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Research Article

Artemisinin (ART) Drug Delivery Using Mixed Non-ionic Surfactants and Evaluation of Their Efficiency in Different Cancer Cell Lines

Elnaz Asgharkhani[1], Aazam Najmafshar[2], *Mohsen Chiani[3]

[1]*Basic Science Department, Science and Research Branch, Islamic Azad University, Tehran, IRAN*
[2]*Biochemistry Department, Pharmacy Faculty, Isfahan University of Medical Sciences, IRAN.*
[3]*Pilot Nanobiotechnology Department, Pasteur Institute of Iran, Tehran, IRAN*

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ABSTRACT
This study aims to investigate the effects of different non-ionic surfactants on physicochemical properties of ART niosomes. ART is a natural compound that is used as an antimalarial and chemotherapy agent in medicine. ART has low bioavailability, stability and solubility. In order to solve these problems and enhancing the efficiency of the drug, nanotechnology was used. In the present study, several niosomal formulations of ART prepared using different molar ratios of Span 60 : Tween 60 : PEG-600: ART in PBS. These three formulations were FI (1:1:0.5:0.5), FII (2:1:0.5:0.5) and FIII (1:2:0.5:0.5), respectively. The encapsulation efficiency was measured by HPLC and the drug release was evaluated by dialysis method. The cytotoxicity test was determined by MTT assay. The size, zeta potential and polydispersity index of the vesicles was measured by Zeta Sizer. Stability study was performed within two months. The MTT assay results showed that cytotoxicity effect of these formulations on MCF-7 cell line is better than C6 cell line and the FIII had the best results for both of them. The entrapment efficiencies of the formulations I, II and III were obtained 82.2±1.88%, 75.5±0.92% and 95.5±1.23%, respectively. The results of size, zeta potential and polydispersity index indicated that the size of the vesicles is below 200 nm, their surface charge is about -35 mV and they were monodisperse. Stability and release study indicated that the formulation III has the best stability and release pattern. Therefore, the use of PEGylated niosomal ART can effectively improve its therapeutic index, stability and solubility.

Key words Artemisinin, Drug Delivery System, Non-ionic Surfactant, C6 Cell line, MCF-7 Cell line.

INTRODUCTION

Surfactants actually reduce the surface tension and electrical or mechanical barriers that cause stabilize the emulsions. The nonionic surfactant sorbitan esters (Spans) and polyethoxylated sorbitan esters or ethoxylated spans (Tweens) have many potential pharmaceutical applications. They are often used to improve the stability of water-in-oil-in-water (wow) multiple emulsions. They can provide highly steady noisome with low cost and toxicity. They are also good carriers for both hydrophilic and hydrophobic drugs [Park MJ et al 2003]. When these non ionic surfactants are mixed together, they can provide more stable structures that called emulsion or microemulsion [Tianqing L et al 2007; Dongmei L et al 2000].

Niosomes, non-ionic surfactant vesicles, are now widely studied as an alternative to liposomes, because they alleviate the disadvantages associated with liposomes [Beugin S et al 1998], such as chemical instability, variable purity of phospholipids and high cost [Vora B et al 1998]. Niosomes can be prepared by the same procedure as of liposomes. Most methods require large amount of organic solvent that are toxic to human and environments and have multistep. Most preparation methods even without using organic solvents such as heating method [Mozafari MR et al 2007], and polyol dilution method [Kikuchi H et al 1994], there are problems of using high temperature that is not suitable for heat labile substances. Thus preparation of niosomes using safer components and easier methods that have the least toxic effect on normal cells, have always been considered.

Artemisinin, an endoperoxide sesquiterpene lactone isolated from Artemisia annua L. (Asteraceae), has attracted increasing attention as an important anti-malaria agent for many years. Because of its strong cytotoxic effects, it was reported that ART has special antitumor activity against melanoma, breast, ovarian, prostate, central nervous system, and renal cancer cell lines [Henry CL et al 2013; Liu C et al 2006].

Besides the promising clinical results, the utility of ART is limited in the biological systems due to its toxicity and very low solubility both in aqueous media and oils. ART has an initial burst effect and high peak plasma concentration but it metabolizes quickly in vivo [Chen Y et al 2009]. In addition, it is unstable and easily degrades, mostly by the opening of the lactone ring [Henry CL et al 2013]. Recently, it has been reported that its encapsulation into conventional and PEGylated liposomes is a reasonable method to prolong the

*Author for correspondence: E-mail:

Table 1: Three formulations of ART with different molar ratios.

	Formulation I	Formulation II	Formulation III
Span 60	1.0 M	2.0 M	1.0 M
Tween 60	1.0 M	1.0 M	2.0 M
PEG-600	0.5 M	0.5 M	0.5 M
ART	0.5 M	0.5 M	0.5 M
PBS	10 ml	10 ml	10 ml

Three niosomal formulations of ART were prepared by different molar ratios of Span60:Tween60: PEG600: ART in 10ml PBS by mixning, sonication and homogenization.

Table 2: Physicochemical properties of PEGylated niosomal formulations of atrimisini

Formulations	Span60:Tween60: PEG600:ART (Molar ratios)	Mean diameter (nanometer)*	Zeta Potential (millivolt)*	Entrapment Efficiency (%)*	Poly Dispersity Index (PDI)*
FI	1:1:0.5:0.5	156.4±5.5	-36.3±3.4	82.2±1.88	0.29±0.03
FII	1:2:0.5:0.5	153.6±7.4	-34.4±2.2	75.5±0.92	0.31±0.05
FIII	2:1:0.5:0.5	146.3±2.4	-38.4±3.3	95.5±1.23	0.21±0.03

**Mean ± SD, n=3.*

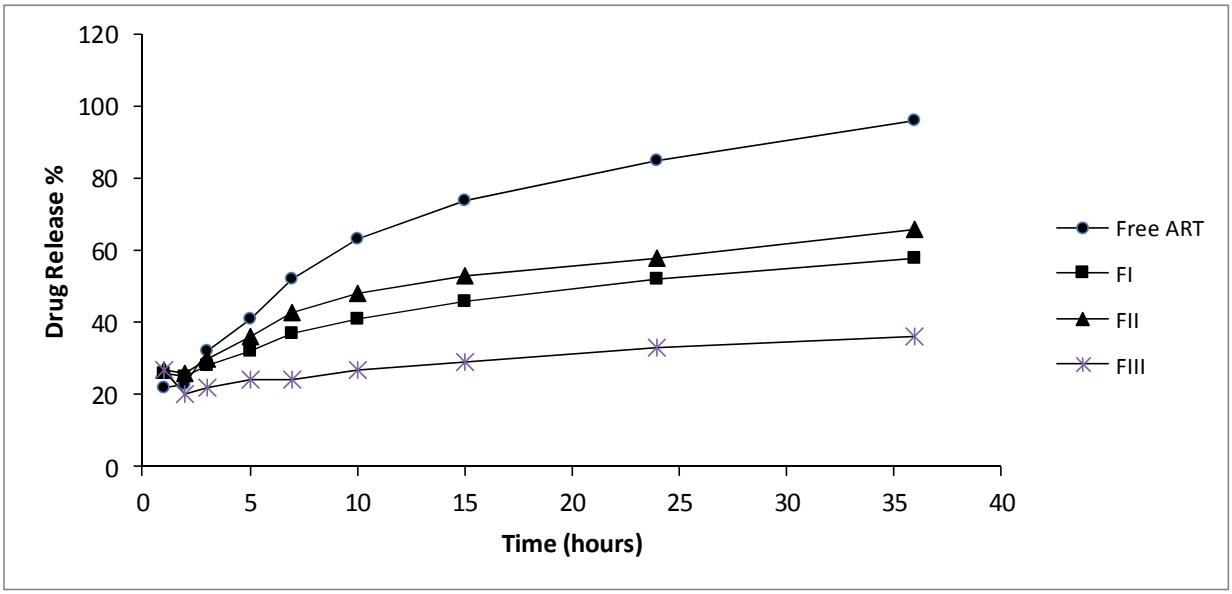

Fig. 1: *Comparison of drug release rate between free drug and PEGylated niosomal formulations (FI, FII and FIII) over 36h using dialysis method.*

circulating time of ART in blood plasma and to enhance the half-life of ART [Isacchi B et al 2011]. Paolina et al prepared a drug delivery system in 2008 using span80, cholesterol and FU-5 (2:5:2 molar ratio), which is used mostly in the treatment of skin cancer [Paolina D et al 2007; Li VHK et al 1987].

In this study different PEGylated niosomal formulations of ART was prepared using a mixture of surfactants and their anti-cancer efficiencies, stability, entrapment efficiency, release pattern were inspected in two model of cancer cell lines (MCF-7 and C6).

MATERIALS AND METHODS

Materials*:* Tween 60, ART and Span 60 were obtained from Sigma chemical Company. Poly ethylene glycol 600 were kindly obtained from Kimiagaran Emrouz Company. RPMI 1640, MTT reagent, Trypsin and Pen/Strep were purchased from Invitrogen Company. MCF-7 and C6 cell lines were bought from cell bank of Pasture Institute of Iran.

Preparing of niosomes: Three niosomal formulations of ART were prepared by different molar ratios of Span60:Tween60: PEG600: ART (1:1:0.5:0.5, 2:1:0.5:0.5 and 1:2:0.5:0.5) in 10ml PBS (pH 7.4, 20mM). These formulation were mixed together by magnetic stirrer (25°C, 300rpm, 2h), sonicated by bath sonicator (BANDELIN electronic, *SOROREX DIGITEC*, Germany, 25°C, 60 W, 10 min) and then homogenized (Heidolph instruments, Germany; 15min, 15000rpm, 25°C, 35 kHz) for increasing the entrapment efficiency and minimizing the vesicle size. These formulations have shown in the table1.

Characterization of vesicles

Determination of entrapment efficiency: After preparing niosomal dispersions, a certain amount of different formulations were centrifuged for 1h at 4°C in 16000× g for separating of unentrapped ART. The concentration of

Table 3: Stability analysis of PEGylatd niosomal formulations of ART during storage at 4±1ºC for 60 days.

Formulation	Entrapment Efficiency (EE%)*	Size (nm)*	Zeta potential (millivolt)*	Poly Dispersity Index (PDI)*
FI				
Day 0	82.2±1.88	156.4±5.5	-36.3±3.4	0.29±0.03
Day 30	78.5±1.54	197.3±2.7	-34.7±1.3	0.36±0.05
Day 60	72.3±2.36	316.3±7.5	-32.5±4.1	0.53±0.11
FII				
Day 0	75.5±0.92	173.6±7.4	-34.4±2.2	0.31±0.05
Day 30	62.4±2.88	283.4±6.7	-33.3±3.5	0.54±1.07
Day 60	54.5±2.92	468.8±5.7	-31.7±2.8	0.78±1.3
FIII				
Day 0	95.5±1.23	142.3±2.4	-38.4±3.3	0.21±0.03
Day 30	92.2±2.21	166.7±3.7	-38.3±6.6	0.28±0.22
Day 60	87.4±1.73	247.8±4.4	-37.6±2.8	0.42±0.07

**Mean±SD, (n≥3)*

unentrapped ART in supernatant was measured by KNAUER smart line HPLC (Berlin, Germany). The RP-HPLC was equipped with a Eurospher C18, 5 µm, 250 × 4.6 mm, 100 Aº column (KENAUER) and an UV detector (KENAUER PDA Detector 2800) set at 216 nm. The mobile phase comprised acetonitrile:water solution (65:35 v/v) and was delivered at a flow rate of 0.6 ml min−1. The injection volume was 20µl and the relative retention time was found to be 9.3 min. The unentrapped ART concentration in all samples was determined using a standard curve generated by known concentrations of ART dissolved in ethanol. The entrapment efficiency of ART in different PEGylated niosomal formulations was calculated according the following equation:

$$\text{Entrapment Efficiency (EE)\%} = \frac{\text{weigth of ART in carrier}}{\text{weigth of ART fed initially}} \times 100$$

Particle size, zeta potential and PDI analysis: The particle size, polydispersity index (PDI) and zeta potential was measured by dynamic light scattering. For these measurements, the dispersions were diluted to about 50 times with PBS. The time-dependent correlation function on the scattered light intensity was measured at a scattering angle of 90◦ and wavelength at 633 nm. Measurements were carried out at 25°C using a Zetasizer, (Nano ZS3600, Malvern Instruments, UK).

In vitro release study: In vitro release studies of ART from vesicles were performed by dialysis method. Two milliliters of all formulations were taken for in vitro drug release studies. Dialysis tube containing ART-loaded niosomes dispersion was placed into a flask containing 100ml phosphate buffer saline (20mM, pH 7.4, 37°C). The release pattern of niosomal formulations of ART, their controls and free ART solution were compared to each other. The flask was stirred at 50 rpm at 37°C for 36h. Five milliliter aliquots of dialysate were taken at predetermined time and replaced immediately with the same volume of fresh PBS, and withdrawn samples were assayed spectrophotometrically at 216 nm.

Stability study: Stability of ART-loaded niosomes in all formulations was studied during 60 days. Appropriate amounts of the three formulations were subjected to stability study by storing at 4±1°C. Every month, 1ml of niosomes suspensions was inspected for their physico-chemical stability. Physical stability was studied by measuring the size, zeta potential and polydispersity by zetasizer instrument. Chemical stability was tested by measuring the entrapment efficiency over the incubation time.

In vitro cytotoxicity assay: The cytotoxicity of PEGylated niosomal formulations of ART, was measured by MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl-tetrazolium bromide blue-indicator dye]–based assay [Plumb JA et al 1989], and compared with Free ART in MCF-7 (human breast cancer) and C6 (glioma, brain cancer) cell lines. Tumor cells were seeded at dilution of 1×10^4 / well in RPMI supplemented with 10 % FBS, 100 units/ml penicillin and 100 mg/ml streptomycin, at 37 ºC in a humid atmosphere containing 5% CO_2, for 24 h . After that, the medium replaced with fresh medium containing the formulations at different concentrations.

After 72h, all media were then removed and 100µl of MTT solution (0.5 mg/mL in RPMI) were added to the wells. The cells were incubated for 3h. MTT was removed and isopropanol was added to dissolve the formazan crystals. The optical density at 570 nm was read using a BioTek ELISA reader. Untreated cells were taken as control; all experiments were carried out for three times and in triplicate.

Statistical analysis of data: Statistical tests were performed with the SPSS software (IBM, SPSS, Statistics 19). Results are expressed as mean ± standard deviation. Statistically significant difference was determined using the Student's t-test and analysis of variance (Anova). A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Entrapment efficiency % (EE%): Entrapment efficiency is an important factor in drug delivery systems. This is especially true for expensive drugs. The EE% of the ART-loaded PEGylated niosomes has shown in table 2. As you see, the most entrapment efficiency was related to FIII formulation (up to 95.5±1.23%).

Table 4: Cytotoxic activity of three PEGylated niosomal formulations of ART against MCF-7 and C6 cell lines (expressed as IC50).

Formulation	Cell line, IC50 (µM)	
	MCF-7	C6
F1	12.8 ± 2.4	21.4 ± 3.9
FII	22.4 ± 4.7	32.2 ± 5.7
FIII	9.6 ± 2.8	18.6 ± 3.7
Free ART	38.4 ± 3.8	46.8 ± 4.1

The cells were treated by different formulations of ART and free ART for 72h using MTT assay. Results was analyzed by Pharm program for determination of IC50 value that indicate the inhibitory activity of free and pegylated niosomal ART against MCF-7 and C6 cell lines.

In vitro release studies: The release studies of ART from PEGylated niosomes were performed using dialysis tube containing 2 ml of ART-loaded niosomes dispersion placed into a flask containing 100 ml PBS. Collected samples in predetermined times, were analyzed by spectrophotometer at 216 nm. The rate of drug release across the dialysis membrane was slower for drug-loaded PEGylated niosomes compared to free drug. The free drug release was approximately 96% in 36 h, while the rate of release for formulations FI, FII and FIII were 58.3%, 66.4% and 36.6%, respectively. The in vitro release profiles are shown in Figure 1.

Stability study: Stability study of three formulation of ART was carried out during storage at 4±1ºC for 60 days. The results showed that the most stability of vesicles is related to formulation III as about 13% of the drug was released from the vesicles within 60 days. Size, zeta potential, PDI and EE% were selected as parameters for evaluation of the stability, since instability of the formulations would reflect in drug leakage and decrease in the percentage drug retained. The PEGylated niosomal formulations were sampled at predetermined time, analyzed for size, zeta potential, PDI and EE%. The results of stability test have been shown in table 3.

In vitro anticancer activity: Cytotoxicity assays are widely used to screen the cytotoxic activity of a compound. MCF-7 and C6 cell lines were evaluated for cytotoxicity assay by MTT assay. Toxicological effects of PEGylated niosomal formulations were much more than the pure drug. The IC50 for the formulations FI, FII, FIII and Free ART against MCF-7 cell line were 12.8 ± 2.4µM, 22.4 ± 4.7 µM, 9.6 ± 2.8 µM and 38.4 ± 3.8 µM respectively. The IC50 of the formulations FI, FII, FIII and Free ART against C6 cell line were 21.4 ± 3.9µM, 32.2 ± 5.7µM, 18.6 ± 3.7µM and 46.8 ± 4.1µM respectively. These have shown in table 4.

DISCUSSION

The membrane structure and layer number of vesicles are very important for the size, stability and application of the vesicles. Tween60 has a large hydrophilic head group with high HLB value that can solve in water and form order bilayer aggregation by hydrogen binding and hydrophilic action but cannot form vesicles alone. Span60 contains long hydrophobic group and hardly dissolved in water but can interaction with ART and form vesicles alone because of long alkyl chain. The balance between hydrophilic and hydrophobic moiety of mixed surfactant are important factors for high entrapment efficiency. Increase the amount of Span 60 leads to high entrapment efficiency and stability of the vesicles. This is due to its stronger hydrophobicity of span 60 than tween 60 that can cause more rigidity in vesicle wall.

In this study, three PEGylated niosomal formulations of ART were successfully prepared by a simple method that mentioned above. Characterization of these PEGylated niosomal formulations indicated that these vesicular carriers could be used as a potential delivery system. Because of small size, high stability and stealth mode of these niosomes (presence of PEG on their surface), they have long blood circulation half-life in the body [Mehrdad H et al 2007]. The particle sizes decreased with increasing Span 60 content, which was due to the critical packing parameter of span 60. Because of its strong cytotoxic activity, it was reported that ART has potential antitumor activity against many cancers [Efferth T et al 2007]. For evaluation of antitumoral activity of these ART-loaded niosomes, their cytotoxicity was evaluated on two models of cancer cell lines, i.e. MCF-7 and C6.

The IC50 is an index to express the effectiveness of a compound in inhibiting biological or biochemical function. It is the half maximal (50%) inhibitory concentration of a substance [Cui Z et al 2006]. This index obtained by MTT assay. The MTT results indicated that ART-loaded PEGylaed niosomes have more antitumoral activity (lower IC50 values) on these cancer cell lines compare to free drug.

CONCLUSIONS

Our findings showed that preparation of noisome using a mixture of non-ionic surfactants and a simple method can provide stable and small vesicles in nano dimensions. The release pattern of ART from these vesicles is very slow without burst effect. These vesicles can be a suitable carrier for delivery of a very range of hydrophobic, hydrophilic or amphiphilic molecules.

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DISCLOSURE

The authors declare that no competing interests exist.

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