



Review Article

Liposome As Mucosal Vaccine Drug Delivery System

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ABSTRACT

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Vaccines are a pharmaceutical product that is used to overcome infectious diseases and are a new therapeutic strategy for chronic disease treatment, recently. However, the parenteral route as the main route of vaccination nowadays has several limitations, so mucosal vaccines rise as a potential alternative for vaccine delivery. A few research found that mucosal vaccines have better immune protection against pathogens. Liposome, a biodegradable particulate drug delivery system, offers the most promising future as a mucosal vaccine delivery system because of its versatility. A liposome can act as an adjuvant by direct stimulation of immune response in several ways. Several liposome properties that affect system uptake after mucosal administration are size, surface charge, and hydrophilicity. To enhance liposome ability as mucosal vaccine, targeted liposomes, such as mannose receptor-targeted liposome, macrophage galactose-type C-type lectins (MGL) targeted liposome, DC-specific intracellular adhesion molecule-3 grabbing non-integrin (DC-SIGN), and M cells targeted liposome then developed.

1. INTRODUCTION

Vaccines are a kind of pharmaceutical product, composed of an antigenic substance that is commonly used to maintain and fight against infectious diseases, and recently, vaccination is a new therapeutic strategy for chronic disease treatment, such as tumor, malaria, and HBV (Ma, 2011; Kraan 2014). Upon a vaccine injection, a human body can react by producing antibodies, a humoral immune response system, that elicits long time protection against pathogens, either bacterial or viral pathogens. Vaccines products are divided into two kinds of general types, a live-attenuated vaccine that elicits a robust immune response and a killed-inactivated vaccine, with a

weaker immune response, containing a killed pathogen, fraction, or protein subunit/substrate/antigen of a pathogenic organism (Tlaxca, 2014). Despite its strong immune response, live-attenuated vaccines have several disadvantages, which are high manufacturing costs, require complicated cold chain storage and distribution, and are harmful to immunosuppressed patients (Henderson, 2011; Tlaxca, 2014). However, subunit vaccines that are poorly immunogenic have a promising future because of better safety and lower risk of contamination during the manufacturing process, incorporation of potent adjuvant into vaccine formulation is necessary to increase vaccine immune response (Ma, 2011; Watson, 2012). Among all of the new adjuvants that have developed, the liposome is gaining a high interest as vaccine adjuvant delivery system.

Up to this day, the main route of vaccine administration is the parenteral route, either subcutaneously or intramuscularly (Neutra, 2006; Kraan, 2014; Henderson, 2011; Wang, 2014). This way of administration has a lot of disadvantages such as low patient compliance, high-risk needle contamination, and a requirement of professional personnel (Wang, 2014). Mucosal vaccines are increasingly developed nowadays to overcome parenteral vaccine limitations. Besides the easier administration and higher patient compliance especially for children, mucosal vaccines offer better health protection against the pathogen (Kran, 2014). Mucosal surfaces, which consists of a large surface area, are the main entry site for pathogen microorganism, and mucosal immunity is an essential and initial immune defense against pathogen microorganism by preventing pathogen microorganism or their toxin binds to the epithelium, hence avoiding further microorganism invasion into the bloodstream (Neutra, 2006; Watarai, 2014; Jiang, 2015; Tada, 2018). However, vaccine administration via injection usually induces poor mucosal immunity, on the other hand, mucosal vaccination through oral, nasal, rectal, or vaginal routes can produce both mucosal and systemic immunity (Neutra, 2006; Kraan, 2014; Wang, 2015). Further, vaccine administration toward a mucosal surface can enhance vaccine safety by avoiding a direct systemic circulation of potentially toxic vaccine components (Gupta, 2015). Therefore, the biggest challenge of mucosal vaccine development is how to deliver an antigen with safer properties toward multiple barriers of the mucosal layer, producing an effective immune response right after administration.

2. MATERIALS AND METHODS

3. RESULTS AND DISCUSSIONS

Liposome as Mucosal Vaccine Drug Delivery System

As previously mentioned, mucosal vaccines have a high potency toward better immune protection against harmful pathogens. Upon vaccination, mucosal vaccines inoculated at lumens mucosa that is rich with dendritic cells, and able to induce not only mucosal but also systemic immune response (Zhen, 2015; Wang, 2015). Due to numerous mucosa-associated lymphoid tissues (MALT) at the generalized mucosal immune network, immune responses will be generated not only at the site of mucosal vaccination but also at another remote mucosal tissue (Wang, 2014).

For example, mucosal vaccination in the gastrointestinal tract produces IgA not only in the intestine but also in the bronchus and genitourinary tract (Gupta, 2015). Although its superior potential for better vaccination practice, mucosal vaccine possesses several challenges at the development stage. For a mucosal vaccine can induce potent immune responses effectively, firstly, the mucosal vaccine must reach the antigen-presenting cell, particularly dendritic cells. However, the mucosal surface is usually protected with a viscous fluid-covering mucus layer that is renewed continuously and contained several enzymes. As the result, the mucosal vaccine will be removed from the administration site quickly, and vaccine antigens' possibility of crossing the epithelial layer and approaching antigen-presenting cells become lesser (Kraan, 2014; Zhen, 2015). Another hurdle that concerned mucosal vaccine development, mainly sub-unit antigen vaccines, is their low immunogenic response after vaccination. A potent adjuvant must be included in a vaccine formulation to overcome this problem (Henderson, 2011; Watson, 2012; Soema, 2015).

An adjuvant acting as an immunopotentiator can be divided into two classes based on its mechanism of action. Storage type adjuvant, such as aluminum hydroxide and aluminum phosphate, lengthen persistent antigen time inside the organism, therefore, inducing durative and effective immune stimulation because of the antigen protection. Other classes of adjuvant, center type adjuvant such as endotoxin and liposome, together with antigen subunits can directly induce immune system cell stimulation (Fan, 2011). The major problem concerned with adjuvant is the reactogenicity properties of the adjuvant such as local reaction within the administration site (Tlaxca, 2014). Aluminum-salt adjuvants are the mostly used adjuvant for sub-unit antigen vaccine formulation, and the only adjuvant approved until 2009 in the United States. Although used widely, alum adjuvant elicits low TH1 response and cell-mediated immunity (Rosekrand, 2011; Watson, 2012). Other adjuvants used nowadays are mutated cholera toxins (CT), E.coli labile toxins, and Toll-like receptor agonists such as CpG DNA and monophosphoryl lipid A (MPL). CT is generally incompatible for human vaccine products because of systemic toxicity, particularly neurotoxicity although CT adjuvant can elicit strong humoral immune system. CTB or modified cholera toxin subunit B is a modification of CT adjuvant for systemic toxicity elimination. On the other hand, CpG ODN adjuvant, stimulate and activate the innate immune system via the TLR9 pathway, and MPL preferentially elicits a cellular immune response via the TLR4 pathway. Despite each desirable property, safety concerns remain, so an invention of a safer vaccine system is still needed (Henderson, 2011; Treniti, 2014).

Liposome, a particulate drug delivery system, is a system that commonly composed of phosphatidylcholine and cholesterol that are usually found in membrane cells. Among all of the particulate systems that have been developed before, liposome offers the most promising future because of its versatility. Lipid component of liposome makes a liposome product have a biodegradable characteristic, producing a product with high safety. Another liposome versatility is liposome capability to entrap both hydrophilic and hydrophobic molecules, enabling entrapment of all kinds of antigens, such as protein, peptide, nucleic acid, carbohydrate, and hapten (Watson,

2012). As a vaccine delivery system, a liposome can act as an adjuvant by direct stimulation of immune response through antigen uptake stimulation by antigen-presenting cells, antigen depot formation, and local inflammation induction (Lockner, 2012; Soema 2015). An antigen-presenting cell, mainly dendritic cells is the main key for adaptive immune system induction. Upon vaccine exposure, the dendritic cell will recognize, internalize, and process antigens, then present antigen to CD4+ or CD8+ cells. Dominant factors that affect antigen uptake by dendritic cells are the size and surface charge of the antigen system. Generally, the subunit antigen size is too small, so the antigen uptake process by dendritic cells is ineffective. Antigen entrapment within a liposome system, therefore, increases the whole particulate size that is comparable with virus size, resulting in higher antigen uptake by dendritic cells (Soema, 2015). On the other hand, the surface charge of liposomes influences liposome-dendritic cell's surface electrostatic interaction, with cationic liposome being the most effective system. The interaction between positive or cationic charge liposomes and negative or anionic cellular membrane cells can promote maturation of dendritic cells. Cationic liposome with higher surface charge elicits a better dendritic cell maturation, antigen uptake, and antigen-specific immune response. Example of lipids that can construct a cationic liposome are 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), octadecenoyloxy(ethyl-2-heptadecenyl-3-hydroxyethyl) imidazolium chloride (DOTIM) and dimethyldioctadecyl-ammonium (DDA), 1,2-dimyristoyl-trimethylammonium propane (DMTAP) (Ma, 2011; Henderson, 2011; Wang, 2012; Soema, 2015, Bernasconi, 2016).

The utilization of liposomes as a vaccine delivery system is already gaining interest for both parenteral and mucosal vaccine development. CAF01 is a liposome vaccine adjuvant system, construct from cationic DDA lipid and immunostimulator trehalose 6,6'-dibehenate (TDB). Incorporating trivalent subunit influenza vaccine (TIV) into CAF01 system successfully improves the induction of both humoral and cell-mediated immune response upon parenteral injection of vaccine compared to TIV alone (Rosekrand, 2011). Another example of liposome vaccine delivery system for infectious disease prevention is HspX-CpG DNA liposome and HspX-MPL liposome. HspX is a specific protein that is highly expressed under stress conditions and has proven to be a useful antigen to induce strong immune protection against latent tuberculosis infection. Among three HspX-containing liposome formulations construct from phosphatidylcholine, HspX-CpG DNA liposome has the most robust specific immune response compared to HspX liposome (without additional adjuvant), and HspX-MPL liposome (Trentini, 2014).

As a novel therapeutic strategy, the liposomal vaccine was developed for non-infectious diseases such as nicotine dependency and tumor/cancer therapy. For nicotine dependency, Lockner et al prepared a liposomal vaccine from 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-sn-3-phospho-(1'-rac-glycerol) (DMPG), cholesterol, and lipid-maleimide linker with or without agonist TLR4 MPL and/or agonist TLR2 S-{2,3-bis(palmitoyloxy)-(2RS(-propyl])-N-palmitoyl-(R)-cysteinyl-alanyl-glycine (Pam3CAG) as vaccine adjuvant. Antigen hapten-protein conjugate AM1-KLH was then mixed with each liposome at room temperature for an hour. In vivo

evaluation using mice showed that liposomal vaccine delivery with Pam3CAG alone has a little advantage and MPL, with or without Pam3CAG, is the most optimal adjuvant for immune response induction (Lockner, 2012). Another novel approach that has recently gained a high interest is cancer vaccine immunotherapy for tumor or cancer treatment. Cancer vaccine therapy can induce a specific response of cytotoxic T lymphocyte (CTL) that can destroy tumors or cancer cells. Compare to chemotherapy and radiotherapy, cancer therapy using a specific vaccine enables to produce more specific tumor/cancer-destroying effect with minimal side effect. Polyriboinosinic: polyriboinosinic acid (PIC) is popularly known as antiviral and anticancer, and also can be used as an adjuvant for the maturation of dendritic cells and increase antigen-specific immune responses. Preparation of PIC cancer vaccine using DOTAP lipid showed a higher CTL response and IFN- γ production compared to DOTAP and PIC alone after an intraperitoneal vaccination.

Meanwhile, the administration of two doses of PIC including DOTAP liposome vaccine successfully induces tumor cell apoptosis and tumor growth suppression (Wang, 2012). Cancer therapy based on liposome vaccine also developed for specific breast cancer treatment using p5 peptide derived from HER2/neu protein, a tumor-associated antigen that is overexpressed in 20-40 % of breast cancer. To increase encapsulation efficiency of p5 peptide within the liposome system, firstly, the p5 peptide was conjugated to maleimide-PEG2000-DSPE before being incorporated into DMPC:DMPG:cholesterol: DOPE containing MPL liposome. Upon subcutan vaccination, liposome consists of 1,2-dioleoyl-sn-glycero-3-phospho-ethanolamine (DOPE), a pH-sensitive lipid that can be used to deliver an antigen into MHC class I pathway and MPL adjuvant combination proven to be useful to induce a higher production of interferon γ (IFN- γ) by CD+8 T cells and CTL response compared to p5 peptide, liposome p5-DOPE or liposome p5-MPL alone (Shariat, 2014).

Liposomes Properties for Optimal Mucosal Immune Responses

Liposome as a mucosal vaccine delivery system faces a lot of challenges. As previously mentioned above, a mucosal surface covered by viscous liquid changes continuously. Upon administration, mucosal-viscous liquid served as the first barrier that can eliminate the vaccine system before absorption takes place. Second, an optimal liposome vaccine system must be able to penetrate into or across mucosal cell epithelium without damaging the cells and reach a circulating antigen-presenting cell or directly interact with mucosa-associated lymphoid tissue (MALT) for eliciting an immune response (Christensen, 2010; Kraan, 2014; Zhen, 2015). Several liposome properties that affect system uptake after mucosal administration are size, surface charge, and hydrophilicity.

Liposome size

Liposome size is a crucial factor that affects the liposome-based vaccine delivery system. Before a system is available for APCs uptake, firstly it must be able to penetrate the mucosal epithelium layer. Generally, liposomes with a size range from hundred nanometers up to 5 μm are

taken by M cells in mucosal tissues, and liposomes bigger than 10 μm are failed to penetrate (Masarini, 2017). M cells or microfold are a special gate at mucosal epithelium that has a specific function at antigen uptake, then presenting the antigen to APCs such as dendritic cells and macrophages (Jiang, 2015). Another way that can be taken by liposome-based-vaccine is to penetrate directly through mucosa tissue that has the tightly layered stratified squamous epithelium. A liposome, called MLL liposome with a size less than 300 nm was made from soy phosphatidylcholine (SPC), mannose-PEG1000-cholesterol conjugated, stearylamine, and monophosphoryl lipid A (MPLA) with bovine serum albumin (BSA) as antigen model and used for a mucosal vaccine. Oral mucosal administration of MLL was successful with small liposome size argued to be a critical factor that affects MLL penetration across the mucus layer and then epithelium of mucosa for immune response induction (Wang, 2014).

Besides affecting mucosal penetration, liposome size also proved to be an essential factor for antigen-uptake by APCs, where smaller liposomes are suitable for dendritic cells-mediated uptake and the bigger liposome for macrophage uptake (Masarini, 2017). Based on several studies, the particle size of the liposome that is desirable for macrophage uptake is around 1000 nm (Wang, 2013). A cationic liposome for intranasal administration with a particle size of approximately 250 nm is proven to be sufficient for pulmonary DC uptake (Henderson, 2011). Liposome size is dominantly affected by liposome preparation methods such as extrusion and sonication method, and lipid composition of liposome is defined by lipid chain length, molecular shape, the fluidity of lipid bilayer membrane and charged lipid incorporation (Soema, 2015).

Surface Charge

The liposome surface charge, assessed by zeta potential is an essential factor to predict colloidal liposome system stability (through electrostatic repulsion) and encapsulation efficiency of antigen from the electrostatic attraction mechanism (Soema, 2015). Based on the surface charge, the liposome-vaccine system can be classified into three liposome types, which are non-ionic liposome, anionic liposome, and cationic liposome, with the cationic liposome, being the most prominent system. Cell surface and viscous-mucus liquid of mucosal membrane have a negative surface charge, so a stronger interaction will happen when the liposome-vaccine system is made into a positive charge. An interaction between the negatively charged mucosal membrane and the positively charged liposome-vaccine system will increase mucoadhesion properties of the system, leading to a reduction in clearance rate and longer resident time at the mucosal membrane (Bernasconi, 2016). Cationic liposome, constructed by DDA lipid is found to be able to increase system permeability in both the cell culture and ex vivo imaging because of the electrostatic attraction between the liposome system and nasal mucosa that has a negative surface charge (Yusuf, 2017).

An interaction between cationic liposomes and cell membrane is also particularly advantageous because the interaction can induce a higher APCs antigen uptake and better APCs maturation. Cationic liposome alone can stimulate dendritic cells as APCs and elicits an expression

of CD 80 and CD86, the co-stimulatory molecules (Vangasseri, 2006). A mucosal vaccine using ovalbumin as a model antigen was successfully made using CLDC or cationic liposome-DNA complexes (CLDC) system. CLDC liposome is made from a combination of equimolar cationic lipid DOTIM and cholesterol, complexes with non-coding plasmid DNA as an immunostimulant. Intranasal administration of CLDC liposome vaccine was able to effectively generate an IgA mucosal response and intrapulmonary T cell immune response (Henderson, 2011).

Liposome surface charges also directly affect the entrapment efficiency of antigens inside the liposome system. Incorporation of negatively charged bovine serum albumin (BSA) antigen into positively charged stearylamine liposome elicits an electrostatic attraction between the two, producing a high entrapment efficiency of antigen within the liposome system. However, the electrostatic attraction of antigen-liposome also obtains a slow release rate of antigen; hence the liposome system is a suitable carrier for depot release system (Wang, 2014). A high interaction between negative charge ovalbumin and CAF01, a liposome-based vaccine adjuvant construct from DDA cationic lipid and TDB also observed, pointed by a homogeneous mixture system with no precipitation or aggregation (Christensen, 2010).

Hydrophilicity

To overcome the first barrier of mucosal vaccine administration, which is a mucus liquid layer covering the mucosal epithelium, the surface of liposome system can be tailored to facilitate a desirable interaction between liposome system and mucus liquid layer. One of the liposome surface modifications is PEG moiety attachment into a liposome surface to increase the hydrophilicity of the system. Attachment of MPC, mannose-PEG1000-cholesterol, a hydrophilic group into the liposome surface increased the compatibility between liposome-based vaccine system and hydrophilic mucus protein and significantly facilitates liposome penetration through mucus barrier (Wang, 2014). Incorporation of D-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS), a hydrophilic moiety into DDA cationic liposome also showed a positive effect on system penetration through mucus layer by producing a more slippery liposome system (Yusuf et al., 2017). A PEG modification of liposomes also elicits a longer liposome residence time in the intestinal lumen because of intense interaction with the mucous layer (Minato, 2003).

On the other hand, a PEG modification on the liposome surface is correlated to lipid bilayer thickness. Hence a higher degree of PEGylated modification produce a thicker liposome layer and reduces the release rate of antigen entrapped (Liau, 2015). Furthermore, a liposome with PEG modification decreases the system uptake by macrophages; hence the Peyer's patches uptake will be decreased too (Minato, 2003).

Despite several adjustments to liposome properties already showing enhancement in the penetration of liposome-based mucosal vaccine, protection of liposomes themselves from the mucosal environment, especially in harsh conditions of the gastrointestinal tract for oral

vaccination remain a challenge (Ogue, 2006; Liau, 2015). Several attempts that have been made to increase the stability of liposome-based mucosal vaccine in an oral environment are mainly based on liposome coating with an outer liposome or another particulate system. As an example, a double liposome system was made with the purpose to increase oral system stability. OVA contained-inner liposome was constructed from soy phosphatidylcholine (SPC), di-palmitoyl-phosphatidylcholine (DPPC), cholesterol, and stearylamine. A lipid mixture of dimyristoyl-phosphatidylcholine (DMPC) and negative charge dimyristoyl-phosphatidylglycerol (DMPG) is then used to form the outer layer of the double liposome system. In vitro release rate and stability test of OVA contained-double liposome system showed a positive result. Despite different testing solution pH (1,2 and 6,8), the system showed a stable release of OVA, and double liposome system was also found to be able to produce higher protection against pepsin solution compared to OVA and OVA contained-single liposome. Lastly, an oral administration of OVA contained-double liposome significantly elicits higher IgA and IgG levels compared to the other systems (Ogue, 2006). Another liposome carrier modification with liposome protection as the purpose is liposome incorporation into a double emulsion (water-in-oil-in-water) system using span 80, tween 80, and Pluronic F127. In a double emulsion system, OVA-contained liposome was found in the internal water phase; therefore the in vitro release profile demonstrates an extended-release of OVA (Liau, 2015). Liposome vaccine also showed potential protection for gastrointestinal environment when formulated into chitosan coated-polyplex-loaded liposome (CS-PLL) containing DNA, a chitosan-DNA complex loaded into phosphatidylcholine (PC), cholesterol and

sodium deoxycholate liposome, then further coated with chitosan. In vivo study of the system using BALB/c mice of CS-PLL demonstrated the highest protection from enzymatic degradation, therefore highest potential to deliver the DNA throughout the intestine (Channarong, 2011). Several research about the liposomes mucosal vaccine can be seen in **Table 1**.

Table 1. Formulation of Liposome-Based Mucosal Vaccine.

Antigen	Administration Route	Liposome composition	Liposome properties	Results
Ovalbumin (Henderson, 2011)	Intranasal	An equimolar cationic lipid DOTIM and cholesterol, complexes with non-coding plasmid DNA (CLDC system)	Approximately 250 nm in particle size and positive surface charge.	The antigen-CLDC complex was able to explicitly target the antigen into pulmonary DCs, and induce both humoral and cellular immunity.
MSP-1 ₁₉ , a <i>P.falciparum</i> surface antigen (Tyagi,	Transdermal	Soy phosphatidylcholine (SPC) and span 80 (86 :14 % w/w)	An elastic liposome with particle size at 122 ± 9,2 nm,	The elastic liposome successfully facilitates the skin penetration of antigen and induces a better humoral and cell-mediated

Antigen	Administration Route	Liposome composition	Liposome properties	Results
2015)			entrapment efficiency at $54,2 \pm 7,23$.	immune response compared to intramuscularly administrated antigen solution and topically administrated antigen-loaded conventional liposome
Ovalbumin (Christensen, 2010)	Intranasal	CAF01 liposome-based vaccine adjuvant consists of cationic lipid DDA and TDB (5:1 w/w)	Not defined	CAF01 slightly increases the transepithelial absorption of antigen and significantly increases transport of antigen within the mucus layer hence increasing the antigen exposure to the MALT system. An in vitro test using Calu-3 cell model also suggests that CAF01 is well-tolerated in the nasal epithelium pointed out by there are no changes in integrity and viability of epithelium after CAF01 administration.
Ovalbumin (Yusuf et al, 2017)	Intranasal	SPC, cationic lipid DDA, and TPGS with molar ratio 4:16:0,1	A cationic liposome with size and surface charge that suitable for cell uptake ($265,9 \pm 51,9$ nm and $56,5 \pm 11,9$ mV respectively)	Intranasal administration of the liposome-based vaccine can induce a higher production of serum IgG ₁ , and sIgA immune response in nasal and vaginal samples compare to free OVA (intranasal and intramuscular)
Pneumococcal surface protein A (PspA) antigen (Tada,	Intranasal	Equimolar (1:1) DOTAP : cholesteryl 3β-N-(dimethylaminoethyl) carbamate (DC-chol)	A cationic liposome-based vaccine adjuvant (size and surface	Co-administration of PspA with DOTAP/DC-chol significantly promotes the antigen by nasal DC; hence an intranasal administration of the system elicits not

Antigen	Administration Route	Liposome composition	Liposome properties	Results
2018)			charge not defined)	only mucosal and systemic specific PspA IgA and IgG immune response but also PsA-specific Th17 immunity, an important immune response to eliminate the bacterial invasion in the host circulation.
Formaldehyde-killed whole cell (KWC) <i>Yersinia pestis</i> vaccine (Baca-Estradad, 2000)	Intranasal	Phosphatidylcholine, cholesterol, and DDA	Positively charged multilamellar liposome	Liposome-based vaccine formulation significantly increases the immunogenicity of KWC vaccine and intranasal administration of liposome vaccine significantly induced higher systemic and mucosal immune response compared to KWC vaccine alone.
pCI-HA10, plasmid encoding hemagglutinin gene (Wong, 2001; Wang, 2004)	Intranasal	1,2-dioleoyl-3-dimethylammonium chloride (DODAC), DOPE and polyethylene glycol	Cationic liposome with average size $98,3 \pm 6,1$ nm.	Liposome carrier provides plasmid DNA protection against nuclease degradation in the lung. Intranasal vaccination of pCI-HA10 liposome vaccine elicits a strong cellular, humoral and mucosal immune response.
Ovalbumin (Minato, 2003)	Oral	Unmodified liposome consists of distearoylphosphatidylcholine (DSPC) and cholesterol; PEG-modified liposome consists of distearoylphosphatidyl ethanolamine-polyethyleneglycol 2000 (DSPE-PEG), DSPC and cholesterol	Multilamellar liposome (MLVs) with particle sizes between 1,7 until 3,3 μ m	Oral vaccination of unmodified and PEG-modified liposome elicits a higher systemic IgG (highest response induced by unmodified liposome) and mucosal IgA immune response (highest response induced by PEG-modified liposome) compared to antigen solution.

Targeted-Liposome as Mucosal Vaccine Drug Delivery System

Mannose Receptor Targeted Liposome

Mannose receptor (MR) is a family member of C-type lectin receptor (CLR) and is excessively expressed by APCs, which are DCs and APCs. The previous study showed that mannosylated liposome was able to generate a lymphatic-targeted vaccine, enhanced antigen uptake by APC, and produced an immune response with long-term memory (Wang2, 2014). Hence, conjugation of mannose moiety into liposomes for mucosal vaccine development gaining a high interest recently. A liposome vaccine called MLLs consisting of soy phosphatidylcholine, stearylamine, monophosphoryl lipid A, and mannose-PEG-cholesterol (MPC) as targeting molecules was successfully developed. In vitro testing of the MLLs revealed that MLLs could facilitate antigen uptake via mannose receptor-mediated phagocytosis more efficiently than conventional liposomes. Upon oral vaccination, MLLs were proven to be a system with high safety and elicited high levels of serum IgG and IgA in several mucosal secretions, that is salivary, intestinal, and vaginal (Wang, 2014). The major drawback of oral mucosal vaccination is rapid clearance of the system by saliva, tongue, and jaws movements, further, penetration of the system is also limited by the anatomic structure of oral mucosal epithelium. Hence, a biodegradable microneedle was further developed to overcome the penetration hindrance of liposome-based mucosal vaccine MLLs. The proMLLs-filled microneedle arrays (proMMAs) has a stable structure, and are rapidly dissolved, releasing the MLLs without alteration in size and OVA entrapment of MLLs. Later, oral mucosal administration of proMLLs produced a robust systemic and mucosal immune response, verifying the system as an effective vaccine adjuvant-delivery system (Zhen, 2015).

The proHBsAg-MLLs microneedle arrays (proHMAs) containing HBsAg, an antigen of the hepatitis B virus further developed. Identical with proMMLs, oral mucosal vaccination of proHMAs also elicited a high level of serum HBsAg specific IgG as a systemic immune response and IgA as a mucosal immune response. Besides, oral vaccination also induced a strong cellular immunity against hepatitis B infection, proven by high levels of CD8+ T cells, IgG2a, and IFN- γ in the vaccinated mice. Furthermore, the proHMAs has high thermal stability even at 40oC for three days; making it a potential vaccine system to overcome the usually complex cold chain handling and transportation, especially in a remote area (Wang, 2015). Lately, MLLs system has also been developed for vaginal delivery, combining the MLLs with stealth lipid A-liposome (SLLs) into the proSLL/MLL-constituted microneedle array (proSMMA). The system proved to have the capability to induce robust humoral and cellular immunity against sexually transmitted pathogens (Wang, 2016).

Macrophage Galactose-Type C-Type Lectins (MGL) Targeted Liposome

Another family member of CLR expressed by DCs and macrophages is macrophage galactose-type C-type lectins (MGL). Generally, CLR can recognize the carbohydrate structure such

as mannose, fucose, and glucan on the antigen surface, with MGL, specifically bind into galactose, N-acetylgalactosamine, and Lewis X. Among those three, galactose is a potent ligand for specific targeting to APCs, the critical cells to induce desirable immune responses (Wang, 2013; Jiang, 2015). Galactosylated liposome-containing OVA as model antigen was successfully made using conjugate galactose-lipid 1,2-didodecanoyl-sn-glycero-3-phosphoethanolamine (DLPE), phosphatidylcholine and cholesterol. Compared to the unmodified liposome, galactosylated liposome showed better macrophage recognition, higher endocytosis by macrophage, and lastly higher induction of TNF- α and IL-6 production, the pro-inflammatory cytokines. Intranasal administration of galactosylated liposome to the BALB/mice successfully produced the highest concentration of sIgA and IgG antibody as mucosal and systemic immune response (Wang, 2013). In addition, in vitro and in vivo testing of similar galactosylated liposomes, especially beta-galactosylated liposomes also showed a positive effect on the facilitation of antigen uptake by DCs. Upon administration, galactosylated liposomes are also able to induce an immune response more efficiently, as proven by lower doses needed than unmodified liposomes. Furthermore, intranasal administrations of galactosylated liposomes induce higher production of serum IgG and more effective anti-tumor immunity (Jiang, 2015).

DC-Specific Intracellular Adhesion Molecule-3 Grabbing Non-Integrin (DC-SIGN) Targeted Liposome

Dendritic cells are one of the APCs found in all tissues and have a specific role in pathogen recognition to initiate a specific humoral and cellular immune response. Moreover, DCs also capable of suppressing unwanted autoimmune reactions making an antigen targeting to DCs become a potential treatment for cancer or autoimmune disorders (van Koyk, 2013). DC-specific intracellular adhesion molecule-4 grabbing non-integrin (DC-SIGN) is a CLR family member that is specifically expressed on DCs and bound into Lewis-type antigens and antigens with high mannose glycans. A PEGylated and non-PEGylated liposome vaccine containing OVA as model antigen with Lewis B and Lewis X glycan as targeting molecules was successfully made. In vitro trial of the system using bone marrow-derived DC (BMDC) revealed that PEG molecule affects the flexibility and orientation of glycan, hence hampering the interaction between glycan and DC-SIGN at BMDC. Specific interaction between both Lewis B and Lewis X glycan and DC-SIGN only happens in non-PEGylated liposome vaccines (Joshi, 2014).

In cancer immunotherapy, a potent tumor-specific CD4⁺ and CD8⁺ T cells response initiated by DCs is needed. Based on that background, DC-SIGN targeted liposome vaccine containing melanoma MART-1 antigen, using Lewis B and Lewis X as targeting moiety was being developed. Glycan-modified liposome showed an enhancement in binding and internalization by BMDC expressing DC-SIGN in humans. Antigen targeting into DC-SIGN has a high potency toward antigen recognition by CD4⁺ and CD8⁺ T cells, particularly when combined with lipopolysaccharide (LPS) as TLR4 trigger. LPS is a prominent component that is needed to increase

the effector T cells response induced by the liposome. Moreover, the TLR4 trigger also plays an important role in DCs maturation for optimal T cells responses (Unger, 2012).

M Cells Targeted Liposome

As mentioned above, M cells are mucosal epithelium gates for antigen uptake prior presented to dendritic cells and macrophages as APCs (Jiang, 2015). A specific uptake by M cells restricts the vaccine interaction with alternative areas of intestinal mucosa; therefore, produces a dominant delivery of the vaccine system to the Peyer's patches (Gupta, 2011). Ulex europaeus agglutinin 1 (UEA-1) is a specific lectin that exclusively binds into α -L-fucose epitope of mouse M cell's apical surface (Clark, 2002; Jepson, 2004; Gupta, 2011). Previously, it was reported that compared to the unmodified liposome, UEA-1-conjugated liposome significantly increases the percentage area of mouse intestinal M cells occupied by the liposome. Administration of UEA-1 conjugated liposome was also specifically targeting liposome to M cells of the intestinal mouse, proven by a higher ratio of M cell to enterocytes/goblet cells percentage area occupied by liposomes (Clark, 2002). UEA-1 conjugated liposome-based vaccine containing hepatitis B surface antigen (HBsAg) was successfully made from DSPC lipid, cholesterol, and N-glutarylphosphatidylethanolamines (NGPE) as a hydrophobic linker to facilitate conjugation of hydrophilic moiety UEA-1 to the liposome membrane. In vitro determination of UEA-1 conjugated HBsAg liposomes vaccine using bovine submaxillary mucin (BSM), a glycoprotein as the biological model showed good liposome-BSM binding in the absence of α -L-fucose, a UEA-1 specific sugar.

Conversely, the liposome-BSM binding was decreased significantly when the α -L-fucose was added to the system. In vivo determination of non-lectinized liposomes using orally vaccinated mice showed a lower immune response compared to intramuscular administration of HbsAg-alum vaccine. However, oral administration of UEA-1 conjugated liposome elicits comparable anti-HbsAg antibody levels to that intramuscularly HbsAg-alum. Furthermore, UEA-1 conjugated liposome also produces the highest amount of mucosal IgA antibody in salivary, intestinal, and vaginal secretion compared to non-lectinized liposomes, intramuscularly given of HbsAg-alum and orally given pure HbsAg (Gupta, 2011).

4. CONCLUSIONS

Liposomes are a promising delivery system for vaccine delivery, mainly mucosal vaccines. For optimal vaccine responses, liposomes vaccines must meet several properties, including not more than 10 μ m of particle size, having a positive surface charge and hydrophilic surface properties. Another way to enhance the mucosal immune responses through liposome delivery is to formulate the liposomes into a targeted system. Through this review, we can conclude that liposomes are a potential delivery system for mucosal vaccines in the future.

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