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LIPOSOMES FOR VACCINE DELIVERY AGAINST INFECTIOUS BURSAL DISEASE IN CHICKENS

MUKMINAH SAKINAH WAHAB

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By

MUKMINAH SAKINAH BINTI WAHAB

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

LIPOSOMES FOR VACCINE DELIVERY AGAINST INFECTIOUS BURSAL DISEASE IN CHICKENS

By

MUKMINAH SAKINAH BT WAHAB

February 2016

Chairman: Professor Mohd Hair Bin Bejo, PhD

Faculty: Veterinary Medicine

Application of liposomes may help to enhance vaccine delivery process. Infectious bursal disease (IBD) is a highly contagious viral disease of chickens which cause immunosuppression and high mortality. Therefore, the objectives of this study were to develop a suitable liposomes for IBD vaccine delivery using a thin lipid hydration method, to determine the safety of the developed liposomes in embryonated specific pathogen free (SPF) chicken eggs and effectiveness of the encapsulation of IBD vaccine in liposomes in commercial broiler chickens. Three experiments were conducted in this study; 1, 2 and 3.

In experiment 1, positively charge liposomes consist of three major components of lipids namely; dipalmitoylphosphatidylcholine (DPPC), cholesterol and sterylamine (SA) was successfully prepared. Thin lipid hydration technique was used for preparation of cationic liposomes. The results showed a significant differences ($p<0.05$) between the particles size of empty cationic liposomes when compared to the size of IBD vaccine with three different mixtures of working seed IBD virus (IBDV) MyHatch UPM93 with cationic liposomes based on ratio; 1:1, 1:2 and 2:1 and identified as Sevac 1, 2 and 3, respectively. The value of zeta potential for the cationic liposomes and Sevac formulations were varying from 17±29.68 mV to 32±21.58 mV. Safety study showed 67% and 33% death of embryo in the liposomes and Sevac 3, but not in other groups. It appears that SA is toxic to the embryonated eggs.

In experiment 2, the method of liposomes preparation and safety of the cationic liposomes in SPF embryonated chicken eggs were successfully developed. 1,2-dioleoyl-3-trimethylmmonium propane (DOTAP) was used in the study to replace SA. A 1:1 and 1:2 ratio groups were selected with two types of live attenuated IBDV namely as Se (IBDV of UPM93 seed virus) and Co (IBDV of UPM93 commercial vaccine). Several methods for the preparation of cationic liposomes in the experiment 1 were modified. The results showed that all embryonated SPF chicken eggs in all groups were survived throughout 7 days post inoculation (pi).

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This further confirmed that SA is toxic to embryonated chicken eggs, whilst DOTAP is safe to be used in preparation of cationic liposomes.

In experiment 3, the effects of IBD vaccine and a safe liposomes mixture on the induction of high and protective IBD antibody titre were determined in commercial broiler chickens at hatchery vaccination via subcutaneous route. The study showed that the chickens in all groups did not exhibit any abnormal clinical signs and gross lesions, except atrophy of the bursa of Fabricius at day 28 post vaccination (pv) for IBD, Covac and Sevac groups throughout the experiment. The bursa weight and bursa to body weight ratio were remained unchanged for the IBD, Covac and Sevac groups compared with the Control group except at day 28 pv. The lesion scoring of the bursa of Fabricius was detected as early as 21 days pv in the Covac and Sevac groups compared to the IBD group at 28 days pv. The IBD antibody titre in the Covac and Sevac groups started to increase at day 21 pv compared to the IBD group at day 28 pv. Despite of low dosage of IBDV in the Covac group (2/3) when compared to the IBD group the induction of IBD titre was remained high in the group. This indicated that the encapsulation of IBDV in liposomes could enhance the induction of IBD antibody.

In conclusion, this study demonstrated that the application of cationic liposomes can enhance the deliver IBD vaccine to the target organ, the bursa of Fabricius, and induce high and protective level of IBD antibody titre with mild bursal lesion. Hatchery or day old vaccination using MyHatch UPM93 strain either with or without cationic liposomes is effective and could induce high and protective level of IBD antibody against IBDV challenged. These applications will give a new dimension in the field of poultry vaccines and vaccination.

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Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Sarjana Sains

LIPOSOM UNTUK PENGHANTARAN VAKSIN MENENTANG PENYAKIT BURSA BERJANGKIT PADA AYAM

Oleh

MUKMINAH SAKINAH BINTI WAHAB

Februari 2016

Pengerusi: Profesor Mohd Hair Bejo, PhD

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Aplikasi liposom boleh membantu untuk meningkatkan proses penghantaran vaksin. Penyakit bursa berjangkit (IBD) adalah penyakit ayam berjangkit yang menyebabkan kehilangan daya tahan imun dan kematian yang tinggi. Oleh itu, objektif kajian ini adalah untuk menghasilkan liposom kationik yang sesuai untuk menghantar vaksin IBD menggunakan teknik penghidratan lipid nipis, untuk menentukan liposom yang dihasilkan selamat kepada embrio telur ayam bebas patogen khusus (SPF) dan keberkesanan pengkapsulan vaksin IBD di dalam liposom kepada ayam pedaging kemersial. Tiga eksperimen telah dijalankan dalam kajian ini; 1, 2 dan 3.

Dalam eksperimen 1, liposom bercas positif mengandungi tiga komponen utama lipid iaitu; dipalmitoylphosphatidylcholine (DPPC), kolesterol dan sterylamine (SA) telah berjaya disediakan. Kaedah penghidratan lipid nipis telah digunakan untuk menghasilkan liposom kationik. Keputusan telah menunjukkan terdapat perbezaan yang ketara ($p<0.05$) di antara saiz zarah liposom kationik yang kosong apabila dibandingkan dengan saiz vaksin IBD dengan tiga campuran berbeza benih kerja virus IBD (IBDV) MyHatch UPM93 dengan liposom kationik berdasarkan nisbah; 1:1, 1:2 and 2:1 dan masing-masing dikenali sebagai sebagai Sevac 1, 2 dan 3. Nilai keupayaan zeta untuk liposom kationik dan formulasi Sevac adalah berbeza daripada 17±29.68 mV kepada 32±21.58 mV. Kajian keselamatan telah menunjukkan 67% dan 33% kematian embrio berlaku dalam Liposome dan Sevac 3 tetapi tidak untuk kumpulan yang lain. Ini menunjukkan yang SA adalah toksik kepada embrio telur.

Dalam eksperimen 2, mengoptimumkan kaedah penyediaan liposom dan tahap keselamatan liposom kationik di dalam embrio telur ayam SPF telah berjaya dihasilkan. 1,2-dioleoyl-3-trimethylmmonium propane (DOTAP) telah digunakan untuk menggantikan SA dalam kajian ini. Kumpulan nisbah 1:1 dan 1:2 telah dipilih dengan dua jenis vaksin IBDV hidup yang dilemahkan iaitu Se

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(benih virus IBDV UPM93) dan Co (komersial vaksin IBDV UPM93). Beberapa teknik untuk penyediaan liposom kationik dalam eksperimen 1 telah diubah. Keputusan telah menunjukkan semua embrio telur ayam SPF dalam semua kumpulan telah terus hidup sepanjang 7 hari pos inokulasi (pi). Ini mengesahkan lagi bahawa SA adalah tosik kepada embrio telur ayam, manakala, DOTAP adalah selamat digunakan dalam penyediaan liposom kationik.

Dalam eksperimen 3, kesan vaksin IBD dan campuran liposom yang selamat untuk menghasilkan tahap perlindungan IBD titer antibodi yang tinggi ditentukan dengan proses vaksinasi ditempat penetasan melalui laluan subkutaneus kepada ayam peaging komersial. Kajian menunjukkan ayam dalam semua kumpulan tidak menunjukkan tanda klinikal yang tidak normal dan lesi mata kasar kecuali atrofi pada bursa Fabricius direkodkan pada hari 28 pv bagi kumpulan IBD, Covac dan Sevac sepanjang kajian. Berat bursa ayam dan nisbah bursa kepada berat badan kekal tidak berubah untuk kumpulan IBD, Covac dan Sevac berbanding dengan kumpulan Control kecuali pada hari ke 28 pv. Penskoran lesi untuk bursa Fabricius telah dikesan seawal hari ke 21 pv dalam kumpulan Covac dan Sevac berbanding dengan kumpulan IBD pada hari ke 28 pv. Titer antibodi IBD untuk kumpulan Covac dan Sevac mula meningkat pada hari ke 21 pv berbanding dengan kumpulan IBD pada hari ke 28 pv. Walaupun dos IBDV yang rendah untuk kumpulan Covac (2/3) apabila dibandingkan dengan kumpulan IBD, titer IBD yang telah dihasilkan kekal tinggi untuk kumpulan tersebut. Ini menunjukkan yang pengkapsulan IBDV dalam liposom dapat meningkatkan penghasilan antibodi IBD.

Kesimpulannya, kajian ini menunjukkan aplikasi liposom kationik dapat meningkatkan penghantaran IBD vaksin ke organ sasaran iaitu bursa Fabricius, dan meningkatkan tahap perlindungan titer antibodi IBD dengan bursal lesion yang rendah. Vaksinasi pada tempat penetasan atau hari pertama umur ayam dengan menggunakan strain MyHatch UPM93 sama ada bersama dengan liposom kationik atau tidak adalah berkesan dan mampu menghasilkan aras antibodi IBD titer yang tinggi dan dapat memberi perlindungan ke atas cabaran IBDV. Aplikasi ini dapat memberi dimensi baharu dalam bidang vaksin dan vaksinasi ternakan ayam.

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I certify that a Thesis Examination Committee has met on 10 February 2016 to conduct the final examination of Mukminah Sakinah Wahab on her thesis entitled “Liposomes for Vaccine Delivery against Infectious Bursal Disease in Chickens” in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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TABLE OF CONTENTS

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LIST OF TABLES

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LIST OF FIGURES

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LIST OF ABBREVIATIONS

ANOVA	analysis variance
attIBDV	attenuated IBDV
caIBDV	classical IBDV
CAM	chorioallantoic membrane
Cho	cholesterol
Covac	commercial IBD vaccine with cationic liposomes
DDAB	dimethyldioctadecylammonium
DNA	deoxyribonucleic acid
DOTAP	1,2-dioleoyl-3-trimethylammonium propane
DPPC	dipalmitoylphosphatidylcholine
dsRNA	double-stranded ribonucleic acid
EID	egg effective dose
ELISA	enzyme link immunosorbent assay
g/mL	gram per milliliter
IBD	infectious bursal disease
IBDV	infectious bursal disease virus

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ISCOM	immunostimulatory complex
LUV	large unilamellar vesicle
MDA	maternal antibody titer
min	minute
MLV	multilamellar vesicle
mL	milliliter
mV	millivolt
MVP	Malaysian Vaccines Pharmaceuticals
ND	Newcastle disease
nm	nanometer
PBS	phosphate buffer saline
PCR	polymerase chain reaction
pi	post infection
pv	post vaccination
RNA	ribonucleic acid
SA	stearylamine
Sevac	IBDV seed virus with cationic liposomes

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SPF	specific pathogen free
SUV	small unilamellar vesicle
Tc	gel-liuid crystal transition temperature
UPM	University Putra Malaysia
UK	United Kingdom
USA	United States of America
UV	ultraviolet
vaIBDV	variant IBDV
vvIBDV	very virulent IBDV
µm	micrometer
ºC	degree celcius

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CHAPTER 1

INTRODUCTION

In Malaysia, the poultry industry is one of the livestock industries that contribute a high profit in the agricultural sector. Products from the poultry are a major source of protein which is readily available, affordable and admissible for the majority of the community worldwide. Statistics output of livestock products in Malaysia were showed that the production of poultry meat is grown significantly from 2005 to 2014 about 52.7% from 0.980 million metric tonnes to 1.496 million metric tonnes (Department of Veterinary Services, 2014). Nevertheless, this industry is inevitable from many challenges especially the issue of emerging and re-emerging diseases.

The diseases are caused by the interaction of many factors where immunosuppression plays an important role causes the frequent problem in chicken production (Hair-Bejo, 2010). One of the common diseases in chickens is an infectious bursal disease (IBD). This disease is a highly contagious immunosuppressive viral disease of chickens causes high mortality and immunosuppression (Whitfill et al. 1995; Hair- Bejo et al., 2004). This disease also affected the poultry in many countries such United States, Africa, India, Japan and Australia (Van den Berg et al., 1991; Van den Berg, 2000). Based on the first location outbreaks in 1957 which occurred in Gumboro, Delaware, United States, thus, IBD also recognized as Gumboro disease (Cosgrove, 1962). In early 1991's IBD outbreak was first reported in Malaysia (Hair-Bejo et al., 1992). Since then, IBD has spread widely throughout poultry farms in the country causing high mortality.

IBD virus (IBDV) belongs to the family Birnaviridae of the genus Avibirnavirus. IBDV has a double-stranded ribonucleic acid (dsRNA) (Nick et al., 1976; Park et al., 2009; Hair-Bejo, 2010). This virus has two serotypes which are serotypes 1 and 2. Pathogenic and replicate in proliferating B cells of the bursa of Fabricius are referring to the serotype 1 strains (Nagarajan et al., 1997) whereas serotype 2 strains are non-pathogenic and may infect chickens. The IBDV serotype 1 can be classified according to their virulence as classical (ca) IBDV, variant (va) IBDV, attenuated (att) IBDV and very virulent (vv) IBDV (Hair-Bejo, 2010). The vvIBDV infection could lead to high mortality and severe immunosuppression in the surviving chickens compared with the caIBDV only caused moderate to severe lesions and lower to moderate percentage of mortality. The vaIBDV only causes immunosuppression and damaged of the lymphoid organs especially the bursa of Fabricius (Kibenge et al., 1988; Hair-Bejo, 2010). The infected chicken is highly susceptible to other pathogenic pathogens and died later due to secondary infection.

Vaccination programmes are important to prevent IBD. The objective of vaccination is to stimulate protective immunity while avoiding disease from the vaccine itself. Early vaccination is necessary for protecting the chicks from the

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disease (Hair-Bejo et al., 2004a), however, maternal antibodies may interfere the IBD vaccines (Muller et al., 2003; Vaziry et al., 2007). The perfect vaccine is stable, safe and easier to apply in a poultry farm. Currently, several types of vaccines are available in the commercial chickens industry including live, killed and subunit vaccines (Muller et al., 2003). Normally, inactivated or killed vaccines are safe but less effective compared with live attenuated vaccines (Van den Ber, 2000; Sarachai et al., 2010). The virus is unable to replicate in the chickens. Hence, adjuvants such as based oil adjuvants (Van den Berg, 2000), liposomes (Li et al., 2013), ISCOM (Rasool, 2008) were used to enhance the immune response. Live attenuated vaccines are usually developed from the field or wild virus attenuated in chicken embryonated eggs or tissue culture (Lauring et al., 2010). The vaccine virus could replicate effectively in the target organ of the chickens and could induce protective immune responses similar to the natural infection. Recently, studies more focusing on the application of technologies, for instance, development of subunit and DNA vaccines (Park et al., 2009). Subunit vaccine is the recombinant technology which expressed structural proteins of IBDV. VP2 has been used for the development of subunit vaccines and has been expressed in a number of systems. Another recombinant technology is DNA vaccines which induce an immune response by transfer naked DNA to a foreign antigen, encoding the target gene into host cells (Fahey et al., 1991). This vaccine can induce an efficient immune response in chickens. However the recombinant technologies are costly and complicated.

There are many vital factors to determine the efficacy of IBD vaccination such as type of vaccine preparation, maternal derived antibody (MDA) in the chicks, the time of vaccination and pathogenicity of the IBDV field challenge. Proper management and vaccination programmes and biosecurity are also important for control and prevention various infectious disease in poultry especially IBD in chickens. Developments of vaccines have undergone variation from attenuated vaccines to DNA vaccines. In recent years, treatments and immunization against various diseases have undergone a transformation due to the vigorous development vaccine research. Furthermore, in order to provide optimal immunization, various efforts have been made to improve the effectiveness of vaccines including developing a specific vaccine for each disease (Shahiwala et al., 2007).

In past several decades, applications of nanotechnology have been most widely studied in various technological and biomedical fields. Nowadays, development of nano-based carrier for vaccine delivery has attracted a lot of interest in an effort to provide effective immunization. Nanoparticles are polymeric particles in the nano-meter scale which can dissolve, entrap or encapsulate materials such as drugs or biologically active materials (Kreuter, 1996). The purpose of the development nanoparticles is to control and manipulate supramolecular assembly (supermolecule) and biomacromoecular structures that are critical to living cells in order to improve the quality of living things. These structures and assembly are in nano-scale including very small creatures like viruses, bacterial and DNA (Mudshinge et al., 2011).

Nanotechnology increasingly lays a significant role in vaccine development. As vaccine development orientates toward less immunogenic compositions,

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formulations that boost antigen effectiveness are increasingly needed. The application of nanoparticles in vaccine formulations improved antigen stability and immunogenicity. However, the application of nanoparticles in vaccine delivery as well as in drug delivery is still at an early stage of development. A number of challenges still remain due to a lack of fundamental understanding. Therefore, rational design combination of the productive nanoparticles with desirable properties, functionalities and efficacy becomes increasingly important. Furthermore, by integrating some other attractive properties such as slow release targeting, alternative administration methods, delivery pathways with novel vaccine systems will fulfil the intention including single-dose and needle free delivery during vaccination process will become practical in the future (Zhao et al., 2014).

In nanotechnology field, liposomes is one of the nano-based carrier which gaining more attention in research. Liposomes are synthetic spheres composed of lipid bilayers that can incorporate with various antigens including viruses (Tseng et al., 2009). Bangham et al. (1965) suggested that these special vesicles are typically used as model membranes due to the structural resembling with the cell membranes (Bangham et al., 1965; Taylor et al., 1995). The liposome size can range from 50 to 5000 nm in diameter (Sharma and Sharma, 1997) meanwhile other researchers stated that the range diameter size of liposomes can be from 20 nm to 20 000 nm (Taylor et al., 1995) or 80 nm to 100 000 nm (Slabbert et al., 2011). Liposomes are produced from biodegradable and biocompatible materials and contain empty space for encapsulate antigens. A broad variety of lipid materials can be used to form liposomes. Various type of phospholipid can be used to produce liposomes either natural or synthetic phospholipids (Taylor et al., 1995). Characteristics of liposomes can be varying in size and charge particles depending on the lipids used and method preparations (Chrai et al., 2011; Slabbert et al., 2011). This nanoparticle can be formed either single or multiple bilayer membranes. Based on the size and number of bilayers, liposomes can be grouped as small unilamellar vesicles (SUV), large unilamellar vesicles (LUV) and multilamellar vesicles (MLV) (Sharma and Sharma, 1997; Chrai et al., 2011).

It is well known that liposomes can be formed spontaneously after hydrated with aqueous solution (Chrai et al., 2011) with an inner empty compartment. This nanoparticle typically composed by phospholipids as a major component and has been widely used as carriers of protein or peptide antigens. Antigenic materials can be encapsulated within the internal empty spaces, reconstituted within the lipid bilayers or attached to the outer surface of the liposomes (Alving, 1991; Jain et al., 2003). Various methods also have been developed for the production of liposomes including thin lipid hydration (Jain et al., 2013), ether injection (Mathai and Sitaramam, 1987), reverse-phase evaporation (Szoka et al., 1980) and detergent dialysis (Jiskoot et al., 1986). However, the most commonly technique used for preparation of liposome formulation in laboratory is thin lipid film hydration method with some modification techniques such as sonication and high pressure homogenizer to improve entrapment efficacy and stability of liposomes (Jain et al., 2003). There are several advantages of liposomes such as sustained release, site specific delivery and reduction in toxicity (Slabbert et al., 2011). Besides, the application of

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liposomes is useful alternative to reduce dosage amount of antigen for intravenous administration (Chrai et al., 2011).

Typically in vaccine production, the antigens itself may be less immunogenic to produce high immune response. Hence, another component is added to intensify the immune response which called as adjuvant (Peek et al., 2008). For instance, aluminium salts was used to function as adjuvants for certain antigens and also applied in several of commercial vaccines (Richards et al., 1996). In the development of vaccines, additional component must have special criteria not only can be adjuvant to enhance immune response but at the same time can be carrier to deliver antigens to the target organ. It was reported that, among the carriers liposomes were proved as efficient drugs and vaccines delivery systems and gaining attention due to their abilities to act as delivery vehicles and adjuvants (Fraley et al., 1980; Muderhwa et al., 1999; Tardi et al., 2000; Tseng et al., 2009).

Generally, live attenuated vaccine has low cost of production and could induce protective immune responses similar to natural infection. However, the disadvantages of live attenuated vaccine are cause high risk of immunosuppression due to severe damages of the bursa of Fabricius (Hair-Bejo et al., 2004a). Besides, interference of maternal antibody (MDA) still a challenge in early vaccination of IBD vaccine (Van den Berg, 2000; Hair-Bejo et al., 2004b; Muller et al., 2012). Application of cationic liposomes as vaccine delivery in the present study could improve the effectiveness of live attenuated IBD vaccine with minimizes vaccine usage. It was the hypothesis of the study that the application of cationic liposomes as IBD vaccine carrier can effectively deliver IBD vaccine to the target organ and induce high IBD antibody titre.

The objectives of the study were:
(i) to develop a stable and suitable cationic liposomes for IBD vaccine delivery using thin lipid hydration method.
(ii) to determine the safety of the developed liposomes in embryonated SPF chicken eggs.
(iii) to determine the effectiveness of the encapsulation of IBD vaccine in liposomes in broiler chickens.There may be a preamble at the beginning of a chapter. The purpose may be to introduce the themes of the main headings.

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APPENDICES

Appendix A

Chemicals and reagents

1. Solvent chloroform/ methanol (4:1 v/v)

Chloroform	200 mL
Methanol	500 mL

2. Stock solution of DPPC (20 mM)

DPPC 0.1468g

Dilute with chloroform to 10 mL. Store at 4ºC.

3. Stock solution of cholesterol (20 mM)

Cholesterol 0.0387g

Dilute with chloroform to 5 mL. Store at 4ºC.

4. Stock solution of DOTAP (20 mM)

DOTAP 0.0699g

Dilute with chloroform to 5 mL. Store at 4ºC.

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Appendix B

Calculation weight of each lipid compound used for preparation of liposomes in experimental 1

DPPC:	Cho:	SA:
7/10 x 500 mg = 350 mg	2/10 x 500 mg = 100 mg	1/10 x 500 mg = 50 mg

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Appendix C

Calculation weight of each lipid compound for stock solution in experimental 2

DPPC in 0.05M (MW: 734.039g/mol)	Cholesterol in 0.05M (MW: 386.65 g/mol)	DOTAP in 0.05M (MW: 698.542 g/mol)
1 mol = 734.039 g 0.05 mol = x g x = (0.05/1) (734.039) = 36.7020 g 36.7020 g in 1 L y g in 0.004 L y = (0.004/1) (36.7020) = **0.1468 g**	1 mol = 386.65 g 0.05 mol = x g x = (0.05/1) (386.65) = 19.3325 g 19.3325 g in 1 L y g in 0.002 L y = (0.002/1) (19.3325) = **0.0387 g**	1 mol = 698.542 g 0.05 mol = x g x = (0.05/1) (698.542) = 34.9271 g 34.9271 g in 1 L y g in 0.002 L y = (0.002/1) (34.9271) = **0.0697 g**

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Appendix D

Calculation weight of each lipid compound for stock solution in experimental 3

DPPC in 0.02M (MW: 734.039g/mol)	Cholesterol in 0.02M (MW: 386.65 g/mol)	DOTAP in 0.02M (MW: 698.542 g/mol)
1 mol = 734.039 g 0.02 mol = x g	1 mol = 386.65 g 0.02 mol = x g	1 mol = 698.542 g 0.02 mol = x g
x = (0.02/1) (734.039) = 14.6808 g	x = (0.02/1) (386.65) = 7.733 g	x = (0.02/1) (698.542) = 13.9708 g
14.6808 g in 1 L y g in 0.0085 L	7.733 g in 1 L y g in 0.005 L	13.9708 g in 1 L y g in 0.005 L
y = (0.0085/1)(14.6808) = **0.1248 g**	y = (0.005/1)(7.733) = **0.0387 g**	y = (0.005/1)(13.9708) = **0.0699 g**

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Appendix E

Cumulative mortality of embryonated SPF chicken eggs throughout the trial

Group	Total no. of SPF eggs	Cumulative Mortality								Total no. of SPF eggs viable	Mortality (%)
		Day (pi)									
		0	1	2	3	4	5	6	7		
A	6	0[a]/6[b]	0/6	0/6	0/6	0/6	0/6	0/6	0/6	6	0
B	6	0[a]/6[b]	0/6	4/6	4/6	4/6	4/6	4/6	4/6	2	67
C	6	0[a]/6[b]	0/6	0/6	0/6	0/6	0/6	0/6	0/6	6	0
D	6	0[a]/6[b]	0/6	0/6	0/6	0/6	0/6	0/6	0/6	6	0
E	6	0[a]/6[b]	0/6	0/6	0/6	0/6	0/6	0/6	0/6	6	0
F	6	0[a]/6[b]	0/6	2/6	2/6	2/6	2/6	2/6	2/6	4	33

a: Total number of eggs dead

b: Total number of eggs inoculated

A: Control
B: Liposomes
C: IBD
D: Sevac 1
E: Sevac 2
F: Sevac 3

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Appendix F

Cumulative mortality of embryonated SPF chicken eggs throughout the trial

Group	Total no. of SPF eggs	Cumulative Mortality								Total no. of SPF eggs viable	Mortality (%)
		Day (pi)									
		0	1	2	3	4	5	6	7		
A	50[a]/5[b]	0/5	0/5	0/5	0/5	0/5	0/5	0/5		5	0
B	50[a]/5[b]	0/5	0/5	0/5	0/5	0/5	0/5	0/5		5	0
C	50[a]/5[b]	0/5	0/5	0/5	0/5	0/5	1/5	4/5		1	80
D	50[a]/5[b]	0/5	0/5	0/5	0/5	0/5	1/5	1/5		4	20
E	50[a]/5[b]	0/5	0/5	0/5	0/5	1/5	3/5	4/5		1	80
F	50[a]/5[b]	0/5	0/5	0/5	0/5	2/5	2/5	2/5		3	40
G	50[a]/5[b]	0/5	0/5	0/5	0/5	0/5	1/5	1/5		4	20

a: Total number of eggs dead

b: Total number of eggs inoculated

A: Control
B: Liposomes
C: IBD
D: Sevac
E: Sevac 2
F: Covac 1
G: Covac 2

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Appendix G

Lesion scoring of the bursa of Fabricius

Score	Category	Descriptions
0	Normal	No lesions.
1	Mild	Mild degeneration and necrosis of lymphoid cells in a few follicles especially in the medulla are visible.
2	Mild moderate	Degeneration and necrosis of lymphoid cells in a few follicles especially in the medulla. The interstitial connective tissue become oedematous and fills with inflammatory.
3	Moderate	Moderate follicular necrosis involving bot the cortex and medulla. Pyknotic nuclei were scattered in the follicles. Interstitial space was obvious and present with heterophils and macrophage and few erythrocytes and fibroblast. Epithelial lining is thickened, vacuolated in some areas.
4	Moderate to severe	Depletion of lymphoid cells in the follicles. Lymphoid cell aggregation found in the cortex of some follicles, necrotic cells and cysts were present in some follicles specially in the medulla. The interstitial space was infiltrated with inflammatory cells and well packed with fibrinous connective tissues. The intra and extra follicular areas might be hyperemic and hemorrhagic. Epithelium was thickened, corrugated and vacuolated in some areas.
5	Severe acute OR Severe chronic	Moderate to severe atrophy of bursal follicles with cellular necrosis and degeneration involving both cortex and medulla. Follicular cysts with fibrinous exudates and cell debris were frequently observed. The interstitial connective tissues were obvious, oedematous and infiltrated with mild to moderate inflammatory cells. The epithelial lining of the of Fabricius was thickened and vacuolated. Severe follicle atrophy with cysts formation within the follicles and epithelial lining of the organ. Remarkable infiltration of fibroblast in the interstitial area. Lymphocytes and macrophages infiltration were commonly observed.

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Appendix H

Body weight of the chickens throughout the experiment

	Days Post Vaccination				
Groups	**0**	**7**	**14**	**21**	**28**
Control	44±1.7^{a}	198±15.7^{bq}	500±43.4^{c}	998±67.6^{d}	1350±78.4^{ep}
IBD	44±1.7^{a}	177±15.3^{bpq}	440±16.5^{c}	998±68.0^{d}	1570±156.0^{epq}
Covac	44±1.7^{a}	166±11.0^{bp}	438±38.5^{c}	1056±97.6^{d}	1646±122.0^{eq}
Sevac	44±1.7^{a}	182±10.3^{bpq}	488±38.2^{c}	1040±50.5^{d}	1652±119.0^{eq}

* Values are mean ± SEM
^{abcde} Value with different superscripts within column differ significantly at $p<0.05$
^{pq} Value with different superscripts within row differ significantly at $p<0.05$

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Appendix I

Bursa of Fabricius weight of the chickens throughout the experiment

	Days Post Vaccination				
Groups	**0**	**7**	**14**	**21**	**28**
Control	0.10±0.03^{a}	0.53±0.13^{a}	1.28±0.21^{bpq}	2.17±0.33	2.70±0.55^{cq}
IBD	0.10±0.03^{a}	0.50±0.16^{a}	1.56±0.40^{bpq}	2.80±0.88^{c}	1.52±0.45^{bp}
Covac	0.10±0.03^{a}	0.55±0.17^{a}	1.21±0.29b^{p}	2.73±0.68^{c}	1.38±0.27^{bp}
Sevac	0.10±0.03^{a}	0.53±0.14^{a}	1.75±0.11^{bq}	1.83±0.76^{b}	1.58±0.31^{bp}

* Values are mean ± SEM
^{abc} Value with different superscripts within column differ significantly at $p<0.05$
^{pq} Value with different superscripts within row differ significantly at $p<0.05$

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Appendix J

Bursa to body weight ratio of the chickens throughout the experiment ($x10^{-3}$)

	Days Post Vaccination				
Groups	**0**	**7**	**14**	**21**	**28**
Control	2.19±0.67	2.68±0.63	2.59±0.57	2.19±0.41	1.99±0.30^{q}
IBD	2.19±0.67^{b}	2.82±0.90^{b}	3.53±0.80^{b}	2.78±0.77^{b}	0.96±0.25^{ap}
Covac	2.19±0.67^{b}	3.28±0.94^{b}	2.75±0.51^{b}	2.60±0.71^{b}	0.84±0.16^{ap}
Sevac	2.19±0.67^{b}	2.92±0.74^{b}	3.59±0.27^{b}	1.78±0.78^{a}	0.96±0.23^{ap}

* Values are mean ± SEM
^{ab} Value with different superscripts within column differ significantly at $p<0.05$
^{pq} Value with different superscripts within row differ significantly at $p<0.05$

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Appendix K

Lesion scoring of the chickens throughout the experiment

	Days Post Vaccination				
Groups	**0**	**7**	**14**	**21**	**28**
Control	0.0±0.00	0.2±0.45	0.2±0.45	0.4±0.55	0.4±0.55
IBD	0.0±0.00[a]	0.2±0.45[a]	0.4±0.55[a]	0.8±0.45[a]	1.6±0.55[ab]
Covac	0.0±0.00	0.4±0.55	0.4±0.55	1.0±1.22	1.8±1.30
Sevac	0.0±0.00	0.4±0.55	0.4±0.55	1.8±1.79	1.4±0.55

* Values are mean ± SEM
[abc] Value with different superscripts within column differ significantly at $p<0.05$

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Appendix L

IBD antibody titer of the chickens throughout the experiment

	Days Post Vaccination				
Groups	**0**	**7**	**14**	**21**	**28**
Control	2958±1090.0^{b}	609±224.0^{a}	199±139.0^{a}	37±15.3^{a}	29±9.5^{ap}
IBD	2958±1090.0^{b}	388±263.0^{a}	190±106.0^{a}	55±29.7^{a}	2933±1810.0^{bq}
Covac	2958±1090.0^{b}	711±603.0^{a}	106±57.3^{a}	258±428.0^{a}	3572±1270.0^{bq}
Sevac	2958±1090.0^{b}	602±528.0^{a}	57±19.5^{a}	436±719.0^{a}	3101±1730.0^{bq}

* Values are mean ± SEM
^{ab} Value with different superscripts within row column significantly at $p<0.05$
^{pq} Value with different superscripts within row differ significantly at $p<0.05$

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Mukminah Sakinah binti Wahab was born in Kuala Terengganu, Terengganu, Malaysia on 15th March 1987. She is the eldest child in a family. She did her primary education in Sekolah Kebangsaan Tengku Ampuan Intan, Hulu Terengganu from 1994 to 1998 before she transferred to the Sekolah Kebangsaan Rengas Bekah, Kuala Terengganu until 1999. Then, the author continued her secondary education in Sekolah Menengah Agama (Atas) Sultan Zainal Abidin, Kuala Terengganu where she sits for her Penilaian Menengah Rendah (PMR) and Sijil Pelajaran Malaysia (SPM) examination from 2000 until 2004.

After that, she continued her study to Sekolah Menengah Kebangsaan Padang Midin, Kuala Terengganu until she finished Sijil Tinggi Pelajaran Malaysia (STPM) examination from 2005 to 2006. In the next year, the author continued her study in Bachelor of Science (Chemistry) in Universiti Sains Malaysia from 2007 until 2010. Then she was pursuing her study in Master of Science program in immunology focusing on nanotechnology field in Faculty of Veterinary Medicine, Universiti Putra Malaysia.

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LIST OF PUBLICATIONS

Journal

Mukminah-Sakinah, W., Hair-Bejo, M., Omar, A. R. and Aini, I. (2015). Hatchery Vaccination Using Liposomes as Vaccine Delivery against Infectious Bursal Disease in Broiler Chickens (accepted to Journal of Animal and Veterinary Advances).

Proceedings

Mukminah-Sakinah, W., Hair-Bejo, M., Omar, A. R. and Aini, I. (2013). Infectious Bursal Disease Vaccine Delivery via Topical Application in 18-Day-Old Specific Pathogen Free Embryonated Chicken Eggs. In: Proceeding of World Poultry Science Association (Malaysian Branch) Scientific Conference 2013; 30^{th}-1^{st} December 2013. Faculty of Veterinary Medicine, Universiti Putra Malaysia, Selangor, Malaysia.

Mukminah-Sakinah, W., Hair-Bejo, M., Omar, A. R. and Aini, I. (2015). Liposomes as Vaccine Carrier in Hatchery Vaccination against Infectious Bursal Disease in Broiler Chickens. In: Proceeding of 2^{nd} World Poultry Science Association (Malaysian Branch) Scientific Conference; 21^{st} - 22^{nd} September 2015. Kuala Lumpur Convention Centre, Kuala Lumpur, Malaysia.

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