solution of Fe^{2+} and Fe^{3+} was slowly added drop-wise to NaOH solution with bubbling argon gas (TIG) and vigorous stirring. A black precipitate was produced immediately. SPIONs were separated by magnetic decantation and washed with ethanol (LabScan) and ultrapurified water. Finally, the black particles were re-dispersed in ultrapurified water (6 mg/mL).

Preparation of MLs-Dox

To prepare MLs-Dox, the mixture of phosphatidylcholine and cholesterol in a 2:1 molar ratio was dissolved in 4 mL of chloroform/methanol solution (10/1 v/v) in a round-bottom flask. Then, this solution was applied onto a rotary evaporator and the organic solvent was removed slowly under reduce pressure (260-400 mmHg) at 43°C. One ml of Fe_3O_4 (6 mg/ml) and 1 mL of doxorubicin solution (3 mg/ml) were added drop-wise and the mixture was stirred for about 40 min in an ice bath (0°C) until a stable emulsion formed. Then the sample was then subjected to sonication for 15-30 min to prepare unilamellar MLs-Dox. The MLs-Dox was filtered thought 0.22 μm polycarbonate membranes and separated by magnetic decantation.

Characterization of MLs-Dox

The MLs-Dox were diluted and their size distributions were determined by dynamic light scattering (DLS) using the Zetasizer ZS (Thermo Scientific, Thailand). Approximately 1 ml of sample was filled into a cuvette and measured with the following specifications: measurements per sample prepared in water, viscosity of water 0.89 cP, temperature 25°C.

Transmission electron microscopy (TEM) was performed for analyzing the structure of the MLs-Dox. The images were made with appropriately diluted particle dispersion in MilliQ water. The samples were dropped on carbon-coated copper grids and dried under vacuum. Observation of the samples was performed at 80 kV with a transmission electron microscope (JEM-2010, JEOL, Japan).

Magnetization curve for the MLs-Dox was measured by using a super conducting quantum device (SQUID) magnetometer (PPMS-9, Design) with maximum magnetic field of 7 Tesla (T), sensitivity of 10^{-6} emu at temperature of 300°C. To determine the magnetic property, samples were cooled in a zero-applied magnetic field, from room temperature down to the measuring temperature. Each sample was dried and fixed on the quartz holders, which were placed in the magnetometer. Magnetization result of MLs-Dox was determined by applying an increasing magnetic field.

Prior to characterization of the SPIONs and Mls-Dox by X-ray diffractometry, the sample was pre-dried at reduced pressure, and then heated to 80°C for 2 h. The X-ray patterns were taken in an X-ray diffractometer, JDX-3530 (JEOL, Japan) with Cu Kα radiation, selected by means of a secondary graphite monochromator. The divergence stilt was 1° and the receiving slit was 0.15°. The starting and final 2° angles were 0° and 90°, respectively.

Doxorubicin encapsulation efficiency

To determine the encapsulation efficiency, non-encapsulated doxorubicin was separated by dialysis and quantified by fluorescence spectrometry with excitation wavelength at 485 nm and emission wavelength at 591 nm. Briefly, the coarse MLs-Dox (1 mL) was dialyzed (MWCO 8000) against 50 mL of distilled water. Complete equilibrium of non-encapsulated doxorubicin across the dialysis membrane was assumed when the concentration of doxorubicin in the dialysis medium did not increase for 2 h. After equilibrium, the concentration of non-encapsulated doxorubicin was equal to that of free doxorubicin which can go through dialysis membrane. The total doxorubicin was measured and encapsulation efficiency was calculated by the following formula.

$$\% \ Encapsulation = \frac{(DOX_{initial} - DOX_{free})}{DOX_{initial}} \times 100 \qquad (1)$$

Cytotoxicity evaluation of MLs-Dox

For the cytotoxicity evaluation, Hela cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% foetal bovine serum and 100 IU/mL penicillin G and 100 µg/mL streptomycin at 37°C in humidified 5% CO_2 atmosphere. All reagents were purchased from GibThai (Bangkok, Thailand). For cytotoxicity assays, cells were seeded for 48 h in standard 96-well plates at 1 x 10 cells per well. Then the culture medium was discarded and the cells were treated with 200 µl MLs-Dox solutions with 0.0, 0.1, 0.2, 0.3, and 0.5 millitesla (mT) for 0.0, 10.0, 20.0 and 30.0 min, respectively, and incubated at 37°C in humidified 5% CO_2 atmosphere for 96 h. Cell viability was determined using a tetrazolium dye (MTT) assay. The cells were rinsed twice with conc. phosphate buffer solution (PBS) pH 7.4 and incubated for 4 h in 200 µL containing 0.5 g/L of MTT at 37°C in humidified 5% CO_2 atmosphere for 3 h. Then the medium was poured, washed twice with PBS, and then replaced by 200 µL of dimethylsulfoxide (DMSO) to dissolve the formazan crystal formed by viable cells at room temperature. Absorbance was measured at 540 nm using a multiwell microplate reader (Labsystem iEMS Reader MF, Scientific Sciences, Thailand). The cytotoxicity was determined as the concentration that resulted in a reduction in cell viability. All the experiments were performed in six determinations.

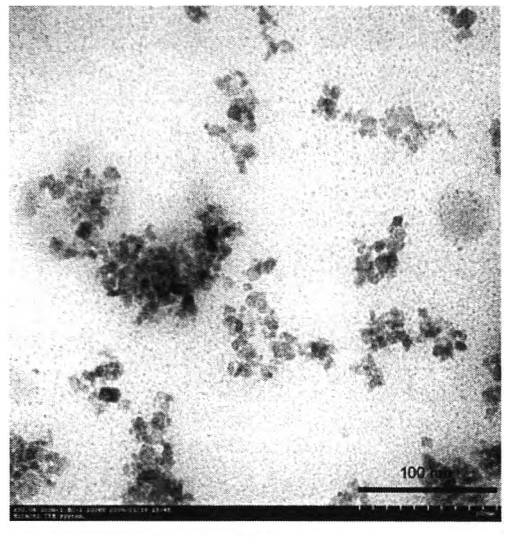
Results and Discussion

Several preparation methods had been developed, which included sonication, hand shaking, and ethanol

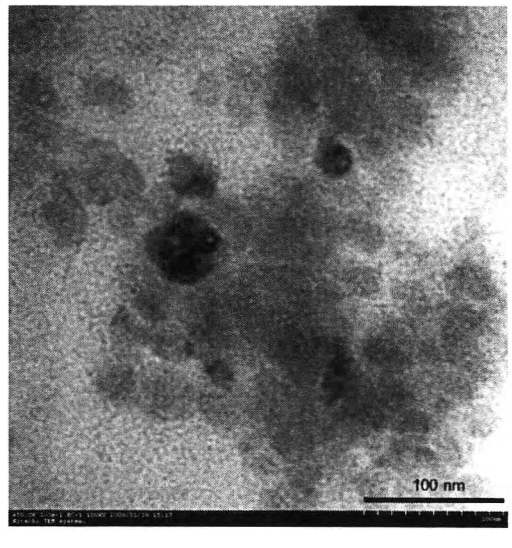
injection method [31-33]. All methods gave either low encapsulation efficiency (drug or SPIONs) or unstable liposomes with large particle size and distribution. A sonication method is the effective method for preparation liposomes with water soluble drug and SPIONs. Figure 1 shows the TEM analysis of SPIONs and MLs-Dox. After preparation, the particle size and morphology of MLs-Dox were determined and had narrow uniform distribution. The particle size and morphology of MLs-Dox were determined by dynamic light scattering technique and found to be 45.1 ± 7.5 nm with narrow size distribution.

Figure 2 illustrates the magnetization curve of the MLs-Dox measured in term of the saturation magnetization. The magnetization of these particles was 670 emu/g and the magnetization curve with an absence of hysteresis at room temperature demonstrated the superparamagnetic behavior of MLs-Dox as same as SPIONs [28,31].

Representative power XRD patterns of SPIONs and MLs-Dox are represented in Figure 3. From the XRD analysis, it was found that the peaks correspond to the structure of magnetite phase (Fe_3O_4). The line positions were the same for all samples, indicating no



(a)



(b)

Figure 1 Transmission electron microscope images of (a) superparamagnetic iron oxide nanoparticles and (b) magnetoliposomes loaded doxorubicin

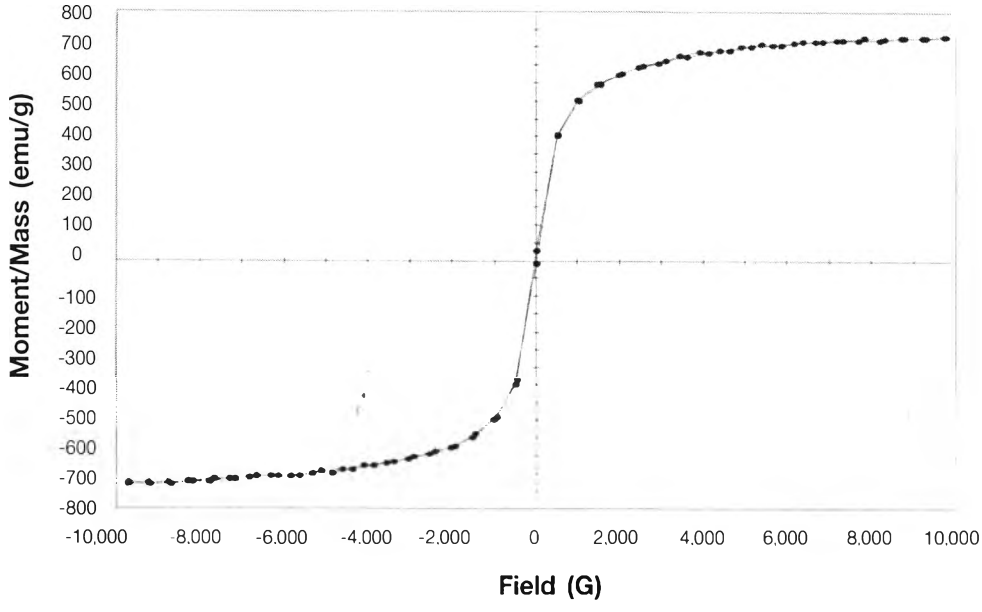


Figure 2 Magnetization as a function of external magnetic field for magnetoliposomes loaded doxorubicin at 300°C. The external magnetic field was swept from +10,000 to -10,000 G

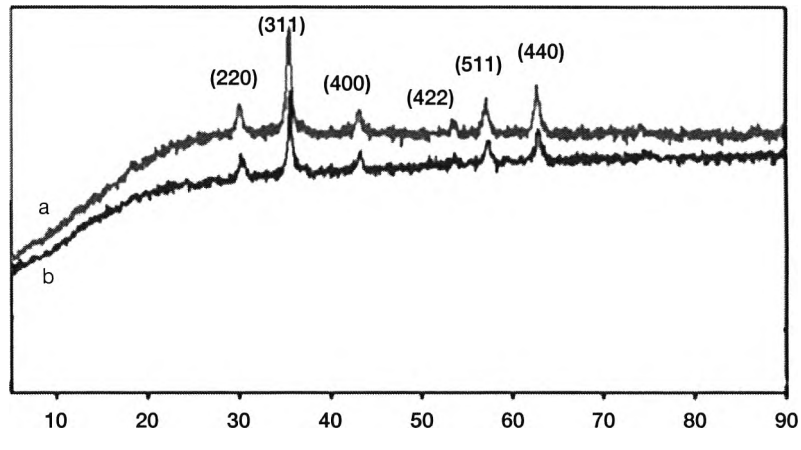


Figure 3 X-ray powder diffraction profile of (a) naked superparamagnetic iron oxide nanoparticles and (b) magnetoliposomes loaded doxorubicin

significant structural variations between the samples. From the absence of 210 and 300 peaks in the XRD pattern, it could be concluded that separate maghemite (γ-Fe_2O_3) was not present in our samples. Our XRD results agreed well with the previous reports [27,31].

The concentration of the encapsulated drug was determined by fluorescence spectrometry with excitation wavelength at 485 nm and emission wavelength at 591 nm. The MLs-Dox had a high average encapsulation efficiency of 81.6 ± 4.9%.

Figures 4 and 5 summarize the effects of magnetic strength and applied time of magnetic field on Hela cell lines. The cytotoxicity activity is shown in term of percent viability. The free doxorubicin was used as a control group. The aim of this biological evaluation was to determine if MLs-Dox still presents an antineoplastic activity and also to study the effect of strength and applied time of magnetic field on their cytotoxicity. According to the results, we chose to perform a cytotoxicity assay over 96 h in order to study the activity

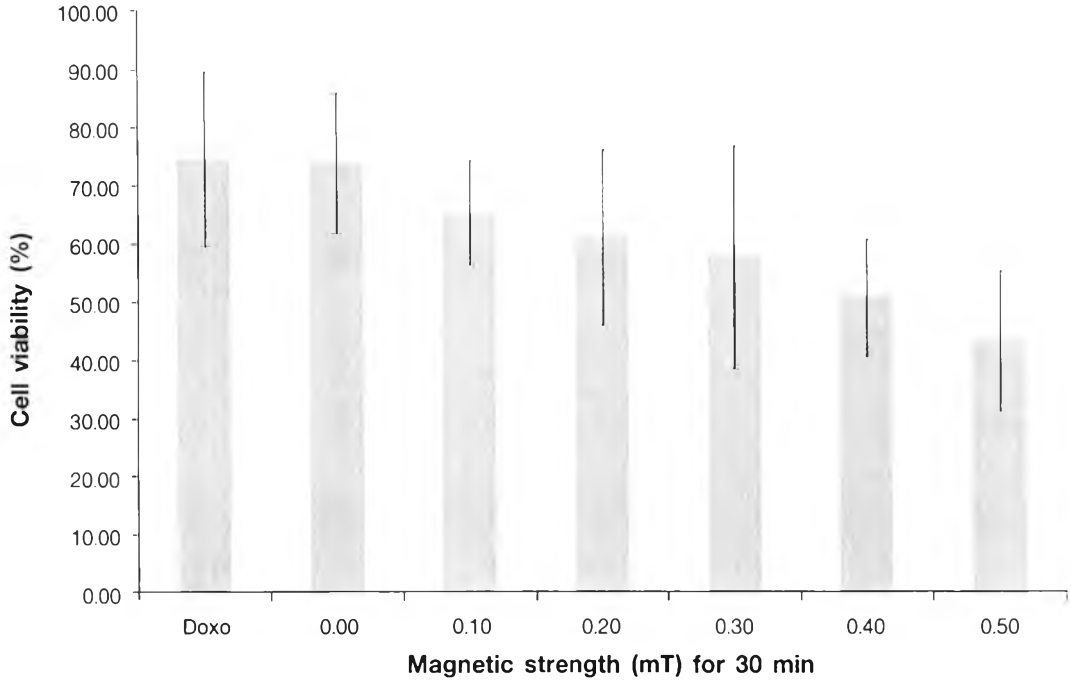


Figure 4 Effect of magnetic strength on cytotoxicity of magnetoliposomes loaded doxorubicin in Hela cell lines

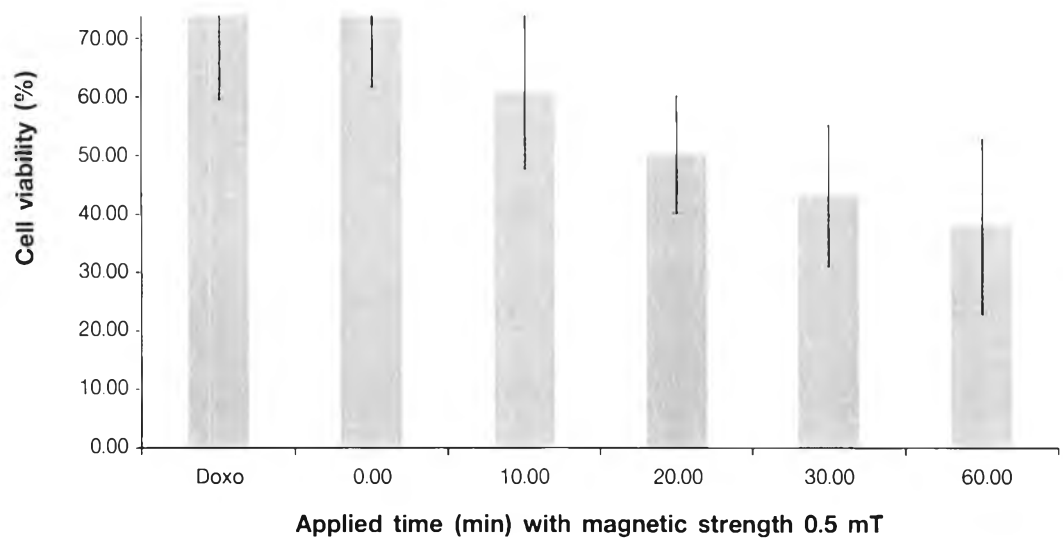


Figure 5 Effect of applied time of magnetic field on cytotoxicity of magnetoliposomes loaded doxorubicin in Hela cell lines

of the whole drug introduced in the medium. This duration corresponds to two cycles of division of each cell, which guaranties the validity of the results. We compared the cytotoxicity of MLs-Dox (0.40 μg/mL) without or with varied strength and applied time of magnetic fields. We demonstrated that neither doxorubicin nor MLs-Dox adjusted for the same doxorubicin concentration produced any significant cytotoxicity on Hela cells (74.67% vs 73.82% viability). We found that the cytotoxicity of MLs-Dox was increased according to the strength and applied time of magnetic field. The percent viability of Hela cell treated with samples and magnetic field 0.5 mT for 10.0, 20.0, 30.0 and 60.0 min were 60.79%, 50.18%, 43.10%, and 37.84%, respectively. For the effect of magnetic strength, we found the cytotoxicity of MLs-Dox on Hela cells was 73.82% (0 mT), 65.24% (0.1. mT), 60.94% (0.20 mT), 57.59% (0.30 mT), 50.69% (0.40 mT), and 43.10% (0.50 mT).

The results showed that the cytotoxicity of MLs-Dox was significantly increased according to the magnetic strength and applied time of magnetic field compared with doxorubicin and MLs-Dox without applied magnetic fields.

Conclusion

In this study, we synthesized and conducted cytotoxicity of MLs-Dox by varying the strength and applied time of magnetic field on Hela cell lines. The size of MLs-Dox was 45.1 ± 7.5 nm with narrow size distribution and its magnetization is 670 emu/g. The percentage of doxorubicin encapsulation was 81.6 ± 4.9. The antitumor of MLs-Dox against Hela cells demonstrated that when the magnetic strength and applied time of magnetic field increased the cytotoxicity of MLs-Dox was also increased. The results of this study suggested that MLs-Dox should be able to archive the good magnetic carriers for targeting doxorubicin delivery.

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