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Archaeosome Made from Lipids Extracted of *Acidianus brierleyi* as a New Drug Delivery System

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Archaeosomes are a novel generation of liposomes that are made from polar ether lipids extracted from the Archaea. Archaeosomes are more stable in the presence of acidic or alkaline pH, bile salts, high temperatures and phospholipase in comparison with liposomes and have found application in drug, vaccine and gene delivery. The aim of this study was to formulate archaeosomes using lipid extracted from *Acidianus brierleyi* and evaluation of physic-chemical properties. The lipids were extracted from *A.brierleyi* and analyzed by High Performance Thin-Layer Chromatography (HPTLC). Archaeosomes were prepared using film method and methylene blue was used as a drug model. Then they characterized by Differential Scanning Calorimetry (DSC), and their particle sizes were also determined. The release and permeation of methylene blue was carried out using dialysis membrane and rat skin, subsequently. HPTLC analysis of the extracted lipids revealed that the glycerol ether was the major lipids with more than 70 percent probability. Results of particle size assay showed a mean size of 109.83 nm. The results of DSC showed the shift in melting point of methylene blue on loading that is a good indication of methylene blue loaded archaeosome. The addition of cholesterol improved the ability of archaeosome to encapsulate methylene blue. Encapsulation efficiency was 81.66 ± 2.88% and there was about 100% release after 24 h. Also the drug release pattern followed Peppas model. The results demonstrated that archaeosome may be a suitable new type of nano-particle that can be used as drug delivery system.

Key words: Archaeosome, *Acidianus brierleyi,* Differential scanning calorimetery, High performance thin-layer chromatography.

Liposomes are spherical vesicles with concentric phospholipid bilayers that are formed spontaneously in aqueous solution and are important as a drug delivery system for their biodegradability and biocompatibility. Liposomes have many advantages such as, lack of toxicity, prolonging release of active pharmaceutical agents, ability to entrap both lipophilic and hydrophilic drugs, enhancement of drug penetration, increased circulation life times of drug and protecting encapsulated agents from metabolic processes[1-6]. In spite of these advantages, a major limitation to the development of liposomes as drug delivery system is their instability, especially during their transit to the site of action. Archaeosomes are a novel generation of liposomes that are made from polar ether lipids extracted from the Archaea or synthetic archaeal lipids. Many Archea live in harsh environment including low pH, high salt concentrations and high temperatures[7]. Conventional liposomes are made from ester phospholipids such as phosphatidylcholine. The ether linkages are more stable than esters in wide range of pH[8, 9]. Archaeosomes exhibit higher stabilities in several conditions, such as high

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temperatures, alkaline or acidic pH, presence of bile salts, and are more resistant to oxidation, chemical, enzymatic hydrolysis[7, 9, 10]. Thermal stability allows heat sterilization of archaeosomes without losing of the encapsulated substance[11] and they can be prepared and stored in the presence of air and oxygen without any degradation. Due to their biocompatibility and stability in many conditions, they can be used in biotechnology including drug, vaccine, and gene delivery [8].

Acidianus brierleyi was firstly isolated from an acidic thermal spring in Yellowstone National Park, Wyoming, USA. Its growth is chemolithotrophic by means of oxidation or reduction of S^o or oxidation of ferrous iron and has remarkable capacity in bioleaching[12, 13]. The objective of the study was to prepare archaeosomes using lipid extracted from *A. brierleyi* as new drug delivery and characterize their physico-chemical properties.

MATERIALS AND METHODS

Methylene blue, dipotassium phosphate dibasic (K_2HPO_4), magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), ammonium sulfate ($(NH_4)_2SO_4$), potassium chloride (KCl), calcium nitrate tetrahydrate ($Ca(NO3)_2.4H_2O$), sulfur, cholesterol, chloroform and methanol were purchased from Merck, Germany. Sephadex G-25 and yeast extract were obtained from Sigma, Germany and QUELAB, Canada, respectively. Sulfuric acid was provided by UNI-CHEM®, Germany. *Acidianus brierleyi* was kindly donated by National Iranian copper Industries Co. Sarcheshmeh, Kerman, Iran.

Extraction of lipids from *A. brierleyi*

A.brierleyi cells were grown in 9K-medium, which contained (gram per liter): $(NH_4)_2SO_4$ (3), K_2HPO_4 (0.5), $MgSO_4.7H_2O$ (0.5), $Ca(NO3)_2.4H_2O$ (0.01) and KCl (0.1). Sulfur (10 gram per liter) and 0.1% yeast extract were added to the basal medium and adjusted to pH 1.7 using sulfuric acid. Cultures were incubated in rotary shakers (IKA® KS4000i, Germany) for 7 days at 70°C. Cultures were filtered for removing the sulfur and then sulfur free cells were lyophilized. Extraction of lipids from lyophilized cells was carried out by stirring with chloroform-methanol (2:1, v/v) for 1h at room temperature. The suspension was passed through a sintered glass filter, and the residue re-extracted for an additional hour. Combined filtrates were evaporated, taken up in chloroform-methanol-water (60:30:4.5, v/v/v), and passed through Sephadex G-25 for removal of nonlipid contaminations[14].

High Performance Thin-Layer Chromatograph (HPTLC)

HPTLC was performed on glass bucked silica gel 60 F 254 (Merck) plates of 10×10 cm with the help of CamagLinomat-IV applicator (E.MerckKGaA). All plates were first activated by heating in 150 °C for 30 min. Different developing solvent including chloroform-methanol-water (65:25:4, v/v/v), chloroform, diethyl ether (9:1, v/v) and chloroform-methanol-water (60:10:1, v/v/v) were used[14]. 25µl of samples were spotted on the plates with a Hamilton syringe and chromatography was performed.

Preparation of Archaeosomes

Archaeosomes were prepared from the extracted lipid using thin film method. Briefly, 1% methylene blue was added to the lipids solution that extracted from *A. brierleyi*, and then the mixture was evaporated in a rotary evaporator (Heidolph, Germany). When the thin film was formed in the round-bottom flask, it was hydrated with phosphate buffer. The suspension was agitated by vortex for 30 min and then sonicated for 45 min[1, 2]. Also formulations containing 20 mg cholesterol together with the above mentioned compounds were prepared.

Measurement of archaeosomes size

The average diameters of archaeosomes were determined using a particle sizer Qudix, ScatterO Scope I system (Korea) at 25 °C[15].

Differential Scanning Calorimetry (DSC)

The calorimetric analysis was performed in order to determine the properties of lipids previously structured in the archaeosomes and the effect of methylene blue on the thermograms was also evaluated. The DSC curves were recorded using a DSC-1 Mettler Toledo oven with a temperature range of 0 to 200°C for 6 min[15].

Evaluation of the loading efficacy

The archaeosomal suspension was centrifuged at 20000 rpm for 15 min (VS-35SMTI, Korea). The supernatant was analyzed at 660 nm using a spectrophotometer (Biochrom WAP Biowave II).

***In vitro* drug release studies**

In vitro methylene blue release from the archaeosomes was determined using dialysis membrane method and a specially designed Franz diffusion cell. Samples were put in a dialysis bag (BETAGEN, width 40 mm). The receptor chamber was contained 22 ml distilled water and was continually stirred using a magnet stirrer at 37°C. An aliquot of 3 ml of sample was withdrawn from each batch at definite time intervals (0.5, 1, 2, 3, 4, 6, and 24 h) and replaced with the same amount of distilled water to maintain sink condition. Then, the concentration of released methylene blue was monitored using a UV spectrophotometer at 660 nm[15].

***In vitro* skin permeation**

Male Wistar rat skin was used as model membrane. The skin was hydrated by immersion in water for 24 h before the experiments. The skin was mounted on the Franz-type diffusion cells with the stratum corneum side facing upward into the donor compartment. The donor compartment was filled with methylen blue containing archaeosomes. An aliquot of 3 ml of sample was withdrawn from the receptor compartment at 0.5, 1, 2, 3, 4, 5, 6, and 24 h and replaced with the same volume of distilled water at 37°C to maintain the volume constant. The amount of methylene blue in the receptor phase was assayed using above mentioned UV spectrophotometer apparatus at 660 nm[16].

Drug release kinetics

The release mechanism was evaluated using different kinetic models such as zero order, first order, Higuchi and Korsmeyer-Peppas. The data were reported as mean ± SD and frequently as percents.

RESULTS

Approximately 100 mg lipid was obtained from each 2 gram cells. Total lipids extracted were identified by HPTLC analysis. According to the results, nine, three and seven spots were detected at 254 nm on the plates using different solvent systems containing chloroform-methanol-water (65:25:4, v/v/v), chloroform, diethyl ether (9:1, v/v) and chloroform-methanol-water (60:10:1, v/v/v), respectively (Fig 1-3).

Results of particle size determination showed a mean size of 109.83 nm. Particle size distribution of methylene blue containing archaeosome is shown in Fig (4a, b).

The results of DSC for methylene blue, archaeosomes without methylene blue and methylene blue containing archaeosomes are shown in Fig (5a, b and c), respectively. The DSC curves of methylene blue showed peaks in the

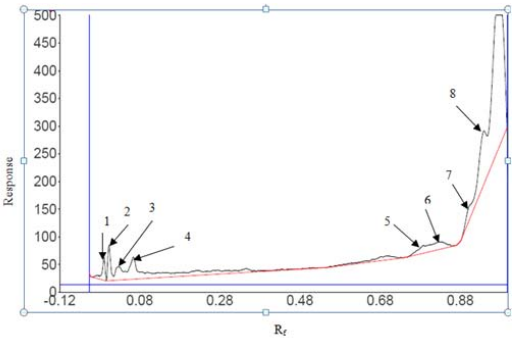


Fig.1. Chromatogram of lipids in solvent system containing chloroform-methanol-water (65:25:4, v/v/v) scanned at 254 nm using camage HPTLC scanner

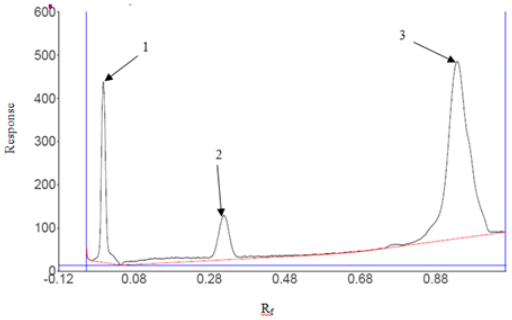


Fig. 2. Chromatogram of lipids in solvent system containing chloroform, diethyl ether (9:1, v/v) scanned at 254 nm using camage HPTLC scanner

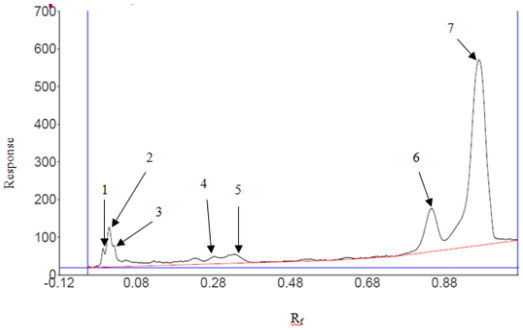


Fig.3. Chromatogram of lipids in solvent system containing chloroform-methanol-water (60:10:1, v/v/v) scanned at 254 nm using camage HPTLC scanner

region 90-110 ° C. The shift in melting point of methylene blue indicated the possible interaction of methylene blue with lipids during the preparation of archaeosome.

Encapsulation of methylene blue in formulations without cholesterol was less than 10%, while the results showed by adding cholesterol, 81.66 ± 2.88% of the methylene blue was encapsulated in archaeosomes. The result of *in vitro* skin permeation showed that methylene blue could pass through skin model and the value of release from archaeosome in 0.5, 1, 2, 3, 4, 5, 6 and 24h was 44±0.00%, 60±3.3%, 75±8%, 85±8.5%, 90±4.5%, 90±0.5%, 90±7.5% and 100±1%,respectively, whereas no release was observed from dialysis membrane. Also the results indicated that the methylene blue was released from archaeosome according to Peppas model (RSQ= 0.999% and MPE=2.446%).

DISCUSSION

Archaeosomes are a new generation of liposomes that are prepared from natural Archeal membrane lipids. They show high stability to acidic or alkalin pH, low or high temperature, high pressure and oxidative conditions. Archaeosomes have potential application in drug and vaccine delivery (8).

According to the results shown in the HPTLC spectum (Fig 1), nine spots were observed at 254 nm on the plate with solvent system containing chloroform-methanol-water (65:25:4, v/v/v). As illustrated from the figure, some components could not move far in this solvent system so two peaks at R_f: -0.01 and R_f: 0.00 were detected with concentration of about 2.51% and 3.51% of the total. The third at R_f: 0.03 with 3.06%, the forth at R_f: 0.07 with 6.21%, the fifth at R_f: 0.79 with 3.32%, the sixth at R_f: 0.83 with 3.33%, the seventh at R_f: 0.90 with 3.24% , the eighth at R_f: 0.94 with 15.43% and the ninth at R_f: 0.98 with 59.33% of the total were detected. This solvent system was employed by Fager*et al* in 1977 and Langworthy *et al* in 1977 for separation of phosphatidylcholine, lysophosphatidyl-ethanolamine and for determination of glycolipids and acidic lipids[14, 17]. It is suggested that the points

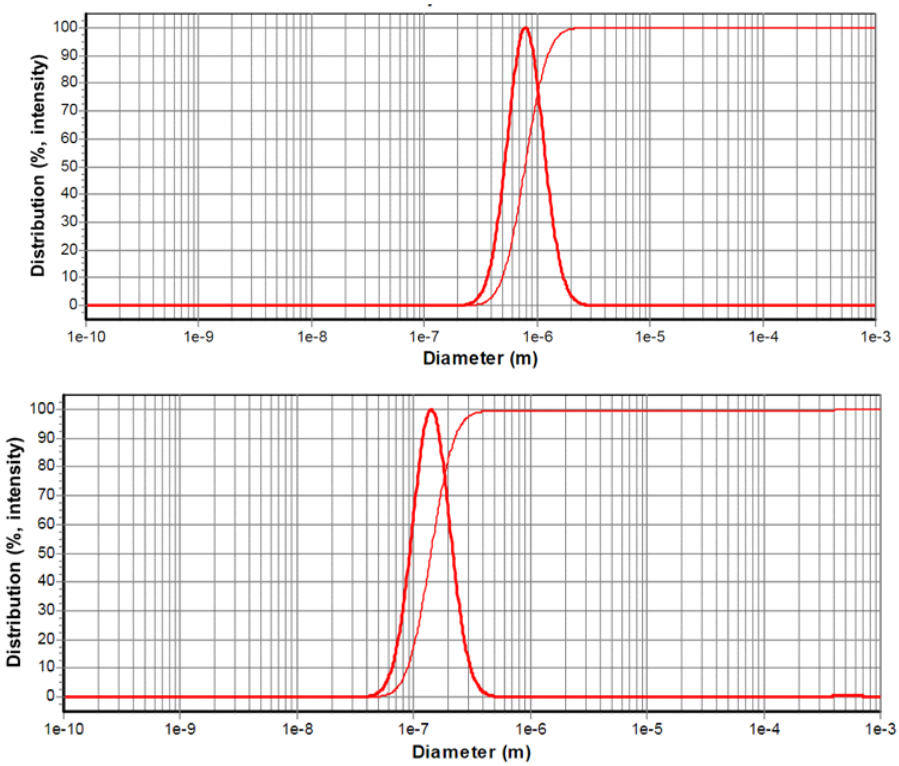
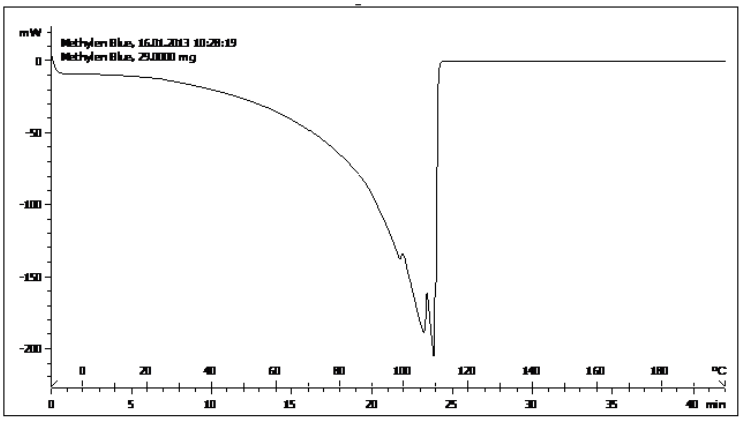
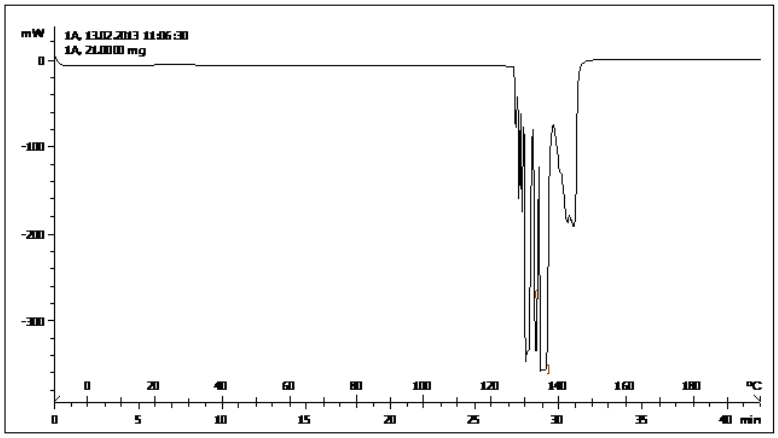


Fig. 4. a). Particle size graphs of methylene blue containing archaeosomes befor sonication. b). Graph of particle size of methylene blue containing archaeosomes after 45 min sonication

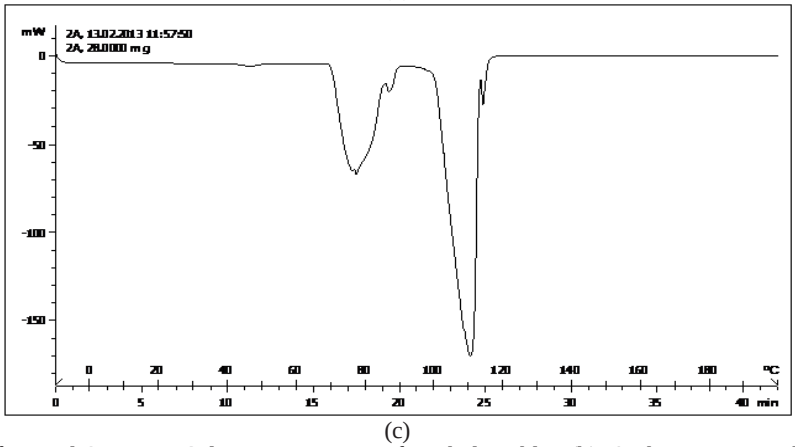
3-9, may be evidences for the presence of phosphatidylcholine, lysophosphatidyl-ethanolamine, glycolipids or acidic lipid. However, still some components were not eluted from spotting place. Moreover, Minnikin *et al* in 1971 employed this solvent system for detecting lipids in the bacterial membranes. The results revealed the presence of three major phospholipids including, diphosphtidylglycerol, phosphatidylethanolamine and phophatidyleglycerol [18].



(a)



(b)



(c)

Fig .5. (a). Differential Scanning Calorimetry spectra of methylene blue (b). Archaeosomes without methylene blue and (c). Methylene blue containing archaeosomes

Another HPTLC plate was developed with solvent system mixture of chloroform, diethyl ether (9:1, v/v). Again some components did not move in this solvent system and a peak at R_f: 0.00 with 15.94% was present (Fig 2) showing less tendency of the lipids to this solvent system. The second component was at R_f: 0.32, with 9.57% and the third at R_f: 0.94 with 74.48% of total. This solvent system has been used by Langworthy *et al* in 1977 for separation of lipids containing glycerol ethers. It is suggested that the second and third component may be the lipid containing glycerol ethers[14].

In order to check the availability of other lipids, another HPTLC plate was developed with solvent system containing chloroform-methanol-water (60:10:1, v/v/v). As indicated in the Fig 3, seven spots were detected at 254 nm on this solvent system. The first component was at R_f: -0.00, meaning that this solvent system was not suitable for detecting this component. It is about 1.56% of the total. The second component at R_f: 0.01 with 4.70%, the third at R_f: 0.02 with 1.72%, the forth at R_f : 0.28 with 2.09%, the fifth at R_f : 0.34 with 3.17%, the sixth at R_f : 0.85 with 13.90% and the seventh at R_f : 0.97 with 72.86% of total were observed. This solvent system was used by Langworthy *et al* in 1977 for separation of lipids containing glycerol ethers.[14] According to their results, spots 2-7 can be lipids composed of glycerol ethers.

The results of measurement of size showed that the particle size reduced from 897.33 nm to 109.83 nm after 45 min sonication. So it is suggested that sonication may be a key parameter in reduction of size of particles. Decreasing in the particle size is an important factor for improving the performance of poorly soluble drugs[19]. It has been previously investigated that sonication time is an important factor in the liposome preparation. By increasing the sonication time, the particle size was reduced from 969 nm to 677 nm[20].

DSC analysis of methylene blue, archaeosome and methylene blue loaded archaeosome is illustrated in Fig 5a, b and c. As shown in Fig 5a, methylene blue has thermal peaks at 95- 110 ÚC that is melting point of the compounds. Comparing Fig 1b, Fig 1a with Fig 1c that is DSC pattern of methylene blue loaded archaeosome, it can be concluded that methylene blue is loaded successfully on the polymer.

The results clearly showed that archaeosome without cholesterol had low entrapment efficiency, (less than 10%), while encapsulation increased with adding of cholesterol (81.66 ± 2.88%). It is suggested that cholesterol may reduce the fluidity and increase the stability of archaeosomes prepared from lipid extracted from Archea. Cholesterol has been used as helper lipid in the liposome formulation to give further rigidity to the bilayer that may improve *in vitro* and *in vivo* stability of liposomes[21]. The results of skin permeation indicated that archaeosome could enhance the permeation of the methylene blue, while no release was observed from membrane dialysis. It appears that, the increased permeability of the methylene blue by archaeosome may be related to the composition of their lipids that are similar to stratum corneum lipids, which are likely to most readily enter stratum corneum lipids and fuse with endogenous lipids. The mechanism of enhanced drug uptake into the stratum corneum is unclear. It is possible that the archaeosomes penetrate the stratum corneum, by adhering onto the surface of the skin and then destabilizing, fusing or mixing with the lipid matrix, loosening the lipid structure of the stratum corneum and increased skin partitioning of the drug[22, 23].

Barbeau *et al*. in 2011 prepared and evaluated archaeosomes based on synthethic Archaeal tetraether lipid and compared them with conventional liposomes. They used carboxyfluorescein as a drug model. The results showed that 70% of the encapsulated carboxyfluorescein was lost within 3h, while a significant improvement in stability was observed with archaeosome, which released only 20% at the same time. They coated the archaeosome with a polyethylene glycol (PEG) in order to achieve a stabilizing nanovector and demonstrated that small proportions of PEGylated archaeolipid added to liposomal formulations increased stability and allowed slow release of the encapsulated dye[9]. Gonzalez *et al*. in 2009 investigated the archaeosome that was made from *Halorubrum tebebquichense* total lipid as a new source of adjuvancy and for entrappment of bovine serum albumin. According to their results, the encapsulation efficiency was approximately 34% and the mean sizes of archaeosomes were 564±22

nm. Their results also indicated that archaeosomes prepared with total polar lipid from this Archea could be successfully used as vaccine delivery system[24]. Also Gupta *et al.* in 2008 investigated delivery of molecules to cancer cells using liposomes from bacterial culture that they were economically comparable with the synthetic lipids. The results showed that these bacterial liposomes had potential applications in delivery of hydrophilic molecules to cancer cells [25].

Our findings indicated that the mechanism of drug release was according to Peppas model. Peppas in 1985 introduced a semi-empirical equation to describe drug release from polymeric devices[26]. This model is dependent on the fraction of drug released at time, rate constant and release exponent [27]. The nature, physicochemical properties of the drug molecule and the enhancers are very important factors in enhancing the skin permeation of the drug[16]. Methylene blue is a polar compound and the stratum corneum layer of skin represents the rate limiting barrier for transdermal delivery of polar compounds. It is believed that follicular shunt route is responsible for the permeation of polar molecules and flux of large polar molecules that have difficulty in diffusing across the stratum corneum. However it is generally accepted that these routs comprise a fractional area for permeation of approximately 0.1% of total permeation. Therefore, penetration enhancement techniques have been focused on increasing transport across the stratum corneum rather than via the appendages[23]. According to our results, archaeosome can be employed as penetration enhancer for polar compounds and drugs.

Further studies in this field, it is suggested to trace the archaeosome in the cancer cell for determining the potential application in delivery of molecules. In the end, the objective is to use archaeosome as new drug delivery system that can be employed for treatment of different disease including cancer.

CONCLUSION

The results of this study indicated that it is possible to prepare a novel liposomal formulation from Archaeal lipids. Regarding to the results, entrapment efficiency was dependant on the presence of cholesterol. In addition, archaeosome could improve permeation properties of the drug model. It is concluded that archaeosome may be regarded as nanodrug delivery vehicle for treating and preventing of diseases and the entrapped drug molecule can be targeted on tissues, cells or intracellular components.

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