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The development and in vitro evaluation of Coenzyme Q10 long-circulating liposomes

Shuoye Yang

College of Bioengineering, Henan University of Technology, Zhengzhou, P. R. China

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To prepare Coenzyme Q10 (CoQ10) long-circulating liposomes by three most commonly used preparative methods, dry-film, reverse phase evaporation and ethanol injection. The resulting samples were contrasted through morphology observation, particle size and zeta potential analysis. Free drugs and liposomes were separated using protamine aggregation method and entrapment efficiency was determined. HPLC assay for determination of CoQ10 in vitro was developed, and its release property was studied by using dialysis method. The results showed that liposomes prepared by dry-film method were of best quality and stability. The HPLC assay developed was specific, rapid and reliable, which can be used to determine lomustine and iohexol in vitro accurately. In vitro release tests showed that the release profile of CoQ10 from liposomes at 37 ℃ fitted to the Weibull release kinetics, with the equation of ln ln [1/(1-Q)]=2.0352lgt-4.6143. The formulation and preparative method can be used to prepare CoQ10 long-circulating liposomes with high entrapment efficiency and good release behavior.

Key words: Coenzyme Q10, long-circulating liposomes, Preparative methods, Encapsulation efficiency, in vitro release.

INTRODUCTION

Coenzyme Q10 (CoQ10) is one of ubiquinone compounds, as an endogenous fat-soluble molecule, it plays an essential role in the production of cellular energy in mitochondria in the form of ATP [1].CoQ10 consists of a redox active quinoid moiety and is also a vitamin-like substance present in many organisms. Through functioning as an electron carrier in the electron transport chain, CoQ10 can help to energy conservation, while the reduced form of CoQ10 is capable of scavenging free radical oxygen intermediates [2]. Furthermore, CoQ10efficiently decreases the damage from peroxidation and free-radical-induced oxidative reaction to the membrane phospholipids, mitochondrial DNA and membrane proteins [3, 4]. Thus its ability to scavenge free radicals makes CoQ10 a promising potential anti-cataract agent.

Nowadays, potential health benefits and therapeutic value of CoQ10supplementation have been well recognized, and it has been widely consumed as one kind of food supplement or drug. It was reported that insufficiency of CoQ10 causes a series of health disorders resulting from mitochondrial dysfunction. Numerous studies have indicated thatCoQ10 deficiencies maybe lead to cardiovascular disease, neurodegenerative disorders, diabetes, and even cancer [5,6]. However, as a synthesized compound inside the human body, the synthesis activity of CoQ10 would be reduced by increasing age [7]. On the other hand, despite the nutraceutical and pharmaceutical benefits, the application of CoQ10 is largely hampered as a therapeutic agent due to its low bioavailability with high variability after oral administration [8]. The incomplete bioavailability of CoQ10 should attribute to its extreme hydrophobicity, large molecular weight, instability to light and thermolability, especially the insignificant solubility in aqueous media might be the critical factor for its poor bioavailability and poor delivery properties [9]. To promote the uptake by peripheral tissues and the blood brain barrier and improve the oral absorption of this bioactive molecule, numerous approaches have been undertaken to enhance the solubility of CoQ10, involving the use of oil solutions, solid dispersion system, nano- and micro-emulsions and the self-emulsified drug delivery system [10-12]. Among the above novel formulations, colloidal drug delivery systems, such as liposomes, are regarded as the effective means to obtain improved bioavailability in comparison with liquid formulations [13]. Liposomes are preferable because they have good characteristics such as easy delivery, no interference with vision, stabilizing effect and high drug loading efficiency. Nevertheless, liposomes are generally rather unstable and tend to degrade or aggregate under ordinary conditions, leading to leakage of entrapped drug during storage or after

To whom all correspondence should be sent:
E-mail:yangshuoyecpu@163.com

administration. To prevent these disruptive influences, a number of research efforts have been made, including surface modification by using long-circulating materials, which is an effective way to improve liposomal stability both in vitro and in vivo.

The present study was undertaken to develop a new CoQ10 long-circulating liposomes, with DSPE-PEG (2000) as long-circulating material. The liposome prepared by three different methods was investigated with particle size and zeta potential as assessment index. The protamine aggregation method was developed to determine the encapsulation efficiency, and their in vitro release characteristics were investigated in details, to provide elementary experiment reference for the practical use in clinic.

MATERIALS AND METHODS

Chemicals and reagents

CoQ10 was purchased from Aladdin Industrial Corporation, soybean phospholipid (SP) was purchased from Shanghai Tywei Pharmaceutical Co., Ltd, DSPE-PEG (2000), DPPG and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Protamine Sulfate injection was provided by Shanghai No.1 Biochemical Pharmaceutical Co., Ltd, vitamin E (VE) was purchased from Guangzhou rekon Food Chemicals Co., Ltd. Methanol and n-hexane were of chromatographic pure and purchased from Tianjin Shield Specialty Chemical Ltd. Co. and Tianjin Kemiou Chemical Reagent Co., Ltd., respectively. Trichloromethane was purchased from Luoyang haohua Chemical Reagent Co. Ltd. All other reagents were of analytical grade.

Preparation of liposomes

Dry-film method (DF). Briefly, CoQ10, SP 98, DPPG, DSPE-PEG (2000) and cholesterol were dissolved in 10ml chloroform and the solution was evaporated for about 15min at 25 ℃. After the dried film was formed, N2 gas was used to remove there siduary solvent. The dried film was hydrated with deionized water. After sufficient hydration, the film was suspended by vortexing. The liposomes were then sonicated.

Reverse phase evaporation method (RPE). A lipid mixture of CoQ10, SP 98, DPPG, DSPE-PEG (2000) and cholesterol was dissolved in chloroform, deionized water was added to the lipid solution to form an emulsification automatically, and subsequently the organic solvent was dried under vacuum through attaching to a rotary evaporator.

Ethanol injection method (EI). In brief, lipids and drugs were dissolved in 8 ml ethanol, and the ethanol was removed in rotary evaporator leaving behind about 4 ml solutions. Next, a constant volume of deionized water was added to the ethanol solution. Liposomes formed spontaneously after further evaporation of the residual solvent.

Finally, the liposome suspension was further disrupted by using ultrasonic probe. Resulting liposomes were sterilized by extruding through a 0.22 μm sterile filter. Cationic liposomes were prepared the day before the experiment, stored overnight at 4^{o}C.

The liposomes prepared above were all semi-transparent solutions with light yellow color. The processing operations were performed under shade environment due to CoQ10 is not to be exposed to daylight.

Morphology observation

The morphologies of liposomes were observed using transmission electron microscopy apparatus (JEM-200CX); samples were negatively stained with 1% phosphotungstic acid.

Particle size and zeta potential measurement

Long-circulating prepared by different methods were stored at 4^{o}C for 3 months. Samples were taken on the 0 day, 1 and 3 months for particle size and zeta potential analysis to assess the stability of vesicles. For determination process, each sample was dispersed in deionized water to a final volume of 3 ml, their particle size and zeta potential were analyzed using Laser particle analyzer (Malvern Zetasizer 3000HS, Malvern, UK). Volume-weighted Gaussian size distribution was fit to the autocorrelation functions and particle size values were obtained.

Encapsulation efficiencies (EE) determination

Protamine aggregation method was developed to separate free drugs and liposomes; EE was determined by using high performance liquid chromatography (HPLC).

Effect of protamine dosage on separation for liposomes. The proper amounts of protamine sulfate injection (10 mg·ml-1) was taken and diluted by different folds. 100 μl liposomes and 100 μl dilution were taken and mixed, placed for about 3 min, centrifugated with the relative centrifugal force 350×g for 10 min after diluting with 5 ml purified water precisely. The supernatant was selected to measure absorbance by using an ultraviolet spectrophotometer at 540 nm, signed as A1. Another 100 μl liposomes were mixed with purified water directly, centrifuged and collected the supernatant to determine turbidity, signed as A0, the clarity was

calculated according to the formula: (A0-A1)/A0 ×100%. By the same method, the clarities of supernatant from the series of dilutions by separate liposomes were determined.

Effect of protamine dosage on EE. 100 μl liposomes and 100 μl protamine sulfate were taken and mixed; the free drugs and liposomes were separated as described above. The supernatant was collected for HPLC analysis. The chromatography separation was performed with a DiamonsilTMC18 Column (200mm×4.6mm, 5μm), the flow rate was 1.5 ml·min-1, with column temperature of 30 ℃, the mobile phase was methanol-n-hexane (4:1), the drug was detected at 275 nm for determining the content of CoQ10. By the same method, EE of CoQ10 from the series of dilutions by separating liposomes were determined.

Determination of recovery of CoQ10. Different concentrations of CoQ10 solutions (80%, 100% and 120%) were prepared with methanol. The proper amounts of blank liposomes were mixed with CoQ10 solutions, 100 μl mixtures was taken to separate by protamine sulfate and centrifugation. The supernatant was collected for HPLC analysis to determine the recovery.

EE determination. 100 μl liposomes were taken precisely and mixed with 100 μl protamine sulfate, after centrifugation the supernatant was collected for HPLC analysis to determine the amounts of free drug (W1). Meanwhile, another 100 μl liposomes were taken and dissolved by 4 ml methanol; samples were taken to determine the total amount of lomustine (W0). EE was calculated according to the formula: EE=(1-W1/ W0) ×100%.

Development of release assay method for CoQ10

Specificity. The reference solution of CoQ10, demulsification solution of blank liposomes (no CoQ10 contained) and demulsification solution of liposomes were taken and injected for HPLC analysis, respectively.

Linearity. The CoQ10 testing solutions of different concentrations were prepared. The calibration curve samples were assayed in triplicate, using concentration (C) as abscissa (X) and peak area as ordinates (Y).

Recovery. The proper amounts of blank liposomes were mixed precisely with different volumes of stock solution, after filtering through a 0.45 μm sterile membrane, filtrate was collected for determination. Recoveries were calculated by comparing the mean concentration obtained from the tested solutions with that of the neat standard samples.

Study on the in vitro release of CoQ10 in long-circulating liposomes

Assay method. Dislysis method was applied to determine release rate [14,15]. 1000 ml release medium was taken to dissolution glass at predetermined temperature, 1 ml liposomes were precisely transferred into the semipermeable membrane with leak sealing ends and located in release solvent. Release medium was agitated by stirring blades and sampled at 10, 30min, 1, 2, 4, 6, 8and 12 hours after experiment initiating. 5 ml sample was collected and filtered through a 0.45 μm membrane, filtrate was selected to determine at 37°C.

CoQ10 content at each time point was determined by HPLC analysis, meanwhile, the proper amounts thermosensitive compound liposomes was demulsificated by methanol for the total drug amounts (W), the accumulative release amounts and release percent were calculated according to the formula:

$$Q_n = C_n V_0 + \sum_{i=0}^{n-1} C_i V_i \qquad \text{Accumulative release percent}(\%) = Q_n / W \times 100\%$$

Noting: Qn was the accumulative release amounts at each time point, Cn was the measured concentration at each time point, V0 was the bulk volume of release medium, Vi was the sampling volume, Ci was the measured concentration at time point i, W was the total drug amounts in liposomes.

Release medium investigation. The in vitro release feature of CoQ10 was assessed using purified water, HCL and phosphate buffer (PBS, pH7.4) as release solvent, respectively, with the stirring blades rotation speed of 50 r·min^{-1}.

Mathematical model fitting. The in vitro release data of CoQ10 was processed by using zero-order kinetics, first-order kinetics, Higuchi, Ritger-Peppas and Weibull equations, and the data obtained from all the time points set was selected to simulate.

RESULTS

Morphology of long-circulating liposomes

The morphologies ofliposomes were observed by transmission electron microscopy. All the liposomes were spherical particles with integrated bilayers and fingerprint-like surface on the liposomes was distinct. There was no difference in the morphology of the samples prepared by different methods.

Characterization of liposomes prepared by three methods

As shown in Tables 1 and 2, for particle size analysis results, there was significant difference between liposomes prepared by three methods, the lipid vectors prepared by RPE experienced great size increase during storage, by contrast, the EI-liposomes remained more or less unchanged over long periods, while the size distributions were most uneven (Polydispersity index, PDI were higher than 0.4). Among the three vehicles, samples produced by DF method showed better stability and distribution feature within 3 months, without the tendency to aggregate and become larger. For zeta potential analysis results, samples prepared by DF method possessed lower potential, as well as were more stable compared with their counterparts prepared by the other two methods. Based on an overall consideration, DF method was selected as the best alternative to prepare liposomes.

Table 1. Results of particle size (nm) analysis for liposomes prepared by three methods (n= 3). Data represent average ± standard deviation.

Days	DF	PDI	RPE	PDI	EI	PDI
0	165.6±1.4	0.291	171.2±0.7	0.194	163.2±2.1	0.438
30	215.8±4.1	0.243	459.5±28.8	0.448	166.3±5.7	0.410
90	188.5±4.4	0.121	515.6±16.8	0.330	165.3±4.0	0.409

Table 2. Results of zeta potential (mV) analysis for liposomes prepared by three methods (n=3). Data represent average± standard deviation.

Days	DF	RPE	EI
0	-27.7±0.9	-29.5±3.2	-4.02±0.4
30	-33.9±0.6	-25.7±1.4	-17.5±2.0
90	-11.0±0.3	-1.26±0.6	None

Development of protamine aggregation method

Effect of protamine dosage on separation for liposomes. The clarities determination results were summarized, we can conclude that free drugs and liposomes are well separated (Clarities were higher than 95%) by using very low amounts of protamine sulfate (0.5 mg).

Effect of protamine dosage on EE. The results showed that while protamine dosage was between 0.1-1.0 mg, EE of CoQ10 determined were 82％-90％, thus there were little effects of protamine dosage on the EE of CoQ10.

Determination of recovery. Through HPLC analysis and calculation, recovery of CoQ10 were 100.4 %, 104.5 % and 99.6 %, RSD=4.41 % (n=3).

EE determination results. The EE of CoQ10 liposomes for the three batches were 94.0 %, 94.2 % and 91.4 %.

Development of release assay method for CoQ10

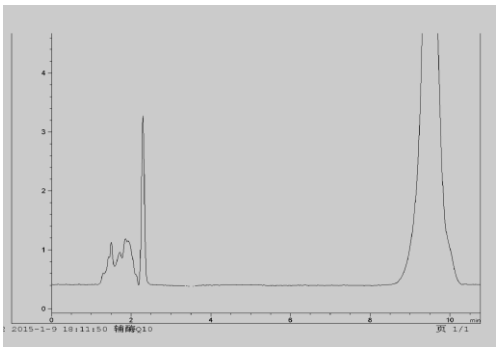
The HPLC chromatograms of CoQ10 were shown in Fig 1, it was indicated that the retention time (RT) of CoQ10was about 11.0 min, lipid and other pharmaceutical necessities were eluted within 4 min, without interfering the determination.

Further, the calibration curve of CoQ10 was calculated as: A=9.9232×C+1.9675(r=0.9999), indicating the good linear relationship from 5.0 to 150.0μg·ml-1.The recovery were 97.0％, 102.3％ and 101.5％, with RSD of 1.74％, 1.61％ and 0.63％ (n=5).

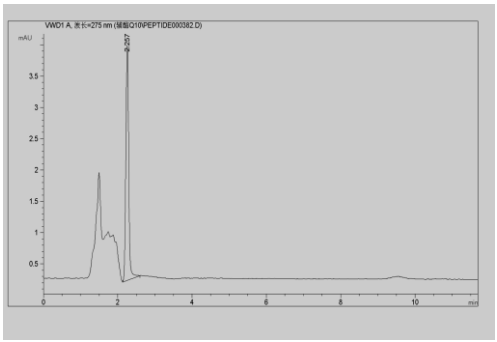
Study on the in vitro release of CoQ10

Release condition investigation. The release curve of CoQ10 in three medium were shown in Fig 2. It can be drawn that the accumulative release percents calculated in different medium all reached over 80 % at the last time point. However, it should be noted that the release of CoQ10 was affected by the medium to some extent; CoQ10 was released relatively slower within 8 h in HCL, but became more rapidly in subsequent time, PBS has also great influences on release characteristic and properties. Purified water showed little effects on the release of CoQ10 from liposomes. Therefore, purified water was selected as release medium in order to make complete release.

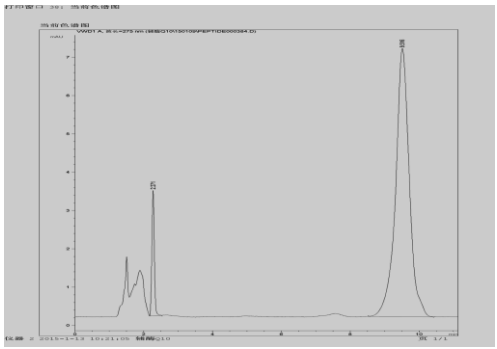
Release results of CoQ10 in liposomes. The release curve of liposomeswere shown in Fig.3. We can conclude thatCoQ10 in different batches of samples was released with nearly the same property, and release rates increased continuously along with the time.



A



B



C

Fig. 1. Chromatograms of CoQ10 standard (A), blank liposome (B) and long-circulating liposomes (C).

Mathematical model fitting

As shown in Table 3, through regression model parameters analysis, the drug release from liposomes were with closest agreement with the Weibull release kinetics, with the equation of lnln [1/(1-Q)]=2.0352lgt-4.6143 (r=0.9986).

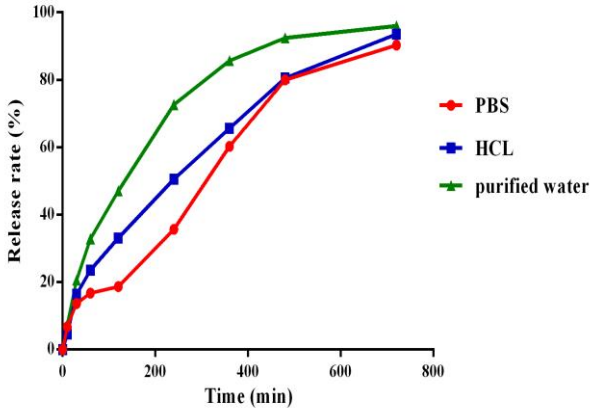


Fig. 2. Effects of mediums on in vitro release of CoQ10.

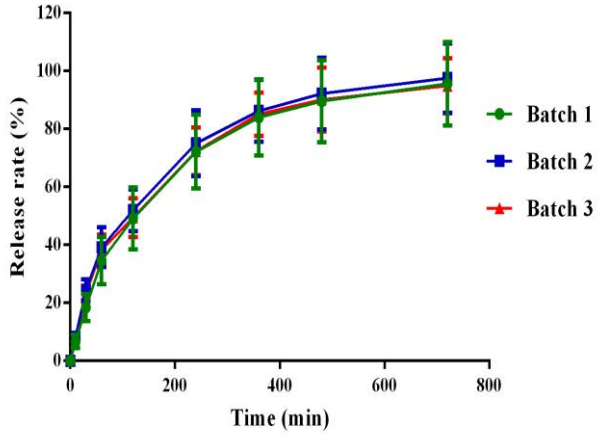


Fig.3. The release curves of the CoQ10 in liposomes, with purified water as the release medium at the rotation speed of 50 rpm.

Release results of CoQ10 in liposomes. The release curve of liposomes were shown in Fig.3. We can conclude thatCoQ10 in different batches of samples was released with nearly the same property, and release rates increased continuously along with the time.

Table 3. The different release models of CoQ10 in liposomes in vitro.

Model	Equation	k	C	r
Zero-order kinetics	Q% = kt + C	0.0013	0.2007	0.9051
first-order kinetics	ln(100-Q)=kt+C	-0.0044	4.4926	0.9958
Higuchi equation	Q% = kt½ + C	0.0398	0.0108	0.9818
Ritger-Peppas equation	lnQ=klnt+C	0.6181	0.7615	0.9772
Weibull equation	lnln [1/(1-Q)] = k lgt + C	2.0352	-4.6143	0.9986

DISCUSSION

Particle size and zeta potential are generally considered as most important parameters in liposomes quality evaluation [16,17], stable particle size indicated liposomes did not show to aggregate and cluster swimmingly during the use or deposit. On the other hand, conventional lipid carriers with low zeta potential were more negatively charged, which were capable of absorbing and binding to protamine sulfate molecule better, leading to

efficient separation from free drugs in EE determination. For the preparative methods selection, although as many as a dozen methods have been used to prepare long-circulating liposomes in recent research, most of them, such as extrusion method, freeze thawing method, etc., were less frequently employed in report or only used as supplement and substitution for the three methods investigated in the present study. It was suggested that long-circulating vectors prepared by DF method had more advantages over ones produced by the other two from the results of particle size and zeta potential analysis.

EE is one of the most important evaluation parameters for liposomal carriers. In general, the free drugs and liposomes are separated, and the amounts of free drug as well as the total amounts are both determined to calculate EE [18, 19, 16]. As to the EE determination for CoQ10 liposomes, organic solvent extraction was widely used in literature, in which mixed solutions composed of tween 80 and other organic solvent were used for multistage separation by solubilizing and eluting liposomes. However, as a multistep approach, this method was more labor-intense to process, and more numerical error would be brought for determination. Protamine sulfate is one kind of polycation macro molecule and composed of basic amino acids. After mixing with liposomes protamine sulfate can adsorb their surface through electrostatic interaction, thus the density of liposomes would be increased and may be separated effectively with free drugs by lower centrifugal force. For the advantages of quickness, simple operation and high efficiency, furthermore, the separation is based on electrostatic attraction and independent of the drugs enveloped in liposomes, thus this method is applicable for EE determination of most of the drugs.

For the CoQ10 assay method, UV spectrophotometry was also used in literature, nevertheless, lipid necessities in formulation could not be effectively separated from drug by UV method, as well as lead to erroneous results. Furthermore, it was reported that methanol-anhydrous alcohol mixed solvent (with the ratio of 5: 95, 1: 9 or 1: 1) was the frequently used mobile phase, in this paper the mixed solvent was also used and the results indicated that these two organic solvents could be well miscible with each other, and CoQ10 was eluted within 3 min. However, chromatographic peak shapes became abnormal, while pharmaceutical necessities were not completely separated from drug. Methanol-n-hexane (4:1) mixed solvent was studied through experiments, by which pharmaceutical necessities could be completely separated from drug, with longer retention time and symmetrical peak. Through large experimental validation and comparison, it was selected for in vitro release determination.

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REFERENCES

1. U. Singh, S. Devaraj, I. Jialal, *Nutr Rev*, **65**, 286 (2007).
2. B. Frei, M.C. Kim, B.N. Ames, *Proc Natl Acad Sci USA*, **87**, 4879 (1990).
3. K.S. Echtay, E. Winkler, K. Frischmuth and M. Klingenberg, *Proc Natl Acad Sci USA*, **98**, 1416 (2001).
4. C.H. Huang, J.P. Sipe, S.T. Chow, R.B. Martin, *Proc Natl Acad Sci USA*, **71**, 359 (1974).
5. M. Mancuso, D. Orsucci, L. Volpi, V. Calsolaro, G. Siciliano, *Curr Drug Targets*, **11**, 111 (2010).
6. S. Suzuki, Y. Hinokio, M. Ohtomo, M. Hirai, A. Hirai, M. Chiba, S. Kasuga, Y. Satoh, H. Akai, T. Toyota, *J Diabetologia*, **41**, 584 (1998).
7. R. Wajda, J. Zirkel, T. Schaffer, *J Med Food*, **10**, 731 (2007).
8. P. Balakrishnan, B.J. Lee, D.H. Oh, J.O. Kim, Y.I. Lee, D.D. Kim, J.P. Jee, Y.B. Lee, J.S. Woo, C.S. Yong, H.G. Choi, *Int J Pharm*, **374**, 66 (2009).
9. N.K. Swarnakar, A.K. Jain, R.P. Singh, C. Godugu, M. Das, S. Jain, *J. Biomaterials*, **32**, 6860 (2011).
10. K.H. Bhandari, M. Newa, J.A. Kim, B.K. Yoo, J.S. Woo, W.S. Lyoo, H.T. Lim, H.G. Choi, C.S. Yong, *Biol Pharm Bull*, **30**, 1171 (2007).
11. T.R. Kommuru, M. Ashraf, M.A. Khan, I.K. Reddy, *Chem Pharm Bull*, **47**, 1024 (1999).
12. A. Palamakula, M.A. Khan, *Int J Pharm*, **273**, 63 (2004).
13. A.S. Monem, F.M. Ali, M.W. Ismail, *Int J Pharm*, **198**, 29 (2000).
14. C.H. Huang, J.P. Sipe, S.T. Chow, R.B. Martin, *Proc Natl Acad Sci USA*, **71**, 359 (1974).
15. S.C. Semple, A. Chonn, P.R. Cullis, *J. Biochemistry*, **35**, 2521 (1996).
16. B. Ma, S. Zhang, H. Jiang, B. Zhao, H. Lv, *J Control Release*, **123**, 184 (2007).
17. M. Ramezani, M. Khoshhamdam, A. Dehshahri, B. Malaekeh-Nikouei, *Colloids Surf B Biointerfaces*,**72**, 1 (2009).
18. L. Ciani, A. Casini, C. Gabbiani, S. Ristori, L. Messori and G. Martini, *J. Biophysical chemistry*., **127**, 213 (2007).
19. H. Farhood, N. Serbina, L. Huang, *Biochim Biophys Acta*, **1235**, 289(1995).