Chapter 5

Liposomes Used as a Vaccine Adjuvant-Delivery System

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Additional information is available at the end of the chapter

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Abstract

Liposomes, a kind of bilayered vesicles formed by self-assembly of phospholipid molecules in an aqueous medium, are widely used as a vehicle for delivering various therapeutic agents due to their high biocompatibility, diverse and high-loading capacity, and relative ease for preparation and surface decoration to engender multifunctional features. Also, liposomes are a useful carrier for delivering vaccine antigens forming a versatile vaccine adjuvant-delivery system (VADS), which can efficiently fulfill both functions of adjuvancy and delivery when the liposomes are modified with specific functional molecules, such as lipoidal immunopotentiators, antigen-presenting cell (APC) targeting ligands, steric stabilization polymers and charged lipids. In this chapter, liposomes used as a VADS are introduced, including the preparation processes for liposomes, the evaluation methods toward different immunological responses, and also the measures for tracking *in vivo* of the vaccine-carrying liposomes, to provide reader with wide information as a reference related to the liposomal VADS.

Keywords: lipid vesicle, bilayer membrane, vaccine adjuvant-delivery system, vaccination, immune response, mucosal immunity, humoral immunity, cellular immunity, toll-like receptor, pathogen-associated molecular pattern

1. Introduction

Vaccination proves to be the most cost effective and best prophylactic strategy against many types of diseases, such as pathogenic infections, cancerous lesions, and even rheumatoid arthritis [1]. The vaccine concept was first introduced in the late eighteenth century by Edward Jenner, an English physician and scientist who was the pioneer in development of the world's first smallpox vaccine against the fatal virus [1, 2]. All living organisms are continuously exposed to substances including those called pathogens, which may invade, reside in, and eventually damage the organisms as their hosts. Fortunately, the invading pathogens can be prevented by the

organisms in several ways: with physical barriers, for example, or with chemicals that repel or kill invaders; moreover, in vertebrates (the animals with backbones), mammals and, especially, primates (including humans), the pathogens can be further controlled by a more advanced protective system called immune system, which is a complex network of organs containing different types of cells, such as T cells, B cells and antigen-presenting cells (APCs) [including mainly dendritic cells (DCs) and macrophages (MPs)] [2, 3]. Generally, the specific surface structures called antigens (Ags) on the pathogens are first recognized by APCs, which are specialized in uptake and processing the pathogens into fragments to present Ags bound to MHC molecules on APC surface as an epitope, which can interact with and stimulate T cells and B cells into cytotoxic T lymphocytes (CTLs) and plasma cells, respectively [4]. While the plasma cells can secret the Ag-specific antibodies in a substantial amount to neutralize the pathogens into harmless non-infectious organisms, CTLs release the cytotoxins, perforin, granzymes and granulysin, which work together to trigger a series of the caspase cascade and cause apoptosis (programmed cell death) of the pathogen-infected cells, erasing finally the cell-hidden pathogens. This process for pathogen defense will also imprint Ag features in immune system setting up so-called immune memory, which allows the pathogen-experienced survivors to rapidly initiate the immune response toward, and thus erase, the reencountered pathogens bearing the identical Ags [2]. Such ability of immune system obtained after experience to defend pathogens defines the concept of immunity, which underlies the fundamental mechanisms for the Ag-based vaccines to be developed and employed for prophylaxis of various pathogens, including the alien microbes such as viruses, bacteria, fungi, parasites, and even treatment of the neoplasma and cancerous lesions in the body [2].

The conventional vaccines are mainly used for prophylaxis of the infectious diseases and are usually made of live attenuated or inactivated pathogenic organisms, which, after administration, can effectively stimulate the body immune systems to set up robust immunity in recipients against the related microbes [5]. However, the vaccines based on the live attenuated pathogens possess, per se, the potential to cause detrimental infections due to the possible mutation occurrence in the engineered organisms and thus may lead to severe outcomes; while the vaccines made of inactivated microorganisms may stimulate the rather weak and even target-deviated immune response. To enhance the potency of the inactivated microorganism-based vaccines, the products are often added with alum, which were introduced as a vaccine adjuvant in the second decade of last century by Glenny and colleagues [6, 7]. Though alum, together with the undefined complex components, can enhance the efficacy of certain vaccines, it often causes adverse stimulus reactions and even, gives rise to serious side effects. To erase these drawbacks associated with the whole pathogen-based vaccines, researchers have recently developed the subunit vaccines, which contain only the essential antigens with the well-defined components and, thus, are anticipated to be a safe product without the potential risks confronted by the conventional vaccines [8]. Unfortunately, due to lack of other microbial components, which may not only protect the antigens but also be a pathogen-associated molecular pattern (PAMP) for mammalian immune systems, the subunit vaccines are usually unstable and often induce insufficient immune responses against pathogens [9, 10]. To overcome these weaknesses of subunit vaccines, numerous types of nanoparticles with composition mimicking the components of pathogenic organisms have been developed as a vaccine

carrier forming the so-called vaccine adjuvant-delivery systems (VADSs), which can protect antigens from the environmental damage, deliver ingredients to specific lymphocytes, and even enhance, as an adjuvant, the initiation of Ag-specific immune responses [8, 11–14].

Recently, among different types of vaccine carriers, such as emulsions, poly(lactic-co-glycolic acid) (PLGA) particles, silico nanocarriers and virus-like particles (VLPs), liposomes, the vesicles made up of lipid bilayers, were first described by Bangham et al. under an electron microscope in the early 1960s [15], and have ever since attracted much research interests in the development of novel drug delivery system (DDS) or VADS, due to their high biocompatibility, diverse and high loading capacity, and the ease for preparation and surface decoration to engender unique structures bearing the desired functions [16]. In fact, owing to their ability to entrap water- and lipid-soluble molecules in their aqueous and lipid phases, respectively, liposomes have been used since 1970 as a delivery system in therapeutics for a great variety of pharmacologically active agents, including antimicrobial and anticancer therapeutics, vaccines, metal detoxification chemicals, DNA/RNA fragments, enzymes and hormones [17]. As confirmed by researchers, agent delivery with liposomes can circumvent many of the problems associated with direct drug use, for instance, toxicity as a result of indiscriminate drug action, premature drug inactivation or excretion, and inability of drugs to reach the target intracellularly. For medical application, liposomes have proved able to be safely administered by various routes, including the intravenous, intramuscular, subcutaneous, intrathecal, intratracheal, oral, intranasal, and topical (skin and a variety of mucosal tissues) routes, having met with considerable success with several liposome-based products, including several vaccines, already licensed for clinical use in different countries [18].

Particularly, as a vaccine carrier, liposomes have the intrinsic adjuvant properties, which were established as early as 1974 by Gregoriadis and coworkers when strong humoral immune responses to liposome-entrapped diphtheria toxoid were observed after injection into mice, while, unlike other adjuvants, no granulomas at the site of injection were noticed [19–21]. Moreover, there were no hypersensitivity reactions in preimmunized animals when the antigen was entrapped in liposomes and given by intravenous or intra-foot pad injection [20]. In the ensuing years, extensive work in this laboratory and elsewhere has shown that liposomal adjuvanticity applies to a wide variety of bacterial, viral, protozoan, tumor, and other antigens [19, 22]. Now it is generally accepted that liposome can always function the role of adjuvanticity regardless of the type of association of the antigen with liposomes, such as being entrapped within the vesicles, attached onto their surface, and even simply mixed together [23, 24]. To be efficiently recognized and thus taken up by antigen-presenting cells (APCs), liposomes have been explored to be decorated with PAMP molecules and/or the molecules as ligands matching the receptors expressed on the surface of the aimed immunocytes, thus forming a multifunctional targeting VADS [11, 12, 25–27]. To date, various multifunctional liposomes have been developed as a novel VADS targeting APCs to enhance vaccine immunostimulating capacity by utilizing the specific binding affinities between functional molecules on the carrier and special features expressed or engendered by the immune cells. For example, recently, multifunctional liposomes have been successfully constructed being anchored with a toll-like receptor (TLR) ligands, including lipid A for TLR4, CpG-ODN for TLR9, and the synthetic molecules with a distal of mannose group for the C-type receptors on APCs,

such as dendritic cells (DCs), macrophages (MPs), and even been fabricated into microneedles for penetration of skin and mucosa to further enhance delivery efficiency [24]. These kinds of multifunctional liposomes as a VADS proved highly effective in both targeting delivery of vaccine to APCs and enhancing antigen presentation of APCs to T-cells fulfilling a dual function of delivery and adjuvancy for vaccines [11, 24, 25, 27, 28].

2. The components and structure of liposomes used for delivery of vaccines

Common liposomes are the vesicles made up of one or more concentric lipid bilayers alternating with aqueous spaces [17, 21]. The components of liposomes are mainly amphiphilic lipids and include phospholipids such as phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylglycerol (PG), sphingomyelin (SM), or other lipidic amphiphilies such as polysorbate 80 (SPAN80, nonionic surfactants), often supplemented with cholesterol (CHO) and other charged lipids such as stearylamine (SA), N[1-(2,3-dioleyloxy) propyl]-N,N,N-triethylammonium (DOTMA), 1,2-dioleyloxy-3-(trimethylamonium propane) (DOTAP), and 3 (N,N,-dimethylaminoethane)-carbamyl cholesterol (DMACHO). **Figure 1** shows the molecular structure of some representative lipids.

At ambient temperature, depending on the nature of the lipids, the liposome bilayers may be in a "fluid" or "rigid" state: the fluid state is manifested when liposomes are made with amphiphilic lipids that have a gel-liquid crystalline transition temperature (T_c)— the temperature at which the acyl chains melt—below ambient temperature, whereas the rigid state requires liposomes to be made of amphiphilic lipids with a T_c above ambient temperature [21]. Although liposomes as

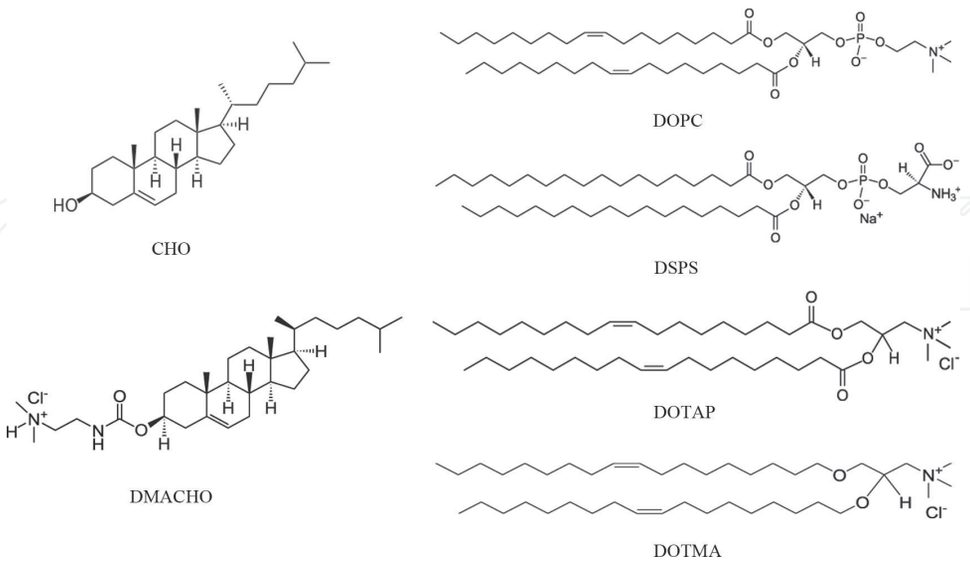

Figure 1. The molecular structure of typical lipids most often used for preparing liposomes. Abbreviations: DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DSPS, 1,2-distearoyl-sn-glycero-3-phospho-L-serine; DOTAP, 1,2-dioleyloxy-3-(trimethylamonium propane); DOTMA, N[1-(2,3-dioleyloxy) propyl]-N,N,N-triethylammonium; CHO, cholesterol; DMACHO, 3(N,N,-dimethylaminoethane)-carbamyl cholesterol.

an agent carrier bear a common weakness of instability, the pegylation (modification with PEG) to produce a steric stabilization effect, charging with ionic lipids to engender an electrostatic repulsion, and/or lyophilization to form a dry entity renders the liposome-based VADS the stability completely satisfying the shelf-life requirements for a medicinal product [29].

To date, various types of liposomes have been developed for delivery of drugs or vaccines, including unilamellar vesicles, multilamellar vesicles (MLVs), multivesicular liposomes (MVLs) (**Figure 2**) and the liposomes with special structural features, such as cochleates [25, 30], bilosomes [31], niosomes [32], the inorganic nanoparticle-cored liposomes such as phospholipid bilayer-coated aluminum nanoparticles (PLANs) [28], and the interbilayer-crosslinked multilamellar vesicles (ICMVs) [33]. Unilamellar liposomes are a spherical chamber/vesicle, bounded by a single bilayer of an amphiphilic lipid or a mixture of such lipids, containing aqueous solution inside the chamber. Small unilamellar liposomes/vesicles (SUVs) have sizes up to 100 nm; large unilamellar liposomes/vesicles (LUVs) may have sizes more than 100 nm up to few micrometers (μm), and SUVs or LUVs are often used for specific site-targeting delivery of drugs or vaccines. Multilamellar liposomes (MLVs) consist of many ᶜoncentric amphiphilic lipid bilayers analogous to onion layers, and MLVs may be of variable sizes up to several micrometers, while multivesicular liposomes (MVLs) are characterized by their unique structure of multiple nonconcentric aqueous chambers surrounded by a network of lipid membranes. Both MLVs and MVLs, with multilayers and multi sub-spherules, respectively, can be employed for sustained delivery of small chemical drugs or other biological agents, e.g. the MVL-based techniques have been developed into a platform of so called DepoFoam for manufacturing the extended-release medicinal products, which may release drugs over a desired period of time from 1 to 30 days [34]. Notably, DepoFoam has already been used in the FDA-approved commercial products, including DepoCyte® (cytarabine liposome injection), DepoDur (morphine

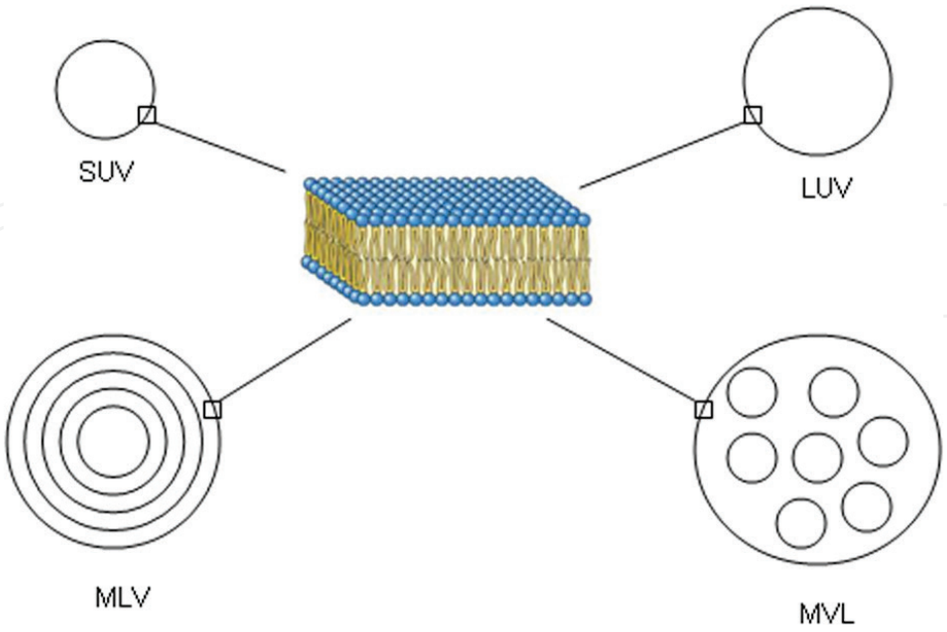

Figure 2. Schematic structure of different types of liposomes. Abbreviations: SUV, small unilamellar vesicles; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; MVL, multivesicular liposomes.

sulfate extended-release liposome injection), and EXPAREL® (bupivacaine liposome injectable suspension) [18].

3. Preparation and characterization of Ag-loaded liposomes

Since first discovery of liposomes, researchers are always trying to set up different ways for preparation of the liposomes that meet the therapeutic demands [35]. Indeed, a variety of methods for production of the drug/vaccine delivery liposomes have been developed and are now so great that no one laboratory has hands-on experience with all of them [21]. Notably, different methods fit different liposomes for entrapping different agents, and, indeed, none of them should be regarded as a versatile procedure that can be employed once and for all to prepare any types of liposomes for entrapping drugs [36]. Moreover, certain agents such as the small compounds that are neutral and nonionizable can hardly be encapsulated in liposomes with a high efficiency by any one of the established processes. Summarily, liposomes used for vaccine delivery can be prepared with high encapsulation efficiency (EE) for Ags by exploiting two strategies: using unique procedures such as emulsification-evaporation or -lyophilization, and constituting special carriers such as charged vesicles. Herein, the method of thin film dispersion-extrusion and the method of emulsification-evaporation or -lyophilization are introduced because they can be safely used to encapsulate Ags without causing chemical/mechanical damage to labile biological agents [11, 36–39].

The method of thin film dispersion-extrusion is regarded as the simplest way to prepare the liposomes with a control size and can be used for entrapping almost all categories of agents if a high EE is not required [15, 36]. This method involves several steps including dissolving lipids organic solvents in a round-bottom flask, removing organic solvent by rotation and evaporation to make a thin lipid film lining the inside walls of flask, rehydration with an aqueous medium of the lipids for dispersion by agitation to engender heterogeneous liposomes, and extrusion several times of the coarse liposomes through a series of polycarbonate membranes with size-defined pores, just obtaining the homogeneous liposomes with a size similar to that of the final membrane pores.

3.1. Materials and equipment used for preparation and characterization of Ag-loaded liposomes

The main materials include sterile ultrapure water, chloroform, phosphate-buffered saline (PBS: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 in 1000 mL distilled H_2O, pH of 7.4 adjusted with 0.1 M HCl), the PBS sterilized by autoclaving, soy phosphatidylcholine (SPC, molecular weight of 775.04, Avanti Polar Lipids, Inc., Alabaster, Alabama, USA), cholesterol (CHO, molecular weight of 386.65), OVA (ovalbumin) or other Ags, 10 mg/mL of SPC stock solution in chloroform, and 10 mg/mL of CHO stock solution in chloroform, and 2 mg/mL OVA (or other Ags) in PBS containing, or not, 50 mM sucrose, N_2 gas tank, liquid nitrogen, Triton-X100,: Sephadex G-50 (medium) gel (Pharmacia Company), Coomassie Brilliant blue G-250 (Sigma-Aldrich), Bradford reagent: Dissolve 50 mg of Coomassie Brilliant Blue G-250 in 50 mL ethanol, add 100 mL 85% (w/v) phosphoric acid (H_3PO_4). For charged or stealth

liposomes to be used for carrying Ags, 1,2-dioleyloxy-3-(trimethylammonium propane) (DOTAP, a cationic lipid), soy phosphatidylserine (SPS, an anionic lipid), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)$_{2000}$ (DSPE-PEG$_{2000}$) may be included.

The equipment and apparatus include 50-mL round-bottom flask, vortex mixer, 10-ml LIPEX® Extruders (Transferra Nanosciences Inc., Burnaby, B.C. Canada), membranes with the pore sizes of 400, 200, 100, and 50nm (Avestin, Ottawa, ON, Canada), a rotary evaporator, a probe or water bath sonicator with working frequency of 20 kHz, a vacuum desiccator, and a freeze-dryer, Malvern Zetasizer Nano ZS90 (Malvern instruments Ltd., Malvern, Worcestershire, UK), A FEI Tecnai G2 Spirit—transmission electron microscope (FEI Company, Hillsboro, Oregon, USA) and Vitrobot™ Cryo-TEM sample preparation instrument, a Micro CS150NX Ultracentrifuge (Hitachi, Japan) or other equivalent, 8453 UV-Vis spectrophotometer (Agilent Technologies, Inc., Santa Clara, California, USA) or other equivalent.

3.2. Preparation of Ag-loaded liposomes by thin film dispersion-extrusion method

1. To prepare 2 mL SPC/CHO liposomes (1:1, molar ratio) with lipid concentration of 10 mg/mL, 1330 μL of 10 mg/mL SPC stock solution and 670 μL of 10 mg/mL cholesterol stock solution are mixed in a 50-mL round-bottom flask in a chemical hood, and then organic solvent is removed on a rotary evaporator linked to a recycling water vacuum pump with flask dipped in a water bath at 35–40°C under reduced pressure. (For charged liposomes used for vaccine delivery, the lipid components can be replaced with SPC/CHO/DOTAP (4.5:4.5:1, molar ratio), or SPC/CHO/SPS (4.5:4.5:1, molar ratio); for stealth liposomes, the lipid components may be SPC/CHO/DSPE-PEG$_{2000}$ (9.5:9.5:1, molar ratio), or other combinations depending on the carriers to be used.)
2. The opening of the lipid film-adhering flask is covered with a piece of stretched parafilm to prevent the entry of dusts or contaminants and poke few small holes in the parafilm by a needle, and then the lipid film-adhering flask is put in a vacuum desiccator overnight to remove organic solvent residue.
3. Then 2 mL of PBS containing 2 mg/mL OVA (model Ag) is added to the dry lipid film-containing flask, which is vortexed repeatedly for 10 s on maximum speed until the lipid materials are all suspended in the solution.
4. The sample is sonicated for 5 min at the power of <100 W to disperse all visible precipitates and obtain milky crude liposome sample of mostly MLVs.
5. Repeatedly, the liposome sample is extruded with high pressure N_2 gas to pass through the 400 nm-pore polycarbonate membranes 11 times using the Extruder, which is then re-equipped with polycarbonate membranes of the pore size, in sequence, of 200, 100, and 50 nm, each followed by the due 11-cycle extrusion, and, consequently, resulting in the liposomes with a size of about 420, 220, 120, and 60 nm after each of the 11-cycle extrusion through the corresponding membranes.
6. The liposome preparation is stored in a vial filled with N_2 gas at 4°C in the dark.

3.3. Preparation of Ag-loaded liposomes by emulsification-evaporation/lyophilization method

1. To prepare 2 mL of SPC/CHO liposomes (1:1, molar ratio) with lipid concentration of 10 mg/mL, 1330 μL of 10 mg/mL SPC stock solution and 670 μL of 10 mg/mL cholesterol stock solution are mixed in a 20-mL ample, which contains 4 mL cyclohexane resulting in 6 mL of lipids dissolved in cyclohexane/chloroform (3:1, v/v) to be used as oil phase (O).
2. And 6 mL of O is mixed with 2 mL of 2 mg/mL OVA PBS with (for emulsification-lyophilization method) or without (for emulsification-evaporation method) 50 mM sucrose as water phase (W), and then using an ice/water bath to control the temperature under 30°C, the mixture is emulsified with a sonicator with work frequency of 20 kHz at the power of 50 W to make a W/O type emulsion.
3. By emulsification-evaporation method, the W/O emulsion is evaporated at 35–40°C under a slightly reduced pressure to remove slowly all the organic solvents, and then sample is supplemented with appropriate amount of PBS to obtain finally 2 mL of liposome preparations; otherwise, by emulsification-lyophilization method, the following steps should be completed.
4. The obtained 8 mL of W/O emulsions are mixed with 12 mL of PBS containing 50 mM sucrose and mildly emulsified using homomixer at 5000 rpm for 1 min to form a W1/O/W2 double emulsion, which is quickly subdivided and filled into 5-mL freeze-drying vials with a fill volume of 1 mL per vial.
5. The emulsion-containing vials are immediately put into an ultra-low temperature refrigerator and frozen at −85°C for 4 h, and then transferred into a freeze-dryer and lyophilized with a program as follows: primary drying at −45 and −20°C for 2 h periods, respectively; and secondary drying at 20°C for 4 h.
6. After lyophilization, the vials are immediately filled with nitrogen gas, sealed, and stored at 4°C in the dark.
7. For use, an appropriate amount (0.1–1 mL) of water is added into the dry powder-containing vial, resulting in the Ag-loaded liposomes with lipid concentration of 10–1 mg/mL.

3.4. Characterization of the Ag-loaded liposomes

3.4.1. Test of size and zeta potential

The Ag-loaded liposomes may be characterized in size (mean diameter), zeta potential (ζ), morphology, and structure.

The size (mean diameter) and surface charge (zeta potential, ζ) of the multifunctional liposomes with or without antigen are tested using a Malvern Zetasizer ZS90 (Malvern, Worcestershire, UK) referring to the user manual.

Test of liposome size by DLS (dynamic light scattering) is usually performed using samples with lipid concentration of 1 mg/mL. Thus, 0.2 mL of the prepared liposomes is diluted with 1.8 mL of PBS (with or without sucrose) in a polyacrylate or quartz cuvette and placed in

Zetasizer ZS90, and the particle size is determined by photon correlation spectroscopy (PCS) at 25°C at an angel of 90°, to give the intensity, number or volume mode, which can be used to evaluate the particle size distribution profile which is automatically summarized as parameter of polydispersity index (PDI), while the size is often presented with the average one plus PDI.

For ζ test by electrophoretic light scattering (ELS), 0.7–1 mL of the diluted sample is pipetted into the polyacrylate or quartz cuvette, which is then carefully inserted with the "Dip" cell bearing electrode pair, placed in Zetasizer ZS90 and determined by PCS at 25°C at an angel of 90°.

3.4.2. Cryo-TEM of multifunctional liposomes

The electron microscope is a type of microscope that uses a beam of electrons to create an image of the specimen and has up-to-date the greatest resolving power, allowing it to see nano-sized objects in fine detail [40]. It is large, expensive pieces of equipment, generally standing alone in a small, specially designed room and requiring trained personnel for operation. Recently, the cryo-transmission electron microscopy (cryo-TEM) has gained great development and allows the observation of specimens that have not been stained or fixed in any way, just showing them in their native environment at, notably, even near-atomic resolution [40]. Since early 1990s, the cryo-TEM has been more and more employed by researchers to observe the morphology and structure of the agent-loaded liposomes. Since the liposome surface is always hydrophilic, the hydrophobic carbon grid that used to carry liposomes should be first converted to hydrophilic nature by glow discharge. For cryo-TEM, the sample-holding grid is usually freezed in liquid nitrogen at −196°C for 10 min and transferred to a cryo-holder, which is maintained at ultralow temperature using a liquid nitrogen storage box, and then inserted in the microscope for imaging in the ultralow temperature [39].

The following protocol may apply to observation of the Ag-loaded liposomes by cryo-TEM [39].

1. Using a pipette, 3 μL of the sample is applied to an EM grid that has been converted to hydrophilic nature by glow discharge.
2. After being blotted with filter paper to remove excess sample, the grid is plunged into liquid nitrogen at −196°C for 10 min, and then transferred to a cryo-holder, which can be maintained at ultralow temperature using a liquid nitrogen storage box.
3. Then the cryo-holder is inserted into the EM column that is maintained at liquid nitrogen temperature (77 K), and the liposome sample is imaged at the ultralow temperature.

3.4.3. Determination of EE of the Ag-loaded liposomes

The EE of the Ag-loaded liposomes may be estimated by the following Eq. (1).

$$EE\ (\%) = \frac{\text{Total Ag} - \text{Free Ag}}{\text{Total Ag}} \times 100\% \quad (1)$$

Free Ag may be separated from Ag-associated liposomes by ultracentrifugation and quantitatively determined with the classical Bradford protein assay method [41]. For free Ag separation, the liposome sample is centrifuged at 100,000 × *g* in an ultracentrifuge for 1 h, and then after collection of the free Ag-containing supernatant, the liposomal pellet is suspended with PBS for

washing and centrifuged again. The supernatants are mixed together to adjust protein concentration at a range of 0.1–25 μg/mL, and Ag quantification may be carried out by the following procedure.

1. Five standard solutions (1 mL each) containing 0, 0.1, 0.4, 1.6, 6.4, and 25.6 μg/mL BSA are prepared.
2. And 800 μL of each standard and sample solution (containing for < 25 μg/mL protein) is pipetted into a clean, dry test tube. Protein solutions are normally assayed in duplicate or triplicate.
3. Then 200 μL of Bradford dye reagent concentrate is added to each tube followed by vortex for mixing.
4. The tubes are incubated at room temperature for at least 5 min. Absorbance will increase and changed nonlineally over time, and samples should be incubated at room temperature for not more than 1 h.
5. The standard and sample solutions are transferred into cuvette and measured immediately of the absorbance at 595 nm.
6. The standard curve is obtained and used to quantify free Ags.

3.4.4. Assay of Ag integrity in liposomes using SDS-PAGE

During incorporation in liposomes, the Ag may be damaged due to its possible confrontation of the organic solvents and intense mechanic sonication and therefore should be scrutinized of the integrity. Usually, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is used to test the integrity of the Ags entrapped in liposomes that are freshly prepared or have been stored in certain conditions for some time for evaluating the stability of the products [42]. The protocol of SDS-PAGE test of the Ags loaded in vaccine carriers has been described in detail in an open access book chapter and is suggested as a reference for investigators [39].

4. *In vitro* and *in vivo* evaluation of the Ag-loaded liposomes

4.1. Materials and equipment used for evaluation of the Ag-loaded liposomes

For assay of the immunity elicited *in vitro* and *in vivo* by vaccination with Ag-loaded liposomes, the following materials should be at hand: HyClone Roswell Park Memorial Institute (RPMI) 1640 medium and fetal calf serum (FCS) by Thermo Fisher Scientific (Waltham, MA, USA), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)) and TMB (3,3,5-tetramethylbenzidine) by Sigma-Aldrich (St. Louis, MO, USA); LysoTracker-red and (4′,6-diamidino-2-phenylindole) DAPI (Thermo Fisher Scientific Inc., Waltham, MA, USA) for label cellular organelles of lysosome and nucleus, respectively; and the biological agents for

assay of immunoglobulins and cytokines, such as IFN-γ and IL-4 assay kits; goat anti-mouse IgG-horse radish peroxidase (HRP), IgG1-HRP, IgG2a-HRP (or IgG2c, if C57BL/6 mice are used) and IgA-HRP with sales package of 200 μg per 0.5 mL, PE-conjugated anti-mouse $CD8^+$ mAb (monoclonal antibody) and FITC-conjugated anti-mouse $CD4^+$ mAb and other fluorescently labeled immunological agents for assay by eBioscience (San Diego, USA), BioLedend (San Diego, USA), or Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA).

For assay of the immunity elicited *in vitro* and *in vivo* by vaccination with Ag-loaded liposomes, the following equipment will be used: a microplate reader (μQuant™, BioTek Instruments, Inc., Vermont, USA), a fluorescent microscope (Olympus IX83, Japan) or other equivalent, a laser scanning confocal microscope (LSCM) (Leica TCS SP5, Wetzlar, Germany) or other equivalent, flow cytometry (BD FACSVerse™, San Jose, CA, USA) or other equivalent, and the flow cytometry data analysis software of FlowJo (Tree Star, Ashland, OR, USA).

4.2. *In vitro* evaluation of vaccine adjuvancy and delivery ability of the Ag-loaded liposomes through cellular experiments

It is believed that after antigen uptake, APCs with normal functions will evolve from immature, antigen-capturing cells to mature, antigen-presenting and T cell-priming cells, and meanwhile able to convert antigens into immunogen epitope and express molecules such as cytokines, chemokines, costimulatory molecules and proteases to initiate an immune response. Thus, the ability of delivery of Ag to APCs is one of the main functions designed to the liposomal carriers and is usually evaluated through assaying enhancement of cellular uptake of the fluorescent agent/Ag-loaded liposomes using mouse bone marrow-derived macrophages (BMMPs) and dendritic cells (BMDCs), both of which are confirmed to be ideal APCs to evaluate vaccine adjuvancy and delivery efficiencies and can be generated from bone marrow precursors as follows [43, 44].

1. The femur bones are first isolated from the euthanized mouse, and then immersed in 75% ethanol for 2 min for disinfection
2. After rinsing thrice with sterile PBS, both ends of femur bones are removed, and bone marrow is flushed out with ice-cold PBS using a hypodermic needle attached to a syringe.
3. The leukocytes obtained are washed thrice with PBS and then transferred into bacteriological petri dishes and cultured in 10 mL of complete RPMI containing 10% heat-inactivated FBS and in the presence of 50 μM 2-mercaptomethanol and 20 ng/mL GM-CSF for 3 days in a cell culture chamber.
4. On days 4 and 6, the culture medium is replaced with the fresh one containing identical ingredients.
5. On day 8, the nonadherent cells in culture medium are used as BMDCs.

Similarly, BMMPs can be obtained using the above protocol with a little modification in Step 4 by replacing GM-CSF with M-CSF, and in Step 5, and, notably, the adherent cells instead of nonadherent cells in culture medium are collected as BMMPs.

The cellular uptake of the fluorescent agent/Ag-loaded liposomes may be assayed by following processes.

1. APCs (BMDCs or BMMPs) are seeded in a 24-well plate with 1 mL of 10^5 cells/well and incubated in a cell culture chamber with 5% CO_2 at 37°C for 24 h.
2. Then each cell well is supplemented with 50 μL of calcein (1 mM)/Ag-loaded liposomes, mixed homogeneously, and incubated continuously in a cell culture chamber at 37°C for various times.
3. After the incubation and removal of the unassociated agents or liposomes through centrifuge at 800 g for 5 min and washing thrice with PBS, the cocultured cells (10^5 cells/mL) are analyzed by flow cytometry for uptake of liposomes with data analysis using FlowJo software; otherwise, a fraction of the cells is imaged using a fluorescent microscope; or to observe the intracellular localization of the agents delivered by liposomes in APCs, the following steps are carried out
4. The cocultured cells are isolated and incubated for 30 min in pre-warmed media (37°C) containing 50 nm LysoTracker-red and DAPI to identify lysosome and nuclei, respectively.
5. After washing thrice, the APCs are observed in a LSCM, and the dissociation of green dots (calcein-liposomes) from red dots, with blue nuclei for cell localization, is considered as the hallmark of lysosome escape by agents delivered by liposomes.

APC activation and maturation induced by the Ag-loaded liposomes can be evaluated by assay of the immunological cytokines and the surface biomarkers secreted or expressed by the APCs in the activated states. The secretion of cytokines, such as tumor necrosis factor (TNF), various interleukins (ILs), IFN-γ and even nitric oxide (NO), is usually enhanced or altered to different degrees by the activated APCs for induction of the subsequent specific immune responses, whereas CD40, CD80, and CD86 are known to be expressed at an enhanced level on the activated APCs and involved in, through binding receptors on T cell surface, providing costimulatory signals necessary for T cell activation and survival, and therefore may be used as markers for evaluating APC activation state. The levels of CDs on surfaces of the activated APCs may be assayed by several steps as follows:

1. The cells seeded in a 24-well plate with 1 mL of 10^6 cells per well are supplemented with 50 μL of soluble Ags (as a control), Ag-loaded liposomes at the dose of 20 μg Ag for up to 30 h for stimulation.
2. After stimulation, the cells are labeled with different color fluorochrome-conjugated Abs against surface biomarkers, such as CD40, CD80, and CD86, at 37°C for 30 min.
3. The cells are quantitatively assayed of the CD levels by flow cytometry.

The levels of cytokines, such as TNF, various ILs and IFN-γ, secreted in culture medium by the activated APCs are first separated in the supernatants by centrifugation and quantified with enzyme-linked immunosorbent assay (ELISA) kits according to product performance

guidance, while the level of secreted NO in cell culture supernatants is usually measured by colorimetric assay with Griess reagents, which react with nitrite forming a colorimetric compound which in turn reflects NO amount in samples [45].

The antigen presentation profile of the liposome-activated APC with MHC-I or -II is usually determined by measuring APC-primed $CD8^+$ and $CD4^+$ T cell proliferation using Ag-specific T cells as the following protocol.

1. To obtain Ag-specific T cells, C57BL/6 mice are immunized subcutaneously in the scruff of the neck with daily dose of 100 μg OVA plus 10 μg Poly (I:C) at injection volume of 100 μL [46], and mice are repeatedly immunized at the same site, at approximately 24-h intervals for 4 consecutive days.
2. Seven days after the first immunization, Ag-specific $CD8^+$ T cells are negatively selected from the spleen of Ag/Poly (I:C)-immunized mice by magnetic bead adsorption using MagniSort® isolation kit.
3. The isolated $CD8^+$ T cells are prelabeled with CFSE (1 μM final concentration for 10^6 cells) at 37°C for 5 min, and then cocultured with the Ag-loaded APCs that are generated by stimulation with free Ag (used as a control) or Ag-loaded liposomes at the ratio of T cell to APC of 10:1, at 37°C for 48 h [47, 48]. To determine MHC II antigen presentation ability of APCs, CD8-depleted splenocytes are prelabeled with CFSE and cocultured with OVA-loaded APCs at the ratio of T cells to APCs at 10:1 at 37°C for 48 h.
4. The proliferation of $CD8^+$ T cells and $CD4^+$ T cells, which, respectively, reflected the MHC I and MHC II antigen presentation by APCs, is determined by measuring the fraction of live cells with decreased CFSE intensity ($CFSE^{low}$ cells) using flow cytometry.

4.3. *In vivo* evaluation of vaccine adjuvancy-delivery ability of the Ag-loaded liposomes through vaccination experiments

4.3.1. Animal vaccination with the Ag-loaded liposomes

The experimental animals, such as mice, rats, rabbits and dogs, can be used as a vaccination model for Ag-loaded liposomes. Though rats, rabbits and dogs can be used for vaccination of Ag-loaded liposomes, mice are a preferred model due to their relatively low price, small body saving liposome samples, and, most importantly, their immune system bearing much-known backgrounds [49]. The vaccination routes are diverse and can be summarized into two ways: injection and noninjection, and the former includes intradermal, intramuscular, intramucosa and intravenous injection, while the later includes intraoral cavity, intravaginal, oral-uptake administration. Notably, intramucosa is a newly developed pathway and has been investigated only for vaccination of the microneedle vaccines, which have also been invented only recently but developed rapidly to show big advantages over the conventional liquid vaccines in the several aspects including the administration convenience, dosage saving, and, especially, efficient delivery of Ags [12–14, 24, 26, 27, 50–52]. The Ag dose given to mice in the formulation of liposomes is often in the range of 0.5–20 μg for injection, but of 5–100 μg for intracavity administration.

4.3.2. Sample collection

Collection of samples, including blood, secreted fluids in reproductive ducts and respiratory tracts, and even contents in digestive lumen and cavities from mice vaccinated with the Ag-loaded liposomes is necessary to obtain the data on a wide range of parameters, such as immunoglobulins, interferons, interleukins and cytokines, to evaluate humoral and cellular immune responses to vaccines. The sample collection from the vaccinated mice is suggested to be carried out 2–4 weeks after vaccination, when the immune responses are thought to be in a prime period. The protocols for collection of various samples, including blood, saliva, pulmonary, respiratory, vaginal and intestinal secretions, are described in detail in an open access book chapter recently published by Springer and suggested here as a reference and guide to performing the corresponding experiments [26].

4.3.3. Isolation of splenocytes from mouse spleen

Proliferation profile of the immunocytes from treated mice reflects the level of the immune responses to a liposomal vaccine and can be sketched using the different types of immunocytes and their fractions in lymphoid tissues, such as spleen, lymph nodes (LNs) and thymus to show the pathways along which the immune responses elicited by a liposomal vaccine have progressed and developed. Therefore, isolation and assay of immune cells from lymphoid tissues are often carried out as one of the fundamental mechanisms underlying the immunological efficacy of certain types of vaccines. Usually, organ, tissue, or cell isolation should be performed with aseptic manipulation in a sterile cabinet or under a sterile condition to avoid contamination for keeping cells alive.

Isolation of mouse splenocytes from spleen is described as follows:

1. The spleen from the abdomen-anatomically opened mouse is isolated using a small pair of hemostatic forceps, and transferred into the cell strainer (200-mesh with pore size of 70 μm) in the Petri dish containing 2 mL sterile PBS.
2. The spleen is ground slightly with the plunger of a 2-mL syringe by grinding circular movements to release the splenocytes into the Petri dish through passing the strainer.
3. Periodically, liquid from outside the strainer is pipetted to wash out the cells from within the strainer. Continuously, the spleen is slightly mashed until all that remains is the white connective tissue of the outer membrane.
4. The splenocyte suspensions are collected, filtered through the strainer twice if necessary, and transferred into a 5-mL tube, and the Petri dish may be washed out with 1-mL PBS twice to maximize the cell recovery.
5. The splenocyte suspensions are centrifuged at 800 × *g* for 5 min at room temperature and discard the supernatant by decant.
6. The splenocyte pellet is fully resuspended in 1-mL red blood cell lysing solution (containing 155-mM NH_4Cl, 12-mM $NaHCO_3$, 0.1-mM EDTA) and left for just 2 min at room temperature to lyse the red cells.

7. The suspensions are diluted immediately with 4 mL PBS, and centrifuged at 800 × *g* for 5 min to discard the supernatant, and the pellet is resuspended in the cells completely in a full volume of PBS and washed twice with PBS following each centrifuge.
8. Finally, the cells are suspended in a proper medium (e.g. RPMI-1640) to a final known volume, and live cells are counted using a hemacytometer under a light microscope after Trypan blue staining.

Mouse lymphoid node immunocytes are isolated using the protocol as follows [28].

1. The anesthetized or euthanized mouse is put on its side to localize the region of the superficial (e.g., inguinal or brachial lymphoid node) to be harvested.
2. After disinfection with chlorhexidine or other disinfectants of the skin region that covers the lymph node, the skin is opened with a tiny incision (about 5 mM) with scissors.
3. The incision is stretched with two forceps to find the lymph node, which may appear grayish or darker than the surrounding fat.
4. The fascia (thin membrane covering the fat and tissue) on top of the lymph node is pinched with one pair of forceps and pulled lightly without breaking the surrounding tissue, then the lymph node is clamped with the second forceps from the underneath and is removed with the first forceps by breaking the fascia.
5. The lymph node is place in a 5- or 10-mL tube containing PBS and should immediately sink to the bottom of the tube, validating that a lymph node but not fat tissue has been extracted.

The obtained lymph nodes can be further subjected to lymphocytes isolation or histological section for immunological assay.

The immune cell proliferation can be tested by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method.

1. MTT solution is prepared by dissolving 50 mg MTT in 10 PBS followed by filtering through 0.2-μm membranes for sterilization.
2. A cell suspension is adjusted to contain 0.1–1.0 × 10^6 cells/mL in a medium and is seeded with 100 μL of cells per well in a 96-well plate, or with 500 μL of cells per well in a 24-well plate is seeded, with or without the compound to be tested.
3. The seeded cells are cultured for 24–96 h at 37°C and 5% CO_2 in a humidified incubator, and then 10 μL of 5 mg/mL MTT in PBS is added to each well of a 96-well plate, or 50 μL to each well of a 24-well plate.
4. The plate is incubated at 37°C and 5% CO_2 for 3–4 h (resulting in purple precipitates within cells, and the intracellular precipitates can be more precisely visualized under a light microscope).
5. The culture medium is carefully removed using a pipette (after centrifuge at 800 *g* for 5 min for nonadhesive cells), and Dimethyl sulfoxide (DMSO) is added at the amount of

100 μL per well for a 96-well plate, or 500 μL per well for a 24-well plate, which is incubated at room temperature for 2 h in the dark to fully dissolve the intracellular precipitates. (Note: Longer incubations with DMSO in the wells may result in precipitate or turbidity that can increase background. If precipitate is observed, warm the plate at 37°C for 10–20 min and agitate to dissolve the precipitate.)

6. The plate optical absorbance is finally read at a wavelength of 560 nm with a reference wavelength of 650 nm.

4.3.4. Humoral immunity assays

The antigen-specific antibodies, such as IgA, IgG, and IgG2a (or IgG2c in case of samples from C57BL/6 mice), produced in mice vaccinated with Ag-loaded liposomes can be assayed using the method of enzyme-linked immunosorbent assay (ELISA) in a microplate reader (μQuant™, BioTek Instruments, Inc., Vermont, USA) [25, 28]. There are five types of ELISA, including indirect ELISA, direct ELISA, sandwich ELISA, competitive ELISA and ELISPOT, and only a few differences exist amid these ELISA protocols, with the main ELISA principle and lots of procedures being the same. The level of anti-Ag antibody is often determined by the conventional indirect enzyme-linked immunosorbent assay (ELISA) protocol as follows:

1. A 96-well microtiter plate is coated per well with 100 μL of Ag (capture protein) (1 μg/mL) in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and sealed and incubated at 4°C overnight.
2. Each well of the plate is aspirated with pipette to discard the contents and washed three times with 300 μL of PBS containing 0.05% v/v Tween-20 (PBST). After the last wash, the plate is inverted and blotted against clean paper towels to remove any remaining PBST.
3. Each well is blocked by adding 200 μL of PBSTO (PBST containing 1 mg/mL OVA, or protein other than the Ag in liposomes) and then covered and incubated at 37°C for 1 h to prevent the nonspecific binding sites in the coated wells and then blotted on paper towels after discarding of water.
4. Thereafter, 100 μL of samples, which have been collected from mice and diluted appropriately with PBSTO, is added to each well of the plate, sealed, incubated at 37°C for 1 h.
5. After being washed thrice with 200 μL of PBST and blotted on paper towels after last wash, each well is added with 100 μL of the HRP (horseradish peroxidase)-conjugated secondary antibodies that have been diluted with appropriate amount of PBSTO (1:2500) and sealed for incubation at 37°C for 1 h.
6. After being washed with 300 μL of PBST for five times and blotted on paper towels after last wash, each well is supplemented with 100 μL of the freshly prepared TMB (3, 3′, 5, 5′-Tetramethylbenzidine) substrate solution (containing 1 mM TMB, 3 mM H_2O_2 and 0.2 mM Tetrabutylammonium Borohydride (TBABH)) and incubated at room temperature in the dark for 20 min for color development [25, 53].

7. And 20 min later, each well is added with 100 μL of 1 M H_2SO_4 solution to terminate the color development and then immediately determine the optical absorbance at 450 nm, with a reference wavelength at 570 nm, using an automated microplate reader (μQuant™ microplate spectrophotometer, BioTekInstruments, Inc., Winooski, Vermont, USA).

Notably, in above Step 6, TMB substrate solutions should be freshly prepared and used in less than 10 min, because they are chromogenic reagents for peroxidase to develop an intense blue color that can be read directly (at 650 nm), or a deep yellow color (read at 450 nm) after stopping with an acid solution.

And 1 mM TMB substrate solution (containing 1 mM TMB, 3 mM H_2O_2) can be freshly prepared as follows:

1. Preparation of Solution A: Potassium citrate buffer solution (CBS) is prepared by dissolving 2.15 g citric acid in 40 mL H_2O, which is then adjusted of pH 4 with 1 M KOH and diluted with H_2O to 50 mL, and then 50 mL of CBS is then mixed with 15 μL of fresh H_2O_2 (30%), resulting in Solution A.
2. Preparation of Solution B: A total of 10.8 mg TBABH (Tetrabutylammonium Borohydride) is dissolved in 5 mL dimethyl acetylamide (DMA), into which 50.3 mg TMB is then added and dissolved by vertex, resulting in Solution B.
3. Preparation of 1 mM TMB substrate solution: Just before use, 8 mL of Solution A is completely mixed with 200 μL of Solution B, resulting in 8.2 mL 1 mM TMB substrate solution.

4.3.5. In vivo tracking of the Ag-loaded liposomes after vaccination

To track *in vivo* vaccines delivered by liposomes, both *ex-vivo* approaches and *in-vivo* imaging techniques can be used, including *ex-vivo* biodistribution, autoradiography, MRI, optical imaging, PET and single-photon emission computed tomography (SPECT), all of which have their specific advantages and limitations. Consequently, selection of the tracking method should be based on the distinct features of the nanocarriers as well as the specific aims of the experiments [54, 55]. Here, this section introduces a simple fluorescent method that is conveniently applied for tracking vaccine delivery system. For this, the fluorescent agents, such as calcein and sulforhodamine B (SRB), are efficiently entrapped due to their water-soluble properties as a label in the Ag-loaded liposomes, enabling both *ex-vivo* and *in-vivo* imaging approaches to be used for tracking the vaccines.

For *in-vivo* imaging, usually, 0.5, 1, 2, 4, and 8 h after administration of the fluorescent agent/Ag-loaded liposomes via intradermal, intramuscular, intra-mucosal or any other feasible route, mouse is imaged using animal *in vivo* imaging systems; otherwise, mouse is exposed, by anesthetic and anatomic operation, of mucosa, lymph nodes (LNs), or other region wherein vaccines are expected to be delivered by liposomes, to lights with the wavelength matching fluorescent agent entrapped in liposomes for automatic imaging using a camera or a smart phone with lens [24].

For *ex-vivo* imaging and assay, at 0.5, 1, 2, 4, and 8 h after vaccination mice with fluorescent liposomes, the organs, such as spleen, liver, kidney, heart, lung and even brain, and main draining LNs that collect the fluids from the administration site and nearby regions are dissected from the vaccinated mice for further histological section assay. To observe distribution of fluorescent liposomes, the freshly harvested organs and draining LNs should be immediately embedded in the optimum cutting temperature (OCT) compound for cryosection and observation under a fluorescence microscope (IX83, Olympus, Japan). The procedure for cryosectioning is relatively simple and can be performed rather quickly, using the material including necessarily frozen embedding media of OCT, empty slide box, dry ice, cryostat, which is essentially a microtome inside a freezer, disposable blades, specimen mount, slides, and optionally poly-L-Lysine, gelatine, and agarose.

1. The fresh, unfixed tissue sample, up to 2.0 cm in diameter, is frozen in OCT in a suitable tissue mold in liquid nitrogen or at −80°C in an ultralow temperature refrigerator. Certain soft tissues, such as brain, are optimally frozen in M-1 medium at −3°C.
2. The tissue-embedded OCT is fixed and frozen onto the specialized metal grids fitting onto the cryostat.
3. Sections of 5–15 μm thickness are cut in the cryostat at −20°C. If necessary, the temperature of the cutting chamber may be adjusted up or down of ±5°C, depending on the tissue under study. A camel hair brush is useful to help guide the emerging section over the knife blade.
4. Within 1 min of cutting a tissue section, the section is transferred to a room temperature microscope slide by touching the slide to the tissue, avoiding freeze-drying of the tissue. Poly-L-lysine-coated or silanized slides improve the adherence of the section. And to evaluate tissue preservation and orientation, the first slide of each set may be stained with toluidine blue (1–2% w/v in H_2O), hematoxylin, and eosin, or any aqueous stain.
5. The sample-loaded slide is immediately immersed into an appropriate fixative, such as in precooled acetone (−20°C) for 10 min, which is then poured off and allowed to evaporate from the tissue sections for less than 20 min at room temperature, and then rinsed thrice with plentiful PBS.
6. The slide is stained of nucleus with DAPI for immediate observation. Otherwise, the slide may be covered with a layer of OCT to prevent freeze-drying and stored the rest of the sample at −70°C for further assay. For long-term storage, a moistened tissue should be added to the container with the block to prevent desiccation (particularly in a frost-free freezer).

In addition, the draining LN immunocytes that had taken up the liposomes can be sorted out by distinct fluorescent Ab staining using flow cytometry assay as follows.

1. The harvested draining LNs are gently mashed and filtered through a strainer with 70-μm mesh to isolate immune cells.
2. The resulting cells are stained at 4°C for 2 h with different fluorescent dye-conjugated Abs against CD11b, CD11c, F4/80, and CD169.

3. The stained cells are analyzed by flow cytometry to estimate the dLN cells that had engulfed either nanocarriers and their fractions based on fluorescence of liposomes and cells, which can be distinguished according to expressions of $CD11c^{+}F4/80^{-}$ by DCs, $CD11b^{+}F4/80^{-}CD169^{+}$ by subcapsular sinus macrophages (SSMs), $CD11b^{+}F4/80^{+}CD169^{+}$ by medullary sinus macrophages (MSMs), and $CD11b^{+}F4/80^{+}CD169^{-}$ by medullary cord macrophages (MCMs) [24].

4.3.6. In vivo assay of activation of lymphocytes and generation of memory lymphocytes

The immune induction effects by vaccines on lymphocyte activation, memory lymphocyte differentiation, and Ag-specific $CD8^{+}$ T cell production, are also often evaluated by flow cytometry.

1. Three weeks after final immunization with the Ag-loaded liposomes or the control formulations, the treated mice are anesthetized with chloral hydrate and aseptically isolate the splenocytes and LN cells, which, respectively, are seeded in the 24-well plate and cultured at a concentration of 10^{6} cells/mL at 37°C in a cell incubator with 5% CO_2.
2. After 24-h incubation, the cells are co-cultured with OVA (10 μg/mL) for further stimulation at a concentration of 10^{6} cells/mL at 37°C in a cell incubator with 5% CO_2 [25].
3. After 72-h incubation, the cells are washed (10^{5}/mL) and stained with fluorescence-labeled anti-mouse Abs (1 μg/mL), including CD4, CD8, CD69, H-$2K^{b}$-SIINFEKL, CD44 and CD62L for labeling T cells, and CD45R (B220), CD80 and IgD for labeling B cells, at 4°C for 1 h.
4. After washing thrice with PBS, the cells are resuspended in 1 mL PBS and assayed by flow cytometry to measure the percentages of effector memory T cells ($CD44^{high}CD62L^{low}$), central memory T cells ($CD44^{high}CD62L^{high}$), memory B cells ($B220^{+}CD80^{+}IgD^{-}$), and also the Ag-specific $CD8^{+}$ T cells ($CD8^{+}$SIINFEKL-MHC-I^{+}) [56].

Also, the supernatants of the splenocytes culture as well as the sera (1:10 dilution) from treated mice may be subjected to sandwich ELISA assay of the levels of IFN-γ, granuzyme B, IL-4, or other cytokines [25].

4.3.7. Assay of Ag-specific cytotoxicity of CTLs

Production of the Ag-specific cytotoxic T lymphocytes (CTLs) in treated subjects is the required immunity induced by a vaccine to erase the intracellular pathogens and is thus a desired function of a vaccine that has been purposely designed. The *in vivo* Ag-specific cytolysis of induced CTLs in the vaccine-treated mice is regarded as the Ag-specific cytotoxicity of CTLs and is often analyzed using the CFSE-labeled cells bearing the Ag epitopes [57]. Herein, using OVA as the model Ag, the process for assay of the Ag-specific cytotoxicity of CTLs in the vaccinated mice is described as follows [24]:

1. Mice are vaccinated twice with an interval of 3 weeks with the OVA-liposomes by the desired route.

2. The syngeneic splenocytes are isolated from naive mice, washed with PBS, and supplemented with 3% fetal calf serum (FCS) and 5 mM EDTA at 2×10^7 cells/mL in PBS.
3. The splenocytes are equally divided into two populations, which are incubated with CFSE at concentrations of 4 μM ($CFSE^{high}$) and 0.4 μM ($CFSE^{low}$), respectively, for 5 min at room temperature, followed by washing twice with cold PBS.
4. The $CFSE^{high}$ splenocytes are pulsed with 1 μM $OVA_{257–264}$ (SIINFEKL) peptide for 1 h at 37°C in the dark to be target cells identifiable to the OVA-specific CTLs, and then after washing thrice with PBS mixed with $CFSE^{low}$ splenocytes as nontarget cells at equal numbers (2×10^7 total cells).
5. The mixture of the two splenocyte populations is intravenously injected through tail vein into the mice that should have received the second vaccination of OVA-liposomes for 6 days.
6. After 15 h, the draining nodes and spleen from recipient mice are taken to isolate the cells for measuring by flow cytometry the *in vivo* cytolysis effect of CTLs, which is estimated by the loss of the $CFSE^{high}$ antigen-pulsed population relative to the control $CFSE^{low}$ population.

4.3.8. Prophylactic efficacy of the Ag-loaded liposomes tested using pathogen challenge

The prophylactic efficacy of liposomal vaccines can be evaluated using the pathogen challenge experiments using the animals, which should be the hosts that infectious pathogens can lodge at and proliferate in. And the animal models fitting host-pathogen interaction studies are well summarized in a previous paper [58], and the interested readers can refer to the paper for choosing the subjects for carrying out the related research. Notably, the pathogen challenge experiments should be conducted in the laboratory with the necessary biosafety levels (BSLs) that can absolutely guarantee the full protection of the investigators and the complete eradication of the propagation of the pathogens from the laboratory [59]. The requirements of BSLs of the laboratories for carrying out pathogen challenge experiments on various types of pathogens with different infection and lethality abilities are rigorously defined in the guideline book of *Biosafety in Microbiological and Biomedical Laboratories*, which can be freely downloaded from the CDC website and should be at hand for every researcher who conducts related experiments (https://www.cdc.gov/biosafety/publications/bmbl5/bmbl.pdf).

To carry out the pathogen challenge experiments, the model animals with the proper age are vaccinated once or twice at a proper interval by the aimed administration route with the Ag-loaded liposomes at the dose of low, middle and high levels, respectively. And 3 weeks after the (second) vaccination, the animals are challenged with the pathogen in a stock at the challenge dose of 10–50 folds of LD50 (lethal dose 50%) [60]. The animals are weighed once and observed twice daily for certain days to observe the protection efficacy, and the animals may be euthanized before reaching the moribund state due to humane concerns.

5. Conclusions

Liposomes are widely used as a vehicle for delivering various vaccines due to their numerous advantages, including high biocompatibility, diverse and high loading capacity, inherence adjuvanticity, relative ease for preparation and for surface decoration to engender a multifunctional carrier. Particularly, when the liposomes are combined with specific molecules, such as ligands to TLRs, the steric stabilization polymers, and the mannosylated compounds, the carriers are indeed functioning as a stable and effective vaccine adjuvant-delivery system (VADS) to play both the roles of a vaccine adjuvant and a vaccine vehicle. The liposomal VADS can efficiently deliver vaccines to the lymph nodes and even target the APCs to elicit robust humoral as well as cellular immunity against the invading pathogens bearing the surface features matching the loaded antigens, as confirmed by the *in vitro* and *in vivo* experiments, including testing the cellular uptake of liposomes by APCs and the activation of lymphocytes by liposomes, *in vivo* tracking of liposomes, assaying secretion of cytokines and immunoglobulins with liposome stimulation, all of which are described with key steps and key points in this chapter. As researchers are continuously focusing their research interests and efforts on the development of liposomal vaccines, many of the now unknown mechanisms underlying the elicited immunity will eventually be brought to light and may play a crucial role in rational design of the optimal VADSs. Obviously, various types of the liposome-based vaccines may finally be approved by the authorities to enter markets as new products against numerous life-threatening diseases, when many advanced platforms are managed and set up to further explore and constitute the diverse functional liposomes as an effective vaccine adjuvant-delivery system (VADS).

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