Evaluation of DOTAP liposomes as vaccine adjuvant: Formulation, in-vitro characterization and stability

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ABSTRACT

Liposomes are small, spherical, self-closed vesicles. This study evaluates the chaenhancement prepared by lipid film hydration method comprised of varying molar concentrations of phosphatidylcholine and cholesterol and dropwise addition of a defined concentration of DOTAP. A solution of Newcastle disease virus vaccine was added and sonicated to form multilamellar vesicles. The optimum condition was 12.5:12.5:1(w/w) of phosphatidylcholine:cholesterol:DOTAP ratio with a maximum percent encapsulation efficiency of 63.3 %. Liposomes was characterized for morphology, particle size and zetapotential. Antibody titre, lymphocytes and globulin was assessed. The stability of the liposome was assessed by storing for four weeks at 4ºC, 28 ºC and at freeze-thaw temperatures. The mean size was 100 nm as confirmed by PCS and TEM images. The zeta potential of optimized liposomes prepared by this method was +24 mV. Haemagglutination inhibition, haematology and biochemical tests showed that the cationic liposomes induced higher immunity than La Sota® commercial vaccine. Stability studies of the TEM and PCS showed slight alteration in the shape and size of the liposomes after one month of storage at 4 ºC and 25 ºC. Results suggest that DOTAP-based liposome might be for boosting immunity against ND virus in chickens whereas storage of the liposomes at room temperature may not be favourable.

Introduction

Liposomes are small, spherical, self-closed vesicles of colloidal dimension which consist of amphiphilic lipids, enclosing an aqueous core. The lipids are predominantly phospholipids which form bilayers similar to those found in biomembranes. In most cases the major component is phosphatidylcholine. The processing conditions and the chemical composition determine whether liposomes are formed with one or several concentric bilayers [1]. Liposomes have been employed in various applications of life sciences [2]. Twenty-five years after the discovery of their immunological properties, liposomes now appear a major candidate adjuvant with a liposome-based vaccine (against hepatitis A) being licensed for use in humans [3,4]. Vaccines based on novasomes (R) (non-phospholipid liposomes formed from single-chain amphiphiles, with or without other lipids) have been licensed for the immunization of fowl

against ND virus and avian reovirus [5]. Articles on inactivated hepatitis B virus and surface antigens of influenza virus have shown that there are licensed liposomal vaccines. Others such as tumour antigens encapsulated in liposomes, liposomal interleukin-2, muramyl peptides encapsulated within liposomes, polymerized liposomes and immunostimulatory CpG oligonucleotides encapsulated in liposomes are presently undergoing clinical trials. Liposomes offer a number of advantages as carriers of vaccines because they are biodegradable and non-toxic, can elicit both humoral and cell-mediated immunity, and can be prepared entirely synthetically [6,7]. Cationic liposomes have a positive surface charge. They can improve the immune response against co-administered vaccine and protect antigen from clearance in the body. They can fuse with negatively charged cell membranes and deliver the antigen endosomally. One of the most frequently used cationic lipids in formulating liposomes is 1,2-dioleoyl-3-trimethylammonium propane (DOTAP). In this study, DOTAP-based liposome was used to entrap a standard ND vaccine for possible enhancement of immunogenicity and stability. The results suggest that the DOTAP-based liposome might be suitable as a potential candidate for boosting immunity but not as a thermostable vaccines against ND virus in chickens.

MATERIALS and METHODS

Preparation of dry lipid films

To obtain the dry film, 196 mg of phosphatidylcholine, 96.7 mg of cholesterol and 50 µg of DOTAP were weighed and dissolved in 3 ml of chloroform/methanol system (2:1) in a round bottom flask. The solvent mixture was evaporated at room temperature and the flask rotated until a smooth, dry film on the wall of the flask was obtained [8].

Hydration of dry lipid films for the cationic liposomes

The Newcastle disease vaccine La Sota$^{(R)}$ from NVRI, Jos was reconstituted with physiological saline by dissolving a vial in 40 ml of phosphate buffer solution. A 5 ml volume of the reconstituted vaccine was used to hydrate each dry film to form the various cationic liposomes or niosomes and 0.2 ml/bird was administered.

Transmission electron microscopy (TEM)

The prepared cationic liposomes were processed by using copper grids to adsorb the particles from the suspension, staining in 2.5 % uranyl acetate for 30 s and drying. The specimens were observed under JEM – 1010 Transmission electron microscope (JEOL, Japan) operated at 80 Kv at X 3400 and X 10500 magnifications [9].

Vesicle size, zeta potential and polydispersity index

The z-average vesicle diameter, zeta potential and polydispersity index of the cationic liposomes or niosomes in phosphate buffer solution (pH 7.4) were determined by photon correlation spectroscopy using nanosizer 3000 HS, Malvern instruments (Malvern, England, U.K). Zeta potential was calculated from the mean of three runs. Each sample was diluted with bi-distilled water and the electrophoretic mobility determined at 25 ^{o}C and dispersant dielectric constant of 78.5 and pH of 7. The obtained electrophoretic mobility values were used to calculate the zeta potentials using the software DTS Version 4.1 (Malvern, England) and applying Henry's equation [10].

$$UE = \frac{2\varepsilon Zf(K)}{} \quad (1)$$

3η

where Z is the zeta potential, UE the electrophoretic mobility, ε the dielectric constant, η the viscosity of the medium and $f(Ka)$ is the Henry's function.

The polydispersity index was determined as a measure of homogenity.

Lyophilization of the delivery systems

DOTAP-based cationic liposomes were first frozen slowly at -10 ^{o}C and then freeze-dried for 7 hours under vacuum at - 40 ^{o}C. The resulting lyophilized cakes were bottled, sealed tightly and stored at 28 ^{o}C and freeze (-20 ^{o}C) -thaw (4 ^{o}C) temperatures respectively for four weeks maximum. They were rehydrated to their original dispersion volumes with PBS (pH 7.4) during primary and secondary immunization of the birds.

Stability assessment using the haematology of the birds

A hundred birds (100) birds were divided into five groups of twenty birds. The birds were immunized at three weeks and six weeks for primary and booster doses with Newcastle disease formulated vaccines stored at 28 ^{o}C and -20 ^{o}C/4 ^{o}C for seven weeks. This was assessed one week after primary immunization and secondary immunization respectively for antibody using haemagglutination inhibition test and also lymphocyte circulation.

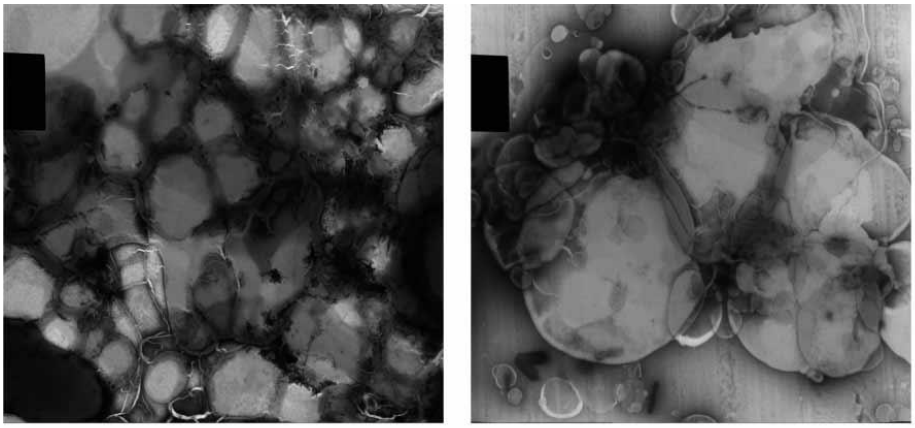
Data analysis

Data was fed into the SPSS statistics program (SPSS Inc, version 16.0) applying a one-way analysis of variance (ANOVA) test with least squared difference (LSD) multiple comparisons at $p < 0.05$.

RESULTS AND DISCUSSION

Photomicrographs of the cationic liposomes using transmission electron microscopy

The technique used for the formulation of the cationic liposomes was lipid film hydration technique (hand shaking method) which formed films on the wall of the flasks and on hydration with phosphate buffer solution (pH 7.4) produced thick, gel-like, milky colloidal dispersion. The photomicrographs of the vesicles were studied by means of transmission electron microscopy at x3600 and x10500 magnifications. From the negative staining electron micrographs, Figure 1a, 1b showed the vesicles of the DOTAP-based cationic liposomes at mags. of x3600 and x10500. The vesicles were spherical and multilamellar. The vesicles appeared tightly packed. The net surface charge of the liposomes was modified by the incorporation of positively charged DOTAP lipid. Liposomes are spherical vesicles in which an aqueous volume is entirely enclosed by a membrane composed of phospholipids. When these lipids are exposed to an aqueous environment, interactions between themselves (hydrophilic interactions between polar headgroups and van der Waals' interactions between hydrocarbon chains) and with water lead to spontaneous formation of closed bilayers [11]. At a high cholesterol concentration, the gel state is transformed to a liquid-ordered phase [12]. An increase in cholesterol content of the bilayers results in a decrease in the release rate of encapsulated material and therefore an increase of the rigidity of the bilayers obtained [13,14].



A at magnification of x3400 B at magnification of x 10500

Fig. 1: Transmission electron microscope images of DOTAP-based cationic liposomes at a mags. of x3600 and x 10500

Particle size and zeta potential of the ND vaccine encapsulated liposomes

The particle sizing using a Zetasizer nano series (Malvern Instruments, U.K) was measured using dynamic light scattering (DLS). This technique measures the time-dependent fluctuations in the intensity of scattered light which occur because the particles are undergoing Brownian motion. Analysis of these intensity fluctuations enables the determination of the diffusion coefficients of the particles which are converted into a size distribution. The particles of the DOTAP-based cationic liposomes at 25 ^{o}C and at 4 ^{o}C were between the ranges of 10 - 1000 nm as seen in Figures 2a, 2b. Temperature differences after formulation did not have significant differences on particle sizes. The sizes of the particles were smaller than the particles in the photomicrographs because a probe sonicator was used during the particle sizing which further reduced the sizes. The average zeta potential of the DOTAP-based cationic liposomes was 24 mV as seen in Figure 3. Polydispersity index (PI) measures the width of the particle size distribution. Consistently high values of PI indicate either an aggregated or poorly prepared sample. The polydispersity index for DOTAP-based cationic liposomes is 0.00917. If the PI was above 0.2, the PI loses its significance as an accurate measure of the width of the size of distribution, but can still be useful for comparative purposes [15]. Thus, liposomes formed from mixtures of saturated phosphatidylcholine with cholesterol which are 100 nm or smaller in size, exhibit circulation half-lives of several hours and longer.

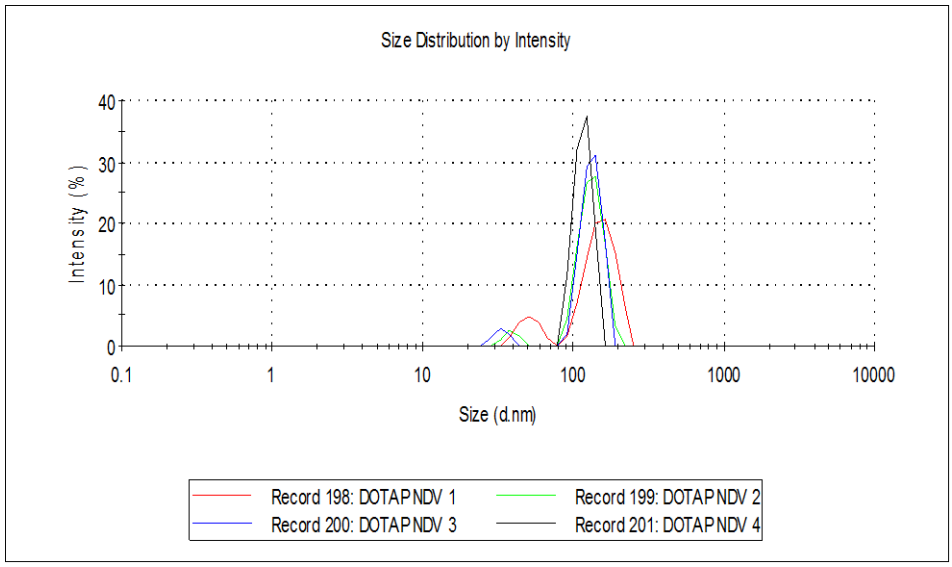


Figure 2a: Particle size distribution of DOTAP-based cationic liposomes at 4 ºC using photon correlation spectroscopy.

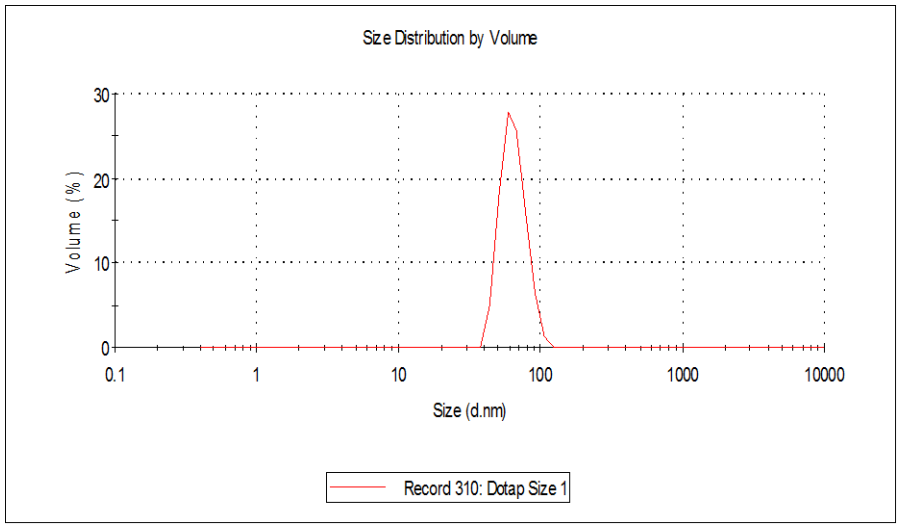


Fig 2b: Particle size distribution of DOTAP-based cationic liposomes at 25 ºC using photon correlation spectroscopy.

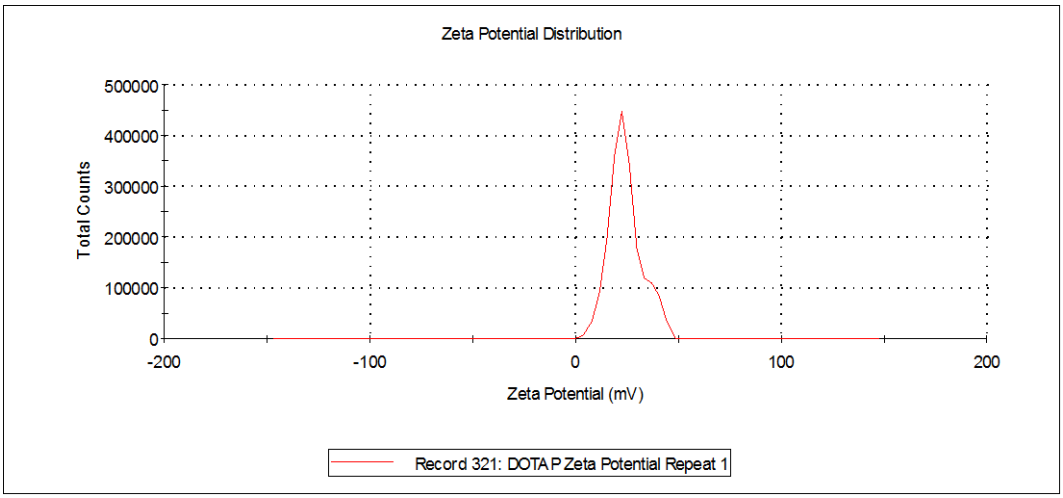


Figure 3: Zeta potential distribution of DOTAP-based cationic liposomes using photon correlation spectroscopy.

Immune response of the birds

All birds screened prior to administration of vaccine were negative for HI antibody. All control (unvaccinated) birds had no antibody throughout the experiment. The immune response of the birds is shown in Table 1. After primary vaccination, the La Sota® vaccine group had a higher mean antibody titre (log2) and standard deviation of 5.50 $\pm$ 0.67 while the liposomal ND vacine group had an antibody titre of 5.30 $\pm$ 0.56. After secondary vaccination the chickens further seroconverted, and the liposomal ND vaccine had a higher mean antibody titre (log2) of 9.60 $\pm$ 0.95, which was significantly higher than that for the La Sota† vaccine group with (log2) 6.00 $\pm$ 0.63.

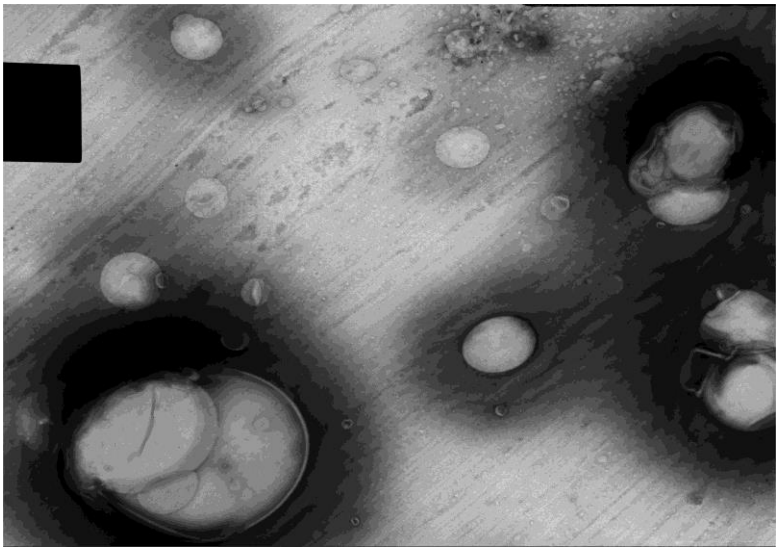
Table 1. immune response of birds after primary and secondary immunization

Treatment groups	**Number of chickens**	**Primary immunization (log_2)**	**Secondary immunization (log_2)**
Unvaccinated control	**20**	**0.0^A**	**0.0^A**
Liposomal ND vaccine	**20**	**5.30 0.56^B**	**9.60 0.95^C**

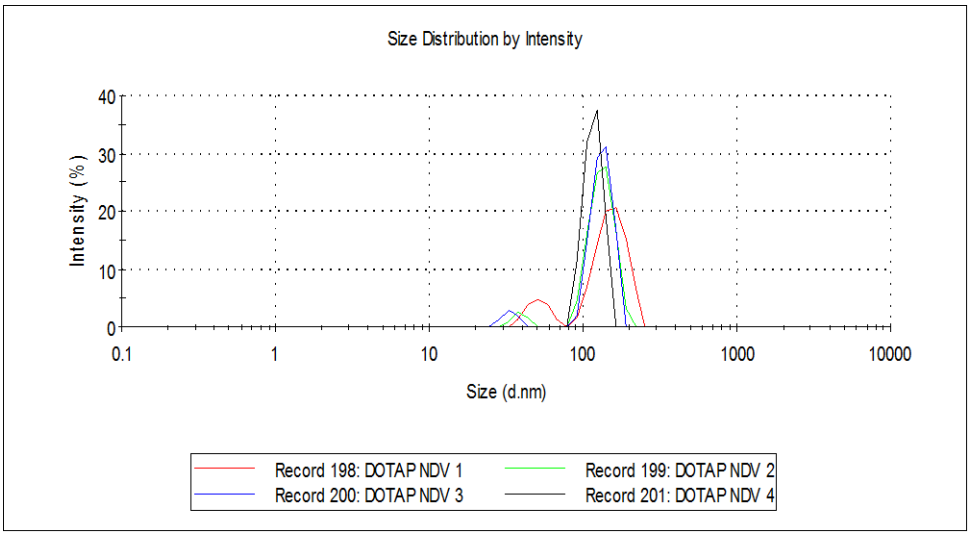
La Sota®vaccine	**20**	**5.50 0.67^{B}**	**6.00 0.63^{B}**

Stability studies

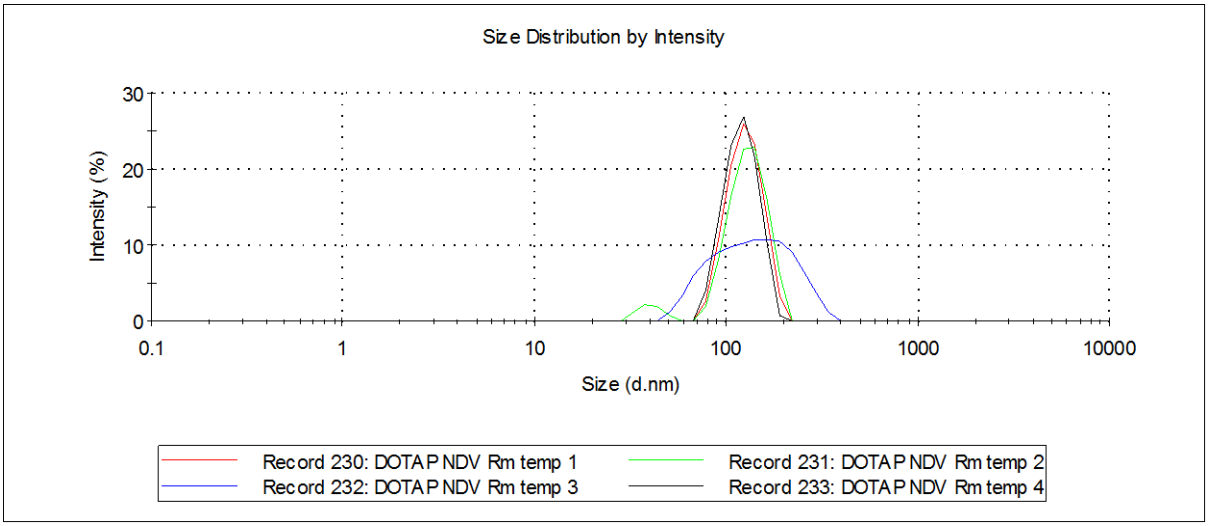
The Newcastle disease vaccine delivery systems were stored for one month at 28 ^{o}C and 4 ^{o}C. Figures 4a and 4b show DOTAP-based cationic liposomes which looked physically altered and disordered under the transmission electron microscope. The vesicles are aggregated and not spherical. Table 2 shows the haematological profile of the birds when immunized with vaccines stored at ambient and freeze-thaw conditions. The total leucocyte count profile showed stability of the vaccines when compared to the negative control [13.85$\pm$1.31], with the profile of the birds given DOTAP liposomes [31.23$\pm$2.51] as the most stable.



TEM images of DOTAP-based cationic liposomes after one month at a magnification of x10500 using transmission electron microscopy



Particle size distribution of the DOTAP-based cationic liposomes after one month at 4ºC
Z –average (d.nm): = 117.2; zeta potential (mV): 6.85 ±1.16; PdI = 0.833 ± 0.059



Particle size distribution of the DOTAP-based cationic liposomes after one month at 25 ºC.
Z. av (d.nm): 490.6 ± 82.69; zeta potential (mV): -9.29 ± 0.681; PdI: 0.614 ± 0.063

Table 2: Haemotological profile of the birds after storage of vaccines at ambient and freeze-thaw conditions

Leucocytes x1000/ul	Total white blood cells	Heterophils	Lymphocytes	Monocytes	Eosinophils	Basophils
Negative control (unvaccinated)	13.85 ± 1.31	3.39 ± 0.54	9.76 ± 1.26	0.25 ± 0.06	0.35 ± 0.05	0.09 ± 0.04
DOTAP (28 ºC)	26.43 ± 2.25	8.49 ± 0.51	15.21± 3.41	0.30 ± 0.10	0.40 ± 0.04	0.04 ± 0.04
Positive control (La Sota) 28 ºC	26.71 ± 2.98	5.15 ± 0.57	21.11 ± 2.70	0.21 ± 0.10	0.10 ± 0.06	0.00 ± 0.00
DOTAP (-20 ºC/4ºC)	31.23 ± 2.51	6.05 ± 1.86	26.24 ± 2.21	0.41 ± 0.14	0.12 ± 0.07	0.00 ± 0.00
Positive control (La Sota) -20 ºC/4ºC	26.69 ± 2.69	10.89 ± 1.65	16.89 ±1.67	0.17± 0.07	0.50 ± 0.17	0.05± 0.05

DISCUSSION

Photomicrographs

Liposomes are spherical vesicles in which an aqueous volume is entirely enclosed by a membrane composed of phospholipids. When these lipids are exposed to an aqueous environment, interactions between themselves (hydrophilic interactions between polar headgroups and van der Waals' interactions between hydrocarbon chains) and with water lead to spontaneous formation of closed bilayers [16]. The phosphatidylcholine which is a zwitter-ionic or non-ionic lipid was used as the basic lipid for the preparation of the cationic liposomes. The fluidity of the liposomal bilayer, when it is made from a single lipid depends on the lipid phase transition temperature (Tc) and its relative position compared to room temperature [17]. When the room temperature is increased and reaches Tc, the membrane passes from a solid-gel phase, where the lipid hydrocarbon chains are in an ordered state, to a fluid liquid-crystalline phase, a disordered state, where molecules have more freedom of movement. Membrane permeability is highest at the phase transition temperature, and is lower in the gel phase than in the fluid phase. Non-ionic lipids (like phosphatidylcholine) in conjunction with cholesterol (tightly packed membranes) as well as sterical stabilization (steric barrier) reduce the levels of protein binding. Thus, liposomes formed from mixtures of saturated phosphatidylcholine with cholesterol which are 100 nm or smaller in size, exhibit

circulation half-lives of several hours and longer. In some cases circulation lifetimes can be further increased by inclusion of a polyethylene glycol (PEG) surface coating. The polymer acts as a steric barrier and reduces the level of plasma protein binding and uptake by phagocytic cells [18,19]. This actually forms the basis of sterically stabilised or 'stealth liposomes'. In serum, the lipid molecules can be transferred from the liposomal membrane to plasma high density lipoprotein (HDL). This is particularly true in the case of "fluid" liposomes, such as those made from dioleyl-phosphatidylcholine (DOPC), which disintegrate and release their contents within few minutes after their intravenous administration [17]. Following the substitution of DOPC by high phase-transition temperature lipids such as distearoyl-phosphatidylcholine (DSPC), the bilayer becomes "rigid" at 37 ^{o}C, and consequently resistant to lipoprotein attack. Membrane fluidity can also be controlled quite accurately by supplementing the lipid bilayer with cholesterol, a mechanism that results in enhanced membrane stability,[20] by mixing two or more lipids, or by manipulating the hydrophobic/lipophobic character of the bilayers, for example with the use of fluorinated lipids [21,22].

Particle size

Particle size is one of the determining factors for macrophage clearance when liposomes are administered *in vivo.* Particle sizes greater than 500 nm are marked for clearance by opsonins and subsequently phagocytosed by macrophages. Larger liposomes are rapidly removed from blood circulation. Sonication is therefore necessary for producing smaller vesicles in the nano range and for unilamellarity. Charge neutral liposomes or niosomes with tightly packed membranes exhibit increased drug retention and circulation half-life *in vivo* [23]. The tight packing reduces the binding/insertion of proteins, which destabilise the membrane and mark the liposomes or niosomes for removal by phagocytic cells. Since the particle sizes of the cationic liposomes or the niosomes had an average size of < 500 nm, they will escape phagocytosis and circulate longer in the blood stream. When given by the oral route, liposomes have to survive the "detergent effect" of bile salts and phospholipase activity. Physicochemical properties of particles influence greatly their rate of uptake by the intestinal tract. The two main factors are the size and the nature of the matrix used to make the particles. Particles are described as crossing either at the level of Peyer's patches or through the enterocyte layer [24,25]. Absorption takes place primarily, but not exclusively at the level of the M-cells. Uptake is very fast and is the result of a transcellular mechanism through normal enterocytes and specialized M-cells or to a lesser extent across paracellular pathways through the tight junctions between cells [26]. Microfold cells are specialized for endocytosis and subsequently transport the particulates to the adjacent lymphoid tissue (Peyer's patches in the gut). Therefore, after the particle binds to the M-cell apical membranes, the particulates are rapidly internalised and offered to the continuous lymphoid tissue. Once internalised, the particles are phagocytosed by macrophages and distributed in the whole body [27,28,29]. Size and hydrophilicity of the particles have been clearly described as important factors influencing intestinal absorption [30]. The particles can be retained within the lymphoid tissue or they can be internalised by phagocytic cells and subsequently transported to another lymphoid tissue through the lymphatic vessels that innervate the Peyer's patches dome area. The bilayer permeability is a measure of the flux or rate at which a solute works its way from an aqueous compartment, through a bilayer, and out into the aqueous compartment on the other side. It depends on the membrane fluidity and on the nature of the solute. Positively charged particles

are better positioned to interact with the negatively charged mucin [31,32]. Since the size of the cationic liposomes or niosomes is similar to the size of a pathogen it would be taken up by antigen presenting cells [33,34] and subsequently delivered into these cells. The cationic liposomes would protect the antigen against degradation on mucosal surfaces, and enhance the uptake in mucosa associated lymphoid tissue.

Stability studies

When conventional liposomes are administered *in vivo*, they are rapidly cleared from the blood circulation by monocytes and macrophages. Formulating the liposmes with a cationic like DOTAP increases the physical stability of the liposome and the potential for enhanced interaction with cells (Maintaining the physical properties of the vesicles can be difficult without lyophilisation and cryoprotectants such as mannitol, glucose and trehalose. Cryoprotectants increases stability from hydrolysis. Small amounts of antioxidants during processing may stabilize the suspension and limit oxidation of the product. The particle size distribution changed after one month of storage probably due to degradation of the components. Figures 23a - 24b represent particle size distributions, zeta potentials and polydispersity indices of the DOTAP-based cationic liposomes or Span 60-based niosomes after one month storage at 25 ^{o}C and at 4 ^{o}C. The particle size distribution was multimodal. The zeta potential was also greatly affected on storage. The zeta potentials of DOTAP-based cationic liposomes at 4 ^{o}C and 25 ^{o}C were -6.85 mV $\pm$ 1.16 and -9.29 mV $\pm$ 0.681 while that of Span 60 were -13.9 mV $\pm$ 0.897 and -5.76 mV $\pm$ 0.857 at 4 ^{o}C and 25 ^{o}C respectively. The negative charges of the cationic liposomes could be due to the gradual degradation of the lipid membrane by the presence of membrane destabilizing components, presumably lysolipid and free fatty acid generated by hydrolysis of the lipid [35]. The permeability properties of these amphiphilic carriers determine how well the vaccine is retained in the liposome or niosome interior. The electrical potential at the membrane surface will affect the ability of charged ions to cross. Negatively charged lipids will repel anions from and attract cations to, the lipid-water interface. The more ordered and hence tightly packed the membrane, the less permeable [36]. Liposomal aggregation, bilayer fusion and drug leakage are the main problems of physical stability encountered in any liposomal formulation which could greatly affect the shelf life of liposomes or niosomes. Drug leakage will also depend on both liposome composition and vaccine characteristics. Large polar or ionic water soluble drugs will be retained much more efficiently than low molecular weight, amphiphilic compounds. In general, membranes composed of saturated phospholipids (with acyl chain of $C \geq 16$) and/or membranes which contain a sufficient amount of cholesterol are the least permeable ones. From the pharmaceutical point of view, the physical and chemical properties of liposome or noisomal particles are critical parameters affecting the performance of the drug loaded liposomes *in vitro* and *in vivo*. Unfortunately, liposomal formulations do not meet the required standards for long term stability of pharmaceutical preparations if they are stored as aqueous suspensions [37]. The encapsulated drug tends to leak out of the bilayer structure and the liposomes might aggregate or fuse on storage. These processes can cause a change in the pharmacokinetic profile of the encapsulated drug and therefore reduce the reproducibility of the therapeutic effect which shortens the storage time for the liposomal preparations, where an acceptable shelf life is prerequisite for the successful introduction of liposomes into therapy. As an alternative to storing aqueous dispersions, freeze-drying of the cationic liposome or noisome was introduced. Lyophilization increases the stability and the shelf life of the

finished product by preserving it in a relatively more stable dry state, especially if the drug is not stable in the aqueous suspension. Some liposomal products in the market or clinical trials are provided as lyophilized powder forms,[37] accompanied with labeling that calls for reconstitution with water or saline for injection) [38]. Freeze drying is a technique that consists of 3 separate steps. First the product is frozen, then sublimation (primary drying) starts which is followed by secondary drying process [37]. Liposome integrity on freeze drying may be protected against leakage, aggregation and fusion in the presence of properly selected cryoprotectants which is an important factor that governs the efficiency of the process of freeze drying of liposomes. For liposome stabilization, usually sugars such as glucose, sucrose, lactose and trehalose are used as cryoprotectants, although other types of excipients have also been reported to exert cryoprotective effects [38]. In Table 8, the DOTAP-based cationic liposomes were prepared by lipid film hydration technique without cryoprotectants, hydrated with the Newcastle disease virus vaccine solution and then lyophilized. The dry flakes were weighed and bottled in vaccine vials. The effects of accelerated temperatures on the stability of the formulations were assessed immunologically. The positive control (commercial vaccine) was also given the same treatment. At three weeks of age, the birds were given their primary immunization and immune response assessed using haemagglutination inhibition test. Tresults showed that reezing process may fracture or rupture vesicles and freeze-thaw conditions may lead to accelerated degradation of the vaccine formulation. Keeping the lipid formulations in the dry form helped to keep the structure intact and did not diminish the immune response of the cationic liposomes.

Freeze-thaw changes

However, from the result, the presence of high circulating lymphocytes in the blood is an indication of cell-mediated immunity. The principal function of the lymphocyte is its immunologic activity. In terms of their response to the antigen, the T-cells are primarily responsible for the cell-mediated response of the chicken. The life span of lymphocytes varies from three to four days. Lymphocytes in the animal body are constantly in a state of circulation and recirculation. Recirculation occurs at a relatively constant rate so that the numbers of lymphocytes entering and leaving the blood are approximately equal, and the number in the blood remains fairly constant in the healthy animal. Because of this constant recirculation and the fact that there are populations of lymphocytes with different life spans, it is not possible to determine precisely the total number of lymphocytes in an animal body at any given time. In conclusion, DOTAP-based cationic liposomes at -20 ^{o}C/4 ^{o}C after primary immunization showed an appreciably level of circulating lymphocytes which was higher than the other vaccine treatment groups.

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