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Original Article

Preparation and Characterization of Nanoliposome Containing Isolated VP1 Protein of Foot and Mouth Disease Virus as a Model of Vaccine

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Abstract

Foot-and-mouth disease (FMD) is an acute and highly contagious disease in livestock, such as cattle, sheep, and pigs, leading to a lot of economic losses. The current FMD vaccines formulated by inactivated whole-virus and adjuvant successfully reduce disease outbreaks in many regions of the world. Immunological studies on FMD viruses revealed that the dominant epitope in arising neutral antibody response is amino acid residues constructing the G-H loop, constituting a surface loop of the structural protein, termed VP1. Liposomes as one of the most well-known vehicles are considered an important carrier in vaccine development, and their function is used to encapsulate purified VP1 protein based on their size, charge, and lipid content. Accordingly, the VP1 protein was isolated from the FMD virus. This study aimed to compare four methods of VP1 protein encapsulation in the liposome and the extruding effect, as follows: 1) VP1 protein was dissolved in dimethyl sulfoxide and added to the lipid film hydrated by ethanol, 2) the lipid film was hydrated by VP1 protein with 7M urea, 3) the lipid film was hydrated by VP1 protein and freeze-thawed, and 4) the lipid film was hydrated by VP1 protein. The highest encapsulation efficiency was 91% in the second method which purified protein-containing urea. The VP1 protein in the prepared liposome (1, 2-dimyristoyl-sn-glycero-3-phosphocholine: 1, 2-dimyristoyl-sn-glycero-3-phosphocholine: cholesterol) released more than 90% of protein content after 240 h.

Keywords: Encapsulation, FMDV, Liposome, Purification, VP1 protein

1. Introduction

The foot-and-mouth disease (FMD) is a contagious disease for livestock, mostly cattle, swine, sheep, and goats, leading to death in young animals and less production in adults and fertility in breeding stock (1). Symptoms of FMD appear as vesicles on the mouth, feet, and tongue (2, 3). FMD is also caused by the FMD virus (FMDV), a positive-strand Ribonucleic acid (RNA) virus that is a member of the genus *Aphthovirus* and *Picornaviridae* (4). The FMD virus has extremely variable RNA, leading to serotypes of O, A, C, Asia 1, SAT 1, SAT 2, and SAT 3, and a wide range of subtypes. A and O strains, the most common serotypes in many parts of the world, are the predominant subtypes of FMDV in Iran (5, 6). Moreover, the FMD virus consists of an icosahedral protein capsid and an RNA genome encoding a single polyprotein, which can be cleaved into four structural proteins (e.g., VP1, VP2,

VP3, and VP4) and eight non-structural proteins (e.g., L, 2A, 2B, 2C, 3A, 3B, 3C, and 3D polymerase) (7, 8). The major neutralizing antigenic site of FMDV is seated in the G-H loop of VP1 protein, consisting of a conserved Arg-Gly-Asp (RGD) motif (9). The VP1 protein has serotype-determining regions (10). The current FMD vaccines are formulated by inactivated whole-virus associated with adjuvants, such as Al $(OH)_3$ and Montanide ISA-206 (11) to control the FMD in Western Europe and South America. The liposome is a kind of adjuvant to deliver an antigen and increase its immunogenicity (12) with a vesicular structure associated with an aqueous core and phospholipid bilayer encapsulating the antigens and drugs (13). Liposomes could also be engineered in different sizes, and nano-sized liposomes are very important adjuvants in vaccinology, termed nanoliposome (14). In the present study, the VP1 protein was isolated and purified from the FMD virus by the electroelution method. Nanoliposome formulation, containing 1, 2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG), 1, 2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), and cholesterol were used as adjuvant and antigen delivery systems. Additionally, the VP1 protein was encapsulated in the nanoliposomes using four different methods. Encapsulation efficiency (EE %) of the VP1 protein, the particle size, zeta potential of the nanoliposomes, the effect of extruding on the particle size, and the protein release profile were also investigated in this study.

2. Materials and Methods

2.1. Materials and Equipment

DMPC, DMPG, and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL), and nucleopore membranes were provided from Millipore, Germany. Moreover, Acrylamide, Tris-Base, Dithiothreitol, NaOH, ethanol, methanol, urea, dimethyl sulfoxide (DMSO), (4- (2-hydroxyethyl)-1-piperazineethanesulfonic acid), sucrose, Tricine, Sodium dodecyl sulfate, NaCl, and other salts were provided from Merck, Germany. BIO-RAD criterion™ vertical electrophoresis cell, BIO-RAD model 422 Electro-Eluter, Bio-Rad Trans-Blot® Semi-Dry system, IKA rotary evaporator RV 10 auto V, Avanti ® Mini Extruder, and Elma Ultrasonic Cleaner S30H were also utilized in this study.

2.2. Foot-and-Mouth Disease Viruses

Inactivated FMD virus serotype O 2016, purchased from the FMD Department, Razi Vaccine and Serum Research, was used for VP1 protein isolation.

2.3. Tricine-SDS-PAGE Electrophoresis

According to the method of Hermann Schagger, a discontinuous Tricine-sodium dodecyl sulfate-polyacrylamide gel was utilized to separate virus proteins and determine VP1 protein (25 kDa) in the gel. The initial voltage of 30 V was also applied until the samples completely cross the stacking gel (4%), and the voltage was then increased 10 V per 10 min and maintained on 100 V.

2.4. Electroelution

VP1 protein was purified by electroelution method with a BioRad model 422 Electro-Eluter. The band of VP1 protein (25 kDa) in Tricine-SDS PAGE was cut out and pooled. The VP1 protein was electroeluted with electroelution buffer (Tris base 25mM, glycine 199mM, and SDS 0.1%) for 3 h at 100 mA, and the buffer was then exchanged with the elution buffer without SDS to remove SDS from protein.

2.5. Western Blotting

The functional activity of the purified VP1 protein was confirmed by Western blot analysis. At first, 16% Tricine-sodium dodecyl sulfate-polyacrylamide gel, including 6 M urea was performed, and protein band was transferred to nitrocellulose membrane (poor size 0.4µm) by the Bio-Rad Trans-Blot® Semi-Dry System (30 min, 10 V). The membrane was firstly blocked with blocking buffer (3% bovine serum albumin in 0.01 M phosphate-buffered saline [phosphate-buffered saline]) incubated at room temperature for 1.5 h, and it was then washed by washing buffer (PBS-Tween 0.05%). Thirdly, the membrane was incubated with guinea pig serum polyclonal antibody raised against serotype O FMDV (1:4 diluted) at 4˚C, overnight. Afterward, it

was washed four times with PBS-T and then incubated with HRP-labeled anti-guinea pig conjugate whole IgG (1:10000 diluted in PBS-T) for 1 h at room temperature. Finally, it was washed again as mentioned previously. The reaction was developed with 4-CN/HRP substrate (Merck, Germany).

2.6. Preparation of the Lipid Film

The liposome was formulated, as follows: DMPG: DMPC: Cholesterol (1:16:4 molar ratio, 60 mM). DMPG, DMPC, and cholesterol were dissolved in chloroform in four round-bottom flasks and placed on rotary evaporator vacuum condition, set at 35˚C for 30 min, leading to the lipid film. The performed lipid films were freeze-dried overnight. Four methods were used to encapsulate the purified VP1 protein in liposomes to achieve the highest EE, repeated three times.

2.7. Hydration Methods

The methods are considered as follows: 1) ethanol: the lipid film was dissolved in 3:7 ethanol: HEPES/sucrose 10% buffer containing 10% DMSO and 100 µg/ml purified VP1 protein, 2) urea: the lipid film was dissolved in HEPES/sucrose 10% buffer, containing 100 µg/ml purified VP1 protein and 7M urea to increase the solubility of VP1 protein, 3) buffer/freeze-thaw: the lipid film was dissolved in HEPES/sucrose 10% buffer, containing 100 µg/ml purified VP1 protein, then it was freeze-thawed twice (liquid nitrogen-water 35˚C), and 4) buffer: the lipid film was dissolved in HEPES/sucrose10% buffer, containing 100 µg/ml purified VP1 protein. All the samples were then sonicated for 15 min in an ultrasonication bath.

All four prepared formulations were extruded through nucleopore membranes (400, 200, and 100 nm) to obtain an optimal size of nanoliposomes. The particle size and zeta potential of formulated nanoliposomes were determined by dynamic light scattering and zeta potential analyzer (Malvern). Transmission electron microscopy was also used to characterize the size and morphology of the empty nanoliposomes.

2.8. Determination of Encapsulation Efficiency

The concentration of the total protein and encapsulated protein was assessed using a modified Lowry protein assay (15, 16) to determine EE% of the VP1 protein. Amicon Ultra centrifugal filter (30 kDa) was used to separate unencapsulated protein from nanoliposomes with VP1 protein. In modified Lowry protein assay protocol according to Wang and Smith 1975, 2% Triton-X100, 20% SDS, and 1N NaOH were used to dissolve nanoliposomes. The samples were then incubated at 60˚C for 20 min to disrupt nanoliposomes composition. The EE is calculated as follows:

$$EE\% = \left(\frac{Protein\ concentration\ after\ Ultrafiltration}{Protein\ concentration\ before\ ultrafiltration}\right) \times 100$$

2.9. Determination of the VP1 Release

To evaluate the VP1 release profile, the nanoliposomes were prepared using urea, and the process with the highest EE was diluted 1/10 in HEPES/sucrose 10% buffer and aliquot in the microtubes and incubated at 37˚C in a water bath. At predetermined time intervals (24, 48, 72, 144, 192, and 240 h), microtubes were centrifuged (15 min, 14000×g, 4˚C). Furthermore, the protein concentration of the supernatant was determined by the modified Lowry method, and the release percentage was then assessed based on the following equation:

$$\text{Protein release (\%)} = \left(\frac{P_t}{P_0}\right) \times 100$$

where Pt indicates the amount of VP1 protein released from the nanoliposomes at certain times, and P0 signifies the total amount of encapsulated VP1 protein in the nanoliposome. The protein release profile over time was then graphed in this study.

3. Results

3.1. Purification of the VP1 Protein from FMDV

In the present study, the electroelution method was used to purify the VP1 protein from the FMD virus. The Capsid of the virus Tricine-SDS PAGE method was used to separate the VP1 protein from pentamers.

After protein separation, VP1 protein was cut out and electroeluted; moreover, analytical electrophoresis was conducted for purified eluted VP1 protein (as shown in figure 1), the whole virus, and the eluted VP1 and VP2 proteins. Furthermore, VP1 protein was demonstrated with a molecular weight of 26 kDa, and VP2 protein molecular weight was 24 kDa.

Tricine-SDS PAGE was set up for Western blotting with Guinea pig anti-serum raised against serotype O FMDV, and the result indicated the function of the whole virus, as well as the VP1 and VP2 proteins (Figure 2).

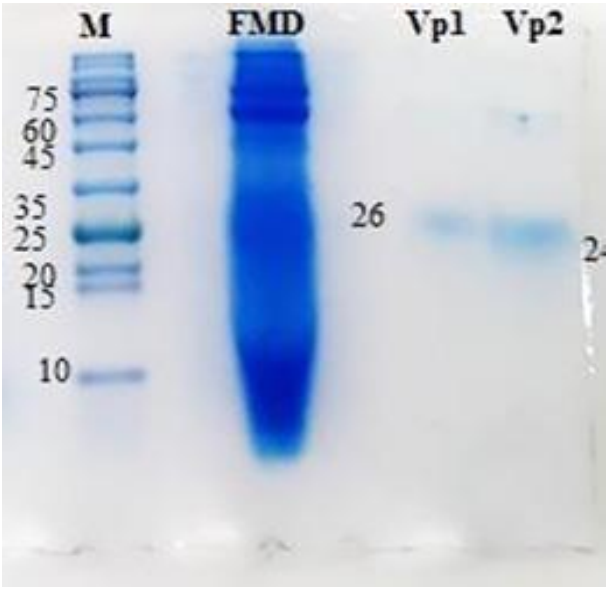

Figure 1. Tricine-SDS PAGE of the whole FMD virus purifies eluted VP1 and VP2 proteins; M is a protein marker

Figure 2. Tricine-SDS PAGE and western blotting of FMD virus and purified VP1 and VP2 proteins

3.2. Liposome Size, Zeta Potential, and Polydispersity Index

The particle size and distribution of the particles were analyzed to compare the average size and polydispersity index of nanoliposomes in the four mentioned methods and compare the extrusion effect on the particle size. VP1 protein was encapsulated in liposomes using four methods so that in one case, they were extruded and in another case, they were not extruded. For each case, liposome size, zeta potential, and polydispersity index were determined as shown in tables 1 and 2, repeated three times with the same overall result. The VP1-encapsulated nanoliposome (urea method) was analyzed by transmission electron microscopy, and the result is shown in figure 3.

For all four processes, the size of the extruded formulations was estimated at 100-110 nm, and the size of the non-extruded formulations was approximately 400-2000 nm. The use of freeze-thaw in nanoliposome preparation without extrusion shows an increase in the particle size and in all four methods, the extruded nanoliposomes displayed monodispersity (PDI<0.2).

3.3. Determination of the Encapsulation Efficiency

Additionally, figure 4 shows the difference in the EE for all methods, with and without extrusion, that extrusion decreases the EE in all methods. In order to determine the effect of different methods on EE, the purified VP1 was encapsulated into the liposome using four methods, namely ethanol, urea, buffer/freeze-thaw, and buffer. Based on the results, the hydrated lipid film has the highest EE (91%) using urea for hydration, the most efficient strategy for the encapsulation of VP1 protein in nanoliposome. EE was repeated three times for each method with the same results.

3.4. Release

in vitro, VP1 protein from nanoliposomes was released by diluting liposomes and centrifugation (14000×g for 15 min) at certain time intervals. Liposomes were diluted 10 times with pH 7.4 HEPES/sucrose 10% and incubated 10 days at 37˚C to mimic physiologic conditions. Figure 5 shows the

profile of protein release, a slow and steady release of VP1 protein from nanoliposome for the experiment, and a release rate of 54% on day 6 and a maximum release rate of about 96% on the day 10. All results repeated three times, and the release curve represents the cumulative results over 10 days.

Table 1. Liposome size, zeta potential, PDI, and EE (%) of VP1 in liposome with extrude

Method	Size (nm)	PDI	Zeta potential (mV)	EE (%)
Ethanol	74±1	0.139	-45.6	24.12
Urea	168±1	0.176	-37.3	73
Buffer/freeze-thaw	123±1	0.02	-37	56.15
Buffer	146±1	0.337	-33.2	29.96

Table 2. Liposome size, zeta potential, PDI, and EE (%) of VP1 in liposome without extrude

Method	Size (nm)	PDI	Zeta potential (mV)	EE (%)
Ethanol	337±1	0.265	-49	58.67
Urea	451±1	0.17	-39.7	91
Buffer/freeze-thaw	854±1	0.84	-37	60
Buffer	455±1	0.782	-37.7	29.96

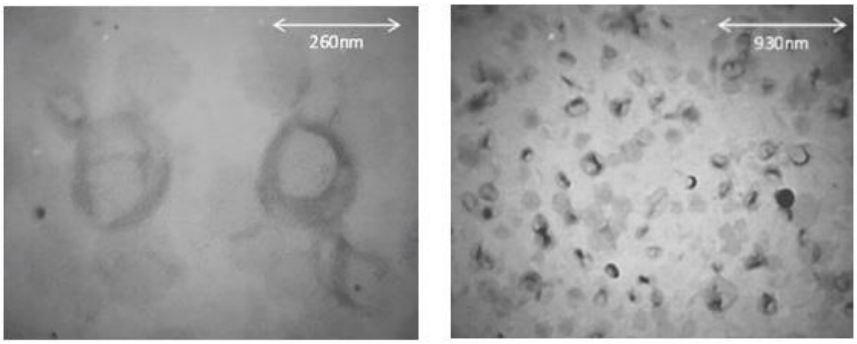

Figure 3. Transmission electron microscopy photomicrographs of encapsulated liposomal by VP1 protein

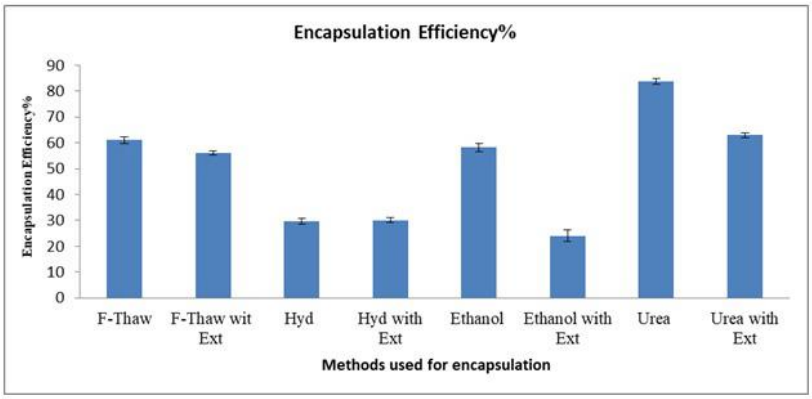

Figure 4. Efficacy of protein encapsulation in four hydration methods demonstrated in extruded and non-extruded cases with error bars showing the standard deviation. *Hydration **Extrude

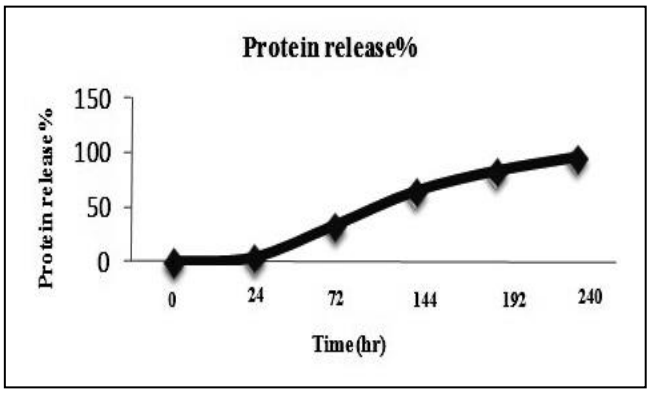

Figure 5. Percentage release of VP
1 protein from nanoliposomes; DMPG: DMPC: Cholesterol (1:16:4 molar ratio, 60mM) incubated at 37˚C over 10 days. The VP1 protein encapsulated in liposome by freeze-thaw method with error bars showing standard deviation

4. Discussion

Vaccination of animals is very important to prevent economic loss in livestock due to extremely rapid replication and intense FMD virus. The current FMD vaccine consists of inactivated binary ethyleneimine purified FMD virus with Montanide ISA 201 or 206 as an adjuvant with some limitations. Animals are protected against FMD; however, 50% of vaccinated animals have a primary infection. This protection should be thus boosted 30 days after vaccination and annual revaccination. Recently, many groups have investigated the various methods to induce protection against FMDV and improve the safety and long-lasting immunity of FMD vaccines. Previous studies on FMDV mainly focused on viral vector vaccines, such as Fowlpox virus expressing VLPs that partially protect swine against FMDV challenge (17), recombinant VLPs, such as purified VLPs expressed in *Escherichia coli* (18, 19), protein vaccines, such as purified VP1 produced in *E. coli* (20), and deoxyribonucleic acid (DNA) vaccines, such as DNA vaccine with P1 and NS 2A, 3C, and 3D, administered by electroporation (21).

In this study, VP1 protein, one of the most important proteins against the FMD virus in immune response, was used as an antigen containing some specific antigenic situations and also located on the FMDV capsid surface. Furthermore, the VP1 protein was separated from the FMD virus by the Tricin-SDS PAGE method and purified by the electroelution process. The important factor in the efficiency of vaccines is selecting an appropriate adjuvant as an immune stimulant and the delivery of antigen to the correct immune cells (22). Liposomes have recently attracted considerable attention in the vaccine delivery system and boost the immune response to specific antigens as an adjuvant. The size scale, charge, lipid composition of liposomes, and antigen EE in liposomes are essential to enhance immune response, vaccine stability, and antigen release from liposomes (12, 22, 23). A study by McLennan et al. (2005) on drug delivery reveals that the average particle size of liposomes smaller than a few nanometers is typically switched into the blood capillaries; however, the larger particles (>150nm) are transferred into the lymphatic capillaries, and the particle size greater than a few hundred nanometers is also stopped for a long period in the interstitial space or transported by dendritic cells (24). The size of nanoliposomes below 50 nm is not stable as liposomes require high lipid for circulation (25, 26).

5. Conclusion

In this study, four methods of protein encapsulation in liposome (DMPC:DMPG:Chol) were performed and size, PDI, Zeta potential and encapsulation efficiency (%) were competed. In addition, the effect of extrusion on size, charge and encapsulation efficiency was investigated. The urea method has highest encapsulation efficiency (91%) and is the best method for encapsulation of VP1 protein in liposome. Extrusion of formulated liposomes will make them size in range of 50-150 nm moreover only 15min sonication make them size in range of 300-850 nm which affect the sort of immune response induction. The result also shows that extrusion does not have significant effect on PDI and zeta potential but that extrusion reduces the encapsulation efficiency (%) of protein in liposomes.

Authors' Contribution

Study concept and design: R. M.
Acquisition of data: M. K.
Analysis and interpretation of data: M. K. and T. E.
Drafting of the manuscript: M. K. and T. E.
Critical revision of the manuscript for important intellectual content: T. E and M. R. A.
Statistical analysis: M. K., T. E., and L. H.
Administrative, technical, and material support: R. M., M. R. A., T. E., and F. G.

Conflict of Interest

The authors declare that they have no conflict of interest.

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