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Original Article

Immunity Evaluation of an Experimental Designed Nanoliposomal Vaccine Containing FMDV Immunodominant Peptides

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Abstract

Foot-and-mouth disease (FMD) is a highly contagious viral disease affecting cloven-hoofed animals. The particular virus causing FMD disease is called FMD virus and is a member of the Aphthovirus genus in the Picornaviridae family. The FMD virus has an 8500 nt long single strain positive RNA genome with one open reading frame (ORF) trapped in an icosahedral capsid protein. This virus genome doesn't have proofreading property which leads to high mutagenesis. It has seven serotypes, including O, A, ASIA, SAT1, SAT2, and C serotypes, as well as many subtypes. Iran is an endemic region for foot-and-mouth disease. Vaccination of susceptible animals with an inactivated whole-virus vaccine is the only way to control the epidemic in many developing countries. Today, conventionally attenuated and killed virus vaccines are being used worldwide. In Iran, animals have been vaccinated every 105 days with an inactivated FMD vaccine. Although commercially available FMD vaccines are effective, they provide short-term immunity requiring regular boosters. A new FMD vaccine is needed to improve immunization, safety, and long-term immune responses. A synthetic peptide vaccine is one of the safe and important vaccines. Peptide vaccine has low immunogenicity, requiring strong adjuvants. Nanoliposomes can be used as new adjuvants to improve immune response. In the current study, nanoliposomal carriers were selected using Dimyristoylphosphatidylcholine (DMPC), dimyristoyl phosphoglycerol (DMPG), and Cholesterol (Chol) as an adjuvant containing two immunodominant synthetic FMDV peptides. The liposomal formulations were characterized by various physicochemical properties. The size, zeta potential, and encapsulation efficiency were optimized, and the obtained nanoliposome was suitable as a vaccine. The efficacy of vaccines has been evaluated in guinea pigs as animal models. Indirect ELISA was

used to detect FMDV-specific IgG. The obtained results indicated that although antibody titer was observed, the amount was lower compared to the groups that received inactivated virus-containing liposomes. In addition, the results showed that liposome was an appropriate adjuvant, compared to other adjuvants, such as Alum and Freund, and can act as a depot and induce an immune response.

Keywords: Guinean pigs, adjuvants, ELISA, Encapsulation Efficiency

1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious and devastating disease of cloven-hoofed animals and can cause significant economic losses. In addition, FMD is characterized by various clinical signs, such as fever; lameness; the formation of vesicles on the feet, mouth, and tongue; decreased milk production; and loss of animal power and fertility. Vaccination of susceptible animals with the inactivated whole-virus vaccine is the only way to control the epidemic in many developing countries (1). The tetravalent vaccine available in Iran produced by the Razi Vaccine and Serum Research Institute, Karaj, Iran (2), contains serotypes A, Asia1, and two strains of serotype O. This vaccine is typically produced from BHK21 cell culture supernatants from foot-and-mouth disease virus (FMDV)-infected cells that were chemically inactivated, purified and formulated with an adjuvant. The use of the inactivated virus as a vaccine has a number of disadvantages, such as incomplete inactivation of the virus, the problem of separating vaccinated animals from infected ones, and the need for booster vaccination every four months. These limitations have prompted researchers to develop a safe alternative vaccine (1, 3). New technologies were adopted to develop alternative vaccines, such as subunit vaccines, recombinant virus vaccines, DNA vaccines, empty capsid vaccines, and peptide vaccines, and identify safe and effective alternatives for conventional methods of FMD vaccine production (4-6). Non-living vaccine antigens, such as peptide vaccines are often poorly immunogenic due to their synthetic nature and require selected strong and safe adjuvants to increase their immunogenicity (7). It should be noted that FMDV contains several neutralizing epitopes and is therefore a good candidate for peptide vaccine (8). In 2014, Zhang, Pan (8) applied FMD virus peptides with Montanide adjuvant and provided 60% protection in cattle. In recent years, liposomes and nanoliposomes have been widely used as an adjuvant for vaccines (9). In 2013, Gao, Feng (10) applied nanoliposome as an adjuvant containing T cell epitope peptide of FMD virus and achieved 25%-60% protection in the mice. Nano-Liposomes act as depots for the slow release of antigen. Features, such as lipid composition, charge, size, size distribution, entrapment, and the status of the antigens within the liposome can be selected to achieve a strong and effective immune response (11).

In the present study, nanoliposomal carriers were used as an adjuvant containing two different peptides. Peptide 1 (VP1 141-161, ATNVRGDLQVLAQKAARTLP) is embedded in a B-cell epitope in positions 141-161 of the FMDV subtype O2016 of the VP1 protein. Peptide 2 (VP1 198-211, EARHKQKIVAPVKQ) is embedded in a B-cell epitope in positions 141-161 of the FMDV subtype O2016 of the VP1 protein. Predictions for peptides 1 and 2 were made using IEDB-AR (http://tools.iedb.org/), and NetMHCpan-2.0 (http:/www.cbs.dtu.dk / services / NetMHCpan) and were synthesized by Chinese Peptide Company (Hangzhou, China). The immunization process was performed on guinea pigs and these animals were divided into 7 groups. The indirect ELISA was used to determine the effect of the vaccine on the animal model.

2. Materials and Methods

The materials used in this study included Dimyristoylphosphatidylcholine (DMPC); Dimyristoyl phosphoglycerol(DMPG); istearoylphosphatidylcholine (DSPC); 1,2-distearoyl-sn-glycerol-3-phospho-(1'-rac-glycerol) (DSPG); and Cholesterol (CHOL); 4-(2-

Hydroxyethyl) piperazine-1-ethanesulfonic acid; N-(2-Hydroxyethyl) piperazine-N′-(2-ethane sulfonic acid) (HEPES), as well as methanol, ethanol, isopropanol (Emsure®), dimethyl sulfoxide (DMSO), and acrylamide. Peptides used in this study were purchased from DG peptides Co., Ltd. (Hangzhou, China) and are presented in table 1.

Table 1. Two synthetic peptides

Peptide	Serotype	Amino acid Position	Amino acid sequence
P1	O2016	141-161	ATNVRGDLQVLAQKAARTLP
P2	O2016	198-211	EARHKQKIVAPVKQ

2.1. Peptide Synthesis

Two different synthesized amino acid sequences corresponded to epitopes in the VP1 proteins as the predominant epitope that elicits the production of neutralizing antibodies by B cells. Peptide stock was prepared by dissolving peptides in DMSO (10mg/ml).

2.2. Formation and Characterization of Nanoliposome

Nanoliposomes were prepared by the dehydration-rehydration method. Lipids were used at a molar ratio of 16:4:5 (DMPC, DMPG, Chol) and dissolved in chloroform. This method involved removing the organic solvent by rotary evaporator (IKA, Germany) and freeze dryer device (Christ, Germany) and formation of a thin layer of lipid film. Afterward, the lipid film was rehydrated by a 10mM HEPES buffer containing 10% sucrose and 10µl peptide (contained peptide was dissolved in DMSO 10 mg/ml) per 1 ml liposomal solution. The prepared liposomes contained 60 mM total lipid.

The hydrated lipids were shaken by rotary evaporator (without vacuum condition) at 35°C (10°C above the transition temperature) or pipetting. The LUV liposomes were sonicated (the 160-W output power, Elmasonic, Germany) for10 min afterward, and the nanoliposomes containing peptide were extruded through 400 nm, 200 nm, and 100 nm polycarbonate membranes employing a mini extruder (Avanti, USA) to form small unilamellar vesicles. Liposomes were stored at 4 ºC under argon. Unencapsulated peptides were removed by a 10 kD dialysis bag (12). The quality of liposome formation was assessed by Dynamic light scattering (DLS) and Transmission electron microscopy (TEM).

2.3. Determination of Encapsulation Efficiency

The evaluation of the encapsulation efficiency of the peptides was conducted using a 10 KDa dialysis bag to separate the peptides which have not been encapsulated in liposomes. The peptide contents were measured by the Lowry method with some modifications (13).

Encapsulation Efficiency (EE %) of peptides were calculated using the following formula:

$$EE\% = \frac{\text{peptide concentration in liposome after dialysis}}{\text{peptide concentration in liposome before dialysis}} * 100$$

2.4. Dynamic Light Scattering (DLS)

The average size of the liposome diameter, polydispersity index (PI), and zeta potential were determined by the DLS method and Zeta Sizer 1000 HSA (Malvern, UK). This technique is based on the Brownian movement which is dependent on the size of the particles (14).

2.5. Transmission Electron Microscopy (TEM)

Nanoliposomes have been described by transmission electron microscopy (TEM) (Eindhoven, NL). Samples were prepared using the process reported by Anderson, Omri (15) A drop of the liposome suspension was floating on a formvar-coated copper grid that was treated with a poly-l-lysine solution. After 3 min, the nanoliposome suspension was replaced by a decrease in negative stain (phosphotungstic acid 2%, w/v, pH 6.5 in distilled water) (15).

2.6. Peptide Release

Nanoliposomes containing peptides were dialyzed to isolate peptides that have not been encapsulated. Liposome formulations containing peptides were formulated using a freeze-thawing protocol and stored in a 10KDa dialysis bag for 14 days in a shaker incubator at 37° C. Sampling of the nanoliposomes was

conducted by removing 0.05ml of the content and replacing it with HEPES/Sucrose buffer performed at predetermined time intervals (16).

The release percentage was calculated by the following formula:

$$\text{Release\%} = \left(\frac{\text{peptide concentration after first dialysis} - \text{peptide concentration after second dialysis}}{\text{Peptideconcentration after first dialysis}}\right) * 100$$

2.7. Animals

Male guinea pigs in the weight range of 250-300 g were provided from the Razi Vaccine and Serum Research Institute, Karaj, Iran, and were kept under clean-air condition. The sera titer was checked by ELISA to be free from antibodies against the FMD virus. They were allotted into seven groups of five each.

2.8. Immunization Groups

In total, 35 guinea pigs were randomly divided into seven groups of five animals each. Seven groups of vaccines injected to animals included group 1) liposome containing peptide; group 2) Combined liposome with an inactivated virus; group 3) combined alum with peptide; group 4) combined Freund with peptide; group 5) combined alum with an inactivated virus; group 6) combined Freund with the inactivated virus; group 7) PBS buffer. The vaccinations groups are presented in table 2.

All groups were immunized by an intramuscular injection of different vaccine formulations (0.5 mL). According to the formulations, 100µg of each peptide and 10µg of inactivated FMD virus (O2016) were inoculated. All groups were injected three times by two weeks intervals.

Breeding was performed, and the serum was collected from all guinea pigs in each groups 2, 3, 5 and 6 weeks after the first injection, and the sera were subjected to indirect ELISA afterwaed.

Table 2. Animal classification table for vaccination

Group	1	2	3	4	5	6	7
Vaccine Formulation	Lip*-pep**	Lip-virus	Alum-pep	Freund-pep	Alum-virus	Freund-virus	Buffer

*Lip: Liposome
**Pep: peptide 1 and 2

2.9. Indirect ELISA Detection of IgG Response in Guinea Pigs

An indirect ELISA was used to detect FMDV-specific IgG response in vaccinated guinea pigs. Blood samples were collected from vaccinated guinea pigs. Micro titer plates with 100 µl/well of 10µg/ml FMD virus in carbonate buffer were coated and incubated overnight at 4°C. After three washes with PBST (containing 0/05% Tween 20) the coated plate was blocked by adding 250 µl per well of 5% skim milk to PBST and incubation at 37°C for 1:30 h. After three rounds of washing, the plates were incubated with serum samples diluted in 1% skim milk (1:50) at 37°C for 1:15 h. After washing with PBST, the plates were treated with rabbit anti-guinea pig horseradish peroxidase-conjugated whole IgG antibody for 1:15 h at 37ºC. After the final washing step, 100µl BM blue Roche substrate (Sigma, St. Louis, MO, USA) was added and the plates were kept for 20 min in darkness at room temperature. The reaction was terminated by the addition of 0.1 M sulfuric acid, and FMDV reactivity was read by a microplate spectrophotometer (BioTek Instruments Inc., USA), based on optical density (OD) at 450 nm.

3. Results

3.1. Liposome Characterization

The size and zeta potential of nanoliposomal formulations are presented in figure 1. Based on the obtained results, the zeta potential of nanoliposomal formulation was negative, and the sizes of formulations were in the range of 120-130 nm. The final

encapsulation efficiency has been measured as well, and the obtained results are tabulated in table 3.

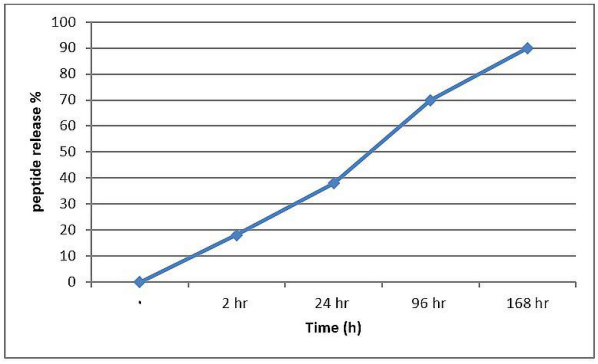

Figure 1. Diagram of peptide release from DMPC: DMPG: Chol liposomes for 7 days

Table 3. Size, zeta, and encapsulation efficiency of nanoliposomal vaccine

	SIZE	ZETA	Encapsulation efficiency %
DMPC/DMPG/CHOL 60 mM	Concentration 60mM		
	129.6±.9	-27.7	67%

3.2. Peptide Release Profile

The peptide release value for DMPC: DMPG: Chol can be found in figure 1. Peptide release of DMPC: DMPG: Chol nanoliposome is shown to be less than 2% after 24 h. After 168 hr, the liposomal formulation released all the peptide content in the release process.

3.3. Antibody Response

In the current study, two synthetic peptide vaccines with different adjuvants, such as nanoliposome, alum, and Freund were tested intramuscularly on guinea pigs. During all tests, animals were kept in isolation, without direct or indirect contact with other animals. Anti-FMDV antibodies have been tested with indirect ELISA in vaccinated animals. The result of IgG titer level is presented in figures 1, 2, and 3. The results showed that anti-FMDV-serotype-O antibodies were detected in all peptide-vaccinated groups two weeks post-vaccination (wpv); however, the amount of antibody titer was not significant. As shown in figure 3, when comparing peptide groups, such as liposome peptide, alum peptide, and Freund peptide, the highest IgG response was related to combined Freund adjuvant with peptide, followed by the liposome containing peptides, and combined Alum with peptide. Anti-FMDV-serotype-O antibodies have been detected in 2 WPVs in virus-vaccinated groups, such as virus Freund, virus alum, and combined virus with nanoliposome (Figure 4). Moreover, the highest level of IgG response was detected in the Freund virus, followed by the alum virus and the liposome virus.

Figure 5 shows that the Freund virus has the highest level of IgG response, followed by the alum virus, liposome virus, Freund peptide, alum peptide, and liposome peptide.

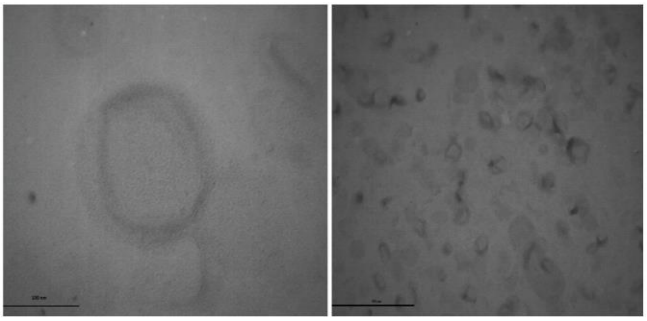

Figure 2. TEM images of nanoliposomes containing peptides, Panel **A:** Nanoliposome with the size of 100 nm, Panel **B:** Population distribution outlook reported for nanoliposomes

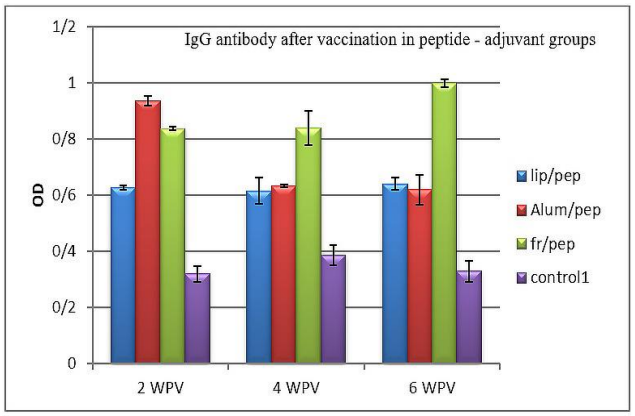

Figure 3. Levels of anti-FMDV specific IgG in pooled sera of different groups of guinea pigs immunized by IM injection three times with two-week intervals. The highest IgG titer was observed in liposome containing peptide, followed by combined alum with peptide, combined Freund with peptide, and negative control group.

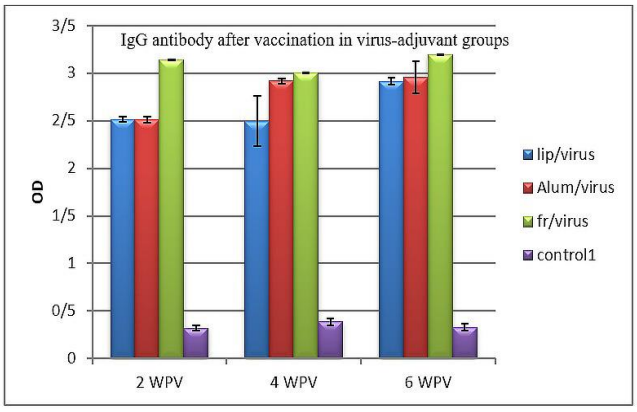

Figure 4. Levels of anti-FMDV specific IgG in pooled sera of different groups of guinea pigs immunized by IM injection applied at three times in two-week intervals. The highest IgG titer was observed in Freund with the virus, alum with the virus, liposome with the virus, and negative control.

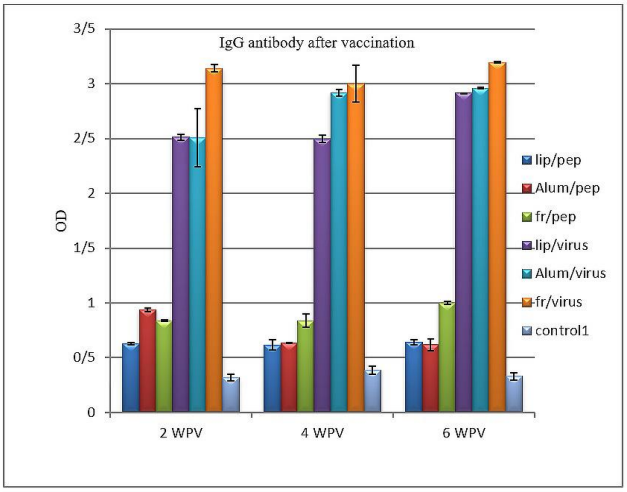

Figure 5. Levels of anti-FMDV specific IgG in pooled sera of the different groups of guinea pigs immunized by IM injection three times in two-week intervals. The highest IgG titer was observed in the Freund virus, followed by the alum virus, liposome with the virus, Freund with peptide, liposome with peptide, alum with peptide, and negative control, in descending order.

4. Discussion

The priority in this study was to use novel adjuvant-containing immunodominant peptides as an FMD vaccine to improve long-term immune response and safety. The selective suitable adjuvant can be beneficially programmed for humoral antibody response, which plays an important role in protecting against FMD. Lipid-based adjuvants, such as liposomes, are thought to induce a powerful immune response (17). The composition of the liposome plays a major role in protecting the cargo against body clearance (18). Phospholipids used for making liposomes include DMPC, DMPG, and CHOL. The results of previous studies revealed that liposomes with an intermediate transition temperature (T_m 25-40°C) could induce a better immune response, compared to liposomes with a high transition temperature (T_m>50° C) (19). The transition temperature for DMPC and DMPG is 25°C, making this composition suitable for inducing an immune response. The application of cholesterol to the liposomal formulations caused significant humoral immune responses (19). The head groups of phospholipids affected the charge of liposomes. Due to the presence of neutral PC (DMPC) and negative PG (DMPG) phospholipid head groups, the liposome revealed a negative charge that could be affected by antigen charges. In the current study, the zeta potential of liposome formulation was -27, which was more stable and appropriate for positive peptides encapsulation (Table 3).

The capillary absorption depends on size, charge, and hydrophobicity. Particle sizes between 10 and 100 nm are eliminated by lymphatic vessels, particles greater than 100 nm remain as a reservoir at the injection site, and particles smaller than 10 nm are extracted by the blood vessels (20). The mean size of liposome formulations is approximately 129 nm (Table 3).

The peptide sequence P1 (an antigen corresponding to the amino acid positions 141 to 161) and the peptide sequence P2 (an antigen corresponding to the amino acid position 198-211 of VP1 in the strain O/2016) were used as a B cell epitope. The nanoliposomes containing peptides are a good candidate for safer and more effective FMD vaccines.

The results of three injections of liposomal vaccine containing peptides revealed that although antibody titer was observed, the amount was much lower, compared to the groups that received inactivated virus-containing liposomes. The results were inconsistent with the reported results of other studies, emphasizing

that amino acid positions 140-160 peptides of VP1 protein could create a high level of humoral immunity (10). Further study of the groups of inactivated viruses combined with Alum, Freund, and nanoliposome showed that the virus-Freund group reached the highest level of antibody titer two weeks after injection. Moreover, six weeks after the first injection, the antibody titer reached the highest level in all three groups containing the inactivated virus.

The stability of the liposome formulation, as a carrier vaccine, is significant for improving the effectiveness of the vaccine delivery mechanism. Therefore, determination of the required amount and time for the release of the trapped portion is important. This *in vitro* stability is important for a nanoliposomal vaccines containing peptides that operate as a depot of antigens, display the antigen for a longer period of time, and trigger the full immune response before the RES clears the released antigen.

figure 1 showed that the release was slow and shown the liposomal formulation containing peptide release all content over seven days. This result is consistent with other studies (15).

In 2014, Saravan, Sreenivasa (21) injected liposomes (VacciMax®) containing inactivated virus and achieved 25-63% protection in the cows being tested. Similar to the study presented in Figure 4, the liposome formulation used in the current study was proved to be a good adjuvant and might induce an immune response. It appears that the liposome has a long-term immune response, compared to the Freund adjuvant and the alum adjuvant.

Results showed that the injection of 10µl (10mg/ml) peptide in 1 ml liposome in liposome containing peptide group lowered the antibody titer, compared to the inactivated virus groups. It seems that the increased amount of injectable peptide up to 2 or 3 times leads to the increase of the IgG antibody level.

Comparison of all the vaccinated groups (Figure 3) showed that the Freund virus had the highest level of IgG response, followed by alum virus, liposome virus, Freund peptide, alum peptide, and liposome peptide, in descending order.

The study of long-term immune response induced by liposomal adjuvant, compared to alum and Freund adjuvants, is recommended for further studies.

Authors' Contribution

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

Ethics

All the procedures were approved by the Ethics Committee at the Tehran Medical Sciences Branch, Islamic Azad University, Tehran, Iran.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Rodriguez LL, Gay CG. Development of vaccines toward the global control and eradication of foot-and-mouth disease. Expert Rev Vaccines. 2011;10(3):377–87.
2. Motamedi-Sedeh F, Soleimanjahi H, Jalilian AR, Mahravani H, Shafaee K, Sotoodeh M, et al. Development of protective immunity against inactivated Iranian isolate of foot-and-mouth disease virus type O/IRN/2007 using gamma ray-irradiated vaccine on BALB/c mice and Guinea pigs. Intervirology. 2015;58(3):190–6.
3. Parida S. Vaccination against foot-and-mouth disease virus: Strategies and effectiveness. Expert Rev Vaccines. 2009;8(3):347–65.
4. Cao Y, Lu Z, Li Y, Sun P, Li D, Li P, et al. Poly(I: C) combined with multi-epitope protein vaccine completely protects against virulent foot-and-mouth disease virus challenge in pigs. Antiviral Res. 2013;97(2):145–53.

5. Guo HC, Sun SQ, Jin Y, Yang SL, Wei YQ, Sun DH, et al. Foot-and-mouth disease virus-like particles produced by a SUMO fusion protein system in Escherichia coli induce potent protective immune responses in guinea pigs, swine and cattle. Vet Res. 2013;44(1):1–13.
6. Shao JJ, Wong CK, Lin T, Lee SK, Cong GZ, Sin FWY, et al. Promising multiple-epitope recombinant vaccine against foot-and-mouth disease virus type o in swine. Clin Vaccine Immunol. 2011;18(1):143–9.
7. Aguilar JC, Rodríguez EG. Vaccine adjuvants revisited. Vaccine. 2007;25(19):3752–62.
8. Zhang Z, Pan L, Ding Y, Zhou P, Lv J, Chen H, et al. Efficacy of synthetic peptide candidate vaccines against serotype-A foot-and-mouth disease virus in cattle. Appl Microbiol Biotechnol. 2015;99(3):1389–98.
9. Su D, Van Rooijen N. The role of macrophages in the immunoadjuvant action of liposomes: effects of elimination of splenic macrophages on the immune response against intravenously injected liposome-associated albumin antigen. Immunology. 1989;66(3):466–70.
10. Gao F, Feng L, Zhang Q, Yan R, Li Y, Li X. Immunogenicity of Two FMDV Nonameric Peptides Encapsulated in Liposomes in Mice and the Protective Efficacy in Guinea Pigs. PloS One. 2013;8(7).
11. Tabrizi MM, Hosseini SA, Akbarzadeh A. Liposome & Nanotechnology Book. 2017.
12. Shariat S, Badiee A, Jaafari MR, Mortazavi SA. Optimization of a method to prepare liposomes containing HER2/Neu-derived peptide as a vaccine delivery system for breast cancer. Iran J Pharm Res. 2014;13:15–25.
13. Smith L. Lowry Determination of Protein X- 100. Anal Biochem. 1975;63:414–7.
14. Smith MC, Crist RM, Clogston JD, McNeil SE. Zeta potential: a case study of cationic, anionic, and neutral liposomes. Anal Bioanal Chem. 2017;409(24):5779–87.
15. Anderson M, Omri A. The Effect of Different Lipid Components on the in Vitro Stability and Release Kinetics of Liposome Formulations. Drug Deliv J Deliv Target Ther Agents. 2004;11(1):33–9.
16. Anzai K, Yoshida M, Kirino Y. Change in intravesicular volume of liposomes by freeze-thaw treatment as studied by the ESR stopped-flow technique. BBA - Biomembr. 1990;1021(1):21–6.
17. Alving CR. Liposomes as carriers of antigens and adjuvants. J Immunol Methods. 1991;140(1):1–13.
18. Li J, Wang X, Zhang T, Wang C, Huang Z, Luo X, et al. A review on phospholipids and their main applications in drug delivery systems. Asian J Pharm Sci. 2015;10(2):81–98.
19. De Serrano LO, Burkhart DJ. Liposomal vaccine formulations as prophylactic agents: Design considerations for modern vaccines. J Nanobiotechnology. 2017;15(1):1–23.
20. Bagby TR, Cai S, Duan S, Thati S, Aires DJ, Forrest L. Impact of molecular weight on lymphatic drainage of a biopolymer-based imaging agent. Pharmaceutics. 2012;4(2):276–95.
21. Saravanan P, Sreenivasa BP, Selvan RPT, Basagoudanavar SH, Hosamani M, Reddy ND, et al. Protective immune response to liposome adjuvanted high potency foot-and-mouth disease vaccine in Indian cattle. Vaccine. 2015;33(5):670–7.