SCVJ
Volume 4, Issue 2, 2023
DOI: http://doi.org/10.55989/XDUS5159
Science View Journal
ISSN (Online): 2734-2638

Thermostability and Immunostimulatory Activity of Cationic Liposome-based Newcastle Disease Virus Vaccine

D. C.Okechukwu[1], P. O. Nnamani[2], A. A. Attama[2], A. L. Onugwu[2], V. C. Okore[1], E. B. Onuigbo[1]

[1]Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Enugu State, Nigeria
[2]Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Enugu State, Nigeria

Email: ebele.onuigbo@unn.edu.ng

Abstract:
Cold storage is a huge challenge in maintaining vaccine potency especially in low and middle income countries (LMIC). The aim of this research was to investigate the heat and freeze-thaw stability of Newcastle disease virus (NDV) vaccine encapsulated in 1,2-dioleoyl-3-trimethyl ammonium propane (DOTAP) cationic liposomes as well as its immunostimulatory activity. DOTAP cationic liposomes were prepared using the thin-film hydration technique and characterized for morphology, particle size, polydispersity index, zeta potential and encapsulation efficiency. Experimental birds were challenged with Herts 33 velogenic strain of NDV. Haemagglutination inhibition test was used to assess the formation of antibodies in the birds. The cationic liposomes were further stored at 28 oC or subjected to freeze-thaw cycles for 7 weeks and then assessed for stability using the same parameters of particle size, polydispersity index, zeta potential, morphology, antibody titre or differential white blood cell count. Using TEM, the morphology of the liposomes was found to be spherical, and using Malvern Zetasizer, particle size was in the nanosize range. Zetapotential and polydispersity indices were positive and below 0.1. The HI test showed that the DOTAP cationic liposomes stimulated a higher titre of antibodies than the commercial vaccine. Heat and freeze-thaw stability studies after 7 weeks of storage showed slight aggregation and elevated particle sizes and wider distribution. The HI titer was appreciably lower after secondary immunization with the DOTAP cationic liposomes stored at 28 oC but elevated for the product kept at freeze-thaw conditions. The immunized birds were 100 % protected from the virulent strain of NDV. In conclusion, the development of the DOTAP-cationic liposomes encapsulating NDV vaccine administered orally was found to be more stable under freeze-thaw conditions than when it was exposed to heat for 7 weeks. The liposome-based vaccine had more immunostimulatory effects than the commercial sample.

Keywords: liposome, DOTAP, cold chain, heat, stability, vaccine, Newcastle disease virus, freeze-thaw condition.

I. INTRODUCTION

In last decade, there has been renewed discussion by vaccine researchers on the value and impact of thermostable vaccines in the global healthcare and economy. Innovation and technological precision are being geared now towards translating concepts and developments into clinical products (Qi and Fox, 2021). However, the long-term storage of Newcastle disease virus vaccines outside of cold chain conditions is still a challenge requiring stabilizing adjuvants that are safe. The socioeconomic impact of poultry production in many LMIC is daunting providing resources for healthcare, education and other social needs (Ngongolo et al., 2021). Poultry is grown both at the backyard of many families as well as commercially, providing protein for dinner as well as employment opportunities. Newcastle disease (ND), is a devastating poultry disease caused by avian paramyxovirus serotype 1 (APMV-1) in the Avulavirus genus, the subfamily Paramyxovirinae, and family Paramyxoviridae (Mansour et al., 2021). The paramyxoviruses isolated from avian species have been classified by serological testing into nine serotypes designated APMV-1 to APMV-9. ND has been designated as APMV-1 which is a single-strand non-segmented negative sense RNA virus (Getabalew et al., 2019). ND is endemic in many tropical low and middle income countries with a huge economic burden (Dzogema et al., 2021). A vast majority of birds are susceptible to infection with ND viruses of both high and low virulence. ND affects the respiratory, digestive and nervous systems of domesticated birds (Ravikumar et al., 2022). Prophylactic vaccination with Newcastle disease virus vaccine is of paramount importance in controlling infections, enhancing food security and reducing economic vulnerability (Lindahl, 2019). The vaccine is given intraocularly which can be administered by poultry farmers without the need of a veterinarian. Newcastle disease virus vectored vaccines can also be used to develop vaccine candidates against existing and emerging infections such as SARS-CoV2, Ebola, HIV viruses (Choi, 2017).

Despite the availability of Newcastle disease virus vaccine for many years, cold storage requirements remain major limitations. The reconstituted vaccine begins to deteriorate rapidly after 1 or 2 hours of exposure if left at room temperature. (Mahmood et al., 2014). Newcastle disease virus is very sensitive to heat, detergents, formaldehyde and oxidizing agents (Schirrmacher, 2017). In LMIC, especially in remote tropical settings with limited resources, there is a huge challenge of adequate protection of chicken from Newcastle disease virus because of lack of electricity needed for cold

SCVJ
Volume 4, Issue 2, 2023
DOI: http://doi.org/10.55989/XDUS5159
Science View Journal
ISSN (Online): 2734-2638

storage (Abdoshah, 2022). Newcastle disease virus vaccine storage should be at 2 oC to 8 oC in the refrigerator, and cold distribution to various poultry farms is equally needed to keep the vaccines viable. Therefore, development of a heat-stable, efficacious NDV vaccine is of paramount importance. For this to be realized, a combinatorial strategy involving stabilization of antigen, optimization of appropriate adjuvants and development of throughput analytical methods is needed (Ozan et al., 2014).

Liposomes are the first generation of lipid nanoparticles. Liposomes are concentric bilayers ranging from 100 nm to 1000 nm encapsulating both hydrophilic and lipophilic drugs making it a versatile drug delivery platform (Tenchov et al., 2021). DOTAP is a single positively charged cationic lipid with great ability to transfect nucleic acids into cells. Cationic liposomes are lipid bilayer unilamellar or multilamellar vesicles with a positive surface charge. These ionizable lipids are less toxic than non-ionizable lipids because they are positively charged inside the cells and neutral in the blood stream (Hou et al., 2021). Vaccine antigens co-administered with cationic liposomes are delivered to antigen presenting cells through different lymphoid tissues. A cationic liposome generally consists of three different domains: a hydrophilic ionizable headgroup which is positively charged usually through the protonation of one (monovalent lipid) or several (multivalent lipid) amino groups; a hydrophobic anchor group composed of a cholesterol or of two alkyl chains (saturated or unsaturated); and a linker such as an ester, ether, amide or carbamate connecting the cationic headgroup with the hydrophobic moiety (Tenchov et al., 2021). The linker which attaches the ionizable headgroup to the hydrophobic anchor may impact the chemical stability and biodegradability of the liposome depending on the nature and length (Zhi et al., 2018). Cholesterol was used as the co-lipid in the preparation of the liposome in a molar ratio of 1:1 with phosphatidylcholine. A study demonstrated that DOTAP alone showed physicochemical stability for two years at refrigerator temperature. When mixed with RNA, the nanostructured lipid carrier was only able to maintain RNA integrity of the complex for five weeks at refrigerator temperature (Gerhardt et al., 2021). Storage sheets from European Medicines Agency showed that mRNA vaccines encapsulated in ionizable cationic lipid were stable in frozen condition up to 6 months at -25 °C, up to 30 days at refrigerator temperature (4 °C), and up to 12 h at room temperature. Walvax (ARCoV) claimed that its COVID-19 mRNA encapsulated in ionizable lipid nanoparticles retained in-vivo delivery efficiency for at least 7 days when stored at room temperature but reduced drastically at 37 °C. Onpattro had also delivered siRNA in lipid nanoparticles with a shelf life of 3 years at refrigerator temperature (2-8 °C), indicating that the extreme cold storage temperature (-80 oC) for COVID-19 mRNA vaccines is dictated by the unstable nature of mRNA, not the instability of LNP (Uddin and Roni, 2021). Cationic lipid nanoparticles based on DOTAP has been discovered as an effective alternative to ionizable lipid nanoparticles to deliver self-amplifying mRNA (Gustavo et al., 2020). Optimization and vector selection play important roles in the development of a stable vaccine. The sublimation of the aqueous product by freeze-drying can play a critical role in the stabilization of the vaccine encapsulated in lipid nanoparticles (Uddin and Roni, 2021). The present study was designed to investigate the heat and freeze-stability of DOTAP cationic liposome encapsulating Newcastle disease virus vaccine as well as its efficacy.

II. Materials and Methods

Materials

Cholesterol and DOTAP (Sigma grade, minimum purity 99%; Sigma Aldrich Chemie, St Louis, Missouri, USA), Phospholipon 90H (Phospholipid GmbH Nattermannallee, Cologne, Riedel-de Haen, Seelze Germany), methanol (extra pure; Scharlau Chemie S.A.), chloroform (Sigma-Aldrich GmbH, Germany), live La Sota® vaccine (National Veterinary Research Institute, Jos, Nigeria).

Methods

La Sota® vaccine containing 200 doses/vial was reconstituted with phosphate-buffered saline by dissolving a vial in 40 ml phosphate buffer solution. Then 196 mg phosphatidylcholine, 96.7 mg cholesterol and 50 mg DOTAP were weighed and dissolved in 3 ml chloroform/methanol system (2:1) in a round-bottomed flask. The solvent mixture was evaporated at room temperature and the flask rotated until a smooth, thin, dry film was obtained on the wall of the flask (Azmin et al., 1985). A 5-ml volume of the reconstituted vaccine was used to hydrate the dry film and agitated gently until multilamellar vesicles were formed.

Transmission Electron Microscopy

The prepared liposome-encapsulated ND vaccines were processed using copper grids to adsorb the particles from the suspension, staining in 2.5% uranyl acetate for 30 sec and drying. The specimens were observed under a JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan) operated at 80 kV at x3400 and x10,500 magnifications.

Vesicle Size and Zeta Potential

The z-average vesicle diameter and zeta potential of the ND vaccine DOTAP liposomes in phosphate buffer solution (pH 7.4) were determined by photon correlation spectroscopy using a nanosizer 3000 HS (Malvern Instruments, Malvern UK). The zeta potential was calculated from the mean of three runs. Each sample was diluted with bi-distilled water and the electrophoretic mobility determined at 25oC and a dispersant dielectric constant of 78.5 and pH 7. The obtained electrophoretic mobility values were used to calculate the zeta potentials using DTS software version 4.1 (Malvern Instruments) and applying Henry's equation (Zetasizer Nano Series, 2005). The polydispersity index was used as a measure of homogeneity.

Vaccine Entrapment Efficiency

DOTAP liposomes were evaluated for vaccine entrapment efficiency. Non-entrapped antigen was separated from vesicle entrapped antigen by centrifugation for 10 min at 3000 rpm.

SCVJ
Volume 4, Issue 2, 2023
DOI: http://doi.org/10.55989/XDUS5159
Science View Journal
ISSN (Online): 2734-2638

The free (unentrapped) antigen was determined in the supernatant by haemagglutination test.

VACCINATION SCHEDULE OF THE BIRDS.

The chicks were raised from 1 day old until termination of the experiment. Handling of the birds and experimentations were conducted following the guidelines stipulated by University of Nigeria Research Ethics Committee on animal handling and use. At 2 weeks of age, all of the birds were tested for the absence of residual haemagglutination inhibiting (HI) antibody. Seven groups of 20 birds each were used in the study: the unvaccinated group, the cationic liposomal ND vaccine group kept at 4 oC, the cationic liposomal ND vaccine group kept at 28 oC, the cationic liposomal ND vaccine group kept at freeze-thaw temperature, the La Sota® vaccine group kept at 4 oC, the La Sota® vaccine group kept at 28 oC and the La Sota® vaccine group kept at freeze-thaw temperature. The different groups were kept in separate rooms before vaccination. At 3 weeks of age, the liposomal ND vaccine groups were given a dose of 0.2 ml/bird of the liposomal ND vaccine orally. The La Sota® vaccine groups were given 0.2 ml/bird of the reconstituted La Sota® vaccine orally. The unvaccinated group served as the control. Two weeks after primary vaccination, all birds were bled from the jugular vein and serum samples assessed for antibody to ND virus by the HI technique (OIE, 2004). Booster vaccinations were done at 6 weeks of age. Two weeks after secondary vaccination, all birds were bled and serum samples assessed for antibody to ND virus. Results of the study are presented as the mean with standard deviation for each of the parameters.

HAEMAGGLUTINATION INHIBITION TEST

A 0.025 ml volume of PBS was dispensed into each well of a plastic V-bottomed microtitre plate. Antigen suspension (0.025 ml) was placed in the first well. Two-fold dilutions of the antigen suspension were made across the plate to the 10th well. A 0.025 ml of 0.6 % (v/v) chicken red blood cells (RBCs) was dispensed to each well. The 11th well served as the control. The solutions were mixed by tapping the plate gently. The RBCs were allowed to settle for about 30 min at 28 oC. When control RBCs had settled to a distinct button, HA was determined by tilting the plate and observing the presence of tear-shaped streaming of the RBCs. The titration was read to the highest dilution giving complete HA (streaming); this represents 1HA unit (HAU), and 4HAU was then calculated there from. The HI titre is the highest dilution of serum causing complete inhibition of 4HAU of antigen. The agglutination was assessed by tilting the plates. Only those wells in which the RBCs stream at the same rate as the control wells were considered to show inhibition.

CHALLENGE EXPERIMENT

The challenge experiment was carried out at 9 weeks of age. The challenge was performed by administering 0.2 ml of 105.5 median embryo lethal dose/ml Herts 33 strain to each bird by the oral route. After challenge, the birds were monitored in 1 week for clinical signs of disease and mortality.

DATA ANALYSIS

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

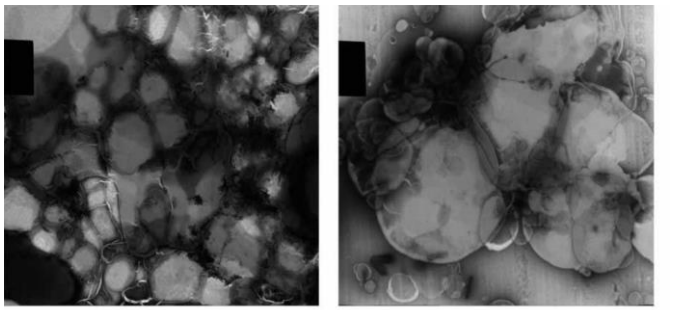
III. Results and Discussion

PHOTOMICROGRAPHS OF THE DOTAP CATIONIC LIPOSOMES

The photomicrographs of the cationic liposomes were seen through transmission electron microscopy (TEM) as near spherical monodisperse liposomes which were closely packed conferring a polygonal shape. The vesicles were formed by hydrating lipid films on the walls of the flasks with phosphate buffer solution (pH 7.4) containing the Newcastle disease virus vaccine and producing milky, thick, gel-like colloidal dispersion. The photomicrographs of the vesicles were studied at x3600 and x10500 magnifications as shown in Figures 1a and 1b. The concentric bilayers composed of phosphatidylcholine as the basic lipid could not be seen because TEM is not the appropriate instrument for observing lamellar vesicles. The phospholipids are said to be amphiphilic in nature because they contain both hydrophilic and hydrophobic elements. They self-assemble spontaneously into ordered lyotropic liquid-crystalline phases in the presence of water (Koynova and Tenchov, 2013). The vaccine which is hydrophilic is expected to be encapsulated at the core of the vesicles. From the photomicrographs, the vesicles appeared ordered relative to each other. The vesicles were not aggregated because of the inclusion of DOTAP which conferred positive charges to the vesicles. Without the cationic lipid or in the presence of negatively charged phospholipids, the vesicles gradually aggregate and decrease in stability. A low positive charge also permits aggregation of liposomes and they interact poorly with target cells (Campbell et al., 2001). The cationic liposome being positively charged is functionally positioned to trigger cell membrane permeability and lead to cellular internalization (Lim et al., 2016). The vesicles after formulation were assumed to be small unilamellar vesicles because the liposomes were sonicated as opposed to large unilamellar vesicles produced by extrusion. The membrane of a liposome is dynamic and in constant motion being more fluidic at the phase transition temperature and this is influenced by the length and degree of saturation of the lipid chain (Montero et al., 2014). It has been well established that cholesterol exerts its impact by controlling fluidity, permeability, membrane strength, elasticity, stiffness, transition temperature (Tm), drug retention, phospholipid packing, and plasma stability (Nakhaei et al., 2021). Cholesterol which is present in the liposome may have controlled the cell shape and structure. It produced a more condensing effect on liposomal packing. Cholesterol could also have contributed to a tightly packed arrangement with phospholipids (Song et al., 2022). An increase in cholesterol content of the bilayers results in an increase of the rigidity of the bilayers obtained and therefore a decrease in the release rate of encapsulated material. The vesicles appeared stiff and this may also be caused by the cholesterol. Phosphatidylcholine also contains saturated fats and, because of their free and higher chemical energy, creates tightly packed tails preventing insertion proteases from destabilizing the

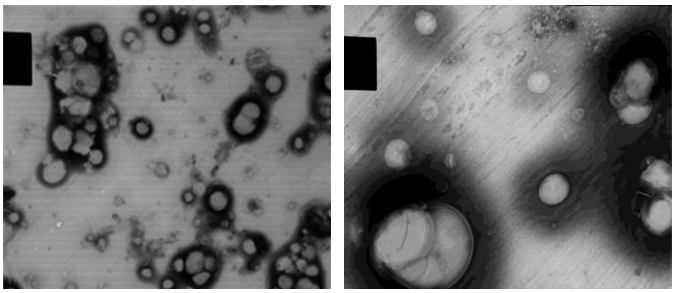
SCVJ
Volume 4, Issue 2, 2023
DOI: http://doi.org/10.55989/XDUS5159
Science View Journal
ISSN (Online): 2734-2638

liposomal membrane and marking them for phagocytosis. Since the liposomes were closely packed under the TEM, it is likely that the phospholipids were below the phase transition temperature (Tm) which formed a rigid ordered gel phase. The permeability of the liposomes is connected to its Tm and controlled by many other factors (Chen et al., 2018).



A at magnification of x3400 B at magnification of x 10500

Figures: 1a and 1b: Transmission electron microscope images of DOTAP-based cationic liposomes at a mags. of x3600 and x10500.



Figures: 1c and 1d: Transmission electron microscope images of DOTAP-based cationic liposomes at a mags. of x3600 and x10500 after 7 weeks of storage at 28 ^{o}C.

The formulated Newcastle disease virus vaccine encapsulated in DOTAP liposomes were further lyophilized without additional cryoprotectants and stored for 7 weeks at 28 oC as shown in Figures 1c and 1d. The shape of the vesicles looked altered and aggregated under the transmission electron microscope. Liposomal aggregation, bilayer fusion and vaccine leakage are the main problems of physical instability of phospholipid vesicles (Garg et al., 2007) and this limits their clinical use. It is suspected that the altered vesicles could be due to the absence of cryoprotectants. Cryoprotectants provide protection by stabilizing biomolecules and cellular structures, minimizing osmotic stress, and limiting the damaging effects of ice formation (Sydykov et al., 2018). Cryopreservation of vaccines is necessary to maintain the physical properties of the vesicles. Cryoprotectants protect vesicular membranes from hydrolysis and oxidation which are the two main mechanisms that cause the destabilization of the membranes of liposomes. This leads to generation of lysophospholipids through free fatty acids production, and eventually formation of phosphoglycerols. The changes in the morphological properties of the liposomes such as its shape, size and lamellarity have also had a major impact on the behaviour of the liposomes in vivo (Robson et al., 2018). The desired goal of vaccine developers is to maintain the physical and chemical integrity of the product during its life on the shelf (Narenji et al., 2017). It is likely that the vaccine was not retained in the core of the liposome due to the heat stress, thus creating changes in physical characteristics the liposomes and modifying the immune response. From the pharmaceutical point of view, the physical and chemical properties of liposomes are critical parameters that affect the performance of vaccine loaded liposomes in vitro and in vivo.

VESICLE SIZE, ZETA POTENTIAL AND VACCINE ENTRAPMENT

The particle sizes of the cationic liposomal vaccine were less than 100 nm and monodisperse as shown in Figures 2a and 2b. The suitability of a nanocarrier for a particular route of delivery depends on the average particle size and the polydispersity index (Danaei et al., 2018). This has an influence on the cellular uptake and internalization of the particles. Size is a critical parameter in determining drug encapsulation in the liposome, and half-life in circulation, with smaller liposomes having more chances of escaping phagocytic uptake. Size of liposomes can affect the rate at which they permeate targeted sites, are cleared from injection sites, and accumulate in the lymph nodes. Smaller liposomes are taken up at lymph nodes via passive transport. It is also characteristic of Th1 cells to be stimulated by smaller-sized cationic liposomes, which form a depot at the injection site due to interaction with the negatively charged interstitial proteins (Tretiakova and Vodovozova, 2022). The formulated cationic liposomes were measured at 4 oC and at 25 oC using a Malvern zeta sizer. Temperature differences after formulation did not have a significant effect on the particle sizes as the particles were <= 100 nm and also monodisperse. The sizes of the particles were small because a probe sonicator was used in sonicating the particles after formulation. Vaccine developers tend to mimic the properties of pathogens with the exception of pathogenicity. They simulate the pathogen's size, shape and surface molecule organization (Bachmann and Jennings, 2010). Particle size has an impact on stability, encapsulation efficiency, drug release and biodistribution. Dendritic cells have been shown to efficiently process small sized liposomes, whereas other immune cells, such as macrophages phagocytose large sized particles (Goya et al., 2008). Therefore, determining the particle size distribution of liposomes could help in predicting their adjuvanticity. Liposome size has been demonstrated to influence the pharmacokinetics and biodistribution of antigen, thereby, impacting the stimulation of the immune response (Manolova et al., 2008).

The average zeta potential of the DOTAP-based cationic liposomes was 24 mV as shown in Figure 3a. It was measured on the Malvern Zetasizer. Large potentials (near +30 mV or -30mV), predict a stable dispersion with the particles repelling

SCVJ
Volume 4, Issue 2, 2023
DOI: http://doi.org/10.55989/XDUS5159
Science View Journal
ISSN (Online): 2734-2638

each other, thus preventing aggregation. Such large potentials provide us with an insight in liposomal stability, circulating half-life, rate of vaccine release, particle cell permeability and fusion with cell membranes. The large positive charge on the cationic liposome will have a beneficial effect on fusion with biomembranes. Choosing the amount of cationic additives has to be interplay between stability and toxicity because cationic additives are toxic at high concentrations. Surface charge has been shown to have effect on immune responses with negatively and positively charged liposomes being immunostimulant while zwitterionic ones are not (Honary and Zahir, 2013).

The polydispersity index for DOTAP-based cationic liposomes was 9×10-3 which indicated a narrow and homogenous size distribution. Polydispersity indices (PDI) measure the width of the particle size distribution. PDI ranges from 0.0 to 1.0. PDI of zero is for a perfectly uniform sample while PDI of 1.0 is a highly polydisperse sample with multiple particle size populations. Values of 0.3 and below for liposomes are considered adequate and indicate a homogenous population of phospholipid vesicles. Consistently high values of PDI indicate either an aggregated or poorly prepared sample.

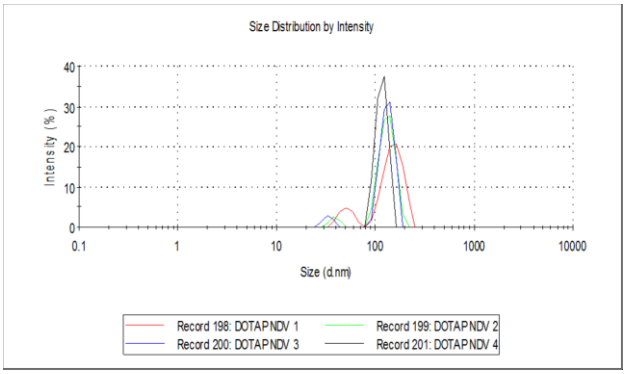


Figure 2a: Particle size distribution of DOTAP-based cationic liposomes at 4 ^{o}C using photon correlation spectroscopy.

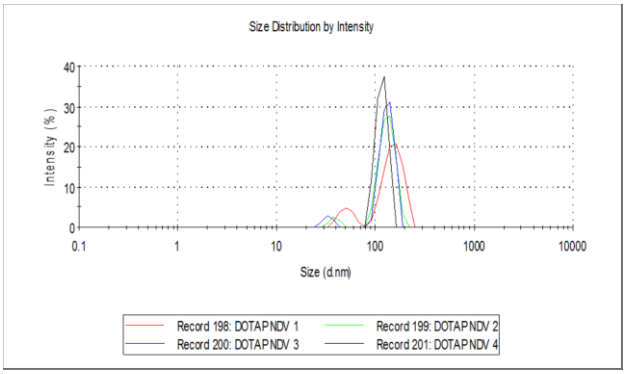


Figure 2b: Particle size distribution of DOTAP-based cationic liposomes at 25 oC using photon correlation spectroscopy.

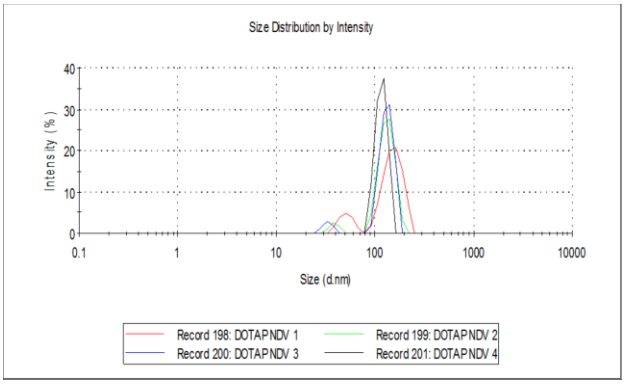
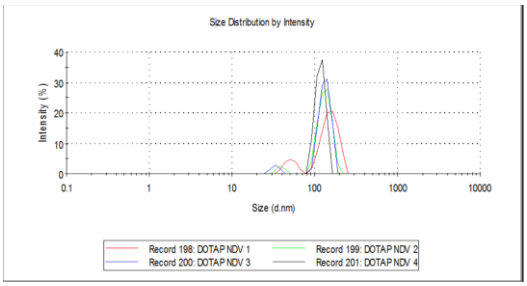


Figure 3a: Zeta potential distribution of DOTAP-based cationic liposomes using photon correlation spectroscopy

An insignificant difference ($P > 0.05$) was found in the mean particle size of the cationic liposomal vaccine stored at 4 oC for 7 weeks, but a significant increase in the particle size was observed for the cationic liposomal vaccine stored at 28 oC for 7 weeks. The size distribution of the cationic liposomes stored at 4 oC was appreciably maintained. Storage at 28 oC may have increased the rate at which the liposomes transited to a nonbilayer phase through intermediates that facilitated fusion (Garg et al., 2007). Our results show that stability and fusogenity of liposomes is temperature-dependent as seen in Figures 2c and 2d. At 4 oC, Zaverage was 117.2 nm; zeta potential (mV) = 6.85 ±1.16; PDI = 0.833 ± 0.059 while the parameters at 28 oC was Zav = 490.6 nm + 82.69 nm; zeta potential (mV) = -9.29 ± 0.681; PDI: 0.614 ± 0.063. No significant ($P > .05$) change in the zeta potential of the liposomal product was observed when they were stored at 4 oC for 7 weeks, but there was an inverse of the zeta potential when stored at 28 oC for 7 weeks. The inversion in the vesicular charge may be a function of temperature as well as time. It could also be due to the fusion of the vesicles. The negative charges of the cationic liposomes at 28 oC could also 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. The membrane becomes more permeable allowing the leakage of the vaccine from the liposome interior. The change in the electrical potential at the membrane surface will also affect the ability of the charged ions to cross the cell membranes. The cationic liposome now turned anionic will no longer be able to transfect proteins and nucleic acids into negatively charged cells. Changes in the particle sizes could also have impacted the chemical and biopharmaceutical behaviour of the product, such as the drug release kinetics, transport across biological barriers, and pharmacokinetics in the human body. The PDI of the liposomes after a long storage at 4 oC or 28 oC was above 0.3 and this would adversely affect the cellular uptake of the liposomes at the Peyer's patches, thus causing differentials in immune responses.

SCVJ
Volume 4, Issue 2, 2023
DOI: http://doi.org/10.55989/XDUS5159
Science View Journal
ISSN (Online): 2734-2638



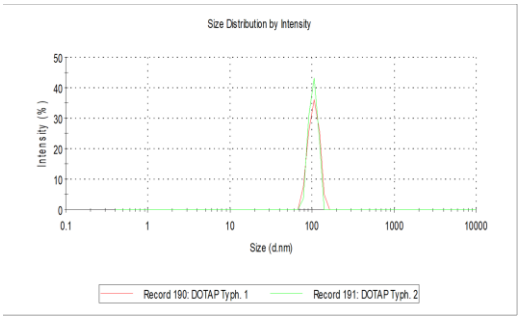
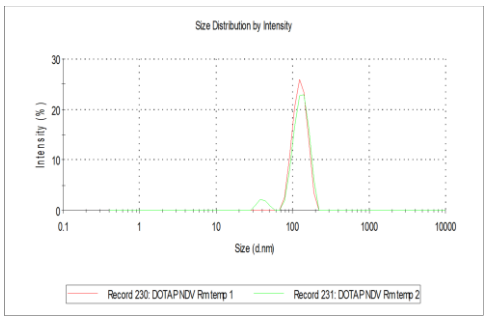


Figure 2c: Particle size distribution of the DOTAP-based cationic liposomes after 7 weeks at 4oC; Zav: = 117.2; zeta potential (mV): 6.85 ±1.16; PDI = 0.833 ± 0.059



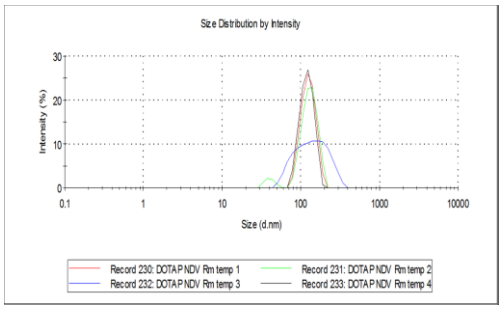


Figure 2d: Particle size distribution of the DOTAP-based cationic liposomes after 7 weeks at 28 ^{o}C; Z_{av}: 490.6 ± 82.69 nm; zeta potential (mV): -9.29 ± 0.681; PDI: 0.614 ± 0.063.

To ascertain the best ratio for the immune response of the birds, the encapsulation efficiency using haemagglutination test was assessed. The different molar ratios used were, 5:5:1; 7.5:7.5:1; 10:10:1 and 12.5:12.5:1 of phosphatidylcholine: cholesterol: DOTAP. The maximum encapsulation efficiency obtained was 63 % from the 12.5:12.5:1 molar ratio. The surface charge of the drug greatly impacts the drug loading efficiency. The zeta potential has also been used to determine the encapsulation of the cargo within the core of the liposome or on the surface. The drug loading can equally affect the zeta potential of the liposomes. For example, increasing the loading of flurbiprofen led to higher zeta potential (Harting et al., 2007). This is most likely due to the location of the drug molecules onto the delivery systems' surface leading to higher zeta potential. In addition, the entrapment efficiency of calcitonin was observed to increase by enhancing the negative zeta potential of the particles (Harting et al., 2007).

IMMUNE RESPONSE

Table 1 shows the immune responses after primary and secondary immunization. After primary immunization, the commercial La Sota® vaccine had a higher cumulative antibody titre (log2) of 5.50±0.67 though not significantly higher than the cationic liposomal vaccine (5.30 0.56). After the secondary immunization, the DOTAP-based cationic liposomes group had a cumulative antibody titre (log2) of 9.60±0.95 which was significantly higher than the commercial La Sota® vaccine (6.00 0.63). The negative control group produced no antibodies throughout the study. Antibody titres above (log 2) 3 are protective. DOTAP cationic liposomes interact with negatively charged molecules on the surface of antigen-presenting cells (APCs) and target antigens for more effective phagocytosis (Mehravaran et al., 2019). DOTAP is used for the transfection of DNA, RNA, nucleotide, proteins and other negatively charged molecules into eukaryotes. It is a leading delivery system in antigen expression which promotes high association with cells in-vitro and has favourable interactions with negatively charged cell membranes (Lou et al., 2020). The DOTAP liposomes will be internalized by immune cells by a classical receptor-mediated endocytosis using cell surface receptors, which contain specific binding sites for, and are able to internalize cationic molecules leading to better uptake and in vivo cytotoxic T-lymphocyte induction and humoral responses (Friend et al., 1996; Felgner et al., 1986). DOTAP liposomes are also potential carriers for oral antigen delivery due to their protective effects on encapsulated antigens and their ability to be taken up by Peyer's patches in the intestine. They provide enhanced antigen processing through their ability to be taken up by antigen presenting cells. The bilayer composition of the DOTAP liposomes could have an effect on the liposome uptake by antigen presenting cells (Ahmad et al., 2019).

TABLE 1: PRIMARY AND SECONDARY VACCINATIONS OF VACCINES KEPT AT 4 ^{O}C

Treatment group	Primary immunization *(log $_2$)	Secondary immunization *(log$_2$)
Negative control	0.00	0.00
DOTAP-based cationic liposomes	5.30±0.56	9.60±0.95
Positive control	5.50±0.67	6.00±0.63

* The antibody titres are expressed in log base 2

SCVJ
Volume 4, Issue 2, 2023
DOI: http://doi.org/10.55989/XDUS5159
Science View Journal
ISSN (Online): 2734-2638

The DOTAP-based cationic liposomes stored for 7 weeks at 28 oC or freeze-thaw temperatures were prepared by the thin lipid film technique, hydrated with the Newcastle disease virus vaccine and lyophilized. The dry flakes were weighed and bottled in airtight vaccine bottles. These were reconstituted during immunization. The effects of the harsh temperatures on the stability of the cationic liposomal vaccine were assessed using the immune responses of the chickens after primary and secondary immunization. The commercial vaccine which served as the positive control was also given the same treatment. At three weeks and six weeks of age respectively, the birds were given their primary and secondary immunization, and immune responses were assessed using haemagglutination inhibition test. The production of antibodies in the sera of the chickens immunized with the lyophilized commercial NDV (with cryoprotectants) vaccine kept at freeze-thaw cycles for 7 weeks had mean antibody titres of log2 5.40 ± 0.60 after primary immunization and log2 7.00 ± 0.95 after secondary immunization, while birds given the lyophilized commercial NDV vaccine kept at 28 oC had mean antibody titres of logs2 4.20±0.58 and 7.60±0.60 after primary and secondary immunization. The antibodies were high even after the long storage conditions. The production of antibodies in the sera of the chickens immunized with the lyophilized DOTAP liposomes (without cryoprotectants) vaccine kept at freeze-thaw cycles for 7 weeks was log2 5.80 0.37 after primary immunization and log2 9.80 0.20 after secondary immunization, while birds given the lyophilized DOTAP liposomes (without cryoprotectants) vaccine kept at 28 oC had mean antibody titres of logs2 0.0 and 5.00 1.88 after primary and secondary immunization. Although, the antibodies of the birds immunized with the lyophilized DOTAP liposomes kept at 28 oC for 7 weeks had the lowest antibody titre, it retained some level of protection. This indicates that the lyophilized commercial NDV vaccine (with cryoprotectants) or lyophilized DOTAP liposomes (without cryoprotectants) kept at exaggerated conditions did not lose its potency at 28 oC or freeze-thaw temperatures but effectively stimulated antibody production.

TABLE 2: IMMUNE RESPONSES OF BIRDS TO VACCINES KEPT AT 28 OC OR FREEZE-THAW CONDITIONS

Treatment group	Primary immunization *(log 2)	Secondary immunization *(log2)
Negative control	0.00	0.00
Positive control at 28 °C	4.20±0.58	7.60±0.60
Positive control at freeze-thaw cycles	5.40±0.60	7.00±0.95
DOTAP-based cationic liposomes at 28 °C	0.00	5.00±1.88
DOTAP cationic liposomes (freeze-thaw cycles)	5.80±0.37	9.80±0.20

* The antibody titres are expressed in log base 2

TOTAL WHITE BLOOD CELL COUNT

Among the parameters evaluated for effect of long storage on DOTAP-based liposomal vaccines were total white blood cell count and differential white blood cell count. In Table 3 the haematological profile of the birds shows that the circulating lymphocytes of the vaccinated birds were significantly higher than that of the unvaccinated birds. The highest number of circulating lymphocytes x1000/uL after primary immunization were produced by birds immunized with DOTAP-based cationic liposomes kept at freeze-thaw conditions for 7 weeks (26.24±2.21) and the least were birds vaccinated with the DOTAP-based cationic liposomes kept at 28 oC for 7 weeks with a mean titre of 15.21±3.41 after primary immunization. The differences were not statistically significant. After secondary immunization, the highest number of circulating lymphocytes x1000/uL were produced by birds immunized with DOTAP-based cationic liposomes kept at freeze-thaw conditions for 7 weeks (16.29±1.06) and the least were birds vaccinated with the commercial vaccine kept at freeze-thaw for 7 weeks with a mean titre of 14.78±0.91. The differences were not statistically significant. The values show robustness of the haematological indices and capacity to protect the birds from the virulent strain of Newcastle disease virus. These values are useful and serve as a guide to interpret the leucocyte count when vaccines are kept at adverse conditions. These results reflect susceptibility of the birds, virulence of the infecting species, nature and severity of the disease process and systemic response of the birds. Low values or very high values could also come from technical errors rather than of physiologic or pathologic changes occurring during disease. The most common errors are those associated with collection, dilution and counting. The presence of high circulating lymphocytes in the blood after primary immunization especially with the group immunized with cationic liposomes kept at freeze-thaw conditions shows consistency with the antibody titres during haemagglutination inhibition test. 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. This may account for the reduced levels of the total leucocyte count after the secondary immunization.

TABLE 3: LYMPHOCYTES OF THE BIRDS AFTER 7 WEEKS OF STORAGE OF THE VACCINES

	Primary immunization (x1000µL)	Secondary immunzation (x1000µL)
Unvaccinated (negative control)	9.76 ±1.26	9.38±1.51
Positive control at 28 °C (La Sota®)	21.11±2.70	15.78±0.86
Positive control at freeze-thaw cycles (La Sota®)	16.89±1.67	14.78±0.91
DOTAP-based cationic liposomes at 28 °C	15.21±3.41	15.08±0.56
DOTAP cationic liposomes (freeze-thaw cycles)	26.24±2.21	16.29±1.06

CHALLENGE EXPERIMENT

The protective ability of the vaccine groups showed a strong association between unvaccination and death of birds. The pattern of morbidity and mortality showed that the unvaccinated group succumbed to the challenge with Herts 33 strain before the seventh day, with all birds showing clinical signs of disease: loss of appetite, torticollis, excess salivation paralysis, greenish and foul-smelling droppings, and weakness. By the seventh day, nine of the 10 unvaccinated birds had died (90% mortality). The birds of the liposomal ND vaccine group and of the La Sota® vaccine group showed no visible signs of disease. There was no mortality in these groups (100 % survival). The cationic liposomal ND vaccine had a significant protective effect on the birds, inducing antibody titres as high as 29 and hyperimmunized the birds against the velogenic strain of the NDV.

IV. Conclusion

The DOTAP cationic liposome containing Newcastle disease virus vaccine was formulated using the thin layer hydration method and subjected to heat and freeze-thaw stresses to ascertain their stability under the harsh conditions. The immune responses obtained from the birds after the stress conditions showed that the DOTAP-based liposomal vaccine was very much stable under freeze-thaw conditions with slightly reduced stability at 28^{o}C. This suggests the possibility of retaining immunogenicity in DOTAP-based cationic liposome vaccine in the tropical atmospheric conditions.

V. Conflict of Interest

All authors have approved the final manuscript, and declared no conflict of interest in the work and publication.

VI. References

Abdoshah, M., Hassanzadeh, M., Masoudi, S., Ashtari, A., Yousefi, A., Nasr, A., 2022. Thermoresistant Newcastle disease vaccine effectively protects SPF, native, and commercial chickens in challenge with virulent virus. Vet. Med. Sci. 8(4), 1539-1546.

Ahmad, M., Nasab, M., Mirahmadi, H., Sharifi, I., Alijani, E., Nikpoor, A., Akhtari, J., Hojatizade, M., 2019. Immunogenicity and protection effects of cationic liposome containing imiquimod adjuvant on leishmaniasis in BALB/c mice. Iran J. Basic Med. Sci. 22(8), 922-931.

Bachmann, M., Jennings, G., 2010. Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. Nat. Rev. Immunol. 10, 787-796.

Campbell, R., Balasubramanian, S., Straubinger, R., 2001. Influence of cationic lipids on the stability and membrane properties of paclitaxel-containing liposomes. . Journal of Pharm. Sci., 90(8), 1091-1105.

Chen, W., Dusa, P., Witos, J., Ruokonen, S., Wiedmer, S., 2018. Determination of the main phase transition temperature of phospholipids by nanoplasmonic sensing. Sci. Rep. 8, 14815. Doi: 10.1038/s41598-018-33107-5

Choi, K., 2017. Newcastle disease virus vectored vaccines as bivalent or antigen delivery vaccines. Clin. Exp. Vac. Res. 26(2), 72-82.

Danaei, M., Dehghankhold, M., Ataei, S., Davarani, F., Javanmard, R., Dokhani, A., Khorasani, S., Mozafari, M., 2018. Impact of particle size and polydispersity index on the clinical applications of lipidic nanocarrier systems. Pharmaceut. 10(2), 57, https://doi.org/10.3390/pharmaceutics10020057

Dzogbema, K., Talaki, E., Batawui, K., Dao, B., 2021. Review on Newcastle disease in poultry. Int. J. Biol. Chem. Sci. 15, 773–789.

European Medicines Agency. More flexible storage conditions for BioNtech/Pfizer's COVID-19 vaccine. Available online. https://www.ema.europa.eu/en/news/more-flexible-storage-conditions-biotechpfizers-covid-19-vaccine(accessed on 17-May 2021)

Friend, D., Papahadjopulos, D., Debs R., 1996. Endocytosis and intracellular processing accompanying transfection mediated by cationic liposomes. Biochim. Biophys. Acta. 1278, 41-50.

Felgner, P., Gadek, T., Holm, M., 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. USA. 84, 7413-7417.

Ismail, M., Abdien, H., Eid, A., 2021. Avian Paramyxovirus Type 1 in Egypt: Epidemiology, Evolutionary Perspective, and Vaccine Approach. Front. Vet. Sci. 8, 647462.

Garg, M., Dutta, T., Jain, N., 2007. Stability study of stavudine-loaded O-palmitoyl-anchored carbohydrate-coated liposomes. AAPS Pharm. Sci. Tech. 8(2).

DOI: http://doi.org/10.55989/XDUS5159

Gerhardt, A., Voigt, E., Archer, M., Reed, S., Larson, E., Van Hoeven, N., Kramer, R., Fox, C., Casper, C., 2021. A thermostable flexible RNA vaccine delivery platform for pandemic response. BioRxiv. doi: 10.1101/2021.02.01.429283.

Getabalew, M., Alemneh, T., Akeberegn, D., Getahun, D., Zewdie, D., 2019. Epidemiology, Diagnosis & Prevention of Newcastle Disease in Poultry. Am. J Biomed. Sci. Res. 3(1), doi: 10.34297/AJBSR.2019.03.000632.

Goya, G., Marcos-Campos, I., Fernadez-Pacheco, R., Saez, B., Godino, J., Asin, L., Lambea, J., Tabuenca, P., Mayordomo, J., Larrad, L., Ibarra, M., Tres, A., 2008. Dendritic cell uptake of iron-based magnetic nanoparticles. Cell Biol. Int. 32, 1001-1005.

Harting, S., Greene, R., Dikov, M., Prokop, A., Davidson, J., 2007. Multifunctional nanoparticulate polyelectrolyte complexes. Pharm. Res. 24, 2353-2369.

Honary, S., Zahir, F., 2013. Effect of zeta potential on the properties of nano-drug delivery systems-A Review. Tropical Journal of Pharmaceutical Research. 12(2), 265-273.

Hou, X. Zaks, T., Langer, R. Lipd nanoparticles for mRNA delivery. Nat. Rev. Mater. 6, 1078-1094.

Koynova, R., Tenchov, B., 2013. Phase Transitions and Phase Behaviour of lipids. Encyclopedia of Biophysics, SpringerNature, Editor Gordon C.K. Roberts, Editor.

Lim, S., Sanden, C., Selgard, R., 2016. Tuning liposome membrane permeability by competitive peptide dimerization and partitioning folding interactions regulated by proteolytic activity. Sci. Rep. 6, 21123. https://doi.org/10.1038/srep21123.

Lindahl, J., Young, J., Wyatt, A., 2019. Do vaccination interventions have effects? A study on how poultry vaccination interventions change smallholder farmer knowledge, attitudes, and practice in villages in Kenya and Tanzania. Trop. Anim. Health Prod. 51, 213-220.

Lou, G., Anderluzzi, G., Schmidt, S., Woods, S., Gallorini, S., Brazzoli, M., Giusti, F., Ferlenghi, I., Johnson, R., Roberts, C., O'Hagan, D. Baudner, B., Perrie, Y., 2020. Delivery of self-amplifying mRNA vaccines by cationic lipid nanoparticles: The impact of cationic lipid selection. J Contr. Rel., 35, 370-379.

Mahmood, M., Siddique, F., Hussain, I., Rafique, A., 2014. Development and evaluation of multivalent vaccines against NDV, AIV, IBD and Mycoplasmosis in village poultry. World Poult. Sci. J. 70(04), 829-838.

Manolova, V., Flace, A., Bauer, M., Schwarz, K., Saudan, P., Bachmann, M., 2008. Nanoparticles target distinct dendritic cell populations according to their size. Eur. J Immunol. 38, 1404-1413.

Montero, N., Martins, A., Reis, R., Neves, N., 2014. Liposomes in tissue engineering and regenerative medicine. JR Soc. Inter. 11(1010), 20140459. Doi.10.1098/rsif.2014.0459.

Nakhaei, P., Margiana, R., Bokov, D., Abdelbasset, W., Kouhbanani, M., Svarma, R., Marofi, F., Jarahian, M., Beheshtkhoo, N., 2021. Liposomes: Structure, Biomedical Applications, and Stability parameters with emphasis on cholesterol. Front. Bioeng. Biotechnol. 9, 705886.

Narenji, M., Talae, M., Moghimi, H., 2017. Effect of charge on separation of liposomes upon stagnation. Iran J. Pharm. Res. 16(2), 423-431.

Ngongolo, K., Omary, K., Andrew, C., 2021. Socio-economic impact of chicken production on resource-constrained communities in Dodoma, Tanzania. Poult. Sci. 100(3), 100921.

Ozan, K., Joshi, S., Smith, D., Middaugh, C., Prusik, T., Volkin, D., 2014. Vaccine instability in the cold chain: Mechanisms, analysis and formulation strategies. Biologic. 42(5), 237-259.

Qi Y., Fox, C., 2021. Development of thermostable vaccine adjuvants. Exp. Rev. Vac. 20(5), 497-517.

Ravikumar, R., Chan, J., Prabakaran, M., 2022. Vaccines against Major Poultry Viral Diseases.

Strategies to Improve the Breadth and Protective Efficacy. Viruses. 14, 1195. https://doi.org/10.3390/v14061195.

Robson, A., Dastoor, P., Flynn, J., Palmer, W., Martin, A., Smith, D., Woldu, A., Hua, S. 2018. Advantages and limitations of current imaging techniques for characterizing liposome morphology. Front. Pharmacol. 9, 80. doi:10.3389/fphar.2018.00080.

Schirrmacher, V., 2017. Immunobiology of Newcastle Disease Virus and Its Use for Prophylactic Vaccination in Poultry and as Adjuvant for Therapeutic Vaccination in Cancer Patients. Int. J Mol. Sci. 18(5), 1103. doi: 10.3390/ijms18051103.

Song, F., Chen, J., Zheng, A., Tian, S., 2022. Effect of sterols on liposomes: membrane characteristics and physicochemical changes during storage. LWT. 164113558. https://doi.org/10.1016/j.lwt.2022.113558.

Sydykov A., Mamazhakypov A., Petrovic A., Kosanovic D., Sarybaer A., Weissmann N., Ghofrani H., Schermuly R., 2018. Inflammatory mediators drive adverse right

DOI: http://doi.org/10.55989/XDUS5159

ventricular remodeling and dysfunction and serve as potential biomarkers. Front Physiol. 9, 609. Doi:10.3389/fphys.2018.00609.

Tenchov, R., Bird, R., Curtze, A., Zhou, Q., 2021. Lipid Nanoparticles ─ From Liposomes to mRNA Vaccine Delivery, a Landscape of Research Diversity and Advancement. ACS Nano. 15(11), 16982-17015.

Tretiakova, D.S., Vodovozova, E.L., 2022. Liposomes as adjuvants and vaccine delivery systems. Biochem. (Mosc) Suppl. Ser. A Membr. Cell Biol. 16(1), 1-20.

Uddin, M., Roni, M., 2021. Challenges of storage and stability of mRNA-based COVID-19 Vaccines. Vac. 9, 1033.

Zhi, D., Bai, Y., Yang, J., Cui, S., Zhao, Y., Chen, H., Zhang, S., 2018. A review on cationic lipids with different linkers for gene delivery. Adv. Coll. Inter. Sci. 253, 117-140.