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ORIGINAL ARTICLE

Artemisinin-loaded niosome and pegylated niosome: physico-chemical characterization and effects on MCF-7 cell proliferation

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Abstract Artemisinin (ART)-loaded niosome and pegylated niosomes were prepared using two different techniques. Nanosized lipid vesicles were physically characterized for entrapment efficacy and stability. Particle sizes were determined and release kinetic of the optimized formulation was carried out by dialysis method. The efficacy of the developed formulation was tested on MCF7 cells and cytotoxicity was accomplished by MTT assay. Common observation was the effect of pegylation on the reduction of vesicle size due to its hydrophilic nature. Span 60 niosomes had slightly larger vesicle size than Span 20 niosomes. Over all the good stability was observed over 60 days. In vitro drug release studies indicate gradual release of niosome over 40 h. similar trend in drug release was observed for most formulation except for the multilammellar pegylated niosomes. Pegylation of niosomes causes increased stability and efficacy of ART. Cytotoxicity (IC_{50}) was evaluated at different time of incubation at 48 and 72 h for selected niosomal formulations. Pegylated ART niosomes show great advantages in term of interaction with MCF-7 cell membrane. Results suggest that pegylated niosomes may be an appropriate candidate for the clinical administration of ART.

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Introduction

Various strategies have been employed to design a targeted drug delivery system. Formulations such as, Lipid vesicles, cyclodextrins, nano and microparticles has been previously studied by our group in order to improve bioactivity of drug molecules (Fathi-Azarbayjani et al. 2010, 2011, 2013). Vesicular lipid systems have gained popularity for their application in cancer therapy (Jing et al. 2016; Pasut et al. 2015). Niosomes are non-ionic surfactant vesicles with flexible lamellar microscopic structure, low toxicity and production cost. Furthermore niosomes are biodegradable, biocompatible and non-immunogenic and can incorporate both hydrophobic and hydrophilic drug molecules. Alternatively polyethyleneglycol (PEG) containing niosomes have also been developed with various applications in the field of drug delivery to help prolong drug circulation time and increase drug accumulation (Palozza et al. 2006; Pasut et al. 2015).

Current anticancer delivery strategies are associated with high levels of toxicity. Artemisinin (ART) is natural compound with potent antimalarial activity and no significant side effect. Recently ART has been explored for its anticancer effect. As a new class of anticancer drugs, ART has low toxicity towards normal cells and high specifity in selectivity killing cancer cells (Lai et al. 2005).

ART is often extensively metabolized and poorly soluble in water. Rapid clearance and erratic adsorption of ART and its low oral bioavailability of 32% is a challenge in pharmaceutical formulation (Navaratnam et al. 2000; Iwunze 2004). ART has an inner hydrophobic core and the

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presence of a lipophilic molecule can help to increase its availability in aqueous environments thus minimizing degradation and loss. ART-loaded peptide micelles have been shown to exhibit great anti tumor effect compared with the free drug (Wang et al. 2012). ART-loaded chitosan nanocapsules were also developed for controlled drug delivery and enhanced aqueous solubility (Chen et al. 2009). Solid dispersion of ART in polyvinylpyrrolidone (PVP) and polyethylene glycol (PEG) 4000 has been formulated for enhanced permeation of this poorly water soluble drug (Shahzad et al. 2013). ART loaded microspheres and nanoparticles have been developed using albumin (Payghan and Bhat 2008; Ibrahim et al. 2015).

The aim of this study was to develop ART-loaded niosome and pegylated noisome and to compare their ability to enhance cellular permeation of ART. ART-loaded niosome and pegylated niosomes were prepared using two different techniques. Nanosized lipid vesicles were physically characterized for entrapment efficacy and stability. Particle sizes were determined and release kinetic of the optimized formulation was carried out by dialysis method. The efficacy of the developed formulation was tested on MCF7 cells and cytotoxicity accomplished by MTT assay.

Materials and methods

Cholesterol, Span 60 (sorbitol mono stearate), Span 20 (sorbitol mono loreate) and ART were obtained from Sigma-Aldrich. Ethanol and isopropanol were purchased from Merck. Poly ethylene glycol 600 was kindly gifted from Kimiagran Emrouz Company. RPMI 1640, MTT reagent, Trypsin and Pen/Strep were purchased from Invitrogen. MCF-7 cell lines were obtained from Pasture Institute of Iran. All other regents were of analytical grade. This study protocol does not contain any human and animal related studies performed by any of the authors.

Preparation of niosomes and pegylated niosomes

Ethanol injection method (EI) and reverse phase evaporation method (REP) were employed for noisome formulation. ART-loaded niosomes were prepared by REP method as reported previously (Vyas et al. 2005). Briefly, a known amount of surfactant (Span 20 or 60) and cholesterol was dissolved in 10 ml of diethyl ether in a clean, dry round bottom flask. Organic solvent was removed using rotary vacuum evaporator above the lipid transition temperature to form a thin film on the wall of the flask. After removal of solvent traces, thin lipid film was hydrated with phosphate buffer saline (PBS) pH 7.4 by injection through a 14 gauge syringe needle at a rate of 0.025 ml/min at 60 °C.

EI method was adopted from a previously reported method (Fang et al. 2001). Briefly, cholesterol and surfactant (Span 20 or 60) were dissolved in ethanol. Niosomes were formed by gradually injecting the lipid solution in to a 18 ml phosphate saline buffer containing ART while stirring. The organic phase was then evaporated under reduced pressure by rotary evaporator (Heidolph, Germany) and allowed to shake at room temperature for 24 h, the solution was sonicated for 15 min and homogenized for 20 min at 12,000 rpm to yield homogenous niosomes. Pegylated niosomes were prepared by adding 5 mg PEG600 to the formulation placed in sonicator for 15 min and finally it was homogenized for 10 min at 12,000 rpm. The composition of different vesicle formulations is listed in Table 1.

Determination of entrapment efficiency

The un-entrapped ART (0.8 mg) was removed following centrifugation at 13,000 rpm for 120 min at 4 °C and estimated by UV-spectrophotometer. The sedimented ART niosome was dissolved in 0.1% Trition X-100 and phosphate saline buffer for 30 min at 37 °C until all membranes

Table 1 Composition and physicochemical properties of ART-loaded niosome and pegylated niosomes

Formulations	Type of surfactant	Method of preparation	Mean diameter (nm)*	Zeta potential (mV)*	EE (%)*	PDI*
N1A	Span 20	EI	306.4 ± 5.5	−13.3 ± 0.7	83.2 ± 1.8	0.29 ± 0.03
N1A+PEG600	Span 20	EI	298.6 ± 6.7	−13.7 ± 0.9	87.5 ± 1.9	0.31 ± 0.05
N2A	Span 60	EI	315.2 ± 9.4	−12.2 ± 1.1	90.5 ± 2.2	0.40 ± 0.06
N2A+PEG600	Span 60	EI	286.3 ± 10.2	−12.9 ± 0.8	92.4 ± 2.7	0.42 ± 0.06
N1B	Span 20	REP	325.4 ± 7.9	−11.2 ± 0.6	85.4 ± 1.3	0.37 ± 0.06
N1B+PEG600	Span 20	REP	321.4 ± 8.3	−13.1 ± 1.0	99.3 ± 2.6	0.32 ± 0.08
N2B	Span 60	REP	335.3 ± 12.4	−12.4 ± 1.2	87.6 ± 2.1	0.31 ± 0.07
N2B+PEG600	Span 60	REP	279.4 ± 11.8	−14.9 ± 0.8	99.8 ± 2.4	0.26 ± 0.03

EI ether injection method, *REP* reverse phase evaporation method, *EE%* entrapment efficiency, *PDI* poly dispersity index

*Mean ± SD, n = 3

were broken and drug was released. The encapsulation efficiency (EE%) was determined using the follow equation:

$$EE\% = \frac{[total\ drug] - [unentrapped\ drug]}{[total\ drug]} \times 100 \quad (1)$$

Particle size and zeta potential

Mean particle size and zeta potential were measured by zeta sizer (Malvern Instruments Ltd). Samples were diluted with Milli-Q water and measurements were carried out at an angle of 165°. Particle size was expressed as nanometer and particle size distribution was expressed as polydispersity index (PDI).

Storage stability study

Storage stability is important for the development of a pharmaceutically acceptable product. The ability of selected niosomes to retain the drug was determined at 4 ± 2 °C (fridge) and 25 ± 2 °C (room temperature, RT) for a period of 60 days. Drug leakage was observed by measuring encapsulation efficacy of the vesicular formulations. Drug leakage was measured every 15 days at two different temperatures. A 0.5 ml of lipid vesicle suspensions was withdrawn, centrifuged and the amount of drug leached out was estimated by spectrophotometer (n = 3).

In vitro release study

In vitro drug release was carried out by dynamic dialysis method. A dialysis membrane sac was filled with 1 ml (0.8 mg) of niosomal ART suspension and placed in 25 ml phosphate buffer saline (PBS) on a shaker at room temperature for 40 h. At predetermined intervals, 1 ml of the release media was withdrawn and replaced with an equal volume of the fresh media. The samples were diluted and centrifuged and the supernatant was assayed for drug content spectrophotometrically. Data were given as mean ± standard deviation (SD) based on 4 independent measurements.

Cell cultures

Human breast cancer cells (MCF-7) were purchased from national cell bank, Pasteur Institute of Iran. The MCF-7 were cultured in a standard medium (RPMI-1640, Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotic–antimycotic in a 50-cm^3 flask in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. In order to ensure the accuracy of the test conditions, we performed live/dead assay before each experiment, using Trypan blue staining and neubauer lam to determine the cell counts quantitatively. Cells were removed from the culture flasks by means of trypsin (0.25%) and EDTA solution, resuspended in RPMI-1640-10% FBS, and counted.

Viability test (MTT assay)

Cytotoxicity of the drug loaded niosomes and PEG-niosomes were measured by MTT assay to determine the viable MCF7 cells count based on the mitochondrial conversion of the tetrazolium salt, 3-[4,5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide (MTT). MCF 7 cells were trypsinized, resuspended in RPMI+10% FBS, counted, and carefully seeded at a concentration of 1×10^4 cells per 96 well plate. The supernatant was removed and the different concentrations of the niosomal formulation and pegylated niosomes and pure drug as control and blank (only cell culture) were put into the cells and incubated at 37 °C in 5% CO_2 for 24, 48 and 72 h. The medium was removed and replaced with 100 µl of fresh medium. Then MTT reagent (100 µl, 0.5 mg/ml) was added to each well for 4 h at 37 °C. The medium was replaced with 100 µl of DMSO. Plates were shaken at 600 r/min for 10 min and the absorption of the colored solution was quantified at 570 nm using an ELISA plate reader (BioTek ELx800). Cell survival fractions were calculated as percentage of the untreated control. IC_{50} (the half maximal inhibitory concentration) values were derived from the concentration–response curves.

UV-spectrophotometer

The quantitative determination of ART was performed using a UV-spectrophotometer. Drug concentration was measured at a wavelength of 195 nm by spectrophotometer (Model UV-160 IPC, SHIMADZU Company).

Statistical analysis

Data were expressed as mean ± standard deviation (SD) of at least three replicates. Statistical analysis was carried out employing one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test to determine the differences between treatment groups. A value of $P < 0.05$ was considered statistically significant.

Results

Vesicle size

Table 1 presents mean diameter of different niosomes. Mean diameter was in the range of 279.4–335.3 nm. Common observation was the effect of pegylation on the reduction of vesicle size due to its hydrophilic nature. Span 60

niosomes had slightly larger vesicle size than Span 20 niosomes. A rational explanation is the number of C–H in the alkyl chain may influence vesicle size. This could be due to the long alkyl chain length in Span 60 (C16) than that of Span 20 (C10) (Shaker et al. 2015). Results also indicate a dependence of vesicle size on the method of preparation. Niosomes prepared by the EI method form monolayer vesicles due to the rapid evaporation of the solvent resulting in a decrease in the vesicle size (N1A 306.4 ± 5.5 nm). RPE method produces multilamellar vesicles (MLV) with larger size (N1B 325.4 ± 7.9 nm).

Zeta potential and PDI

Zeta potential and PDI of the formulations are listed in Table 1. PDI is an important characteristic of colloidal system homogeneity. Zero value indicates mono disperses and normal size distribution of the prepared niosomes while a value of 1 indicates poly disperse vesicle. A low PDI <0.4 was observed with all formulations indicating a small size distribution and low aggregation. The zeta potential of lipid vesicles is a measure of the overall electric charge repulsion and stability. The zeta potential value of the niosomes range from −11.2 to −14.9 mV can account for the relatively good stability of niosomes to provide sufficient electrostatic repulsion to prevent vesicle aggregation. The negative value for zeta potential indicates negative charge on the surface of the niosomes (Khan et al. 2016).

Encapsulation efficacy

Entrapment efficacy (EE) of niosomes is listed in Table 1. Pegylated niosomes (N1A+PEG 600 87.5 ± 1.9%) had higher EE when compared to non-pegylated niosomes (N1A 83.2 ± 1.8%). Hydrophilic nature of PEG could influence the surface structure of the niosomes. Pegylation decreased vesicle size and caused an increase in encapsulation efficacy. It was also found that MLVs prepared by RPE (N1B 85.4 ± 1.3%) where capable of higher drug loading as compared to the monolayer niosomes prepared by EI method (N1A 83.2 ± 1.8%).

From Table 1 it is observed that EE of Span 60 niosomes (N2A) were 90.5 ± 2.2% and that of Span 20 niosomes (N1A) were 83.2 ± 1.8%. Span 60 niosomes had slightly higher entrapment efficacy when compared to Span 20 niosomes. Non-ionic surfactans of sorbitan esters (Spans) are hydrophobic surfactants with low hydrophilic–lipophilic balance (HLB) number. The HLB value of Span 60 and Span 20 is 4.7 and 8.6 respectively. There is a relationship between the HLB value of non-ionic surfactant and the entrapment efficiency of niosomes. Non-ionic surfactants with low HLB value have higher drug entrapment efficacy. This could also be explained by the difference in the gel to liquid phase transition temperature. It is known that Span 60 has higher transition temperature (50 °C) and thus may lead to higher encapsulation rate (Fathi-Azarbayjani et al. 2015; Shaker et al. 2015).

Vesicle stability

Stability profile of pegylated niosomes at room temperature and fridge are depicted in Fig. 1. Niosomes appeared as translucent yellow dispersion without any aggregation or precipitation. It was observed that EI method produced more stable vesicle than the RPE method. Also, stability was higher for niosomes stored in the refrigerator as compared to niosomes stored at room temperature. Over all the great stability results may indicate the placement of ART in the hydrophobic cavity of the vesicle which may help to protect it from degradation.

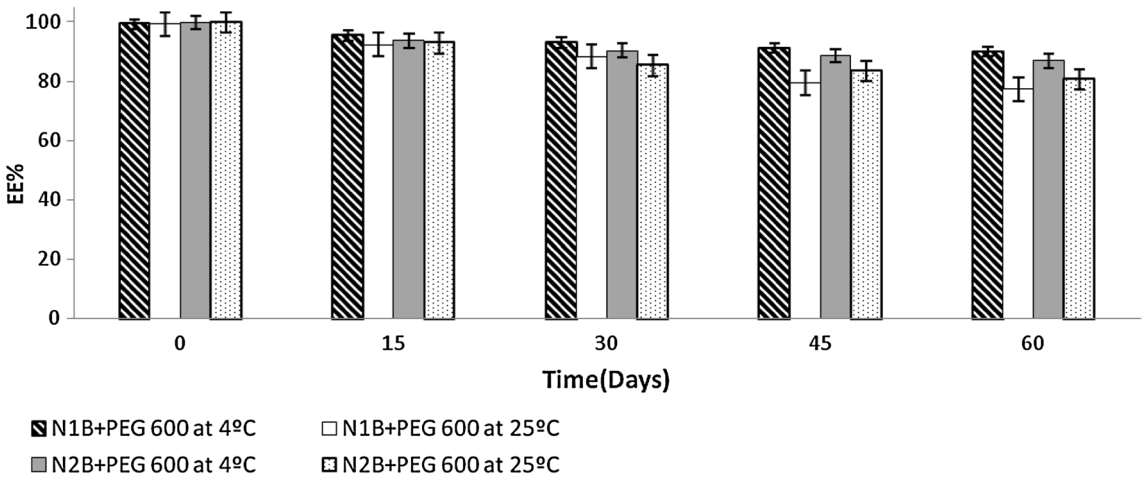

Fig. 1 Physical stability of two formulations of niosomes under different conditions (4 and 25 °C) for 60 days

In vitro drug release

ART release profile investigated over 40 h was cumulatively plotted in Fig. 2. ART released gradually from niosome over 40 h. Similar trend in drug release was observed for most formulation except for the multilammellar pegylated niosomes made with Span 60 (N2B+PEG 600) where drug release was slower. The structure of vesicles prepared by the RPE method may provide a multilammellar barrier for drug release which could explain the slow drug release from this formulation as compared niosomes prepared by the EI method. The free ART was completely released from the bag in less than 3 h (results not shown). The slow drug release from N2B formluation with Span 60 may be due to the hydrophobic nature of the surfactant as compared to the Span 20 used in N1B.

Cell viability test

MTT assays were performed to evaluate the cytotoxicity of ART-loaded niosome and pegylated niosomes. Toxicological effects are significant indicative of drug efficacy. Cytotoxicity was evaluated at different time of incubation at 24, 48 and 72 h for selected niosomal formulations. Cytotoxic activity of PEGylated niosomal formulations of ART against MCF-7 cell line after 48 and 72 h incubation is expressed as IC_{50} in Fig. 3. There was no remarkable cyctotoxic effect of the ART formulations 24 h after incubation (data not presented). By increasing the time of incubation to 48 and 72 h, a greater reduction in cell viability was observed for ART noisome and pegylated niosomes as compare to the free drug. Free ART had a high IC_{50} value of 69.54 ± 0.08 µM with the least potency. This value is within the range of IC_{50} value (IC_{50} 0.17–87.10 µM) reported previously (Efferth and Oesch 2004). It is seen that

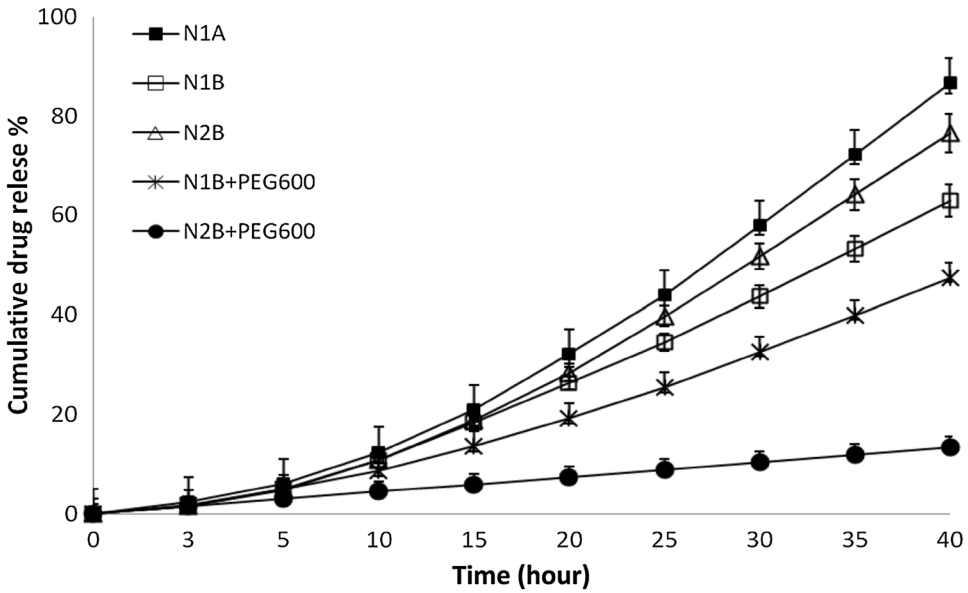

Fig. 2 Cumulative release of different formulations of artemisinin during 40 h incubation by dialysis method

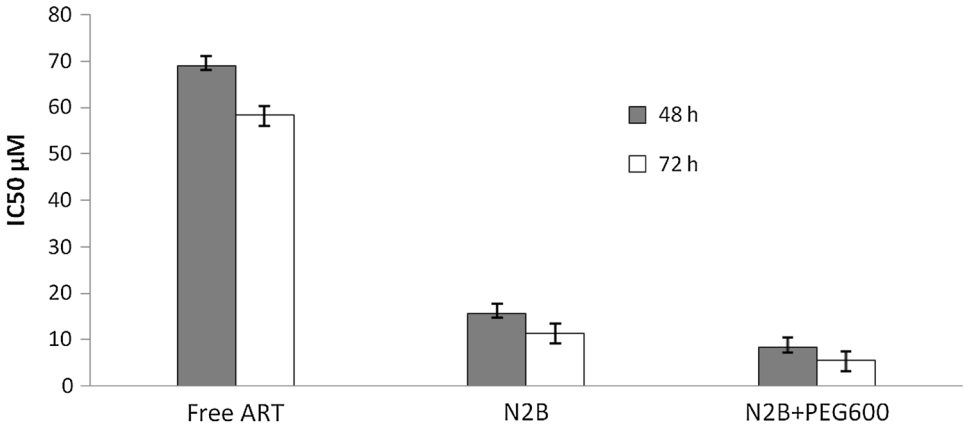

Fig. 3 Cytotoxic activity of PEGylated niosomal formulations of ART against MCF-7 cell line after 48 and 72 h incubation (expressed as IC_{50})

ART-loaded pegylated niosome show lower IC_{50} value of 8.31 and 5.45 μM after 48 and 72 h of incubation. Low IC_{50} of the ART niosomes indicates that its cytotocic activity is much more than the pure drug. This finding show that ART niosomes coated with PEG show great advantages in term of interaction with MCF-7 cell membrane in the in vitro cytotoxicity experiment. In addition, pegylated niosomes have higher drug entrapment that may cause well-defined reduction in cell viability.

N2B formulation had IC_{50} value of 15.71 μM. Low IC_{50} value of this formulae may be due to its high absolute zeta potential value and its small vesicle size which enhance the surface cationic properties of the vesicles and provide better interaction between formulae and cell surface to help provide high intracellular uptake of the drug. (Jing et al. 2016).

Conclusion

ART-loaded niosome and pegylated niosome were prepared, optimized and characterized. Stability and drug encapsulation efficacy was measured. The lipid vesicles (especially pegylated niosome) were able to increases ART cytotoxicity. Results suggest that pegylated niosomes may be an appropriate candidate for the clinical administration of ART. As a conclusion, pegylation of niosomes causes increased stability and efficacy of ART.

Compliance with ethical standards

Conflict of interest This statement is to certify that the authors declare no conflict of interest.

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