

Engraftment of plasma membrane vesicles into liposomes: A new method for designing of liposome-based vaccines

Afshin Samiei ¹, Ali Mohammad Tamadon ², Soliman Mohammadi Samani ², Nicholas Manolios ³, Eskandar Kamali Sarvestani ^{1*}

¹ Department of Immunology, Autoimmune Diseases Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

² Department of Pharmaceutical Biotechnology Faculty of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran

³ School of Medicine, University of Sydney, Sydney, Australia

ARTICLE INFO	ABSTRACT
<p>Article type: Original article</p>	<p>Objective(s): One of the major challenges in the field of vaccine design is choosing immunogenic antigens which can induce a proper immune response against complex targets like malignant cells or recondite diseases caused by protozoan parasites such as leishmaniasis. The aim of this study was to find a way to construct artificial liposome-based cells containing fragments of target's cell membrane. This structure not only mimics the real biological properties of proteins in the cell membrane of target cells, but also may induce the required immune responses, which culminate in eradication of target cells.</p> <p>Materials and Methods: Five different techniques have been investigated to engraft the plasma membrane's vesicles (PMVs) derived from a characterized <i>Leishmania</i> parasite into liposomes. The most efficient method was tested again on the PMVs derived from well-known breast cancer cell line SK-BR-3. The percentage of engraftment was determined by two-color flowcytometry after staining the engrafted dioctadecyl-3,3,3'-tetramethylindocarbocyanine Dil-labeled liposomes with FITC-labeled PMVs.</p> <p>Results: Among the investigated techniques, freeze-drying method with 91±2% and 90±3% of engraftment for <i>Leishmania</i> and SK-BR-3 derived PMVs, respectively, showed superiority over the other methods. In addition, after 9 weeks storage in refrigerator, freeze-dried fused particles kept their original size (660±350 nm) and fusion efficiency (94±3%).</p> <p>Conclusion: Among five different engraftment techniques, freeze-drying is preferred over the other methods due to its simplicity, more fusion efficiency and stability of produced particles during storage.</p>
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Introduction

Despite the ability of live attenuated vaccines to elicit long lasting immunity, many attempts were made to design subunit vaccines with immunogenic plasmid DNAs, proteins and peptides to avoid the side effect of live vaccines and overcome the limitation of their large scale production (1-7). However, previous studies showed these kinds of vaccines barely induced protective immune response even after administration with adjuvant, especially in people at risk of infection with parasites having a complex life cycle (malaria and leishmaniasis) or someone who needed protective immune response against cancer cells.

For parasitic infections and malignant conditions, lack of induction of a protective immune response by subunit vaccines might be due to the need for mimicking natural course of diseases by designed vaccines or introducing whole immunogenic molecules

of targeted cells in their natural shapes (8, 9).

In the field of vaccinology, liposomes can be considered as an alternative solution to overcome the above mentioned concerns, due to their biodegradability, sustain release of molecules, cell-like structures and their ability to carry both hydrophilic and hydrophobic molecules (10, 11). Depending on their lipid composition, liposomes can also provide potent adjuvanticity properties (12-15). In addition, many biosafety and bulk production concerns associated with live vaccines are avoided by using liposomes. Nowadays, clinical trials are carried out in the field of liposomal vaccine against a wide spectrum of diseases ranging from parasitic infections to malignancies. In addition, liposomal based vaccines are widely used in clinics to protect the host against viral infection (15-20).

On the other hand, membranous vesicular structures such as cell-derived plasma membrane

*Corresponding author: Eskandar Kamali Sarvestani, Department of Immunology, Autoimmune Diseases Research Center, Shiraz University of Medical Sciences, Shiraz, Iran. Tel/Fax: +98- 711- 2304069; email: immunol2@sums.ac.ir

vesicles (PMVs) potentially contain numerous antigens (Ags) that are configured into their natural structure. Therefore, insertion of PMVs into liposomes will confer advantages of presence of Ags with their original configuration in the fused particles. Another important advantage of liposomes fused with cell or pathogen-derived membranous vesicles are the presence of numerous known as well as yet uncharacterized Ags and pathogen associated molecular patterns (PAMPs) in the prepared particles. Moreover, lack of DNA in the final product has fewer safety concerns compared to genetically engineered pathogens or cells that express Ags or costimulatory molecules.

Therefore, it is reasonable to find a suitable fusion method for PMVs engraftment into liposomes, so that in addition to production of artificial cells recapitulating the complexity of cell membrane structure of target cell, the original conformation of proteins will also be maintained in the fused particles. To reach this goal several methods were investigated, including reverse phase evaporation vesicles (REV), polyethylene glycol (PEG), PEG plus sonication, sonication alone and freeze-drying techniques.

Materials and Methods

Liposome preparation

Liposomes were prepared as described previously(21). Briefly, DSPC (Avanti polar lipid, inc.USA)/cholesterol (sigma)/DiI (sigma chemical Co., St. Louis, USA) at a molar ratio of 2:1:0.2 were dissolved in chloroform/methanol (3:1, v/v). The solvent was dehydrated in rotary evaporator (Heidolph, Germany) at 60°C and rotation speed of 90 to 100 RPM in order to form a thin lipid film. The lipid film was rehydrated in the solution of HEPES/NaCl (20 mM: 150 mM, pH 7.2) and suspension was extruded 11 times through 400nm Millipore polycarbonate filter by mini-extruder (Avanti polar lipid, inc. USA). The particles were sized with particle size analyzer (Nano-ZS, Malvern, UK) and their zeta potential was determined.

PMVs preparation

Crude PMVs was prepared using the protocol developed by M. Jett *et al* with minor modifications (22). Briefly, *Leishmania major* strain (MRHO/IR/75/ER) (8×10^8 /ml) and SK-BR-3 cell line (prepared from cell bank of Pasture Institute-IRAN) (1×10^6 /ml) were frozen and thawed for 10 times (60 sec in liquid nitrogen and 6 min in water bath 37°C) at the presence of 50 µl/ml of protease inhibitor cocktail (Sigma, St. Louis, MO, USA). It was vortexed thoroughly with high power 5 times (each time for 10 sec). Then, the samples were sonicated on ice seven times (each time 4 second) at 70% power by probe sonicator (SONOPULS, BANDELIN, Berlin, Germany). The suspension was centrifuged at 700g

for 10 min. Supernatant (crude PMVs) was collected and washed two times with HEPES/NaCl buffer at 4000 g for 10 min. The amount of proteins and lipids were determined using Bradford protein assay method and Stewart lipid assay method, respectively (23, 24). PMV vesicles were sized by particle size analyzer (Nano-ZS, Malvern, UK).

Flowcytometry protocol for evaluation of fusion efficiency

DiI with emission wavelength near 570 nm was chosen as a liposome tracer. This fluorescent color was detected by FL2 channel of flowcytometer. Two membranous macro-molecules (gp63 as a *leishmania* parasite surface Ag and HER-2 as a SK-BR-3 cell line surface molecule) were selected for tracking PMVs. Anti-gp63 antibody (Serotech), anti-HER-2 antibody or Herceptin (Roche) were used as primary antibodies. Mouse FITC-conjugated anti-rat IgG and FITC-conjugated anti-human IgG (Sigma chemical Co., St. Louis, USA) were used as secondary antibodies. This fluorescent color was detected by FL1 channel of flowcytometer. To determine the percentage of fused particles which are stained with both DiI and FITC, 100 µl of the final materials derived from fusion reactions were washed twice with PBS by centrifugation at 4000 g for 10 min. Pellets were resuspended in 100 µl anti-gp63 antibody (1/100 dilution) or anti-HER2 antibody (1/20000 dilution) and incubated for 30 min at room temperature. Tubes were washed twice with PBS and FITC-conjugated Abs were added (1/50 dilution) for 30 min at room temperature. The tubes were washed again three times with PBS and the pellet was resuspended in 0.5-1 ml PBS. Flowcytometric evaluation of these samples was carried out by BD flowcytometer (Becton Dickinson, US) equipped with Cell Quest pro software and data were analyzed using WinMDI software.

Fluorescent microscopy protocol for evaluation of fusion efficiency

After staining of DiI contained fused particles with anti-gp63 antibody (Serotech) or anti-HER-2 antibody (Roche), fused particles were also examined by fluorescent microscope (Olympus IX 81, Japan). Band-pass filters for FITC and DiI were ex-WIB (460-495 nm) and ex-WIG (530-550nm) while emission filters were 510-550 nm and 575-625 nm, respectively.

Engraftment of PMVs into liposomes

Five different methods were assessed for engraftment of *Leishmania*-derived PMVs into liposomes. After determining the best method, SK-BR-3-derived PMVs were also tested to evaluate the reproducibility of the developed method for engraftment of cancer cells-derived PMVs into liposomes. Comparison between fusion methods

Table 1. Characteristics of DiI- labeled liposomes and plasma membrane vesicles (PMVs) used in all experiments. In this table, PMVs is a representative of both PMVs derived from both *Leishmania major* parasites and SK-BR-3 cell line. Values are means \pm SD from at least three experiments

Particles	Z-Average (nm)	PDI ^a	Zeta potential (mv)	Lipid conc. mg/ml	Protein conc. μ g/ml	RFU ^b
DiI-Labelled liposomes	400 \pm 50	0.15 \pm 0.02	-19.6 \pm 1.6	7 \pm 2	50 \pm 30	40000 \pm 1000
PMVs ^c	500 \pm 100	0.3 \pm 0.2	-8.9 \pm 2	0.21 \pm 0.1	4000 \pm 500	400 \pm 100 ^d

^a polydispersity index, ^b Relative fluorescent unit, ^c Plasma membrane vesicles, ^dAuto fluorescence

were made with the same batch of characterized liposome and PMVs (Table 1).

Reverse phase evaporation method (REV)

Different lipids including DSPC/cholesterol/DiI at a molar ratio of 2/1/0.2 was dissolved in chloroform/diethyl ether (1:1, v/v) in the round bottom flask. Then, aqueous phase containing PMVs were added to give a ratio of 6/1 (organic/aqueous). After sonication for 5 min at 55°C to produce an emulsion, the flask was connected to rotary evaporator for 60 min with rotation speed of 90 to 100 RPM at 40°C in order to slow the removal of organic solvent. Flowcytometry was used to analyze the rate of fusion.

PEG fusion method

In this method, characterized PMVs (400 μ l, protein amount adjusted to 4000 \pm 500 μ g/ml) and liposomes (200 μ l, lipid amount adjusted to 7 \pm 2 mg/ml) were mixed well with 2/1 (v/v) ratio. The mixture was centrifuged in 4000 g for 10 min and the supernatant was decanted. After resuspending the pellet, 200 μ l of 50% solution of polyethylene glycol (PEG) 1500 (BOHRINGER MANNHEIM, GmbH, Germany) was added at room temperature. To optimize the PEG fusion method, different tubes were kept in different temperature for different times (data not shown). Finally, the effect of different temperature and different incubation times were checked by particle size analyzer and flowcytometry. The best fusion condition for this method was 40°C for 30 min and this condition was chosen for next PEG fusion experiments.

PEG+sonication method

Since after optimization the fused particles produced by PEG method were bigger than expected, bath sonication (BANDELIN, Berlin, Germany) steps were included in the PEG fusion method to reduce the size of particles. In this method, five time bath sonication were included within 30 min of incubation period with 5 min intervals and 1-minute duration at 40°C. Finally, the results were analyzed by particle size analyzer and flowcytometry.

Sonication method

The tubes containing PMVs (400 μ l) and liposomes (200 μ l) with 2/1 (v/v) ratio were vortexed and then were sonicated 5 times in bath sonicator for 1min in 30 min period of their

incubation at 40°C. The produced particles were washed twice with HEPES/NaCl buffer in 4000 g for 10 min. The supernatant was decanted and the Pellet resuspended in HEPES/NaCl buffer. The size of particles was determined by particle size analyzer and the efficiency of fusion was evaluated by flowcytometry.

Freeze-drying method

Freeze-drying protocol was carried out at the presence of 2% (w/v) of sucrose. In this method, vials containing proper amount of PMVs (400 μ l), liposomes (200 μ l) and 2% of sucrose were vortexed and frozen at -70°C one hour before connecting to freeze-dryer (Freeze dryer ALPH A1-2/LD plus, Christ, Germany). After freezing completely, the frozen tubes were placed on freeze-dryer overnight. The freeze-dried particles were resuspended in ddH₂O and analyzed with particle size analyzer to check the size distribution of fused particles or stained to check the percentage of fusion with flowcytometry.

Preparation of fused particles containing SK-BR-3-derived PMVs by freeze-drying method

SK-BR-3-derived PMVs were fused with liposomes using freeze-drying method. Briefly, vials containing proper amount of PMVs (400 μ l) with adjusted protein amount, liposomes (200 μ l) and sucrose (2% w/v) were vortexed and kept at -70°C freezer one hour before connecting to freeze-dryer. After an overnight freeze-drying, the lyophilized particles were resuspended in ddH₂O and checked for size distribution and the efficacy of fusion by particle size analyzer and flowcytometry, respectively.

Statistical analysis

All experiments were repeated at least 3 times. Statistical analysis was performed using the non-parametric Mann-Whitney U test. Results with $P < 0.05$ were considered statistically significant.

Results

Liposomes and PMVs characteristics

The mean size and zeta potential of the prepared liposomes were 400 \pm 50 nm and -19.6 \pm 1.6 mv, respectively (Table 1). DiI concentration in the liposomes was adjusted so that the fluorometric index of 100 μ l of 1/10 diluted liposome suspension reached 40,000 \pm 1000 RFU (relative fluorescent unit), while concentration of lipids in liposomes was 6.5 mg/ml.

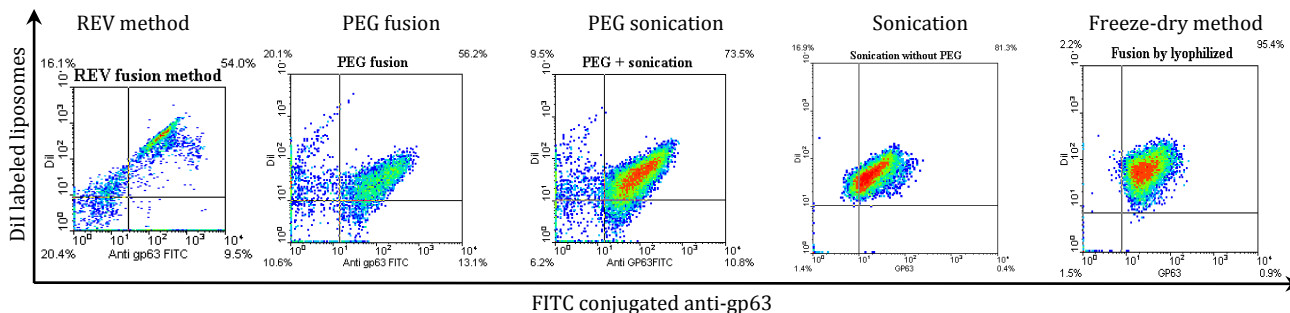


Figure 1. Efficacy of fusion methods investigated by flowcytometry

The mean size and zeta potential of PMVs was 500±100 nm and -8.9±2, respectively. Amount of protein and lipid in the PMVs isolated from 8×10⁸/ml *leishmania* parasite was 3000±350 µg/ml and 130±40 µg/ml, respectively, while those values in PMVs isolated from 1×10⁶/ml SK-BR-3 cells were 4500±500 µg/ml and 250±30 µg/ml, respectively. However, in order to carry out the fusion, the final concentrations of protein and lipid for both isolated PMVs were adjusted to 4000 µg/ml and 200 µg/ml, respectively (Table 1).

Comparison of methods

All five methods of fusion were compared regarding their efficacy in producing the double stained particles (fused particles) in flowcytometric analysis and the size and polydispersity indexes (PDI) of the final products (Figure 1 and Table 2, respectively).

As shown in Table 2, among the investigated methods, REV method showed 54±14% fusion efficiency and led to the formation of big sized fused particles with Z average of 10,000±5000 and uneven size distribution (PDI=0.9±0.5). While the percentage of fusion in PEG method was not significantly different from the REV method (56±8%, P=0.5), the size of fused particles was considerably decreased (4000±2000 nm, P=0.1). In addition, uneven size distribution was not resolved compared to REV method after fusion in the presence of PEG (PDI=0.8±0.2). Bath sonication increased the efficacy of fusion by PEG method as it increased from 56±8% to 71±4 % (P=0.05) while there were no significant reduction in particle size and PDI (P=0.18 and P=0.28, respectively). Of interest, sonication alone had greater impact on the fusion of liposomes and

PMVs compared to PEG method (80±3% and 56±8%, respectively; P=0.05) or PEG + sonication method (80±3% and 71±4%, respectively; P=0.05). The size of fused particles was also smaller than PEG method (700±150 nm and 4000±2000 nm, respectively; P=0.05) and PEG + sonication method (700±150 nm and 2000±750 nm, respectively; P=0.05).

Amongst all tested techniques, freeze-drying method significantly showed higher fusion efficiency (91±2%) in the presence of 2% W/V of sucrose compared to other methods (P=0.05, Figure 1 and Table 2). Interestingly, as shown in Table 2, even after long term storage, the freeze-dried particles kept their integrity according to the Z-average and PDI.

Fluorescent microscopy

Double stained particles were examined by fluorescent microscope. Pictures captured from each excitation and emission filters related to green (FITC) and red DiI colors separately and finally two pictures were merged to each other (Figure 2 a, b and c). Yellow color particles showed the fused particles while un-fused PMV particles remained green (Figure 2-c).

Evaluation of freeze-drying efficiency for fusion of PMVs isolated from human cell line

As a model, isolated PMVs from a well-known human breast cancer cell line SK-BR-3 were fused with DiI-labeled liposome using freeze-drying method in order to evaluate the reproducibility and applicability of this technique to PMVs-derived from other eukaryotic cells. Flowcytometry results show similar efficiency of fusion (90±3%) as shown in the previous experiments done on *Leishmania* isolated

Table 2. Fusion efficacy and size distribution of the fused particles obtained from different methods

Methods	Mean % of fusion efficiency ± SD ^a	Mean Z-Average (nm) ± SD ^a	PDI ^b
REV ^c	54 ± 14	10,000 ± 5000	0.9 ± 0.5
PEG ^d fusion	56 ± 8	4000 ± 2000	0.8 ± 0.2
PEG fusion+sonication	71 ± 4	2000 ± 750	0.6 ± 0.2
Sonication alone	80 ± 3	700 ± 150	0.5 ± 0.18
Freeze-dry (before storage)	91 ± 2	650 ± 180	0.28 ± 0.05
Freeze-dry (after 2 weeks of storage) ^e	92 ± 3	680 ± 190	0.33 ± 0.06
Freeze-dry (after 9 weeks of storage) ^e	94 ± 3	660 ± 350	0.32 ± 0.05

^a Mean ± SD was calculated from three different experiments, ^b Polydispersity index, ^c Reverse phase evaporation method, ^d polyethylene glycol, ^e Lyophilized particles obtained after freeze-drying were kept at 4°C in the sealed 5 ml glass ampoules for two and nine week before checking their physical characteristics

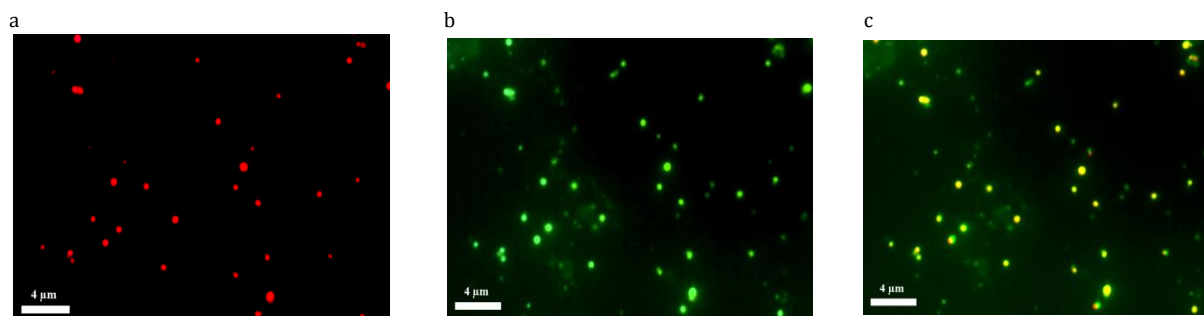


Figure 2. Fluorescence pictures were taken from Dil-labeled liposomes fused with PMVs isolated from *Leishmania major* by freeze-drying method. Fused particles were stained with FITC-labeled anti gp63. A) Picture was taken with excitation filter ex-WIG (530-550 nm) and emission filter (575-625 nm) for detecting Dil fluorescent color. B) Picture was taken with excitation filter ex-WIB (460-495 nm) and emission filter (510-550 nm) for detecting FITC labeled particles. C) Pictures A and B overlaid and fused particles are seen in yellow. Bars showed 4 μ m

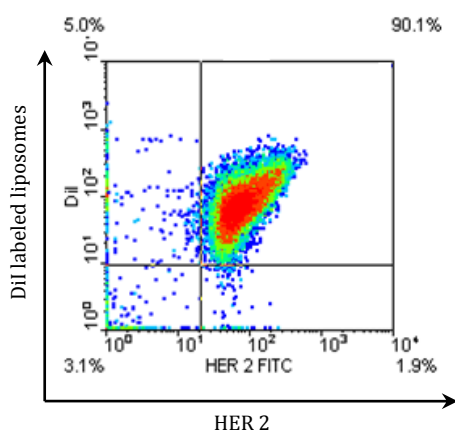


Figure 3. Flowcytometry evaluation of fused particles prepared by freeze-drying method from human SK-BR-3 cell line and Dil-labeled liposomes

PMVs (Figure 3).

Discussion

Among different nano particles, liposomes are still known as more potent carriers in the field of drug delivery and vaccine design due to their flexibility, biocompatibility, structural diversity and adjuvanticity properties. The importance of liposome size, charge and rigidity on immune responses has also been clarified in different studies (25-29). With this information in hand, numerous studies have been conducted to prepare a liposomal based vaccine for infectious and malignant diseases (30). In almost all previous studies liposomes were loaded with peptides or proteins while it has been widely accepted that live attenuated vaccines are more efficient than subunit vaccines in part due to the presence of different immunodominant antigens in their structure. Accordingly, evidence in the field of cancer vaccine suggests that PMVs prepared from tumor cells retain the antigenic character of the tumor from which they are derived (31). Therefore, it is expected that PMV-containing vaccines are able to mimic live attenuated vaccines and elicit a suitable

immune response. In this respect, the advantage of liposome containing Plasma Membrane Vesicle (PMV)-based vaccines in inducing anti-tumor responses has been reported previously (32, 33). Therefore, in the current study, the attempts were focused to find a new method for engraftment of plasma membrane vesicles into liposome based particles so that while maintaining the natural conformation of plasma membrane proteins, fused particles can also be kept for long term without significant changes in their biological and physical properties. On the other hand, use of phospholipids with high T_m in the structure of liposomes can provide longevity to the final fused particles that in turn will grant long exposure with the immune system and thereby, facilitate the process of memory formation after induction of immune responses (34). In addition, in the presence of a proper adjuvant, fused particles will be able to elicit a more powerful immune response owing to the existence of a potent adjuvant.

Considering the above mentioned facts, in the present study, to increase the rigidity of fused particles in order to enhance the possibility of their persistence in the host, liposomes consist of high transition temperature phospholipid (DSPC, $T_m > 50^\circ\text{C}$) were fused with plasma membrane-derived vesicles under the five different condition. Among different evaluated methods, results of REV method were not satisfactory due to the formation of macroscopic particles during evaporating process. In addition, protein aggregation and denaturation that are induced in the presence of organic solvents are among the other disadvantages of REV method. PEG fusion method improved in part some disadvantages of REV method, though uneven distribution of size and the presence of PEG in the final product were the issues remained to be solved. Compared to the above mentioned methods, sonication alone relatively improved fusion efficacy and the size distribution of fused particles and benefited from a simple laboratory process without any requirement for elimination of additional material such as PEG and

organic solvents. Nevertheless, freeze-drying method remarkably was superior in respect to the fusion efficiency and uniformity in size distribution to all other evaluated methods. Other advantages of this technique were simplicity and capability of keeping integrity of fused particles. Accordingly, fusion efficiencies and particles size remained constant, even after long term storage in the lyophilized conditions (Table 2). The simplicity of this technique also makes it compatible for large-scale production when compared with four other methods.

Size of particles is another concern that should be considered when particles used as a vaccine. In fact, it is appeared that vesicle size plays a significant role in biasing the immune response towards Th1 or Th2 immunity (35-37). It was shown that immunogenic particles with 0.5-2 μm in diameter are more potent to induce Th1 response (high levels of IFN- γ and IgG2 antibodies) whilst smaller liposomes (≤ 155 nm) induced Th2 immune responses (36). In this study, regarding the size of fused particles obtained from freeze-drying method (650 ± 180 nm), it is speculated that these particles could be used as a Th1 inducer vaccine in the future studies.

Based on the results obtained from isolated *Leishmania* PMVs, in the present study an experiment was also designed to check whether these techniques are easily applicable to PMVs isolated from SK-BR-3, a typical breast cancer cell line. Interestingly, PMVs isolated from SK-BR-3 cell line showed a similar pattern of size when compared with PMVs isolated from *Leishmania* parasites. It is also showed similar results after fusion with liposomes. Accordingly, results showed high fusion efficiency of freeze-drying method for HER-2 expressing cell line ($90 \pm 3\%$, Figure 3) as well as preserving the conformation of HER-2 protein for binding to its specific antibody.

Conclusion

Given the simplicity and high fusion efficacy of freeze-drying method for engraftment of PMVs into liposomes as well as the preservation of native structure of proteins on one hand and the fact that invented method can be expanded to fusion of liposomes with PMVs isolated from other cell types, freeze-drying technique will open a new window in the field of liposome-based vaccine design. This method might be more important for vaccination against agents with no data on their main immunodominant molecule(s) and therefore, the presence of whole cell membrane constituents (proteins, glycoproteins and glycol phospholipids) could be essential for obtaining a protective immune response. Moreover, including a Th1 inducer adjuvant in the structure of engrafted liposomes could enhance the ability of prepared vaccines against malignant cells and intracellular parasites that almost need Th1 responses for protection.

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