International Journal of Pharmaceutics 516 (2017) 225–230

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Lipid vesicular nanocarrier: Quick encapsulation efficiency determination and transcutaneous application

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ARTICLE INFO

Article history:
Received 15 September 2016
Received in revised form 31 October 2016
Accepted 3 November 2016
Available online 8 November 2016

Keywords:
Encapsulation efficiency
Liposome
Transfersome
Ethosome
Transdermal immunization

ABSTRACT

Nanoscale delivery systems have been widely investigated to overcome the penetration barrier of stratum corneum for effective transcutaneous application. The aim of this study is the development of effective vesicular formulations of ovalbumin and saponin which are able to promote penetration through the skin layers. Three kinds of vesicular formulations have been investigated as carriers, including liposomes, transfersomes and ethosomes, in which cholesterol and/or cationic lipid stearylamine are incorporated. The impact of membrane composition variations on the protein entrapment has been evaluated for each vesicle type. Formulations were characterized for particle size, polydispersity and encapsulation efficiency. The best formulations for each type of vesicle were subjected to in vivo transdermal immunization in mice. Among the three kinds of vesicular carrier, ethosomal nano carrier not only showed the best stability over a two months' storage, but also enabled the highest increase in the titer of serum antibody. In this regard, cationic nano-ethosomes can be considered as a promising vesicular carrier for transdermal vaccines. Meanwhile, we have developed a simple method to determine encapsulation efficiency of vesicular systems, which has potential application as a high throughput screening for vesicular formulations.

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1. Introduction

With the advancement in nanotechnology, much attention of transdermal research has been focused on various kinds of micro/nanometric delivery systems (Kaurav et al., 2016; Matsuo et al., 2013; Schoellhammer et al., 2014), such as microneedles, nanoparticles, liposomes, transferosomes, ethosome, niosomes and cubosomes, etc. In the regime of vaccine encapsulation via novel carrier systems, nano phospholipid vesicles, including liposome, transfersome and ethosome, have stand out as potential candidates owning to their structural and physiochemical similarities to biological membranes. These unique characteristics are highly promising to overcome the challenges faced during transdermal immunization. Comparing with other transdermal strategies, these versatile vesicular structures are easy scalable and cost effective as vaccine recipients and do not require major consideration for safety issue during pre-clinical/clinical trials.

To the best of our knowledge, there are many comparative studies using lipid vesicles to encapsulate small chemical drugs for transdermal delivery(Alvi et al., 2011; Bragagni et al., 2012; Caddeo et al., 2013; Cortesi et al., 2011; El Maghraby and Williams, 2009; Wo et al., 2011), but very few papers are involved in transdermal vaccination (Gupta et al., 2005; Mahor et al., 2007; Rattanapak et al., 2012). As discussed by Cevc, who performed majority of pioneering work in lipid colloids especially transfersomes, that the rate of vesicle transport through skin barrier is strongly sensitive to aggregate deformability and the relative size of a penetrant. (Cevc and Gebauer, 2003) Unfortunately, when we were reviewing the papers on vesicular carrier used for transdermal drug delivery, very few researchers had paid due attention on the size control of the lipid vesicles. It is noted that, some of the vesicles used had micrometers size range or showed too high polydispersity index (PDI > 0.3) (Bragagni et al., 2012; Nasr et al., 2008; Rattanapak et al., 2012), both factors of which would make the vesicles experience much hindrance during skin penetration. Another important aspect is the encapsulation efficiency of lipid vesicles, since its influencing factors and the very importance in immune cell activating after skin penetration remain to be uncovered.

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http://dx.doi.org/10.1016/j.ijpharm.2016.11.011
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On the basis of these premises, we considered it worth of interest to redesign different nano-lipid vesicular system as potential carrier to improve antigen skin permeability. With this aim we have developed, evaluated and compared the effectiveness of three different kinds of lipid vesicular formulations, i.e. traditional liposomes, ethosomes and transfersomes. For each kind of vesicular system, a preliminary screening was performed to evaluate the effect of composition of formulations on protein encapsulation and the best one for each type vesicular formulation was selected for their stability assay and in vivo transdermal immunization in mice.

2. Materials and methods

2.1. Materials

Soy phosphocholine (soy PC), cholesterol, bovine serum albumin (BSA) and ovalbumin (OVA) were purchased from Sigma. Bicinchoninic acid (BCA) protein assay kit was purchased from Beyotime (Shanghai, China). Purified saponin was purchased from Ruibio Co. Tween-20, Triton X-100 and sodium cholate were Aladdin product (Shanghai, China), 3,3′,5,5′-Tetramethylbenzidine (TMB) were product of Oddfoni (Nanjing, China). Horseradish peroxidase (HRP) conjugated goat anti-mouse IgG was Biosharp product (Heifei, China). High-purity water (Milli-Q water, Millipore, Bedford, MA, USA) was used throughout the study. All other chemicals were of analytical grade.

2.2. Methods

2.2.1. Preparation of lipid vesicles

Liposomes and transfersomes were prepared by reverse phase evaporation method (Szoka and Papahadjopoulos, 1978). Lipids were dissolved in 9 mL diethyl ether, followed by emulsification with 3 mL aqueous PBS solution containing ovalbumin (1 mg/mL) and saponin (0.4 mg/mL) by water-bath sonication for 2–5 min. The organic solvent was evaporated in a rotary flash evaporator at room temperature.

Ethosomes were prepared using a modified reverse phase method which has been described by others (Rattanapak et al., 2012; Touitou et al., 2000). Soy PC (20 mg/mL) was dissolved in chloroform in a scintillation vial and a thin lipid film was created by evaporating the chloroform under a stream of nitrogen at room temperature. 0.2 mL ethanol was added to the vial, which was then tightly capped. The solution was vigorously mixed using magnetic stirrer at 45 °C–50 °C for 10 min before the gradual addition of 0.8 mL of peptide (1 mg/mL) and saponin (0.4 mg/ ml) dissolved in PBS. The solution was then mixed for a further 30 min under room temperature.

All prepared milky lipid vesicles were further treated using tip sonicator with ice bath until visually transparent and stored at 4 °C after being filtered with Millex® syringe filter (0.22 μm pore size, Millipore).

2.2.2. Encapsulation efficiency calculation based on direct method

In order to determine the encapsulation efficiency, free unentrapped proteins were removed out using three different methods: ultrafiltration, dialysis or centrifugation. The total protein was determined by Bicinchoninic acid (BCA) protein assay kit after disrupting vesicles with Triton X-100 (Li et al., 2011; Tyagi et al., 2015).

Pipette 0.5 mL of each formulation into Amicon® ultra-centrifugal filter tube (MW cutoff 100 kDa, Millipore) and centrifuge under 10,000 rpm for 10 min and wash the tube with PBS at least 3 times. The remaining solution in tube was removed and supplemented with PBS up to 0.5 mL. Triton X-100 was added to a final concentration of 0.5% (the same condition for the below experiments where needed) and the amount of protein was determined by BCA kit.

Dialyzed 1 mL of each formulation in 100 kDa dialysis tubing (Spectrum Laboratories Inc.) against 200 mL PBS at room temperature for first 3 h then changed into another 200 mL fresh PBS and kept stirring at 4 °C overnight. The dialyzed formulation was removed and added with Triton X-100 for protein determination by BCA kit.

The formulations were spun at 14000 rpm for 60 min. The very top supernatant was carefully transferred and the amount of free protein was determined by BCA kit. In the case of centrifugation, the amount of encapsulated protein would be subtracted free protein from the total protein.

The encapsulation efficiency (EE%) was calculated according to the following equation:

$$EE = \frac{\text{Encapsulated protein}}{\text{Total protein}} \times 100\%$$

The total protein was determined from initial lipid vesicles disrupted by Triton X-100. The experiments were performed in triplicate.

2.2.3. Determination of particle size and size distribution

A 90 Plus particle size analyzer (Brookhaven Instruments) with a 658.0 nm monochromatic laser was employed to measure the size distribution of prepared lipid vesicles. Before conducting measurement, all samples were pre-filtered with Millipore 0.22 μm membrane filter. The vesicle size and polydispersity index (PDI) were measured in triplicate at 25 °C.

2.2.4. Stability assay of lipid vesicles

Selected formulations of the three types of vesicles (liposome, transfersome and ethosome) were stored at 4 °C for up to 70 days. Samples from each formulations were withdrawn at definite time intervals (0, 7, 30 and 70 days) and characterized for their vesicle size and polydispersity index (PDI) (El Zaafarany et al., 2010).

2.2.5. Animal immunization (Mishra et al., 2008)

Female BALB/c mice 6–8 weeks old (18–20 g) were used in all experiments. All animal experiments were conducted in accordance with the national regulations for care and use of laboratory animals. One day before immunization, the hair on abdominal skin was carefully removed after applying depilate cream, which was bought from domestic drug store (for human use, the active gradient is calcium thioglycolate). Totally five groups were included in our experiment, each group contains five animals. The screened formulations were applied onto the surface of mouse skin, 0.2 mL for each animal. In positive control group, mice were subcutaneously injected with 0.2 mL OVA (1 mg/mL) solution with colloidal $Al(OH)_3$ as adjuvant. For negative control, mice were administered with 0.2 mL OVA (1 mg/mL) and saponin(0.4 mg/mL) solution on hairless skin. An equal booster dose of each formulation was administered by the same route after two weeks. Blood samples were collected from tail vein in the following 2, 4, 6, and 8 weeks after primary immunization.

2.2.6. Determination of anti-OVA IgG levels by enzyme-linked immunosorbent assay (ELISA)

The anti-OVA antibody titer in serum was measured by ELISA. Briefly, 96-well ELISA plate were coated 100 μL OVA/well (10 μg/mL in 0.05 M sodium carbonate/bicarbonate coating buffer, pH 9.6) at 4 °C overnight. Then the plates were blocked with 0.5% BSA 200 μL per well for 2 h at 37 °C. Subsequently, the plates were

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