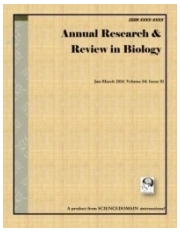

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Application Potential of Liposomal Delivery Systems Prepared by Lipids Extracted from *E. coli* Cultures

Mohammad Kargar[1], Eskandar Moghimipour[2], Zahra Ramezani[2] and Somayeh Handali[1*]

[1]Department of Microbiology, Jahrom Branch, Islamic Azad University, Jahrom, Iran.
[2]Nanotechnology Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

Authors' contributions

This work was carried out in collaboration between all authors. Authors MK, EM and ZR designed the study, performed the statistical analysis, wrote the protocol, Author SH performed the experiment and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: Liposomes are spherical vesicles composed of concentric phospholipid bilayers that can entrap hydrophilic, hydrophobic and amphiphilic drugs. Liposomes can be prepared from natural phospholipids, synthetic lipids or bacterial lipids. Regarding to the easy access to microorganisms in all year round, being economic and possibly growing in various substrates, bacterial lipids can be suitable candidates for preparation of liposomes.
Objective: The aim of the present study was to formulate and evaluate liposomal vesicles prepared by lipid extracted from *E. coli* and loaded with methylene blue as drug model.
Material and Methods: The lipids were extracted from the bacteria *E.coli* and analyzed by High Performance Thin-Layer Chromatography (HPTLC). Liposomes were prepared using film method and then characterized by Differential Scanning Calorimetry (DSC), and their particle sizes were measured. The release of methylene blue was determined using dialysis membrane method.
Results: HPTLC analysis of the extracted lipids indicated that the glycerol ether was the major lipid with more than 70 percent probability. Results of particle size determination

**Corresponding author: Email: handali_s81@yahoo.com;*

showed a mean size of 338 nm. The DSC curve of liposomes without methylene blue was different from methylene blue containing liposomes that indicated the possible interaction methylene blue with lipids during the preparation of liposomes. Encapsulation efficiency was 53.33±2.88% and there was 97.54%±0.00 release after 24 h.
Conclusion: The results of this study may indicate the possible use bacterial lipids in preparation of nano-drug delivery derived system.

Keywords: Liposome; E. coli; differential scanning calorimeter; high performance thin-layer chromatograph.

1. INTRODUCTION

Liposome is a spherical vesicle composed of a bilayer membrane and commercially important drug delivery system for their biodegradability and biocompatibility [1,2,3]. Liposomes have many advantages such as, prolonging release of active pharmaceutical agents, nontoxicity, ability to entrap both lipophilic and hydrophilic drugs, enhancement of drug penetration [4], increased circulation life times of certain drug and protecting encapsulated agents from metabolic processes [5,6].

Liposomes have many applications in the fields of immunology, tumor therapy, vaccine adjuvant, antimicrobial therapy, and delivery of radiopharmaceuticals for diagnostic imaging.

Recently, liposomes have gained potential interest in the field of biotechnology therapeutics as delivery systems for recombinant proteins, antisense oligonucleotides and cloned genes. With the recent development in the field, several companies are actively engaged in expansion and evaluation of liposome products for anticancer, antifungal therapy and for prophylaxis therapy [7-15]. It is demonstrated that liposomes have potential impact in submiocron and nano-scale reaction engineering. In spite of a strong interest for investigating, preparation of liposomes certainly is a challenge for biological engineering [16]. Liposomes can be prepared from natural phospholipids egg or soya or synthetic lipids such as dioleoylphosphatidyl ethanolamine [4]. In fact, these liposome preparation methods require the use of expensive lipids that are commercially available only from a few sources in the world [16]. The cytoplasmic membrane of the *E.coli* includes a high proportion of phosphatidylethanolamine (70-75%) [17].

The aim of this investigation was to prepare and evaluate the liposome using natural lipid extracted from the bacteria *E. coli,* which is extremely economical compared to the synthetic lipids and available in all year.

2. MATERIALS AND METHODS

Methylene blue, nutrient agar, chloroform and methanol were purchased from Merck, Germany and nutrient broth was obtained from QUELAB, Canada. The freeze-dried sealed glass ampoule of *E. coli* (ATCC No. 25922) was obtained from the Iranian Research Organization for Science and Technology, Tehran, Iran.

2.1 Extraction of Lipids from *E. coli*

E. coli cells were grown in nutrient broth (0.5% peptone, 0.3% beef extract/yeast extract, 0.5% NaCl and distilled water) at 37ºC for 24h. Final cell concentrations were 10^8cfu/ml according to the McFarland turbidometry. Extraction of lipids was done according to the Bligh-Dyer method [16]. Chloroform-methanol mixture (1:2) was used as the extraction solvent. Briefly, 3.75 ml of the extraction solvent was added to 1.0 ml of the bacterial culture and vortexed for 10 min. Then 1.25 ml of methanol was added. After vortexing again for 1 min, 1.25 ml of distilled water was added. The final solution was centrifuged at 1000 rpm for 5 min at room temperature. The inferior organic phase containing the lipid was separated using pasture pipette [16].

2.2 High Performance Thin-layer Chromatograph (HPTLC)

HPTLC was performed on glass bucked silica gel 60 F 254 (Merck) plates of 10x10 cm with the help of Camag Linomat-IV applicator (E. MerckKGaA). All plates were first activated by heating in 150ºC 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 [18]. 25µl of samples were spotted on the plates with a Hamilton syringe and chromatography was performed. Different spots were detected by HPTLC.

2.3 Preparation of Liposomes

Liposomes were prepared from the extracted lipid using thin film method [1,2]. Briefly 1% methylene blue was dissolved in distilled water added to the lipids solution, 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 (pH7.4). The suspension was vortexed for 30 min and then sonicated for 15 min [19-21].

2.4 Measurement of Liposome Size

The average diameters of liposomes were determined using a particle sizer Qudix, ScatterO Scope I system (Korea) at 25ºC [22].

2.5 Differential Scanning Calorimetry (DSC)

The calorimetric analysis was performed in order to determine the properties of lipids previously structured in the liposomes and the effect of methylene blue on the thermograms was also evaluted. The DSC curves were recorded using a DSC-1 Mettler Toledo oven with a temperature range of 25 to 200ºC for 6 min [22].

2.6 Evaluation of the Loading Efficacy

The liposomal 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). The incorporation efficiency (EE%) was calculated using the following formula:

$$EE\% = \frac{T - S}{S} x100$$

Where: T: the total of methylene blue and S: the quantity of methylene blue in supernatant.

2.7 *In Vitro* Drug Release Studies

In vitro methylene blue release from the liposome 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 37ºC. 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.

3. RESULTS

Approximately 100 mg lipid was obtained from 100 ml culture. The lipids were identified by HPTLC analysis. According to the results shown in Figs. (1, 2, 3), four, two and three spots were detected at 254 nm on the plate with solvent system 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. The particle size of liposome before and after sonication was 1446 and 338 nm, respectively. The DSC curve of methylene blue free liposome was different from that of methylene blue containing liposomes that suggested that the compound was encapsulated in the liposome and the type of bonding is chemical that is able to change curve thermogram (Fig. 4). The encapsulation efficiency was 53.33±2.88% and release of methylene blue after 0.5, 1, 2, 3, 4, 6 and 24h were 15.4±0.00%, 20±0.00%, 20±0.00%, 50±0.00%, 50±0.00%, 70±0.00% and 97.54±0.00%, respectively.

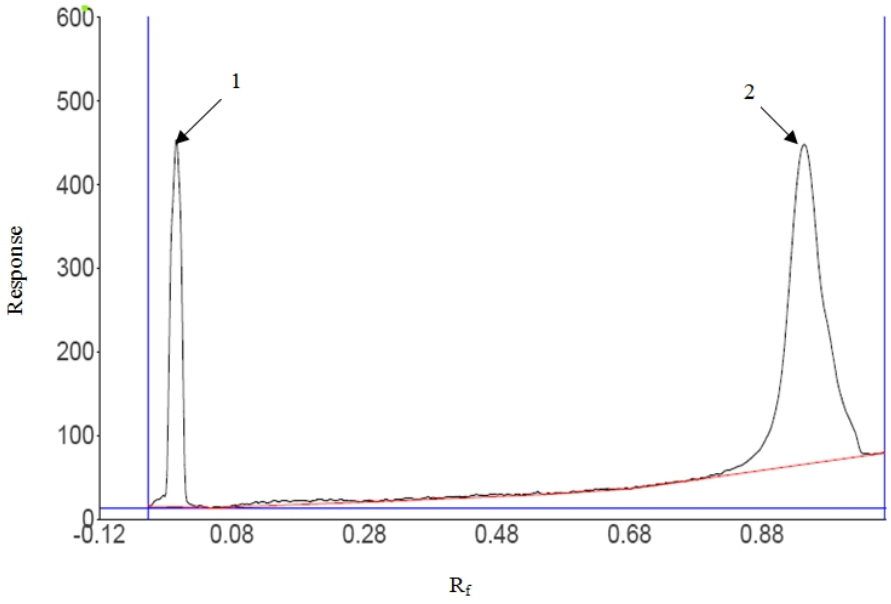


Fig. 1. Chromatography of lipids in solvent system containing chloroform-methanol-water (65:25:4, v/v/v) scanned at 254 nm using camage HPTLC scanner

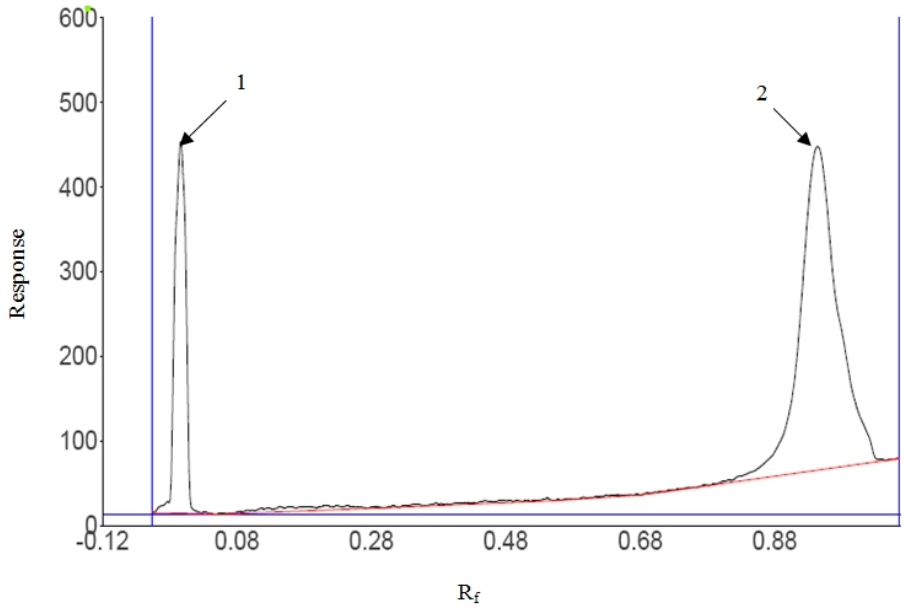


Fig. 2. Chromatography of lipids in solvent system containing chloroform, diethyl ether (9:1, v/v) scanned at 254 nm using camage HPTLC scanner

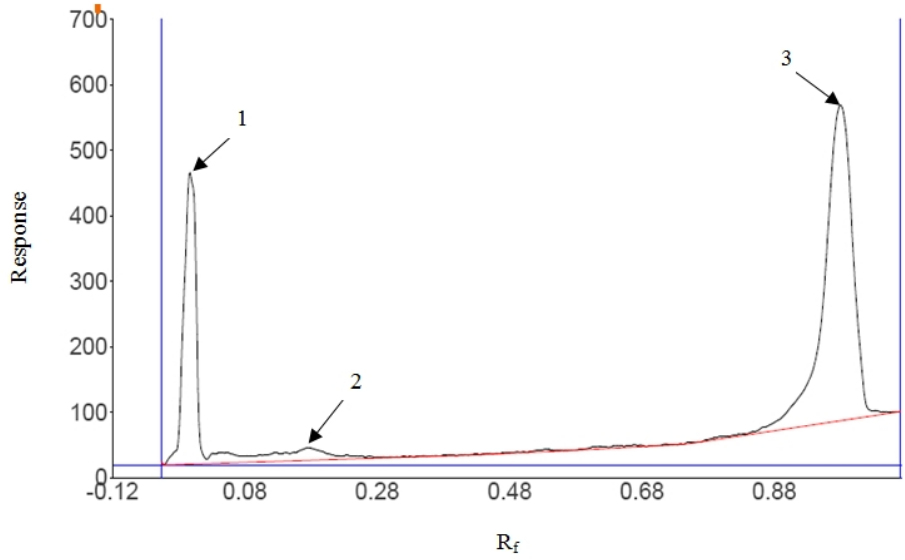
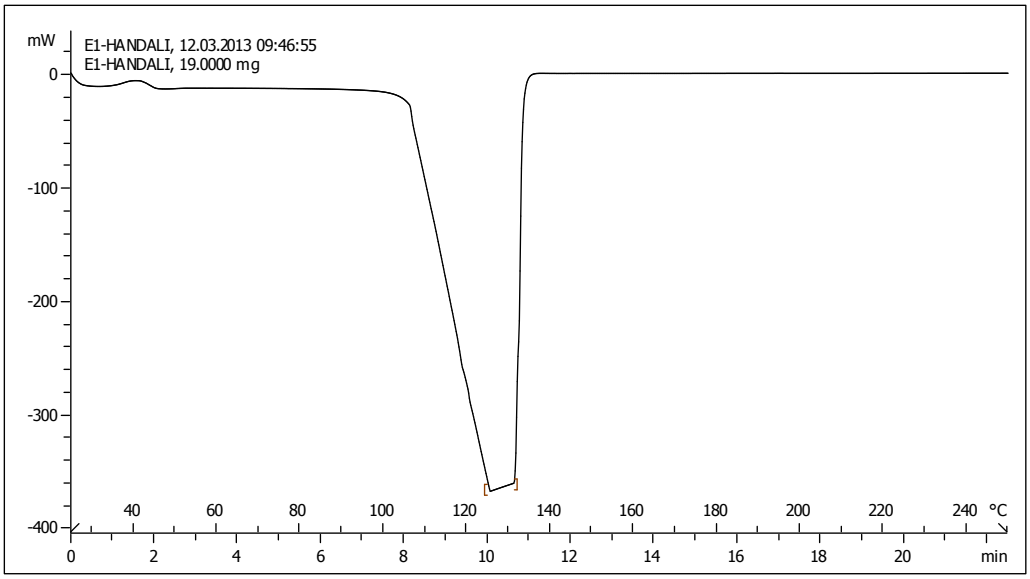
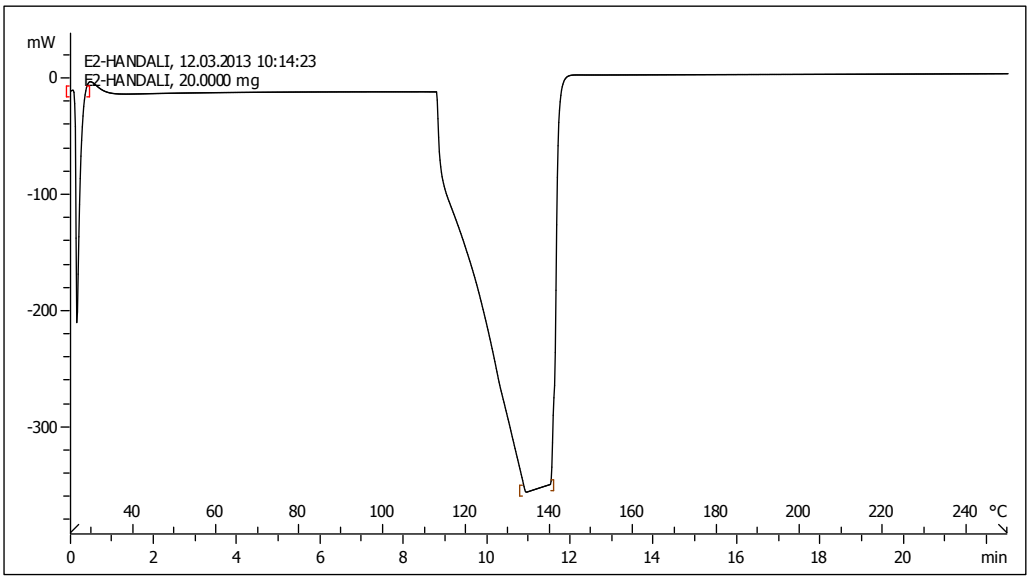


Fig. 3. Chromatography of lipids in solvent system containing chloroform-methanol-water (60:10:1, v/v/v) scanned at 254 nm using camage HPTLC scanner



(a)



(b)

Fig. 4. (a) Differential Scanning Calorimetry of liposome without methylene blue (b) Differential Scanning Calorimetry of methylene blue containing liposomes

4. DISCUSSION

As indicated in Fig. 1. four spots were detected at 254 nm on the plate with a solvent system of chloroform-methanol-water (65:25:4, v/v/v). The first spot was at R_f:-0.00 that means the component did not move. It was about 66.97% of the total. The second component were detected at R_f : 0.15 with 4.23%, the third at R_f: 0.90 with 8.52% and

the forth one at R_f: 0.99 with 20.27% of the total. This solvent system was used by Fager et al. in 1977 and Langworthy et al. In 1977 for the separation of phosphatidylcholine, lysophosphatidyl-ethanolamine and for determining glycolipids and acidic lipids [18,23]. It is suggested that the spots 2, 3 and 4, may be evidences for the presence of phosphatidylcholine, lysophosphatidyl - ethanolamine, glycolipids or acidic lipid. However, still some component did not elute from the spotting place. Moreover, Minnikin et al. in 1971 employed this solvent system for detecting lipids in bacterial membranes. The results revealed the presence of three major phospholipids including, diphosphtidylglycerol, phosphatidylethanolamine and phophatidyleglycerol, that is in accordance with our results. It is well known that in gram negative bacteria phosphatidyle ethanolamine often may constitute 75% or more of the total bacterial lipid [24]. In order to check the availability of other lipids, 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 75.06% was present (Fig. 2). The second component was detected at R_f: 0.95, with 4.23% of total. This solvent system has been employed by Langworthy *et al* in 1977 for separating lipids containing glycerol ethers and the second component may be the lipid containing glycerol ethers [18]. Another HPTLC plate was developed with a solvent system of chloroform-methanol-water (60:10:1, v/v/v). According to the Fig. 3, three spots were observed at 254 nm with 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 was about 27.40% of the total. The second component at R_f: 0.18 with 2.49%, and the third at R_f 0.98 with 70.11% of total were observed. This solvent system was used by Langworthy et al. in 1977 for separation of lipids containing glycerol ethers [18]. According to their results, the components that appear at R_f: 0.18 and 0.98 can be lipids composed of glycerol ethers.

The results of the size monitoring showed the effectiveness of sonication on the size of particles. The particle size decreased from 1446 nm to 338 nm after 15 min sonication. Reduction in the particle size is a key factor for improving the performance of poorly soluble drugs [25]. It has been previously reported that sonication time is an important parameter in the liposome preparation. By increasing the sonication time, the particle size has been reduced from 969 nm to 677 nm [26]. Fig 4a and b show DSC behavior of empty liposomes and methylene blue loaded liposomes, respectively. The DSC curve of liposome without methylene blue showed single endothermic peak in the region 125-130ºC (Fig 4a) relating to its phase transfer, while the DSC curve of methylene blue containing liposomes showed two endothermic peaks at 25ºC and 135-140ºC (Fig. 4b). The shift in the endothermic peak of liposome indicated that methylene blue was successfully encapsulated in the liposome causing a change in its thermal behavior.

The entrapment efficiency of methylene blue in liposome was 53.33±2.88% and release amounts of methylene blue after 0.5, 1, 2, 3, 4, 6 and 24h were15.4±0.00%, 20±0.00%, 20±0.00%, 50±0.00%, 50±0.00%, 70±0.00% and 97.54±0.00 %, respectively. Gupta et al. in 2008 investigated delivery of molecules to cancer cells using liposomes prepared from bacterial culture. They prepared their samples by freeze-thawing method and used methylene blue as drug model. According to their results, the encapsulation value was 91.09±0.11%. They also showed that these liposomes have a potential application in delivery of molecules to cancer cell [23]. According to the results encapsulation efficiency was 53.33±2.88%. It is suggested that it is possible to achieve high loading by changing the method of preparation.

Sprott et al. in 2004 prepared liposome using phosphatidylinositol mannosides extracted from *Mycobacterium bovis* and investigated the adjuvant activity *in vivo*. They encapsulated ovalbumin (OVA) as antigen in the liposome and delivered it to dendritic cells. The results showed the OVA entrapped in the liposome could enhance antibody and cytotoxic T-cell responses. Therefore, they demonstrated that this liposome can be used as vaccine adjuvant. Also, other adjuvant system such as archaeosomes that composed of the polar lipids of Archaea, evoke inflammatory cytokine production by dendritic cells [27]. Gonzalez *et al.* in 2009 utilized total lipids extracted from *Halorubrum tebebquichense* for production of archaeosome as a new source of adjutancy and for entrapment of bovine serum albumin. According to their results, the encapsulation efficiency was approximately 34% and the mean size of archaeosomes was 564±22 nm. Their results also revealed that archaeosomes prepared with total polar lipid from the Archea could be successfully used as vaccine delivery system [28]. Our finding are in accordance with their results that decreased the mean particle size of liposomes to 338 nm after sonication. So, it can be concluded that the sonication is the important factor for decreasing the particle size of nano-particles.

It has been previously reported that total polar lipid-liposomes from *E. coli* and *Saccharomyces cerevisiae* elicit cytotoxicT-lymphocytes (CTL) adjuvant activity. Also, it has shown that *Deinococcus radiodurans* could entrap protein antigen for evoking antibody response. Sprott et al. in 2004 investigated the ability of liposome prepared from the total polar lipids of *Haloferax volcanii*, *Planococcus* spp. and *Bacillus firmus* to elicit and sustain immune responses. Antigen loading liposomes composed of lipids from *H. volcanii*, *Planococcus* spp., and *B. firmus* were 31±2.6, 20±3.7 and 53±4.5 (µg/mg dry weight), respectively. Also average diameters of liposome prepared from *H. volcanii*, *Planococcus* spp., and *B. firmus* were 120±65, 91±51 and 92±48 nm, respectively. According to their results, *Planococcus* liposomes evoked potent non-specific inflammatory cytokine production such as IL-12 and IL-6 by dendritic cells, while *H. volcanii* vesicles evoked little inflammatory cytokines. *B. firmus* liposomes were weak inducers for the CTL response to encapsulated antigen [29]. Unlike to the results of Sprott et al., our finding showed the liposomes prepared from lipid extracted of *E.coli* had relatively high encapsulation efficiency. Barbeau et al. in 2011 prepared and evaluated archaeosomes based on synthetic 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 [30]. Regarding our results, 50% methylene blue was released within 3h. It seems possible that by coating the liposomes with PEG, the stability will be increased. White et al. in 2002 evaluated the physical properties of liposome prepared from *E. coli* polar lipids. According to their results, the mean diameters of liposomes were approximately 150 nm. They showed osmotic up-shifts imposed with NaCl elicited fusion of liposomes, whereas osmotic up-shifts imposed with sucrose did not cause any fusion [17]. Conlan et al. in 2001, immunized mice with lipopeptide antigens encapsulated in novel liposomes (archaeosomes) prepared from *Methanobrevibacter smithii*, *Halobacterium salinarum*, and *Thermoplasma acidophilum* against infection with the facultative intracellular pathogen, *Listeria monocytogenes.* The results of their study indicated that archaeosome-entrapped antigens had great potential as self-adjuvant delivery systems to elicit rapid and prolonged specific immunity against a prototypical intracellular pathogen. When compared to untreated mice, mice vaccinated with lipopeptide contaning archaeosome

as antigen had 8–38 fold fewer *Listeria* in their livers and at least 380–2042 fold fewer *Listeria* in their spleens [31]. Krishnan et al. in 2010 reported that archaeosomes composed of the lipids extracted from *Methanobrevibacter smithii* are potent adjuvants for evoking $CD8^+$T cell response and can be employed for formulating cancer vaccines [32]. Sanchez et al. in 2011 described the formation of stable bilayer vesicles composed of *Pseudomonas aeruginosa* dirhamnolipids (diRL) and dioleoylphosphatidylethanolamine (DOPE), together with the characterization of their fusogenic and delivery properties. For the leakage experiments, a buffer containing 70 mM calcein, was used and the value of encapsulation was 92%. For liposomes stored at room temperature in long-term storage, the value of release was increased. It was suggested that oxidation of oleic acid moieties of DOPE by atmospheric oxygen upon long-term storage may lead to membrane deterioration with the concomitant increase of bilayer permeability. While, liposomes were more stable at 4ºC, temperature at which all the possible adverse effects are delayed. Acidification of DOPE/diRL vesicles leads to membrane destabilization, fusion, and release of entrapped aqueous vesicle contents. According to the results of the study, these liposmes were incorporated into cultured cells through the endocytic pathway, which means that these pH-sensitive liposomes can be employed as delivery system for foreign substances into living cells [33]. It is concluded that the pH can influence the properties of the liposomes. In our work, we have successfully prepared liposomes from lipids derived from bacterial culture. Regarding to the easy access to microorganisms in all year round, to be economic and possibly growing in various substrates, bacterial lipids can be suitable candidates for preparation of liposomes. It is likely by adding nutritional components to the culture medium; we can prepare liposome from lipid bacterial with desirable potential for application in different fields. Utilizing lipids of other bacteria and Archaea, using different methods for preparation of liposomes which may lead to high drug encapsulation, and investigate the physical characteristics that can affect the liposome may be useful for better results. Further studies are needed *in vitro* and *in vivo* in order to evaluate their efficacy and toxicity for treatment of diseases including cancer.

5. CONCLUSION

It is concluded that it is possible to formulate liposome from bacterial lipids. According to the results, loading of some molecules in liposome may exhibit improved release properties. In addition, bacterial liposome's represent low cost in drug delivery systems. They can also be employed for research in various areas such as biotechnology.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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