DESIGNING SPRAY DRIED NANOEMULSION AS VACCINE ADJUVANT

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Pharmaceutical Technology
Department of Pharmaceutics and Industrial Pharmacy
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Academic Year 2015

การออกแบบนาโนอิมัลชันชนิดพ่นแห้งเป็นวัคซีนแอดจูแวนท์

นางสาวทู ทะ เหมียด

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีเภสัชกรรม ภาควิชาวิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2558

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ทู ทะ เหมียด : การออกแบบนาโนอิมัลชันชนิดพ่นแห้งเป็นวัคซีนแอดจูแวนท์ (DESIGNING SPRAY DRIED NANOEMULSION AS VACCINE ADJUVANT) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ภญ. ดร. กาญจน์พิมล ฤทธิเดช, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ภญ. ดร. วิมลมาศ ลิปิพันธ์, 138 หน้า.

ในการศึกษานี้ นาโนอิมัลชันชนิดน้ำในน้ำมันถูกเตรียมเป็นแอดจูแวนท์โดยใช้เทคนิคการทำเป็นเนื้อเดียวกันด้วยแรงดันสูง ส่วนประกอบแตกต่างของน้ำมันจมูกข้าวสาลีเพียงอย่างเดียว หรือร่วมกับวิตามินอีอะซิเตทจะใช้เป็นไขมัน ทวีน80 และสแปน85 จะใช้เป็นสารลดแรงตึงผิวโดยแปรผันความเข้มข้นเพื่อให้ได้นาโนอิมัลชันหมาะสมที่มีขนาดอนุภาคเล็กที่สุด นาโนอิมัลชันเหมาะสมที่ใช้น้ำมันจมูกข้าวสาลีอย่างเดียวจะเป็นตำรับ เอฟเอ ที่มีขนาดอนุภาค 111 นาโนเมตร ความต่างศักย์พื้นผิว -29 มิลลิโวลต์ และน้ำมันจมูกข้าวสาลีร่วมกับวิตามินอีอะซิเตท (อัตราส่วน 0.9:0.1) เป็นตำรับ เอฟบี มีขนาดอนุภาค 100 นาโนเมตร ความต่างศักย์พื้นผิว -24 มิลลิโวลต์ ถูกเลือกสำหรับการพ่นแห้ง มอลโตเดกตรินจะเป็นตัวพา เพื่อศึกษาผลของพารามิเตอร์ระหว่างการพ่นแห้ง ตำรับเอฟบี5-บีแอล ที่มีขนาดอนุภาค 152 นาโนเมตรและความต่างศักย์พื้นผิว -22 มิลลิโวลต์หลังการทำให้คืนรูป ได้รับเลือกให้ศึกษาต่อ บีเอสเอเป็นแอนติเจนต้นแบบที่ใส่ในตำรับ คุณลักษณะของนาโนอิมัลชันและผงพ่นแห้งที่แสดงจากกล้องแบบ ทีอีเอ็ม และ เอสอีเอ็ม ตามลำดับ พบสัณฐานวิทยาของนาโนอิมัลชันเป็นทรงกลม และอนุภาคพ่นแห้งมีพื้นผิวคล้ายรูพรุนและรูปร่างกลม ขนาดของอนุภาคพ่นแห้งวิเคราะห์โดยมาสเตอร์ไซส์เซอร์ พบว่าขนาดอนุภาคของ เอฟบี5-บีแอล เอฟบี5-บีเอสเอ1 และ เอฟบี5-บีเอสเอ3 อยู่ที่ประมาณ 3 ถึง 7 ไมโครเมตร ดังนั้น ผงพ่นแห้งสามารถใช้โดยการสูดดม ในขณะที่ผงพ่นแห้งที่ทำให้คืนรูปสามารถใช้โดยการฉีด การตรวจสอบดีเอสซีเทอร์โมแกรมและความเป็นผลึกของผงพ่นแห้ง พบว่าตำรับผงแห้งทั้งหมดเป็นอสัญฐาน ไม่พบการเกิดปฏิกิริยาระหว่างสารต่างๆในตำรับระหว่างมอลโตเดกตริน บีเอสเอ และนาโนอิมัลชันในผงพ่นแห้งโดยเอฟทีไออาร์ ความสมบูรณ์ของบีเอสเอวิเคราะห์จากเซอคูลาร์ ไดโครอิซึม และเอสดีเอส เพจ สรุปว่ายังคงความสมบูรณ์ของบีเอสเอได้หลังการพ่นแห้ง แต่มีการเลื่อนของพีคในโครงสร้างทุติยภูมิเล็กน้อยซึ่งอาจเกิดจากการสกัดด้วยสารอินทรีย์ปริมาณน้อย นอกจากนี้ความคงสภาพของทั้งนาโนอิมัลชันและผงพ่นแห้งเช่นเดียวกับการทำให้คืนรูปเมื่อเก็บที่อุณหภูมิ 4 องศาเซลเซียสและที่อุณหภูมิห้องโดยวัดขนาดอนุภาคเป็นเวลา 3 เดือน พบว่ามีความคงตัวดียกเว้นพบอนุภาคใหญ่ขึ้นของผงยาที่เก็บที่อุณหภูมิห้อง ซึ่งอาจเกิดจากการเหนียวติดันของมอลโตเดกตรินและใช้ในความเข้มต่ำในการคงสภาพของหยดน้ำมัน การเพาะเลี้ยงเซลล์สองประเภท; เซลล์โมโนไซต์-มาโครฟาจ ของมนุษย์ (ซีอาร์แอล-9855) และเซลล์จมูกของมนุษย์ (ซีซีเอล-30) พบการรอดชีวิตของเซลล์โมโนไซต์ร้อยละ 100 ทั้งใน ตำรับเอฟบี5-บีแอล และเอฟบี-บีเอสเอ1 ส่วนการรอดชีวิตของเซลล์เยื่อบุจมูกร้อยละ 100 พบที่ความเข้มข้น 35 ไมโครกรัมต่อมิลลิลิตร นอกจากนี้ทำการศึกษาการกลืนกินโดยใช้มาโครฟาจ แล้วดูภายใต้กล้องคอนโฟคัลเลเซอร์สแกนนิ่ง ยืนยันการกลืนกินเข้าเซลล์ของตำรับเอฟบี5-บีแอล และเอฟบี-บีเอสเอ1 ดังนั้นจึงสรุปได้ว่าสามารถพัฒนาแอดจูแวนท์นาโนอิมัลชันผงแห้ง โดยใช้บีเอสเอเป็นแอนติเจนต้นแบบและคงความสมบูรณ์ของบีเอสเอได้ ดังนั้นจึงมีศักยภาพเป็นแอดจูแวนท์ชนิดผงได้

ภาควิชา วิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม ลายมือชื่อนิสิต .....

สาขาวิชา เทคโนโลยีเภสัชกรรม ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5676355333 : MAJOR PHARMACEUTICAL TECHNOLOGY
KEYWORDS: NANOEMULSON, SPRAY DRYIING,VACCINE ADJUVANT, MALTODEXTRIN, BSA

HTOO HTET MYAT: DESIGNING SPRAY DRIED NANOEMULSION AS VACCINE ADJUVANT. ADVISOR: PROF. GARNPIMOL RITTHIDEJ, Ph.D., CO-ADVISOR: ASSOC. PROF. VIMOLMAS LIPIPUN, Ph.D., 138 pp.

In this study, oil in water nanoemulsion was prepared as an adjuvant by using high pressure homogenization technique. Various compositions of wheat germ oil alone or with Vitamin E acetate were used as lipid. Tween80 and Span85 were used as surfactants and concentrations were varied in order to obtain the optimized nanoemulsion with the smallest particle size. Optimized nanoemulsion with wheat germ oil alone as FA (formula A) having the particle size of 111 nm, zeta potential of -29 and wheat germ oil with vitamin E acetate (0.9:0.1) as FB (formula B) having the particle size of 100 nm, zeta potential of -24 mV were chosen for spray drying. Maltodextrin (MD) was used as carrier loaded with FA and FB to investigate the effect of parameters during spray drying. FB5-BL was then chosen, which had the particle size of 152 nm and zeta potential of -22 mV after reconstitutions. BSA as a model antigen was loaded into FB5-BL investigation was performed. Characterization of nanoemulsion and spray dried powder was done by TEM and SEM, respectively. The morphology of nanoemulsion was spherical in shape and the spray dried particles had a porous like surface with spherical shape. Spray dried particle size was also analyzed by mastersizer and the particle size of FB5-BL, FB5-BSA1 and FB5-BSA3 was around 3-7 μm. Thus, spray dried powder could be administered by inhalation while reconstituted spray dried powder could be administered by parenteral administration. Thermographic behavior as well as crystallinity of spray dried powder was also examined and all the powder formulation were amorphous in state .There was no interaction between the compositions MD, BSA and nanoemulsion in spray dried powder confirmed by FTIR. BSA integrity was analyzed by circular dichroism and SDS-PAGE. It could be concluded that the integrity of BSA was conserved even after spray drying but a slight shift of secondary structure was note due to the protein extraction by small amount of organic solvents. Moreover, stability evaluation of both nanoemulsion and spray dried powder as well as its reconstitutions were performed at 4°C and at ambient temperature and the particle size of formulations at each time point was analyzed for 3 months. The stability of both formulation at two condition had good results except the spray dried powder at ambient temperature showed increased in particle size. It could be due to slight stickiness and low concentrations of maltodextrin to stabilize the droplets. Two types of cell lines; human monocyte macrophage cell line (CRL-9855) and human nasal cell line (CCL-30) was cultured and cell viability was tested for all optimized formulations. Cell viability testing with monocyte cells gave nearly 100% viability in both FB5-BL and FB5-BSA1. For cell viability in human nasal cells, the concentration of 35μg/ml gave 100% viability. Furthermore, uptake study was also carried out by using macrophage cells. The confocal laser scanning microscopy has confirmed that FB5-BSA1 as well as FB5-BL without BSA was uptake by the cells. It can be concluded that spray dried nanoemulsion adjuvant was successfully developed using BSA as a model antigen with retained integrity of BSA Hence, it could be used as potential powder adjuvant.

Department:	Pharmaceutics and Industrial Pharmacy	Student's Signature	.....
Field of Study:	Pharmaceutical Technology	Advisor's Signature	.....
Academic Year:	2015	Co-Advisor's Signature	.....

ACKNOWLEDGEMENTS

I would like to give all my sincere gratitude to my thesis advisor, Professor Garnpimol C.Ritthidej, Department of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, for her valuable guidance, enthusiasm and encouragement in my research work. She was my inspiration throughout my research work.

Secondly, I would like to thank to Assoc.Prof.Vimolmas Lipipun, who have helped me in all cell studies work with her advice and expertise.

I would like to send my sincere gratitude to Assit.Prof.Nontima Vardhanabhuti, Assit.Prof.Angkana Tantituvanont and Assoc.Prof.Parkpoom Tengamnuay for all the advices given throughout my course work and seminars.

I would like to thank Assoc.Prof.Wiwat Pichayakorn, Dr. Chirasak Kusonwiriyawon and to all my committee members for valuable advice, insightful comments and helpful suggestions in making correction of this thesis book

I am really happy and grateful to be a part of Chulalongkorn University. It was a wonderful experience.

My sincere thanks to all the faculty staff under the Faculty of Pharmaceutical Sciences, Chulalongkorn University and my friends and fellow lab mates from Manu lab who have been with me and cheer me up during this whole study period.

I would like to express my thanks to the lab technicians Mr. Prasong Changmai and Mr. Samreng Thienyen and Miss Raweewan Senasuthum from Manufacturing Pharmacy department for helping me in handling the equipment and repairing the equipment whenever necessary.

Finally, I would like express my love and sincere thanks to my parents for their support, care and kind understanding through out my graduate study.

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List of Abbreviations

°C	Degree Celsius
Cm	centimeter
MD	Maltodextrin
BSA	Bovine serum albumin
WGO	Wheat germ oil
DSC	Differential Scanning Calorimetry
XRD	X-ray powder diffraction
e.g.	Exempli gratia, for example
et al.	Et alii, and others
h	hours
min	minutes
ml	milliliter
µl	microliter
mg	milligram
µg	microgram (s)
µm	micrometer
nm	nanometer
g	gram
r^2	coefficient of determination
SEM	Scanning electron microscope
TEM	Transmission electron microscope
FTIR	Fourier infrared transform spectroscopy
CD	Circular dichroism
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
CLSM	Confocal laser scanning microscopy
w/w	weight by weight
w/v	weight by volume

%	Percentage
CO_2	Carbon dioxide
APCs	Antigen presenting cells
DCs	Dendritic cells
DAPI	4',6-diamidino-2-phenylindole
MHC	Major Histocompatibility Complex
VLPs	Virus like particles
FDA	Food and drug administration
PBS	Phosphate buffer saline
BCA	Bicinchoninic acid
PLGA	Poly (lactic-co-glycolic acid)
PVP	Polyvinylpyrrolidone
PLA	Polylactic acid
TLR	Toll like receptors
GRAS	Generally recognized as safe
ISCOM	Immune stimulating complexes
HSV	Herpes simplex virus
ATCC	American type culture collection
HBsAg	Hepatitis B surface antigen
EMEM	Eagle's minimum essential medium
EDTA	Ethylenediaminetetraacetic acid
NLRs	NOD-like recptors
NOD	Nucleotide binding oligomerization domain receptors
Tg	Glass transition temperature
CFA	Complete Freund adjuvant
CNS	Central nervous system

CHAPTER I
INTRODUCTION

Vaccination is one of the most popular and effective strategies to prevent infectious diseases all around the world. Vaccination works by manipulating the body's immune system and helps the body to get rid of specific pathogens. There are increasing number of vaccines and its quality ranging from gene-based vaccines to protein subunits to protein complexes such as enveloped and non-enveloped virus-like particles and cell-based therapies (Singh and O'Hagan, 1999, O'Hagan and Valiante, 2003). Generally, vaccines can be of two types, i.e., live attenuated vaccines and inactivated vaccines. Live attenuated vaccines work by replicating live organisms via transient infection although, it's easy to produce and cost effective, it has higher intrinsic risks concerned with live pathogens. On the other side, inactive vaccines are safer as its composed of the killed organisms or isolated sub-cellular components but downside is that it has lower and shorter duration of immunity (Martinon et al., 2009). A vaccine design that could provide more protective and long lasting immunity after vaccination is the target nowadays.

Vaccine adjuvant is the component that can boost the effectiveness of vaccines by generating robust immune responses. Generally, adjuvants have the following mechanism of action: (1) it augments the biological or immunological half-life of antigens, (2) raise the antigen delivery to antigen presenting cells (APCs), (3) activation and maturation of APCs and migration to the draining lymph nodes, (4) increase the production of immunomodulatory cytokines and chemokines, (5) cellular recruitment at the site of injection and (6) activation of inflammasome. Adjuvant can be then formulated that favors the T-helper type 1 (Th1) or type 2 (Th2) immune responses to vaccine antigens (Vogel, 2000, Awate et al., 2013). Alum was the first adjuvant approved worldwide and it has been used in large numbers of vaccines for human use for many years. But one downside of alum is that it's not suitable for most antigens and it doesn't have the sufficient potential to provide cell mediated immunity. (Balasse et

al., 2008). Nowadays, there is a progress in inventing the effective adjuvants for subunit vaccine such as emulsion, liposomes, polymeric nanoparticles, immune-stimulating complexes (ISCOMs), and virus like particles (VLPs). Only a handful of these have been marketed yet.

In the past decade, several adjuvants such as MF59 and AS03 have been approved to use and these are oil in water nanoemulsions, which increase the antigen uptake and recruit the immune cells to the injection site (Calabro et al., 2011). So, in this study, our interest was emulsion for several reasons. Nanoemulsion can be of two forms, oil-in-water or water-in-oil with either transparent system (covering size range of 50-200nm) or milky (up to 500nm). The surfactants used in nanoemulsions are approved for human consumption and common food substances, "Generally Recognized as Safe" (GRAS) by the FDA. Nanoemulsions hold a great promise in pharmaceutical field because of their dosage design and pharmacotherapy. Nanoemulsions are only kinetically stable however the long-term physical stability with no flocculation or coalescence makes this formulation more unique. The difference from microemulsion is that nanoemulsion needs (3-10%) of surfactant concentration while microemulsion requires higher concentration of (15-30%) (Bouchemal et al., 2004). Nanoemulsions are also popular because of their efficient delivery system. In the vaccine field, they are not solely delivery systems because some studies have proved that it also has immunomodulatory properties depending on compositions (Mosca et al., 2008). Nanoemulsion can either carry the antigen inside their core for better delivery or can just mixed with antigen. There are few disadvantages of nanoemulsion and that is it can be affected by the environmental pH, temperature and it can undergoes Ostwald ripening during storage which is one of the complicated issues in vaccine development (Sharma et al., 2010).

Nanoemulsions however has a period of long-term stability but mixing the antigen together with it can cause destabilization and unfolding of the antigens. It is due to the presence of hydrophobic phase and an aqueous environment (Dormitzer et al., 2012). There is also a demand for development of better and efficient formulation with better stability and it can be obtained by converting the liquid formulation into solid

powder forms. Several methods are available to perform this and these include spray drying, supercritical drying, lyophilization and emulsion-solvent evaporation method, etc. Of all these methods, spray drying has become one of the most promising methods because of its properties to preserve the conformation of antigens. It is also a liable method to produce the particles with optimized characteristics and narrow particle size range. It is deemed to be one-step and energy efficient process compared to the traditional freeze-drying method (Kusonwiriyawong et al., 2009).

In general, spray drying process can be divided into three steps: nebulization of the liquid by the nozzle using energy to form the droplets, drying of droplets in the drying chamber with warm air and then the particles are moved out from the drying air towards the collector vessel. In order to stabilize these particles, carrier or stabilizer might be needed to add prior to spray drying to prevent denaturation of antigens. According to some studies, disaccharides are the most used carrier and it can retain the activity of the labile materials during storage (Corbanie et al., 2007). Although the droplets are exposed to high temperatures during the spray drying process, antigen degradation cannot be occurred, as the period of exposure time is too short. One of the reasons to depend on the spray drying process is that in developing countries there is shortage of functioning refrigerators and poor transportation to maintain the infrastructure of vaccine. Drying in this situation can improve the stability by reducing the molecular mobility and increasing the shelf-life of the product. (Ingvarsson et al., 2013, Sosnik and Seremeta, 2015).

Several carriers are available for spray drying process such as carbohydrates, starch, gums, semisynthetic cellulose derivatives and synthetic polymers. From the technology view, including the carrier during spray drying will reduce the moisture/water content by acting as a barrier to oxygen and preventing the molecules from degradations. Each of these carriers has their own advantages and disadvantages depending on their physical and chemical properties, cost and efficiency. Particle morphology, droplet size, viscosity, density after drying and stability during storage are mostly dependent on the carrier we choose. In this study, maltodextrin (MD) was used as a carrier, which is a soluble modified starch derivative. It can be used alone or in

combination with other carriers. MD is all-purpose carrier with bulking, film forming properties and it can reduce the oxygen permeation through the wall matrix (Sansone et al., 2011). Moreover, it is low cost, neutral aroma and low viscosity with high solid concentration (Carneiro et al., 2013).

Bovine serum albumin (BSA) was used as a model antigen in developing spray dried vaccine adjuvant. The fact is that spray drying of proteins and peptides are risky as they are potentially sensitive to elevated temperatures. However, several studies have proved that after spray drying the surface of droplets has been preserved (Elversson et al., 2003). Proteins that are spray dried can be adsorbed on the surface or encapsulated within the matrix of the carrier. Particularly, addition of carrier in the spray dried formulation will improve the protein stability by minimizing the protein-surface interactions (Elversson and Millqvist-Fureby, 2006). Possibly, BSA stability and activity can depend on the spray drying parameters.

There are several types of administration for immunization including intranasal, intramuscular, subcutaneous, intradermal and oral. These routes depend on composition and immunogenicity. Intramuscular injection is recommended for adjuvant containing vaccines as subcutaneous or intradermal administration can cause local irritation, skin discoloration, inflammation and granuloma formation. Most dried vaccines can be reconstituted to a liquid phase prior to administration. Balasse showed that a long-lasting IgG secondary immune response against spray dried BSA loaded microparticles have been achieved compared to native BSA (Balasse et al., 2008). Moreover, intranasal route can also be used for spray dried powder vaccines which a promising alternative to intramuscular administration these days. This route can reduce the incidence of needle-stick injuries and also prevent the consequent of blood borne diseases. Nasal cavity is also one of the most effective site because of having low enzymatic activity and high availability of immunoactive sites (Lovelyn and Attama, 2011).

As a contrast, in this study we focused to develop spray dried nanoemulsion as a vaccine adjuvant by using BSA, as a model antigen. It is prerequisite that

physiochemical properties of BSA and nanoemulsion adjuvant should remain unchanged upon rehydration. Spray dried powder as well as reconstituted nanoemulsion was investigated for the physicochemical characteristics and stability. Furthermore, integrity of antigen and in vitro cell study was also performed on human monocyte macrophage cell line (CRL-9855) and nasal epithelial cell line (CCL-30).

The aim of this study are:

1. To develop oil-in-water nanoemulsion by high-pressure homogenization technique and load the nanoemulsion with MD and BSA prior to spray drying.
2. To characterize the nanoemulsion, spray dried powder and its reconstitution as well as to optimize these formulations parameters to obtain the smallest particle size with narrow size distribution.
3. To analyze the thermal behavior of spray dried powder as well as the integrity of model antigen.
4. To determine the protein content in spray dried powder, in vitro release study, cell viability, and cellular uptake study.

CHAPTER II
LITERAL REVIEWS

1. Vaccines

Vaccination is an attempt to protect humans against diseases. The routine vaccination of large population has been only become popular during the 20th century. Some of the diseases such as smallpox have been eradicated because of vaccination. Cases of poliomyelitis have also been reduced to 99% throughout the world. Vaccination of many other diseases has made major headway. Conventional vaccines include live attenuated pathogens, whole inactivated organisms, or inactivated toxins (Plotkin and Plotkin, 2013). However, they have achieved to give immune responses, there are several limitation and adverse effects for more challenging diseases. Drawbacks of those live attenuated pathogens are that in immunosuppressed patients, it can convert into a virulent phenotype and as those live pathogens contain reactogenic components that can cause dreadful side effects. In the past few years, several new developments and approaches have come up with remarkable advantages over the conventional vaccines. Recombinant protein subunits, synthetic peptides and plasmid DNA are some new approaches in the vaccine field with lower toxicity but they are poorly immunogenic to administer alone. Despite of having disadvantages, conventional vaccines were heterogeneous and can give some additional T-cell immune response because of itself containing many epitopes with similar function as adjuvants (Singh and O'Hagan, 1999). For that reason, there is a necessity to have adjuvants with potent, safe and compatible with new groups of vaccines.

2. Vaccine immunology

It is a complex challenge to get the vaccine-mediated protection. The protection against diseases was obtained by the induction of antigen-specific antibodies after vaccination. Furthermore, the long-term efficacy needs the persistence of vaccine antibodies and/ or regeneration of memory cells to be capable

of rapid reactivation on microbial exposure. B-lymphocytes are one of effectors that produced the antibodies and have the ability to bind the specific toxin or pathogen. Another potential effectors are cytotoxic CD8+ T lymphocytes that control the diffusing of infectious agents by identifying and killing the cells or by secreting specific antiviral cytokines. T lymphocytes can be then divided into two types T helper1 (Th1) and T helper2 (Th2). These two subsets release several sets of cytokines. Th1 subset secretes IFNϒ that increase phagocyte-mediated defense against infection particularly with intracellular microbes. Th2 subset secretes cytokines, including IL-4 and IL-5, which produce IgE and eosinophil cell-mediated immune responses and down regulation of Th1 response. Differentiation between Th1 and Th2 is controlled by cytokines in the microenvironment at the place of activation. Both of these B-lymphocytes and T lymphocytes are then control by regulatory T cells that maintain immune resistance. The majority of vaccines gave both B and T cell responses. Additionally, one more cell type is CD4+ T cells which is also needed for antibody responses. They enhance macrophage activation and antibody secretion by B cells (Siegrist, 2013). A vaccine should be able to give all these responses by presenting the antigens to antigen-presenting cells (APCs) bound to antigen receptor major histocompatibility complexes (MHC) (Fig. 1.). Vaccines should also contain something other than antigens to activate APCs (Lima et al., 2004).

To obtain these T and B cell responses by activating antigen presenting cells APCs, dendritic cells (DCs) must be recruited into the reaction. The role of mature DCs is to provide both antigen-specific and delivery of extra signals, ‘danger signals’ to T cells needed to activate native T cells. DCs, monocytes, neutrophils offer a set of receptors against preserved pathogens that are not include in self-antigens and identified as danger. Among these sets of receptors, Toll-like receptors play a critical role. These cells become activated when the pathogen encounter. Then, they produce proinflammatory cytokines and chemokines. This leads to extravasation of monocytes, granulocytes and natural killer cells in that inflammatory environment and monocytes differentiate into macrophages and immature dendritic cells become activated. DCs will be then migrated into draining

lymph nodes and come in contact with native T cells, and differentiate into regulatory CD4+ T cells which are responsible for maintaining immune tolerance. If there are no danger signals, DCs will remain immature (Siegrist, 2013).

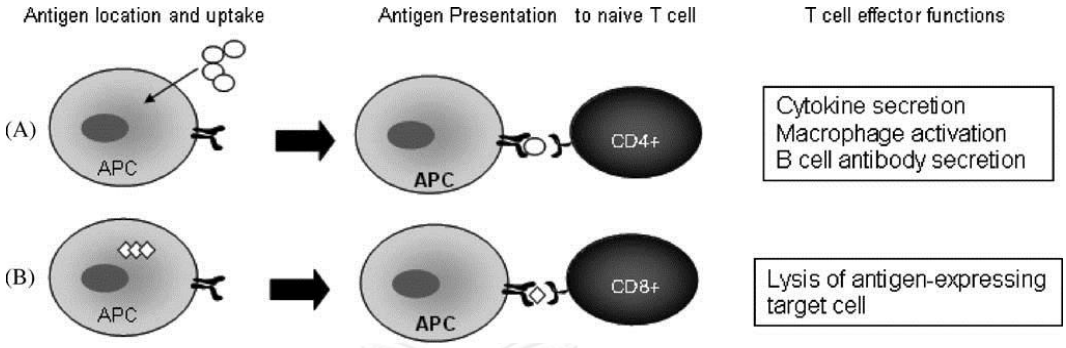


Figure 1 Location of antigen on APCs determines the presentation to different T cells subsets. (A) Extracellular antigens are taken up by APC and present it to CD4+ T cells, which then stimulate lymphocyte for cytokine secretion and activate macrophage for phagocytosis or activate B-lymphocytes to bind the extracellular antigen for elimination. (B) Intracellular antigens will be presented to CD8+ T cells that are able to kill antigen-expressing target cells.

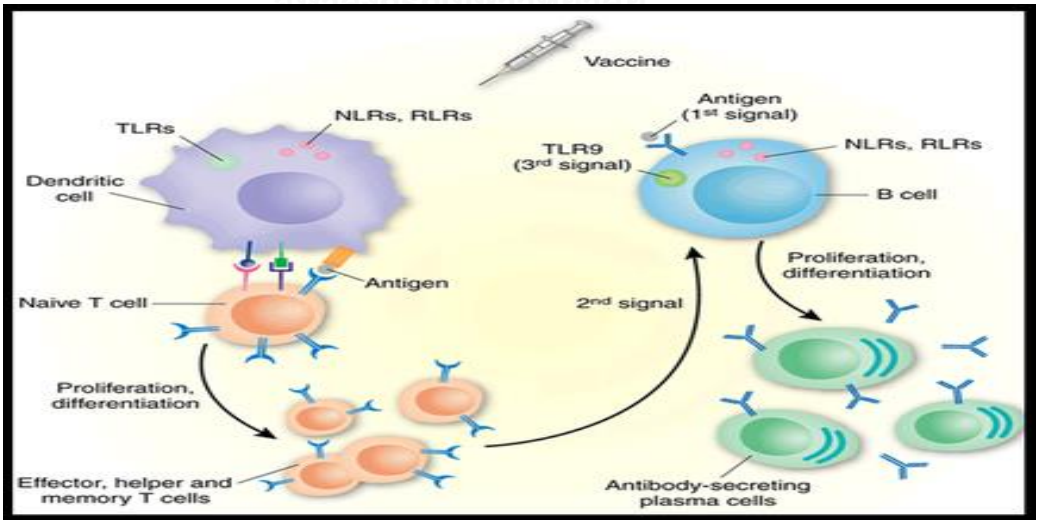


Figure 2 the vaccine response from enhanced vaccine immunogenicity.

3. Vaccine Adjuvants

Adjuvants are agents include into vaccine formulations to enhance the immunogenicity of vaccine antigens. Ramon first discovered the concept by conducting an experiment on horses by injection common substances such as breadcrumbs and tapioca together with diphtheria or tetanus toxoid antigen. He found out that tapioca mixed with antigen gave higher responses compared to antigen given alone. Then, the adjuvant effects of aluminum salts come up with what we called 'alum adjuvants', which are the only adjuvants licensed and widely used in vaccine field (Vogel et al., 1995). Alum however triggers only poor T cell immune responses; better adjuvants with high immune responses and safety might be needed. The adjuvant together with vaccines is also appropriate for newborns, elderly or immune-compromised individuals providing long-lasting immune responses. Adjuvants have limited or no efficacy when they are not properly formulated. The compositions of adjuvant e.g. particle size, charge, etc. are important for its efficacy (Reed et al., 2009).

3.1 Mechanism of action of vaccine adjuvants

Although adjuvants are widely used in both human and animal vaccines, the mechanisms of action by which they provide immune responses are not yet been defined well. In recent years, the development in immunological research has opened up with some mechanism of adjuvants. These following mechanisms are; (1) sustained release of antigen at the site of injection (depot effect), (2) cellular recruitment at the site, (3) increase the production of cytokines and chemokines, (4) enhance antigen uptake and presentation of APCs, (5) activation and maturation of APCs which then increased co-stimulatory molecules expression such as DCs and migrate into the draining lymph nodes, and (6) activation of inflammasome (Awate et al., 2013).

3.1.1 Depot effect at the site of injection

Some adjuvant provides a depot effect at the site injection by slow release of antigen. Adjuvant such as water-in-oil emulsion (Complete Freunds Adjuvants (CFA) and biodegradable micro-and nanoparticles have proved to give depot effect. However, there is no clear evidence that depot effect is needed for adjuvant activity (Awate et al., 2013).

3.1.2 Cellular recruitment by secretion of cytokines and chemokines at the site of injection

Recruitment of innate immune cells at the injection site has been discussed in recent studies. With the help of genome wide microarray analysis, it has been shown that genes encoding cytokines, chemokines, innate immune receptors and interferon-induced genes were enhanced by alum, MF59, and CpG-ODN at the site of injection. Among them, MF59 was a strong adjuvant, which regulated the adjuvant core response genes. Alum recruits cells from blood by modulating complement cascade and building an inflammatory environment (Seubert et al., 2008, Awate et al., 2013). Therefore, most of immune cells can be recruited at the site of injection by the induction of adjuvant and it will migrate the antigen into the draining lymph nodes to give specific immune responses.

3.1.3 Effects of adjuvants on antigen presentation

A proper antigen presentation by MHCs on APCs is crucial step to obtain the adaptive immunity. It has been shown that alum increase the antigen uptake by DCs and change the significance and span of antigen presentation by minimizing the breakdown of internalized antigen. Another study has point out that adsorption of alum on antigen has improved the antigen uptake by the immune cells. MF59 also recruits the immune cells and increases the phagocytosis by internalization of gD2 antigen from type2 herpes simplex virus (HSV) after

intramuscular injection (Dupuis et al., 1999). Antigen size also seems to be a part of antigen presentation efficiency (Brewer et al., 2004, Awate et al., 2013).

3.1.4 Maturation of DCs

Activation of DCs is concerned with the enhancement of adaptive immunity by induction of the MHC II expression, which then leads to higher activity of APCs and increases T lymphocyte activation and differentiation (Coyle and Gutierrez-Ramos, 2001) (Fig.3.). Freund's complete adjuvant, MF59, alum, liposomes, lipopolysaccharide (LPS), AS04, all have shown to modulate the maturation of DCs and increase the adaptive immunity (Awate et al., 2013). However, microparticles with PLGA on bone marrow derived DCs (BMDCs) did not activate co-stimulatory molecules expression but increase the antigen presentation by some other way (Sun et al., 2003).

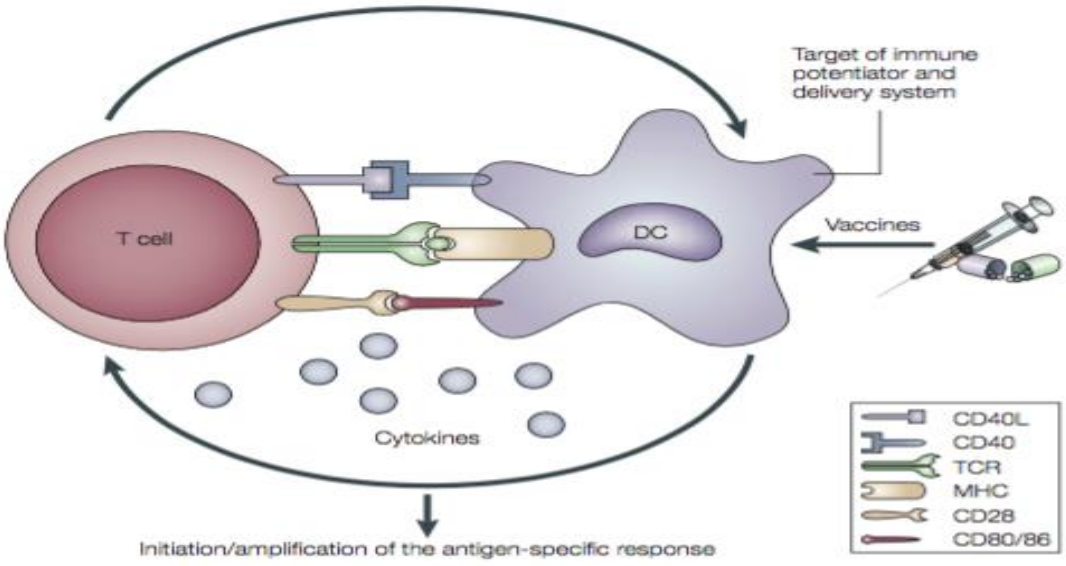


Figure 3 The intimate reaction between APCs and T cells (O'Hagan and Valiante, 2003).

3.1.5 Activation of inflammasome

Inflammasome has become one of the interesting topics when it comes to adjuvant activity. Particulate adjuvant can damage the cells at the site of injection, which then build the proinflammatory environment. These damage cells gave the danger signal and activate the immune system. Inflammasomes are from NLR family, consisting of many other receptors, NODs (NOD1-5), NLRPs (NLRP1-14), NLRP1 (NAIP), NLRC4 (IPAF), and the major histocompatibility complex II trans activator (CIITA) (Martinon et al., 2009). Among them pyrin-domain-containing 3 (NLRP3) from NOD family has been studied widely for adjuvant mechanisms concern with inflammasome receptor. Several studies have showed that alum increases the expression of NLRP3 for IL1β and IL18 secretion. Damage cells at the site of injection also activate the NLRP3 indirectly. But in the case of MF59, one study on NLRP3 deficient mice proved that NLRP3 was not needed for MF59 adjuvant activity. Therefore, we can conclude that adjuvants use various different pathways to give protective immunity with complex mechanisms (Awate et al., 2013).

It is important to understand the mechanism of adjuvant in the vaccine development. The future of vaccine adjuvant is leading toward with enhance quality and quantity of immune response against various antigens. Safety is one of the most important things we should not ignore in developing vaccine adjuvants. Several adverse reactions such as pain at the injection site, malaise, and fever have been reported in clinical trials. So, if we can understand the mechanisms of adjuvants, we can be aware of their safety. Since, most of the adjuvants approved right now are administered via injection, there is a necessity to design and established a good mucosal adjuvant.

3.2 Classification of vaccine adjuvants

Adjuvants can be categorized depending on physicochemical properties or their mechanisms of action. First they can be divided according to their properties to modulate Th1 or Th2 immunity. Or they can also be divided into their capacity to give innate or adaptive immunity (Marciani, 2003). Secondly, they can be classified according to types of vehicles that present vaccine antigens to immune system and it can also give the immunostimulant action (Reed et al., 2013). These vehicles include (1) mineral salts, (2) polymeric microparticles adjvuants, (3) immune stimulation complexes (ISCOM), (4) bacterial derivatives, (5) carbohydrate adjuvants, (6) liposomes, (7) cytokines, (8) virus like particles (VLP), and (9) oil emulsions (Sivakumar et al., 2011).

3.2.1 Mineral salts

Mineral salts such as aluminum containing adjuvants and calcium phosphate are historically used as immunopotentiators in vaccines and they are still widely used. Aluminum compounds include aluminum hydroxide adjuvant, aluminum phosphate adjuvant and alum, all of them are scientific misnomers. Aluminum hydroxide shows higher immune response and adsorption to antigen than aluminum phosphate. Since last decades, a bunch of developments has been done on adjuvant and never been approved for human vaccines because of their toxicity. One downside of alum is that adsorption of antigen depends on the physicochemical characteristics of antigen as this mechanism is occurred by electrostatic attraction, hydrophobic forces, and ligand exchange. The antigen is then released slowly to produce antibodies at the site of injection. The inflammation at the site of injection gathers APCs, which are taken up by phagocytosis. If some antigens remained adsorbed on the alum, it will be then taken into macrophages and dendritic cells and lead to immunopoteintiation effects (Vogel, 2000). But the response obtained from alum is inadequate to produce cytotoxic T lymphocyte activity. There is also some other obstacles concern with alum such as local production of erythema, granuloma and subcutaneous nodules (Sivakumar et al.,

2011). Moreover, alum cannot be frozen or lyophilized (Baylor et al., 2002). Alum activates the eosinophil that initiates the IgE mediated allergic reactions at the site of injections. Although alum is safe and has been used for over 90 years, it is not compatible for small proteins and it needs many boosters dose to obtain optimal immune response.

3.2.2 Polymeric microparticles adjuvants

Biodegradable polymers have been used as sutures and drug carrier for a long time because of its nontoxic and biocompatible nature. They have been widely used in vaccine development in recent years as they are compatible with antigen and have a good permeability and low cost. This type of adjuvant is preferred for control release of antigen and they act as a delivery system. These microparticles have the size range of 1 to 1000 μm and are solid, spherical shape. This kind of controlled vaccine delivery enhances the immunity without giving any inflammation response. Development of tetanus toxoid with PLGA microspheres with suitable stabilizers gave a long lasting release of antigen up to 5 weeks and the antibody titers was higher than the commercial tetanus toxoid vaccine with alum (Sánchez et al., 1999). Another biodegradable polymer is chitosan which is popular because of it high charge density and non-toxicity with potential to use in pharmaceutical applications. It is widely studied as drug delivery system of a large variety of drugs such antibiotics, anti-cancer agents, proteins and vaccines. Chitosan loaded with tetanus toxoid enhance both systemic and local immune response after oral and nasal administration (van der Lubben et al., 2003). So, it has been proved that chitosan can be applied for nasal delivery with various antigens. PLA and PLGA polymer can also be used to improve stability, parenteral and mucosal administration of antigens. Although, the release rate is depend on particle size, porosity of microparticles, molecular weight, loading capacity, cross-linking and the use of proper stabilizing excipients.

3.2.3 Immune stimulating complexes (ISCOMs)

These types of adjuvants contain triterpinod sapnonin such as Quil A, phospholipid and cholesterol. It induces higher cellular immune response by inducing cytokines secretion and acting directly via macrophages. However, Quil A or its pure crude extract QS-21 gives toxicity effects in humans such as haemolysis, granulomas and severe pain at the site of injection (Dalsgaard, 1978, Sivakumar et al., 2011).

3.2.4 Bacterial derivatives

Peptidoglycan or lipopolysaccharides (LPS) are bacterial cell wall components that increase the immune response via modulation of Toll-like receptors (TLR). These lipopolysaccharides include lipid A that is a potent mucosal adjuvant but highly toxic for human use. AS04 is a vaccine adjuvant, approved for human use recently after MF59 includes aluminum hydroxide or aluminum phosphate and lipid A from LPS of gram-negative *Salmonella Minnesota* R595 and purified by mild hydrolytic treatment. AS04 is used as vaccine adjuvant in Cerevarix, a vaccine against human papilloma virus, which is a formative agent of cervical cancer. This adjuvant provides high level of antibody titers with long lasting immune responses by activation of natural killer cells, induction of t cytokine production, optimal antigen presentation and modulation of high Th1 response. Drawbacks of this type adjuvant are reactogenicity, high cost, and problems on consistency of vaccine preparation from bacteria.

3.2.5 Carbohydrate adjuvants

Gamma-inulin obtained from plant root is one of the complex carbohydrates, which proves both humoral and cellular immunity. It activates the macrophages by modulation the complement pathway. This type of adjuvant can be used together with other adjuvants such as algammulin, which is a combination of gamma-inulin and aluminum hydroxide to give higher immune responses of both

Th1 and Th2 (Sivakumar et al., 2011). As it is the carbohydrates, it does not have any side effects concern with alum based adjuvants and can be metabolized facilely into sucrose, glucose and fructose. Other studies have shown that polysaccharide consisting of dextrans, glucomannons, galactomannans, levans, xylans and glucans also possess the activity to induce immune response (Tizard et al., 1988).

3.2.6 Liposomes

Liposomes are composed of synthetic lipid layers that can encapsulate the antigen and served as adjvuants. Hepatitis A vaccine with liposomes has been approved in Europe. Liposomes allow the antigen to get inside the cytoplasma by fusing with cell membrane of macrophages, induct the MHC class I pathway and activate CD8 response (Owais and Gupta, 2000). However, the efficacy depends on the electric charge, composition, and number of lipid layers and method of preparation. It is high cost, poor stability, difficult to manufacture and severe pain at the site of injection (Sivakumar et al., 2011).

3.2.7 Cytokines

Cytokines are soluble proteins with low molecular weight such as IL-2, IFNα and IFNϒ. They control the innate and adaptive immunity with response to antigens. IL12, which is an immunomodulatory cytokine, plays an important role in microbial adjuvants. In one study, immunization of BALB/c mice with IL12 and *Leishmania major* antigens induce both CD4+ Th1 and Th2 responses while antigen alone induce only Th2 activity. Furthermore, clinical study of this vaccine has proved that even 1-4 μg of dose can give fever and flu like symptoms in humans (Scott and Trinchieri, 1997). Cytokines can only be applied in cancer vaccines because of its high toxicity (Sivakumar et al., 2011).

3.2.8 Virus like particles (VLPs)

VLPs are inert, virus shape particles with nothing inside its structure. The desired antigen will be then attached to the particles by genetic engineering technology. These particles will be then presented the antigen to the dendritic cells and enhance the immune response. *Saccharomyces cerevisiae* attached to VLPs has been formulated for recombinant hepatitis B surface antigen (Sivakumar et al., 2011). Moreover, in recent year, human papilloma virus vaccine was approved for clinical use by the production in VLPs design (Aguilar and Rodriguez, 2007).

3.2.9 Emulsions

Oil in water or water in oil emulsions, mostly nano-sized emulsions belongs to this type of adjuvant. Emulsions have been used widely in pharmaceutical field as promising drug delivery system for hydrophobic substances. Freund's complete adjuvant is water in oil type of emulsion consists of paraffin and mixed with killed Mycobacteria. It gives depot effect and slow release of antigen however antibodies production is not sufficient enough to provide immunomodulatory effect. Because of its local irritation and granuloma formation, it is not used currently (Sivakumar et al., 2011). MF59, which is oil in water nanoemulsion containing squalene, has been accepted to use in human after alum adjuvants. It has a high stimulus adjuvant and has a good safety records in clinical trials. It is used in seasonal influenza vaccine and cleared from the injection site independently. The mechanism of MF59 is only partly understood, but it has been shown that it doesn't activate dendritic cells directly, which is primarily important for adaptive immunity. Instead of dendritic cells, it attracts the monocytes, neutrophils and granulocytes, which then modulate the secretion of chemokines and lead to recruitment of immune cells at the injection site. MF59 is even more potent and have strong immune response compare to alum and any other adjuvants ever evaluated (Calabro et al., 2011). Another oil in water emulsion is AS03 that also contain squalene but in addition it consists of alpha tocopherol and modulate high immune response similar to MF59. It increases the production of cytokines, chemokines and directly activates the innate immunity in

draining lymph node, due to the presence of potentiator alpha tocopherol. Possibly, these are not solely delivery system and squalene can be the reason to enhance immune responses. Montanide^{TM} is a large family of oil in water and water in oil emulsions consists of ISA 50V, 51, 201, 206 and 720. ISA 51 and 720 have been applied in malaria vaccines while Montanide 206 and 720 are applied in food-and-mouth disease vaccine (Zhao et al., 2014).

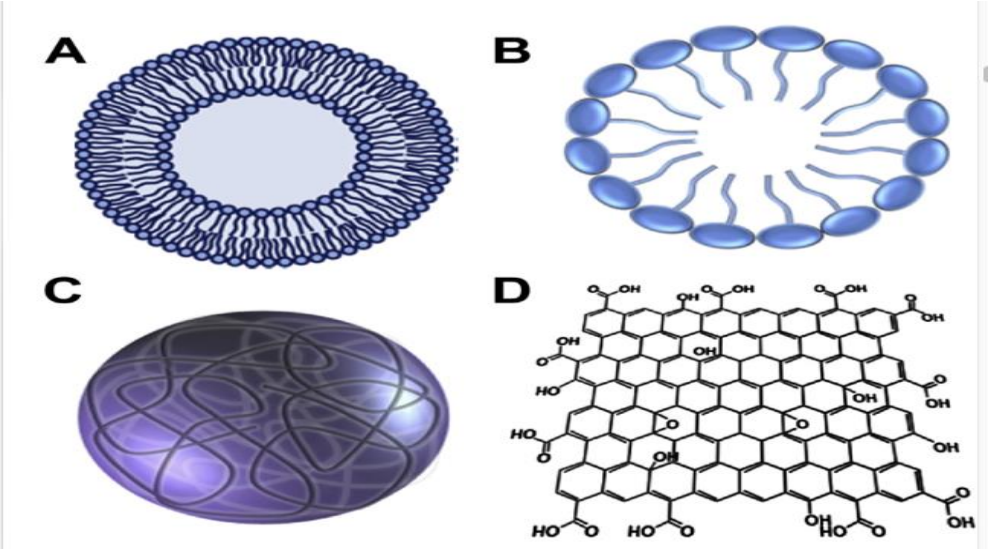


Figure 4 Structure of nanocarriers for antigen as vaccine adjuvant (A) Liposomes, (B) emulsions, (C) Polymeric nanoparticles, and (D) Graphene oxide nanosheets (Kim et al., 2014).

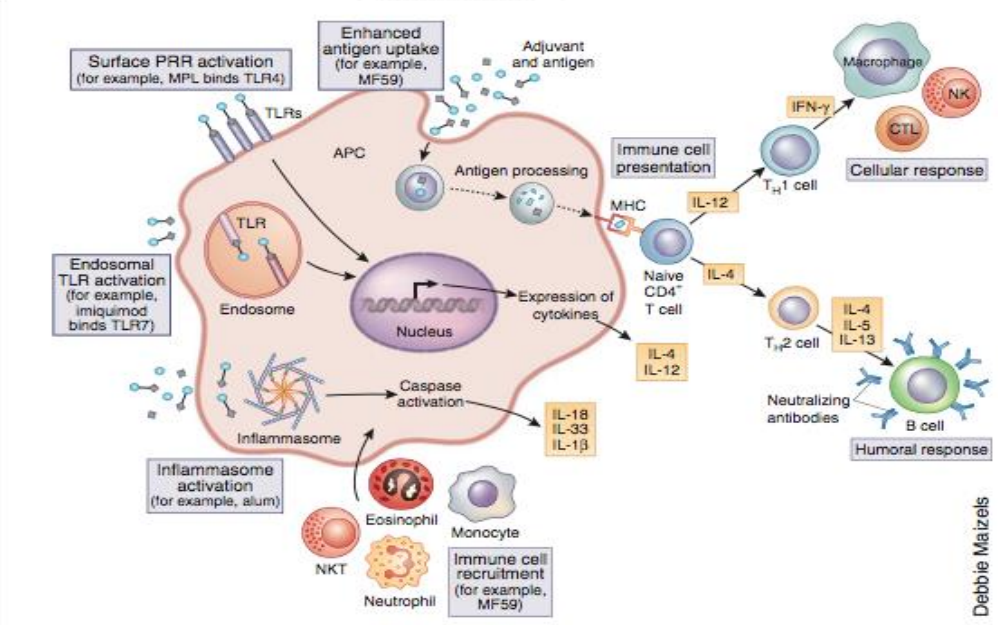


Figure 5 Mechanism of action of different types of adjuvants.

4. Nanoemulsion

With the progress in pharmaceutical technology, dosage forms has become simple system of pills and mixtures to highly classic systems. Nanoemulsions are novel drug delivery system composed of oil and aqueous phase, with the core of particle either oil or water. Natural vegetable oil, synthetic or semi-synthetic oil can be used in nanoemulsion. Nanoemulsion has been widely used for hydrophobic drugs to improve their bioavailability, solubility and protection from enzymatic degradation. It also used as controlled drug release system. Nanoemulsion can be formulated by high energy and low-energy methods. Both methods have been reported to give stable systems and it didn't damage human cells with a good safety profile (Chime et al., 2014).

Surfactants can be of three types; zwitterion, anionic and cationic. A handful of surfactants are Tween80, Tween40, Tween60, Labrasol, Cremophor EL, Cremophor RH40, Poloxamer 124, Poloxamer 188 and Labrafil. Toxicity is also co related with composition of surfactants. Higher amount surfactant can irritate the gastrointestinal lining and skin after oral and topical administration (Thiagarajan, 2011). Therefore, it is important to pick up the correct composition of surfactants to avoid the adverse effects and enhance the stability. Non-ionic surfactants are a good choice as they are less toxic and it is less affected by pH and ionic strength variation however ionic surfactants has some issues with toxicity. Co surfactants usually added to obtain the nanoemulsion to improve the stability, interfacial tension and fluidity. Nanoemulsion in combination with co surfactants can permit the oil to penetrate better. A widely used co surfactants include transcutol, isopropyl alcohol, n-butanol, PEG400, glycerol, propylene glycol, and carbitol (Tenjarla, 1999).

4.1 Methods to prepare nanoemulsions

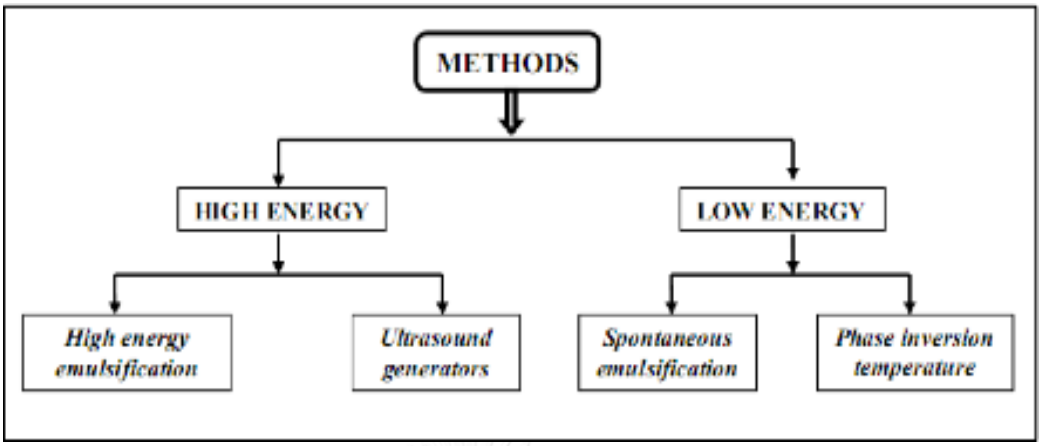


Figure 6 Methods for the preparation of nanoemulsion (Sharma et al., 2010).

4.1.1 High-pressure homogenization method

This technique used the high-pressure homogenizer to produce nanoemulsion with the particle size of up to 1 nm. The mixture of organic phase and aqueous phase are disperse into the inlet container and it will then force the mixture to pass through the small inlet orifice using high pressure form 500 to 5000 psi. With the help of hydraulic shear and extreme turbulence, the small particle size will be obtained. The solution can be put back into the homogenizer as another cycle until the desire particle size and polydispersity index is optimized. This method is widely use for the production of nanoemulsion because of it good proficiency (Rajalakshmi et al., 2011). This type of method can also be scale up to large volumes (Fryd and Mason, 2012). Very high phase volume can lead to coalescence therefore more surfactant can be added to lower the surface tension and smaller particle size will be achieved. It is better to use combination of two or more surfactant to lower the surface tension (Lovelyn and Attama, 2011).

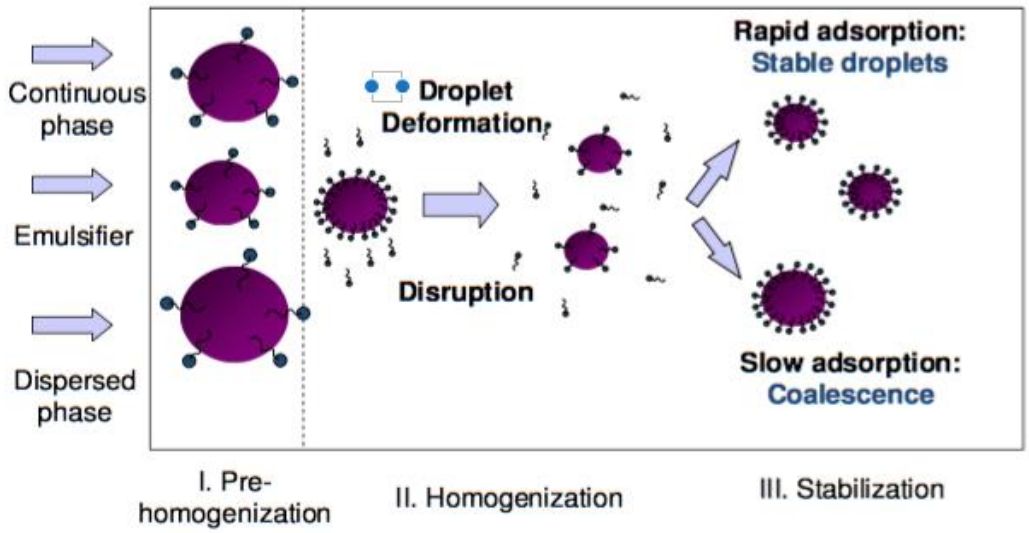


Figure 7 Physicochemical properties of particles undergoing high-pressure homogenization

4.1.2 Microfluidization

Microfluidization is another technique to produce nanoemulsion by using high pressure. A pressure of around 20,000 psi can be applied via the pump. The liquid containing macrosize droplet will pass through the interaction chamber then into the micro channels. Using a high velocity to pass through the micro channels on to the impigmented area lead to the smaller particle size. Like high-pressure homogenizer, variation in the pressure and number of cycles can be optimized to obtain the desired particle size (Chime et al., 2014).

4.1.3 Ultrasonic generators

A probe in ultrasonic generators release ultrasonic waves of 20Hz or higher for the reduction of particle size using cavitation forces. Various in the ultrasonic wave's energy and time can be used to maintain the particle size of your choice. This kind of method can only use as laboratory scale (Sharma et al., 2010, Chime et al., 2014).

4.1.4 Spontaneous emulsification

Store energy inside the system is typically used to form nanoemulsion spontaneously. It is consists of three steps to obtain the nanoemulsion. Firstly, the organic phase containing lipophilic surfactant and the aqueous phase containing hydrophilic surfactant was prepared. Secondly, the organic phase will be added into the aqueous phase under magnetic stirring. The organic phase containing oil will diffuse instantly into the outer side of the aqueous forming the nanodroplets. The magnetic stirring will be maintained for a period of time to obtain the homogenous equilibrated system. At last, water miscible solvent in the aqueous phase will be evaporated for around 45 minutes under the reduce pressure to achieve the droplets disperse in the oil and aqueous phase (Fryd and Mason, 2012).

4.1.5 Phase inversion temperature (PIT)

This method is also called condensation method and it is achieved by changing the curvature of surfactants spontaneously in response to temperature. Nonionic surfactant, polyethoxylated surfactants are widely used because of its properties of having affinities towards both water and oil. When heating this surfactants, it loss it polyoxyethylene group and become lipophilic. All the compositions containing oil, water and surfactants are mixed together at room temperature and heated slowly. At the higher temperature, surfactant will be dissolved in oil phase and phase inversion occurs with very low surface tension. Nanoemulsion then obtained either by cooling down with single phase/ multiphase microemulsion maintain at PIT or diluting with single bicontinous microemulsion with aqueous or oil phase immediately (Solè et al., 2010). HLB of the surfactant is dependent on the formation of nanoemulsion. A drawback is that polyethoxylated surfactants is needed to obtain nanoemulsion and is a complicated method (Chime et al., 2014).

4.2 Advantages and major challenges in nanoemulsion

There is no doubt that nanoemulsion has gained attraction in pharmaceutical as well as in cosmetics because of these following advantages (Lovelyn and Attama, 2011, Chime et al., 2014).

(1) The Brownian motion is sufficient to overcome the gravity caused by small particle size, which eventually has a huge reduction in gravity force. So, no creaming or sedimentation can be observed.

(2) As flocculation is prevented by small droplet size, it makes the system more stable and no separation occurs.

(3) No coalescence is observed as nanoemulsion droplets have elastic properties.

(4) It has been applied as delivery system containing many ingredients inside the system and having the large surface area of droplets gave the efficient penetration.

(5) For the topical use, a suitable oil composition will give the good fluidity, which is pleasant feel on the skin.

(6) Unlike microemulsion, nanoemulsion doesn't need a high amount of surfactants and the surfactants used in nanoemulsion are generally regarded as safe for humans.

(7) Low interfacial tension permits the droplets to deposit uniformly on substrates and wetting, spreading, and penetration all are enhanced.

(8) Nanoemulsion can even be used in the preparation of perfumes without the use of alcohols.

(9) It can also be used in replacement of liposomes and vesicles, which are not that stable as nanoemulsion.

(10) It can be formulated in several dosage forms comprising creams, liquids, sprays and foams. Therefore, various

routes such as intravenous, oral, topical or even mucosal administration can be done.

(11) It optimized the stability of active ingredients inside their core by protecting them from enzymatic degradation and oxidation.

The major challenges to overcome include:

(1) Nanoemulsion production demand special implementation methods for example, use of high-pressure homogenizer, microfluidizer and ultrasonics.

(2) If we use large amount emulsifier, expensive equipment may be needed and it makes the nanoemulsion expensive.

(3) The mechanism of achieving nanosized droplets and the act of surfactant and co surfactants in the formulation are not completely understood.

(4) Stability of nanoemulsion is depend on environmental condition including pH, temperature etc. Moreover, Ostwald ripening can also be a big issue, which occurs due to high curvature of small droplets having good solubility compared to large droplets having low radius curvature (Bouchemal et al., 2004, Devarajan and Ravichandran, 2011, Lovelyn and Attama, 2011, Sharma et al., 2010).

4.3 Nanoemulsion as delivery system

Primarily, nanoemulsion has become a promising delivery system in various fields such as cancer therapy, vaccine delivery, transdermal delivery for cosmetics, intranasal drug delivery, in cell culture technology, ocular and otic drug delivery, gene delivery rector, as non-toxic disinfectant cleaner, for oral delivery

of poorly soluble drugs and it also used as phytopharmaceuticals these days. Nanoemulsion in parenteral delivery system, it is cleared slowly compared to other coarse microemulsion and so it can stay in the body longer and gave the higher efficiency. In ocular and oral delivery, nanoemulsion has high absorption and long-lasting action for poorly soluble drugs (Devarajan and Ravichandran, 2011). After parenteral and oral, intranasal route is one of the most popular routes of administration these days. This route is painless and noninvasive. The systemic drugs can reached the targeted site effectively. Moreover, it is also used as administration of vaccines and immunity is obtained by mucosal antigen (Chime et al., 2014).

5. Drying techniques for liquid pharmaceuticals and proteins

A variety of techniques are available to dry liquid formulations including vaccines to obtain powders with optimized characteristics. Freeze drying is the most widely used techniques in vaccine production but these days several research are undergoing using spray drying method. Another method is supercritical fluid (SCF) drying, which is just become known as alternative.

5.1 Freeze drying

Freeze drying, which is also known as lyophilization includes three steps of drying. It is not only used in vaccines but also in drying of proteins, peptides, liposomes, nanoemulsions, and nanoparticles. But it is a slow and expensive process. As a first step of this process, freezing is done followed by primary drying (ice sublimation) and secondary drying. In the first step of freezing, the liquid formulation is cooled. As the cooling continues, the concentration of the suspension increases and the viscosity increase as well leading to prevention of crystallization. When this liquid solidifies, the formation of amorphous, crystalline or amorphous-crystalline phase occurs. Primary drying step is a second step in freeze drying process in which heat is transmitted to the frozen solution via the tray or vial. Then, the ice sublimes and the vapor is permit to the surface of the sample through the

dried part and from the surface, it is conveyed to the condenser via the chamber. A porous plug will be obtained at the end of this primary drying step. The pores are conferred as the ice crystals. The last step is the secondary drying and it is time to remove the absorb water from the formulation. This water is neither ice during freezing nor water that sublimates off. A freeze dryer comprised of the drying chamber with temperature controlled shelved connected to a condenser chamber through a valve. A series of chambers attached to the condenser chamber and they are preserved at low temperature less than -50C and the pressure can be varied from 4 to 40 Pa during freeze drying. To obtain a good formulation with improve stability, biological activity and safety, it is prerequisite to use the right compositions. The concentration and type of cryoprotectant, the surfactant used and the chemical groups attached to the nanoparticles surface or the polymer used should be able to resist the freeze drying condition (Abdelwahed et al., 2006). Freezing dried products can be used in different administration routes. Lyophilized nanoparticles can be used as oral dosage form of indomethacin-loaded nanocapsules (De Chasteigner et al., 1995). Freeze drying of proteins have some destabilizing effects. Cold denaturation of proteins is due to decrease in hydrophobic effects and nonpolar residues hydration, even this cold denaturation process is too slow to unfold at the time of freeze drying operation (Maltesen and Van De Weert, 2008). The buffer system on freeze drying can crystallize some of its components and causes the change in pH. Sodium phosphate buffer upon freeze drying causes the pH to drop four units. So, the ionic strength can also be varied upon free drying, which can lead to protein instability. Moreover, cooling rate also have impact n crystal formation of water, slow cooling rate leads to obtain larger crystals while rapid cooling achieved smaller crystals with a higher specific surface area (SSA). A large SSA will cause more protein to adsorb on the surface and induce protein denaturation and aggregation (Bhatnagar et al., 2007). Although freezing drying has been used for the manufacturing of protein dry powder and vaccines for parenteral administration, it is the cake formation instead of powders. Therefore, spray freeze drying become an alternative to solve this issue. In this process, the formulation will undergo the spray drying with cryogenic medium and then the primary and secondary drying will be done. One downside is that it is difficult to control the

freeze drying conditions to some extent because of having larger SSA and direct contact with freezing medium (Maltesen and Van De Weert, 2008).

5.2 Supercritical fluid drying

It is one of the new drying methods in pharmaceutical field and it is usually done on laboratory equipment. This process is relied on the anti-solvent properties of a SCF for protein, leads to protein precipitation and water extraction from the formulation. SCF is a single phase with the characteristics of both liquid and gas and the temperature and pressure above their critical points. The density of an SCF is depends on the pressure and it is correlated with solvation power. It means that even a little change in pressure will the change the solvation power immediately. Various SCFs has different temperature and pressure values and it is advisable to apply the exact value on each specific one. Supercritical carbon dioxide is usually applied as it has low critical temperature in response to water and FDA accepts it as safe. Using the carbon dioxide in the solution with protein can lead the reduction of pH if it is not buffered precisely. There are two methods in order to dry the protein by SCF. The first method is SCF is used as drying gas instead of nitrogen in spray drying and drying occurs by the precipitation of protein in the droplets. In this case, SCF loses solvent power and water is extracted to the SCF during drying obtaining the amorphous matrix. In the second method, SCF is dissolved with protein solution and spray dried. SCF increase the atomization of the protein in this case as the drying gas is nitrogen (Maltesen and Van De Weert, 2008). There are also stresses available on the stability of protein like the other methods, however they are not well understood. No advantages over spray drying have been observed so far and it doesn't provide any economic benefits as well.

5.3 Spray drying

Spray drying is applied widely in different industries including food, chemical, biochemical and pharmaceuticals. It is the one step process of converting the liquid into fine powder. There are four stages in spray drying process; (1)

atomization of liquid (2) spray-air contact (3) drying of droplets (4) separation of dried droplets from the drying gas. Fig. 8. Atomization of liquid is depending on several nozzles designs such as rotary atomization, pressure atomization or two-fluid (pneumatic) atomization. The feed liquid is passed into the drying chamber by means of spinning disc or wheel in rotary atomize and in case of pressure atomization, the liquid is passed under pressure and dispersed as it leaves the nozzle. In two fluid nozzles, the feed solution and the atomization air enter from different nozzle, mixed up at the tip of nozzle and break up into a spray. This type of nozzle is widely used in laboratory scale such as Buchi spray dryer 190 (Broadhead et al., 1992).

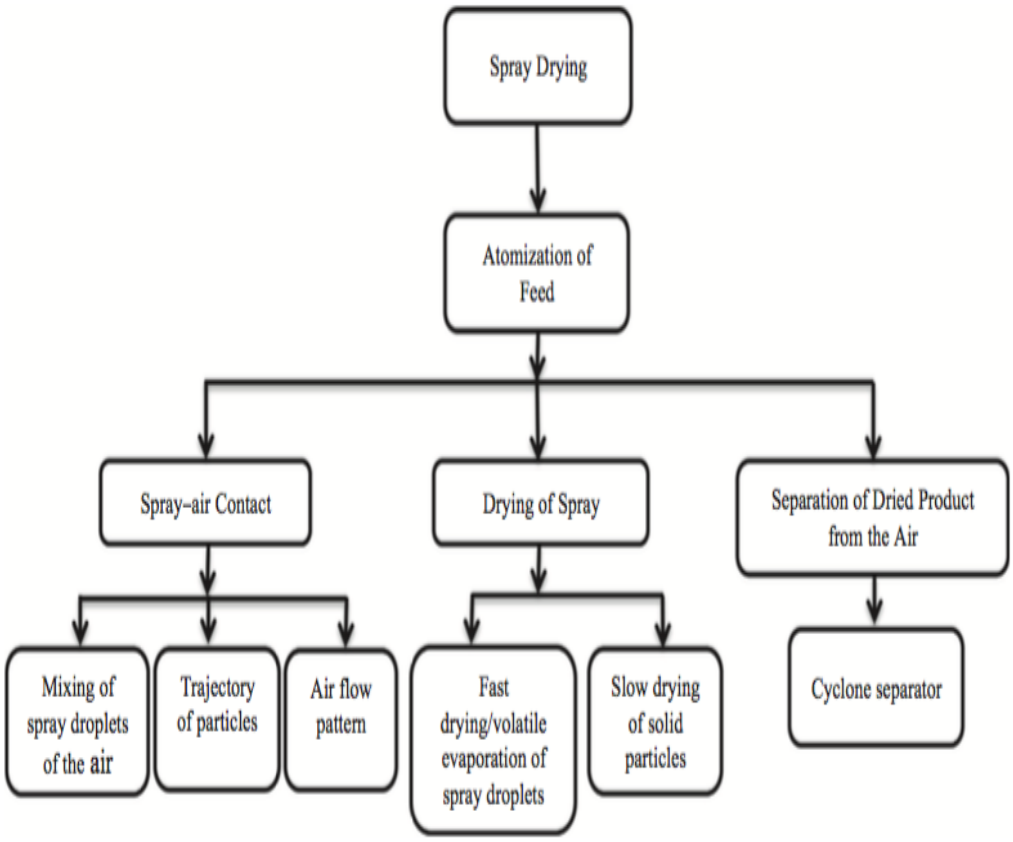


Figure 8 Schematic diagram of spray drying process (Keshani et al., 2015)

Spray dryers work in a co-current manner, with the spray and drying air enters the chamber in the same direction or in a counter current manner where spray and drying air enter from the opposite ends. Co –current is suitable for heat sensitive

materials, as the droplets will come in contact with coolest air. Finally, separation of droplets from the chamber is accomplished by cyclone separator in which product will get in after the drying chamber and there is a collector at the end.

Spray dried powders are spherical in shape and often hollow with a narrow size distribution. Fig. 9. Having a spherical shape indicates that they are free-flowing and hollow nature means a low bulk density to the powders. By optimizing the spray drying conditions, it is easy to change the properties of powder, appearance, particle size, size distribution, bulk density, particle density, porosity, moisture content, flowability, stability, dispersability, friability, and retention of activity as well as smell and taste.

5.3.1 Factors influencing on the spray drying of proteins and pharmaceuticals

Particle size will be increase in response to higher concentration of feed solution and feed rate. Temperature is depends on material to be spray dried. Generally, small particles are dense so the bulk density of it will be higher and as bulk density increases narrow size distribution will be obtained (Broadhead et al., 1992). Investigation on effect of protein types and low molecular weigh surfactants using sucrose and sodium caseinate and pea protein isolate (PPI) as model proteins showed that the amount of protein required to spray dried protein-sucrose solution is depends on the amount of protein available on the surface of the droplets. Its not actually depends on bulk concentrations. Another thing is stickiness, which is due to hygroscopicity of non-crystalline sugars and their thermoplasticity. Maltodextrin, which is usually used as a carrier in spray drying reduce the thermoplasticity and hygroscopicity, which then leads to the reduction of stickiness(Roos, 2009, Keshani et al., 2015). (Woo et al., 2008) has evaluated that lactose and protein together on the surface of spray dried droplets make the surface more rigid in response to high glass transition point.

In spray drying of protein or peptides, there can be of three stress, heat

stress, mechanical stress and adsorption of air-water interface during the operation. The temperature of protein denaturation depend the water content in the formulation. The temperature at which protein denatured increases as the water evaporates during the process. However, thermal denaturation of protein hardly observed because the surface temperature of droplets were maintained at the wet bulb temperature, which is quiet lower than the drying temperature. The mechanical stress here is not a big issue of stability but when it happens together with air-water interface adsorption, aggregation of protein will be seen. Adsorption on air-water interface will change the conformation of protein by exposing the hydrophobic residues and interactions between these residues are the main reason for aggregation.

The complete control over the particle size by varying the parameters and the choice of carrier make the spray drying a great attraction towards vaccines. Spray drying has taken over in pulmonary and nasal administration fields to deliver proteins (Maltesen and Van De Weert, 2008). Therefore, a lot of research has been undergoing on spray drying to obtain better stable formulation as alternative to freeze-drying.

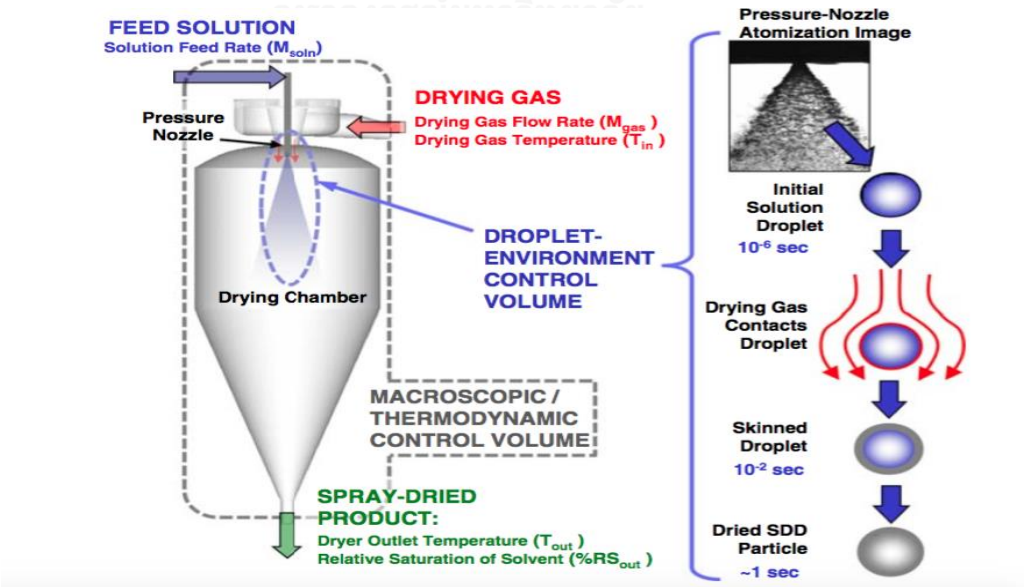


Figure 9 Physical situation of particle during the spray drying process

6. Applications of spray drying

6.1 Excipient manufacturing

Spray dried has been used mainly as the manufacturing of pharmaceutical excipients such as spray dried lactose concentrate. Spray drying of lactose made it directly compressible compared to traditional manufacturing of lactose. Dicalcium phosphate was also spray dried with a saturated lactose solution as a suspension and the amorphous lactose was coated around the dicalcium phosphate crystals were achieved. The product obtained can increase the crushing strength of tablet compared to using the physical mixtures of dicalcium phosphate and lactose. This shows that the amorphous lactose act as a binder for crystalline material.

6.2 Microencapsulation

A microcapsule can be either single coated solid particle or liquid droplet or a matrix with many small fine particles gathers together with wall material. Single coated solid particle can be manufactured by coacervation technique, coating and interfacial tension methods. Spray drying or spray congealing can be used to prepare matrix microcapsules. The vehicle to obtain microcapsules is easily spray dried in a single step. Vitamins A and D, oil soluble vitamins are microencapsulated by spray drying in an emulsion form together with gum Arabic or gelatin solution. Spray drying is also used to microencapsulate a variety of aromatic oils. Moreover, spray drying has also been applied in the production of polymer-coated microcapsules as the aim to cover undesirable taste. This process of microencapsulation has also been used to stabilize volatile compounds. Several binders were investigated for this purpose, including gum arabic, polyvinyl alcohol (PVA), carboxymethylcellulose (CMC), methycellulose and tragacanth. Some researchers have shown that increasing in the solid feed concentration has increased the retention of the volatile components. This theory is that increasing the viscosity slows the mobility of volatiles to the surface of the particle and reduces the internal mixing and loss of volatiles is reduced as well. But increase the viscosity to certain limit can also decrease the retention because of long

resident time and there can be some difficulties in forming the droplets from viscous solution. Phospholipid vesicles of small, unilamellar was also spray dried and the particle size of 18 to 70 μm was obtained. The average size, size distribution and bilayer integrity was preserved even after spray drying and sucrose 0.3M was used as a stabilizer (Broadhead et al., 1992).

6.3 Granulation

This method is another useful technique over wet granulation for tablets, which cannot be compressed directly. Slurry with 50-60% of solids containing filler, binder, disintegrant and coloring agent can be spray dried and mixed up with the active drug just before compression. Advantages of this technique over traditional method of granulation are good flowability, color uniformity, stability, improved hardness and less lubricant needed. Another study has compared high speed mixing, fluidized bed granulation and spray drying for the manufacturing of acetaminophen and ascorbic acid granules. Spray drying in this case gives the smallest granules and it is more relied on the amount of water used in granulation. Furthermore, spray drying is a suitable technique to produce granules in mass as it makes continuous granulation. Spray drying has also been applied for the production of slow release granulations. In comparison with tradition granulation method, spray drying need less binder and give more sustained effect.

6.4 Complex formation

Complex formation referred as forming complexes of various drugs such paracetamol with beta cyclodextrin. This kind of complex is in amorphous state with less than 10 μm, which is lower in particle size than the original drug alone but they have poor flowability and compressibility to form a tablet. But the dissolution rate of this kind of tablets is higher. Complex formation can also improve the bioavailability of poor water-soluble drug such as diazepam (Broadhead et al., 1992). Another research study shows that salicylic acid with acacia by spray drying has also improved the drug solubility and dissolution rate.

6.5 Dried powder in terms of aerosol formulation

Spray drying has a good potential to form powders with controlled characteristics and so it can be applied in aerosol formulation. Spray dried powders inhalation compared to micronized sodium cromoglycate. Spray dried powder gave higher deposition in vitro on the therapeutically important are on the nasal area than mechanically micronized one. The suitable size range of inhaled particles should be around 0.5 to 7 μm. Among that range, 3.3-7 μm have a deposition effect on the bronchioles, and the range from 0.5 to 3.3 μm is effective in the alveoli (Vidgren et al., 1987). As these small particles have inter-particulate cohesive forces with the tendency to agglomerate, dry powders are particularly formulated as drug mixture plus a carrier such as lactose (Broadhead et al., 1992).

6.6 Heat sensitive materials including enzymes and proteins

Several studies have proved that spray drying of heat sensitive materials has preserved its properties. The solution when it comes in contact with hot air, the evaporation occurs. A skin forms at the surface of particle and formation of thickness prevent further evaporation. About that time point, the temperature falling in the particles occurs and that high temperature didn't happen to catch inside the particle and denaturation of heat sensitive materials is restricted. The particles reach a maximum temperature of around 15- 20°C during the process and the period of time of exposure is only 5-30 seconds, which is not enough to denature the thermolabile materials (Deasy, 1984). Some macromolecular drugs including enzymes perhaps streptokinase and enzymes that are used as model protein has also been studied and the activities are remain unchanged. It has been reported that the activity losses during spray drying can be controlled by addition of sugars and salts. In the patent, it has been described that the spray drying of various enzymes by using water insoluble salts and cornstarch as suspenders and thickeners provide generally free-flowing and retained activity as well as improved stability during storage. Thus, it is important to choose the suitable carrier or additives in case heat sensitive materials (Broadhead et al., 1992).

7. Different types of carriers used during spray drying and their effects

Stabilizing excipients as carriers have been used and analyzed in spray drying of vaccines to obtain the thermostable products. Mostly, non-reducing sugars has been used such as trehalose, sucrose and lactose is also applied widely. These sugars form glass instead of crystals and the characteristics of amorphous glass was obtained and allow the protein to form hydrogen bonds. By this way, these sugars act as a water substitute upon rehydration. Non-live vaccine, HepB vaccine with aluminium hydroxide adjuvant was subjected to spray drying in one study. A sugar-glass powder was applied in order to evaluate if it could protect an alum adjuvanted vaccine from denaturation. Both of these formulations after spray drying gave a good stability for at least 2 years at 37C. Even the spray drying used high temperatures, evaporation of moisture occurs but the antigen is not exposed to heat, then the dried particle is migrated from the drying chamber to collector (Chen et al., 2010).

PLGA is a biodegradable polymer and it has been used in a variety of fields as a carrier. Subunit vaccine production from antigenic extract Hot Saline from *Brucella ovis* has been studies by spray dried with PLGA and microparticles size of smaller than 5 µm was obtained. It is applied as pulmonary vaccination for brucellosis (Murillo et al., 2002, Fourie et al., 2008).

Polylactide (PLA) or poly lactide-co-glycolide (PLG) is also widely used polyesters polymers in spray drying. As the other polymers, they are biocompatible and biodegradable and accepted to use in humans. It can prolong the release rates of entrapped antigen in the microparticles. In fact encapsulation of antigen in these kinds of polymers have proven to give high antibody titers and induce the antigen presentation to the immune system (Baras et al., 2000b).

Leucine is another excipient that is used in production of spray dried powder for inhalation. Studies have been investigated on M. bovis BCG and M. smegmatis to produce inhalable powders by using leucine alone as an excipient. It

has been found out that leucine can induce the spray dried powders dispersability (Irngartinger et al., 2004, Wong et al., 2007, Fourie et al., 2008).

PVP has also been evaluated as an excipient to protect the spray dried Newcastle disease vaccine from crystallization. However, not all the remaining water could be removed from the final product after spray drying process. In some studies, the combination of PVP together with the trehalose was also analyzed and it has been concluded that the vaccine titers during 10 months storage at 6 and 25C were maintained. Although, using mannitol, as a carrier did not give a proper stability as it crystallized during spray drying (Corbanie et al., 2007). Amioca® starch/poly (acrylic acid) powder was incorporated with influenza virus adjuvanted with LTR192G. Different ratios of starch and poly (acrylic acid) were varied to see the effect on spray dried powders. Serum IgG titers and HI serum response were increased with inhalation of spray dried powders compared to the liquid vaccine in phosphate buffer. One of the reasons of having high response is the viscosity-enhancing capacity of these powders. Another combination was Amioca® starch and Carbapol® 974P, it shows the enhancement of the intranasal irritation with increase in Carbapol concentration (Coucke et al., 2009).

Maltodextrin (MD) is used in process of microencapsulation by spray drying as core wall material. Maltodextrin is a hydrolyzed starch belongs to same group as glucose and lactose and it is produced by partially hydrolysis of acids or enzymes (Bae and Lee, 2008). Fig. 10. Maltodextrin is a unique polymer and have suitable properties concern with both solubility and viscosity. In addition, it is also applied in lyophilization.

Three different types of maltodextrin is available with different dextrose equivalent. Hydrolysis has an effect directly on the functional group of maltodextrin. The higher the DE, the more starch depolymerization occurs in results of small polymer particle size. According to some results, the concentration of maltodextrin has a high impact in maintaining the stability of protein BSA (Devineni et al., 2007). High dextrose equivalent (DE) of maltodextrin has proved

to protect the encapsulated orange peel oil from oxidation. So, it is important to choose the optimized DE of maltodextrin while using as wall material (Kagami et al., 2003). However, there are not much research studies seen in the production of vaccines using maltodextrin as carrier together with the nanoemulsion adjuvant by spray drying process.

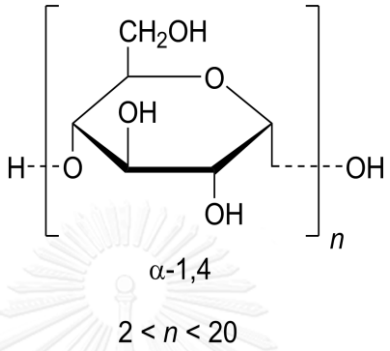


Figure 10 Chemical structure of maltodextrin

8. Structure and application of Bovine serum albumin (BSA) in the field of vaccines

It is a serum albumin protein derived from cows and widely used as a standard protein concentration in experiments. It is also used as a model protein in the vaccine development. BSA has 583 amino acids in length with a molecular weight of 66.5 kDa and estimated alpha helix 54% and beta form 18%.

A bunch of studies have been investigated by spray drying BSA with the appropriate adjuvant or polymer solution to evaluate the immune response, suitable route of administration and integrity of protein. Balasee et al., 2008 has developed the hydroxyethyl starch as a delivery system for BSA and the immune response obtained with the appropriate route has been discussed. The microparticles obtained from their study were below 10 micro m and this allows them to phagocyte by APCs and long lasting antigen presentation to APCs was occurred. Both IgG1 and IgG2

response was obtained with BSA microparticles by intramuscular administration. In another study, PLGA microparticles with BSA with a particle size range between 200 to 1000 nm has obtained IgG2a isotype response with subcutaneous, the oral and intranasal route (Garmise and Hickey, 2009, Gutierro et al., 2002).

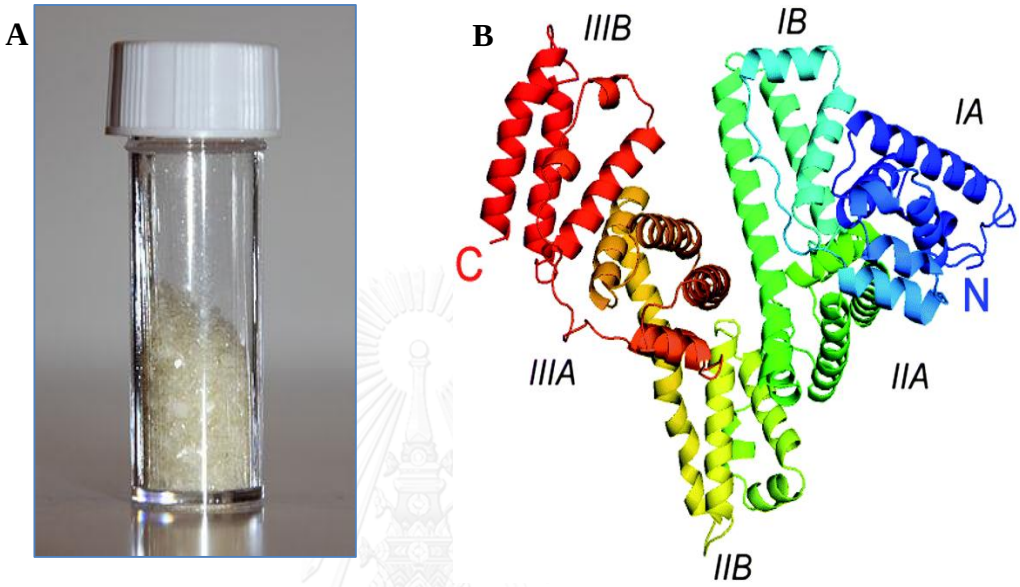


Figure 11 (A) Appearance of BSA (B) Structure of BSA

Spray dried chitosan microparticles together with the protein BSA was also investigated by (Kusonwiriyawong et al., 2009) and it has been proved the integrity of BSA still remain unchanged after spray drying. However, the size of microparticles is depending on the composition and concentration of liquid feed as well as the process parameters. Possibly, proteins accumulate at the surface of the particles and it will have high influence on morphology of the particles. By increasing the solid content and viscosity of the liquid feed with constant process parameters can increase the particle size after spray drying. Therefore, it is possible to spray dried BSA as a model antigen in the development of vaccine adjuvant with the appropriate process parameters and compositions. Furthermore, the immune response obtained upon spray drying of BSA has an influence on the type of administration and the particle size.

9. Parenteral administration for vaccines

The immune type, the strength of immune response and safety of vaccines are relied on the route of administration of vaccines. An appropriate route will make the vaccine potential. However, it is hard to decide the suitable route of administration for the new developed vaccine depending on the available properties and actions of administrations. Simply, a potent immune response will be obtained if the administration route is near the lymph node or afferent lymphatic vessel, we can predict that the upper layer of the skin are better connected with lymphatic units and vessels with more APCs compared to the lower skin. By this theory, the anatomical site having different types of immune cells has influenced the type of immune response obtained upon administration, for example, the most important cells for initiating the immune response is CD8+ T-cell response and it is depend on the presence of DCs. DCs are highly available in the skin, peritoneum, lung, muscles and secondary lymphatic organs.

9.1 Epidermal route

The human epidermis varies in thickness depending on the body area. The upper layer is the *strateum corneum*, comprised of only dead and cornfield keratinocytes, which shed constantly. The inner layer of epidermis, the live cells increase as it reaches the basal membrane that separates the epidermis from dermis. The Langerhans cells (LCs) similar to DCs are highly available in the skin and it also takes part in the defense of the immune system. They can produce the inflammatory cytokines including tumor necrosis factor (TNF-) α, interleukin (IL)-1bβ and IL-18 (Cumberbatch et al., 2001, Tang et al., 1993). Then they activate the macrophages and taken up into the lymphatic vessels. These lymphatic vessels which uptake the antigens and migrate to the lymph nodes are abundantly available in the epidermis area. So, if the vaccines were delivered into this area, it would be really effective as it migrate into the lymph node for further immune responses. Transdermal patches, micro-needle arrays are available for delivery of vaccines easily into the epidermis of the skin.

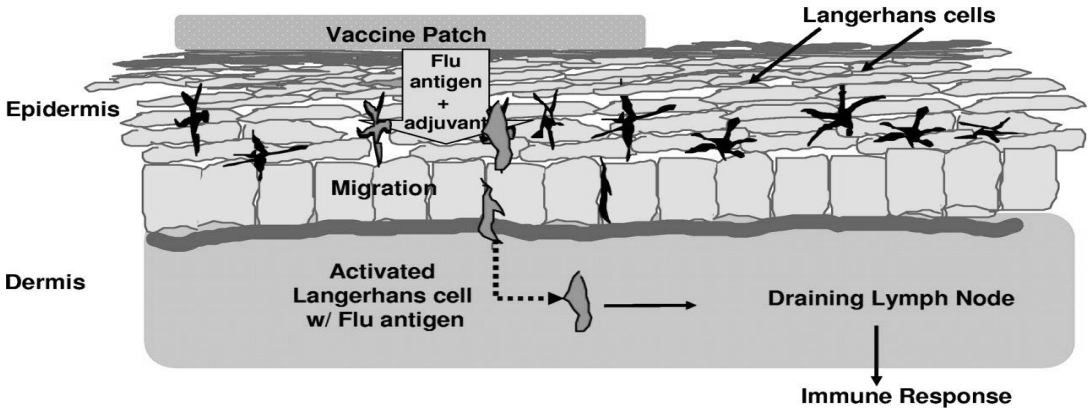


Figure 12 Vaccine patch showing the diagram for epidermal route of immunization

9.2 Intradermal route

Administration of can be done into the dermis area, in which collagen, elastic tissue, reticular fibers, sweat glands, sebaceous glands and hair follicles are present. Generally, thickness of dermis area varies and it is 3- 10 mm thick on the back. The vaccine approved by US-FDA is a flu vaccine (Fluzone®), which is administered intradermally. Another vaccines including the live viral rabies, *vaccinia* small pox vaccine and BCG are given by intradermal route. But his type of route needs special training compared to traditional routes such as subcutaneous and intramuscular routes. The special needle is used for injecting the vaccine by placing the syringe and the needle laid flat on the skin with the tip just inside the skin, not too deep. Fig. 12. This type of route has proved to give a good innate immune response in the skin. As the development of vaccine is mostly depend on the simple delivery method, intradermal route is limited. Therefore, a bunch of research is working to find out an easier delivery method for intradermal route using microneedle arrays (Hickling et al., 2011, Kim et al., 2014, Kim et al., 2012). Possibly, this route of administration can also lower the amount of dose needed to give the immune response compared to intramuscular injection.

9.3 Subcutaneous administration

Subcutaneous tissue is made up of a loose composition of connective and adipose tissue with the blood vessels and nerve bundles. This tissue can take up a large amount of vaccine dose than the dermis area. Vaccines for the disease such as measles, mumps, rubella, varicella, yellow fever, zoster, typhoid and Japanese encephalitis were administered via the subcutaneous route, such below the dermis. The injection into this tissue causes less pain so it is better than the intradermal route. Because of the presence of connective and adipose tissue, which offers static properties, it is suitable for depot effect. Moreover, slow mobility of vaccine can occur, as vascularization is less in that area of tissues and it can lead to vaccine failure. Draining of antigen toward the lymphatic vessels will also slow down, as the blood perfusion is low. Requirement of large amount of dose compared to dermal and epidermal area is the reason to increase the vaccine efficiency to balance the lost caused by low blood perfusion and slow lymphatic draining (Zuckerman, 2000, Poland et al., 1997).

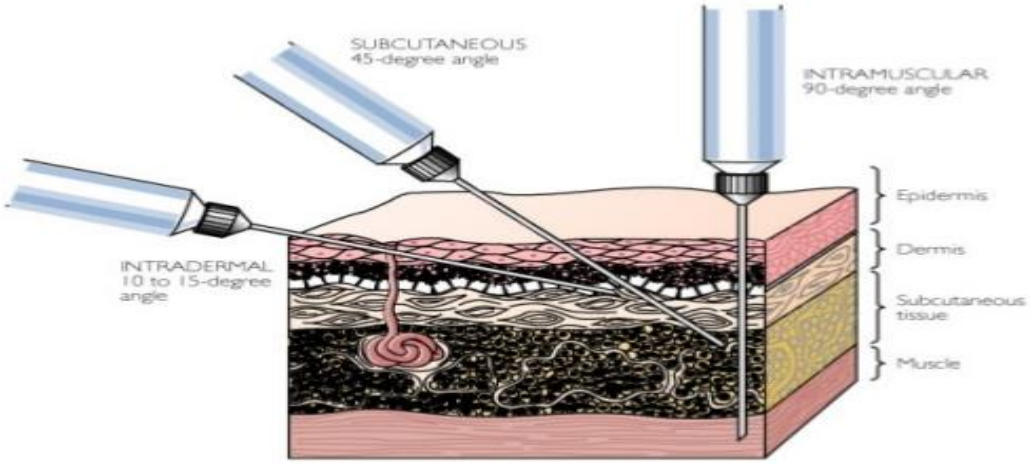


Figure 13 Diagram showing intradermal, subcutaneous and intramuscular route of injection at different angle and positions.

9.4 Intramuscular administration

Intramuscular administration is one of the most widely used in the field of vaccine for almost all kinds of inactivated vaccines. Vaccines containing protein, polysaccharide antigens such as hepatitis A, hepatitis B, meningitis C, and pneumococcus together with adjuvant are administered intramuscularly. MF59 emulsion adjuvant together with a flu vaccine for old patient and viral vaccines for hepatitis B and HPV with the adjuvant AS04 are intramuscularly immunized because these vaccines can cause erythema in the skin, pain and swelling. The typical injection site for intramuscular administration is the *vastus lateralis* muscle (anterolateral thigh) and the deltoid muscle (upper arm). If the vaccine produces local reaction frequently, it is advisable to use different limbs at each time. For several vaccine administrations in young children, injection should be done on thigh as it has a greater mass of muscle. Fig. 13.

9.5 Intravenous administration

Intravenous and intraperitoneal vaccine route has been administered in small animals such as mice for pre-clinical development to evaluate the vaccine potency. In humans, the pre-clinical testing of the live attenuated aporozoites Plasmodium falciparum for malaria, are the only immunogens, which give the protection safely (Roestenberg et al., 2009). The other vaccines tested in mice by intravenous injection have given higher immune response in comparison to subcutaneous and intradermal route (Seder et al., 2013). Intravenous route in humans is mostly for autologous vaccine based on DCs, applied in cancer immunotherapy. This kind of vaccines are prepared by culturing the patient's own blood and the cells are pulsed with tumor antigens. It is then injected intravenously to humans. The only vaccine approved for this kind of treatment is autologous DC vaccine, sipuleucel-T, used in treatment of asymptomatic or minimally symptomatic metastatic castrate resistant prostate cancer (Kantoff et al., 2010).

9.6 Intralymphatic Vaccination

As we have discussed above, the route of administration has a huge influence on the environment, the tissue perfusion and immune cells present around the area of injection. To get the optimum immune response, the route of administration should be chosen properly. Otherwise, the injected vaccine can be cleared by inmate defense mechanism. If the vaccine is carrier toward to the secondary lymphoid organs, the potent immune response will be obtained. By injecting directly into the lymph node, the potent adaptive immune stimulation will be achieved and it is one of the clear out way to obtain the desired immune response. The lymphocytes and APCs are also highly available near the lymph node; this would be really expedient and will lead to APC activation and high antigen presentation. However, this method is not generally used in vaccine administration and it has just been investigated in some pre-clinical studies and human clinical trials for cancer and allergen immunotherapy (Johansen and Kündig, 2015). In mice, this is an invasive procedure where a small incision made in the inguinal region. With the help of small syringe, a small amount of vaccine, around 10-20 µl is injected and the mice were kept under visual control to observe the swelling of the lymph node. At the end, the incision is close with a single stich by surgical sutures. The immune response obtained was about 100- fold higher antigen than the other route administration. In humans, the region chosen for administration is the outer upper quadrant of the groin area and the procedure is monitored by ultrasound and the injection volume for human is around 100 µl (Huppa et al., 2003). After few minutes of injection, 100% uptake of antigen has been observed in human after few minutes of injection. But in subcutaneous route, the proteins are still left at the site of administration even after 24 hours. In future, intralymphatic administration can be an alternative route for vaccine administration (Johansen and Kündig, 2014).

10. Nasal route for vaccines

This route of administration has gained interest in recent as an alternative to parenteral administration because of its potency to give both systemic and mucosal

immune response and it can cut out the use of needle. Dry powder vaccine is mostly use for this kind of administration by inhalation. This route has several advantages including ease of administration, simple formulation and low cost.

10.1 Anatomy and physiology of nasal cavity

The vaccine used the inner nasal surface area to produce an immune response. Generally, the adult human nose has an inner surface area of 160 cm2 and separated into two sides. These two sides are divided by nasal septum. The anterior region is called the nostril, which is also the outer part of the nose, the inner surface is comprised of the squamous epithelium like the normal skin and has nasal hairs. The nasal valve has a diameter of less than 0.25 mm and it is the narrowest part of the nose. The nasal turbinates can be then divided into the lower, middle and upper turbinates. Olfactory bulb is the upper turbinates. The other two of the turbinates are reunited in the nasopharynx, the posterior part of the nose. The olfactory bulb has a direct connection with central nervous system; this pathway can be greatly used for brain delivery. In case of vaccine administration, this pathway is risky because of pathogens, adjuvants and other compounds present in the vaccines (Harkema et al., 2006, Jones, 2001).

The ciliated respiratory epithelium is enclosed with a mucus film. The ciliary while the presence of particles deposited on the mucus is cleared to the posterior part of the nose. In healthy adult, this clearance takes about 10-15 minutes. In nasal administration, this clearance by ciliary is important to obtain the immune response. The time of interaction between the epithelium and the formulation is quiet short but it can be increased with the mucoadhesives. There is low enzymatic activity in the nasal mucosa so vaccination by this route is a good choice for particulate vaccines (Türker et al., 2004).

From the point of physiology, the nose is warm and it humidifies the air after inhalation. It filter, heat and humidity the air before it reaches the lungs. Therefore, the nasal mucosa collects the certain particles size (Harkema et al., 2006). In vivo

examination has shown that the nose took up 80% of particles up to 12.5 µm and 100% of particles greater than 50 µm. The 50 % of small particles around 2-4 µm will pass into the lower airways and distributed throughout the nasal cavity. The nose positioned in the upper respiratory part is composed of bunch of immunocompetent cells as pathogens can enter by this way easily Fig 14. The lymphoid tissue of the nose (nose- associated lymphoid tissue, NALT) and dendritic cells are abundantly present in the nasopharynx and it takes part in giving immunity by nasal administration of vaccines. It enhances the immune response far from the mucosal site such as urogenital tract, and in respiratory tract locally and also gave the systemic immune responses. This type of administration is also potent for vaccination with live-attenuated vaccines for example, intranasal influenza vaccine. It is becoming a popular route of administration for sexually transmitted disease because of its potency to activate immune response in urogenital tract (Jadhav et al., 2007, Jones, 2001).

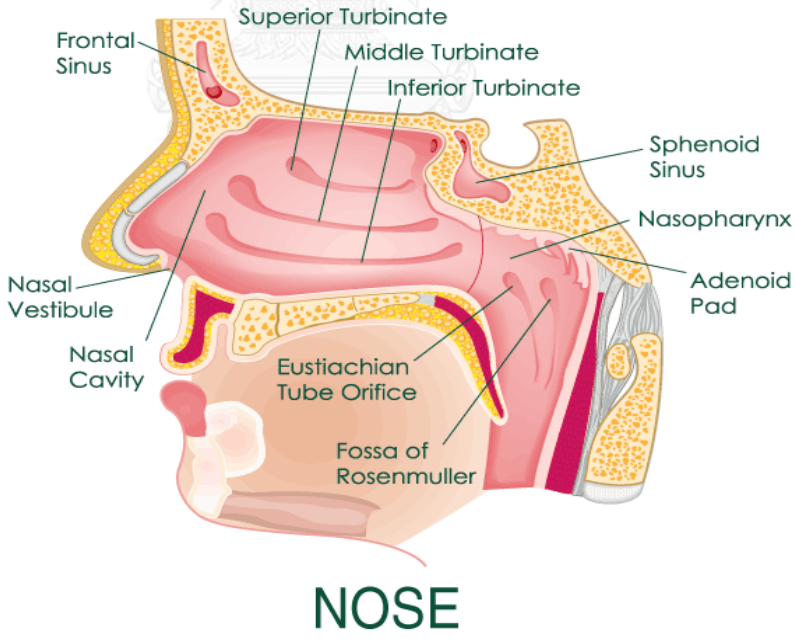


Figure 14 Diagram showing anatomy and physiology of nose

10.2 Formulation demand for nasal administration

To be taken up by antigen presenting cells in the nasal, the vaccines should be in particulate form. So, the antigen formulated with the particulate vaccine carrier would be preferable. Particulate vaccines will be composed of liposomes, immunostimulated complexes, emulsions, and polymeric particles together with the antigen. Particulate vaccines can also protein the antigen from denaturation and instability (Pavot et al., 2012). The particle size of particulate vaccine should be in nanometer range above 50-100 nm so that it will be taken up by the DCs. Particle size smaller than this cannot be processed locally and it will be drained directly into the lymph nodes. The size of particles and the immune response obtained has some dependency on each other. In comparison between 200 nm of particle size and 700 nm, 200 nm gave the higher immune response than larger particles. Optimal uptake by DCs is obtained with the particle size between 200-300 nm. But these nano size particles can carry only a small amount antigen and antigen load can be increased with the use of micro particulate form. These micro-size particles aren't taken up well by DCs in the nasal cavity but it can be engulfed by M-cells in the NALT up to 10 μm size particles. There is a high demand for the formulation with the suitable particle size that can deposit in the nose instead of getting into the lung by inhalation. Most of the nanoparticles tend to agglomerate as they have large surface area, it can be suspend in the solution form and spray into the nose. The spray nozzle of the spray device maintains the spray droplet size, viscosity and surface tension of dispersion medium is also controlled (De Temmerman et al., 2011, Garmise and Hickey, 2009).

Nasal formulation can be solutions or suspensions, which can be administered by nasal sprays, or it can be dry powder formulations that can be administered by passive or active dry powder dispensers. Liquid preparation of nanoparticulate form vaccines has high risk of instability over time because of high mobility of molecules, chemical reactions within the formulation as well as physical instability (Amorij et al., 2007).Therefore, liquid vaccines have to formulate with appropriate pH to maintain the antigen stability and transport under refrigerated

condition. Osmolality plays a great, as for nasal route; pH and osmolality of liquid should be optimal otherwise it can irritate the nose on administration. Buffer ionic strength can also affect the uptake of nanoparticles by M-cells. Hence, liquid preparations for these instable reasons are spray dried or freeze-dried to improve the stability. By converting the liquid into powder slow down the mobility of molecules and therefore intermolecular reactions will not occur. There are stresses and risks available in each drying methods as well, which has been discussed above. During drying, the antigen stabilization is the most important factor and which can maintain by the addition of carrier system. Addition of carrier system into nanoparticulate form will allow the quick antigen release and deposition in the nasal cavity. Dry formulation after administration may cause the physical irritancy depends on the concentration and size of the particles. Nose, is the very sensitive organ and the smell of the formulation should be good as well taste for a better patience compliances. In some formulations, mucoadhesives should be added to increase the retention time between the antigen and formulation and the antigen uptake by the cells. The adjuvant chosen for nasal vaccination should have high safety profile as formulation can enter into the CNS. The best thing about nasal administration is that the dose has to give just once, with one or two boost doses. If the vaccine is in the liquid form, it should be packed in single dose otherwise there can be high risk of microbial contamination. However, using dry powder vaccine can eliminate this high risk of microbial contamination. There are different approaches of nasal subunit vaccines formulation such as mucoadhesives gels, polymeric particles, conjugates/complexes, lipid system, dry powder formulations (Minne et al., 2008, Rajapaksa et al., 2010).

10.1.1 Mucoadhesives gels

Gels have come in use to increase the retention time of antigen contact with the nasal mucosa. A nanogel of cationic type of cholesteryl-group-bearing pullulan with a subunit antigen from *Clostridium botulunum* was administered intranasally to nice and a strong humoral immune response was obtained. The antigen didn't migrate into the brain or accumulate in the olfactory bulb. But the

formulation of the gel needs to be a bit viscous. Therefore, a new type of gel called thermo sensitive gel was investigated and found out that it gives low viscosity at lower temperatures and increased viscosity upon warming in the nasal mucosa. A dry powder formulation together with an excipient that becomes gel upon contact with water can be used. Those formulations will stick on the mucosa and increase the retention time as well. A spray dried nasal vaccine with chitosan glutamate together with virus like particles (VLPs) loaded with Norovirus antigen is undergoing research and shown effective in rabbit models (Nochi et al., 2010, Wu et al., 2012).

10.1.2 Polymeric particles

When it comes to polymer, chitosan is one of the most widely used polymers. Spray drying of chitosan polymer together with the antigen would be the simplest step for the production of nasal vaccines. As after spray drying, the particles obtain will be in micro particulate form. However, normally nasal vaccines aims to produce nanoparticles as antigen carrier for better uptake. Ionic gelation is usually use to produce nanoparticles by dissolving chitosan in acidic media which will lead to positively charged groups and form a complex with negatively charged counterparts such as tripolyphosphate or bile salts to obtain the gel like nanoparticles. Chitosan increases the permeation in several studies as well as owns mucoadhesives properties with adjuvant activity on administration together with antigen. Another polymer suitable for nasal route is PLGA which can be prepared by double emulsion method by loading with hepatitis B surface antigen (HBsAg) and then coated back with chitosan bearing the positive charge over the particles. Cationic particles have provided a great advantage for nasal delivery. Other types of polymers can also be used and coat the particles with chitosan will expect to produce improved humoral immune response. Surface-active substances such as poloxamer and polyvinylaclohol have also enhance the immune response when combine with PLGA particles (Amidi et al., 2007, Gordon et al., 2010).

10.1.3 Conjugate or complexes

This approach has been made to lower the size of vaccine carrier system with the maintain system of soluble small antigen by the use of nanoconjuages to get the immune response. The model antigen ovalbumin was covalently linked to trimethyl chitosan (TMC) and compared the antibody response obtained by ovalbumin antigen alone. In that study, it has been proved that, both nasal uptake and immune response IgA and IgG was increased by conjugate system. It can be concluded that the potential response will only be obtained with the delivery of antigen and adjuvant together in the particulate form (Slütter et al., 2010).

10.1.4 Lipid systems

Liposomes together with antigen tetanus toxoid in addition of mucoadhesives agents such as carbomer, chitosan or hyaluronic acid has been formulated and investigated in mice by nasal vaccination in comparison with the antigen alone. It has been shown that liposomes containing chitosan with liposomes loaded with antigen give high immune response by the increment of uptake. To overcome the physical stability, lipid microparticles can also be formulated instead, which is more stable because of their solid nature. Saraf et al., 2006, studied using HBsAg in lipid microparticles composed of soy lecithin with or without stearylamine (SA), and found out that formulation was uptake by nasal mucosal and result in immune response. Moreover, significant immune response was obtained with cationic particles with SA (Alpar et al., 2005, Kojima et al., 2008).

10.1.5 Dry powder formulations

Almost all particulate forms of vaccines are capable of transformed into the dry powder form, which improved the stability profile. Even the primary particle size of spray dried powder can be quiet small for nasal deposition but it can be improved with agglomeration of powder and dispersion by the device used for nasal spray. Another way to improve the nasal deposition can be by coating the

suspension with the nanoparticulate antigen or a larger carrier particle with the use of spray drying method. Dry powder formulation of alginate microspheres with tetanus toxoid, a spray freeze dried powder of trehalose with anthrax recombinant protective antigen were investigated in rabbit and in mice having good immunity for laboratory use. But the point, the physiology of these rodents compared to human is completely different. Moreover, in some studies, a pipette or a syringe was used to administered the mice and observe the immune response and the dose volume can be around 100 µl. This amount can drain down to the lung giving systemic immune response and the immune response obtained would not be accurate. Immune response may vary depending on whether the mouse is given anesthesia or in awake as in the situation of anesthesia, the higher immune response will be obtained with the drainage into the lungs. So, these things need to consider before conclusion of the results. In another way the use of spray device would be effective to overcome these issues (Harkema et al., 2006, Illum et al., 2001).

There are different devices available for delivery of vaccine to the nasal mucosa. Liquid formulations are normally delivered to the nose via normal spray pumps or nasal drop. Nasal drop system would be suitable for infants and children. Nasal spray are pump system which disperse a single dose into the nose by form of spray mechanically. It is comprised of liquid reservoir, a volumetric dose-metering chamber, the actuation spring and a nozzle to spray the formulation into the nose. As most of the vaccine formulation is in liquid multidose form, using this kind of nasal spray can increase the risk contamination into the formulation and this could be overcome with the seals or silver-coated ring around it. In the modern devices, we can adjust to the desired dose, concentration, and nozzle spray characteristics (Mahajan et al., 2011).

Nasal spray can in two form of system single-dose or bi-dose and these are more suitable for discontinuous use in migraine or vaccination. Another device is called VaxInator, which comprised of cone shaped nozzle connected to a syringe. On pushing the plunger, the liquid came out from the syringe in the form of droplets. The use of this system can overcome the dripping of formulation into the pharynx

and unpleasant taste caused after using the spray pump system (Marx et al., 2010).

Another way is by sniffing the powder vaccine with a nasal adapter so that the patient can inhale the powder. The powder will get disperse with the help the user's airflow. One thing to be note here is these devices also need patient capabilities to induce the sufficient expiration or inspiration airflow. To overcome this a new type of device has been developed, in which a defined volume of air is compressed and upon actuation, it the air from the lower chamber will be dispersed together with the dose. This dispersion may contain propellant. The powder dose and the propellant are in different chamber but connected with each other on actuation (Friebel and Steckel, 2010, Scherließ, 2015).

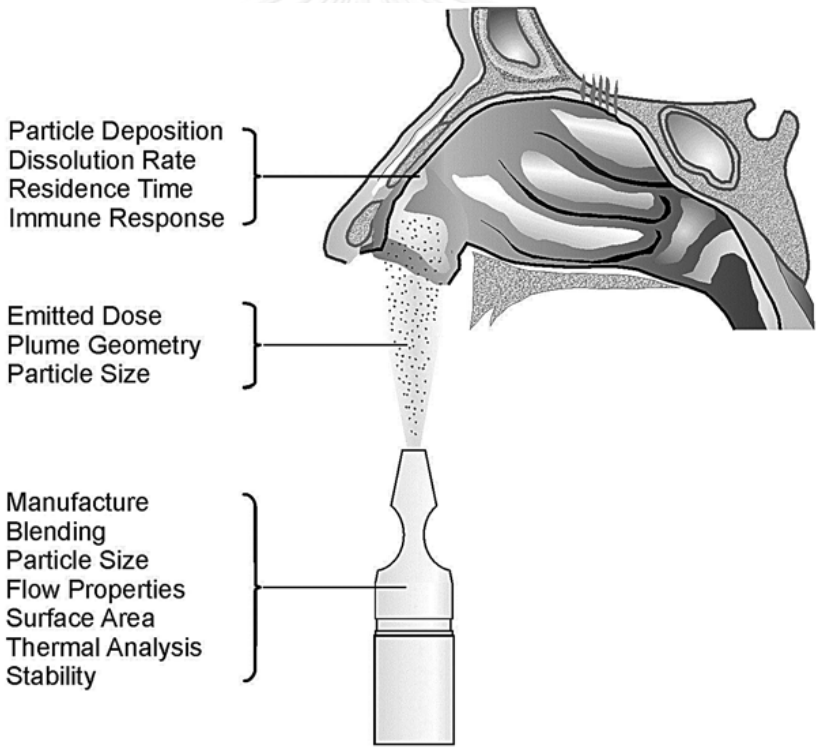


Figure 15 Important parameters in order to characterize the nasal subunit vaccine.

11. Objective of this study

As a conclusion, the objective of this study is to develop the adjuvant in a powder form, which could improve the stability and obviate the need of cold chain.

There is high demand for the adjuvant formulation with high safety profile; this type of adjuvant powder form is possible to overcome these issues. Moreover, in recent years, nasal route has become an alternative way to administer vaccines, this powder form adjuvant would be able to administer in powder form or by reconstituting it for parenteral administration.

In order to develop this type of powder adjuvant, nanoemulsion is prepared and optimized with various influencing parameters. The optimized nanoemulsion was then subjected to spray drying by loading it with maltodextrin and BSA. A set of various parameters was varied to get the optimized condition for spray dried nanoemulsion with the model antigen. The characterization of nanoemulsion, spray dried powder, and spray dried powder reconstitutions were carried out. In addition, spray dried powder were investigated for its thermal behavior, protein integrity, protein content determination and in vitro release study. Cell viability, permeability and uptake study of these spray dried powder was also evaluated in human monocyte macrophage cell line and nasal epithelium cells.

CHAPTER III
MATERIALS AND METHODS

Materials

The following materials were purchased from commercial sources.

1. Model antigen
   - Bovine Serum Albumin (BSA) (Lot # SLBL2091V, Sigma, USA)
   - FITC-BSA (Lot# SLBK3715V, Sigma, USA)

2. Carrier
   - Maltodextrin (Lot# MKBL5377V, Sigma, USA)

3. Solvents
   - PBS (pH7.4)
   - Ultrapure water

4. Chemicals
   - Wheat germ oil (Lot# MKBG6188V, Sigma, USA)
   - Vitamin E acetate (09021, S.Tong Chemicals Co., Ltd)
   - Tween80 (Batch no. G177229, S.Tong Chemicals Co., Ltd)
   - Span85 (Sigma, USA)
   - Dichloromethane
   - Alamar blue reagent (448747, Invitrogen)
   - Phosphotungstic acid (PTA)
   - Micro BCA assay kit (Lot# QG218473A, Thermo Scientific)
   - SIMply BlueTM SafeStain
   - NuPAGE 4-12% Bis-Tris polyacrylamide gel
   - Reducing buffer
   - NuPAGE MES SDS Running Buffer (20x)

- Tris-Glycine SDS Sample buffer (Novex, Invitrogen, Lot no. 677471)

5. Biomaterials
   - Human monocyte macrophage cell line (SC ATCC® CRL-9855^{TM})
   - Human nasal epithelial carcinoma cell line (RPMI 2650, CCL-30 ^{TM}, American Type Culture Collection (ATCC), VA, USA)

6. Cell culture
   - Isocove's modified Dulbecco's medium (Thermo Fisher Scientific)
   - Fetal bovine serum (GIBCO®)
   - 2-mercaptoethanol (Sigma, USA)
   - Hypoxanthine (Sigma, USA)
   - Eagle's minimum essential medium (EMEM) (ATCC®)
   - 0.025% Trypsin in EDTA (Thermo Fisher Scientific)
   - Tryphan blue cell culture tested
   - DAPI (Thermo Fisher Scientific)
   - Wheat germ agglutinin (Thermo Fisher Scientific)
   - Rhodamine

7. Laboratory supplies
   - Centrifuge tubes (Corning®, UK)
   - Microcentrifuge tube (Corning®, UK)
   - Tissue culture flask 25 cm^2 (Corning®, UK)
   - Sterile 96 well plates

8. Equipment
   - Ultraturrax homogenizer (IKA T25 Digial Ultra-Turrax)
   - High pressure homogenizer (Emulsiflex C5, Canada)
   - Magnetic stirrer (MSH-300)
   - Analytical balance (Sartorius analytic)
   - Analytical balance (Metler Toledo)
   - Spray dryer (BUCHI Mini Spray Dryer B-290)

- Nano-ZS zetasizer (Malvern Instrument, Malvern, UK)
- Desiccator
- Transmission electron microscope
  (FEI TECHNAI T20 G2, FEI Company, The Netherlands)
- Malvern Mastersizer 2000 (Malvern Instruments, UK)
- Scanning electron microscope
  (SEM, Joel model JSM 5410 LV, Tokyo, Japan)
- Differential scanning calorimetry (DSC, 2000A, Mettler TA)
- X-ray powder diffractometer (Model JDX- 8030, Joel, Japan)
- Fourier transform infrared spectroscopy (FTIR)
- Spectropolarimeter (Circular Dichroism)
- Centrifuge machine
- Vortex mixer (VELP Scientifica)
- pH meter (METTLER TOLEDO)
- Sonicator bath (Elma®, Germany)
- Spectrophotometer (Microplate reader)
- Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
  (SDS- PAGE)
- Hot air Oven
- Refrigerator
- CO_2 incubator
- Laminar air flow cabinet
- Micropipette
- Inverse phase contrast microscope
- haemocytometer

Methods

1. A. Preliminary studies for composition of nanoemulsions

Nanoemulsion adjuvant was prepared by high-pressure homogenization technique. Nanoemulsion was composed of wheat germ oil (WGO), vitamin E acetate, Tween80 and Span 85. Firstly, the organic phase containing WGO alone or WGO together with vitamin E acetate and Span85 and the aqueous phase containing ultrapure water and Tween80 were subjected to ultraturrax homogenizer of 12,000 rpm at various time duration. The primary emulsion then undergoes high pressure homogenization at different temperatures for 6 cycles at 1000 bar to study the effect of temperatures. Various oil compositions and surfactant were used to obtain the optimized formulation with the smallest particle size. The fixed parameters of (3 min, 40°C) were used at first with various compositions of nanoemulsions. Then, the smallest particle sizes among them were chosen to undergo the next step of optimization.

Table 1. Oil and surfactant compositions with fixed parameter of 3 min of ultraturrax homogenizer and 40°C during high pressure homogenizer

Ingredients	A1	A2	A3	A4	A5
WGO (%v/v)	1	2	3	4	5
Span85 (%v/v)	0.5	0.5	0.5	0.5	0.5
Tween80 (%v/v)	0.5	0.5	0.5	0.5	0.5

The smallest particle size with higher zeta potential negative values obtained from Table.1 was chosen in this step.

As a second step, Vitamin E acetate was include together with WGO in different ratios of 1:0.05. 0.5:0.5, 0.9:0.1, and Tween80 0.5% and Span85 0.5% were kept constant at 3 min of ultraturrax homogenizer (12,000 rpm) and 40°C during high pressure homogenizer at 1000 bar for 6 cycles.

Table 2 WGO and vitamin E acetate at various compositions with fixed parameters of 3 min ultraturrax homogenizer, 40°C during high pressure homogenizer

Ingredients	**B1**	**B2**	**B3**
WGO : Vitamin E acetate (% v/v)	1:0.05	0.5:0.5	0.9:0.1
Span85 (%v/v)	0.5	0.5	0.5
Tween80 (%v/v)	0.5	0.5	0.5

The surfactant concentrations were increased to Tween80 (1% v/v) and Span85 (1% v/v) with the fixed parameters of 3 min ultraturrax homogenizer and 40°C during high pressure homogenizer on two optimized formulations from table 1 (A1 – A5) and table 2 (B1 – B3). These two formulations with the surfactant concentrations of Tween80 (0.5% v/v), Span85 (0.5% v/v) and Tween80 (1% v/v), Span85 (1% v/v) were compared and chosen the two optimized nanoemulsion formulation from this in response to particle size, zeta potential and polydispersity.

The next step is to vary the parameters of ultraturrax homogenizer and the temperature during high pressure homogenizer in preparing the nanoemulsion. The parameters design is shown in table 3:

Table 3 Parameters used in optimizing nanoemulsion

3 min	40°C	A, B	A, B
	50°C		
	60°C		
5 min	40°C	A, B	A, B
	50°C		
	60°C		
7 min	40°C	A, B	A, B
	50°C		
	60°C		

After these variation in the parameters, the nanoemulsion with smallest particle size consisting of WGO alone as FA or WGO with vitamin E acetate as FB, two formulations were appointed for spray drying.

B. Preparation of spray dried powder

The optimized nanoemulsions were loaded with various concentrations of maltodextrin by using magnetic stirrer for 15 minutes. Various parameters were used during spray drying with the constant aspirator rate of 100% in order to optimize the best condition. The formulation that meet the requirement was chosen by evaluating the smallest difference between the particle size of nanoemulsion adjuvant before spray drying and

after reconstitution of spray dried powder. Spray dried powder yield was also taken into account.

Table 4 Parameters used in optimizing the spray drying condition

Parameters (optimization step)	
Maltodextrin (MD)	3%, 5% w/v
Inlet temperatures	110°C, 130°C
Pump rate	20%, 30%
Aspirator capacity	100%

Table 5 study design of spray drying process with various concentration of maltodextrin

Inlet Temp:	Pump Rate (%)	MD (% w/v)		
110°C	20	3	FA1	FB1
110°C		5	FA2	FB2
110°C	30	5	FA3	FB3
110°C		3	FA4	FB4
130°C	20	3	FA5	FB5
130°C		5	FA6	FB6
130°C	30	5	FA7	FB7
130°C		3	FA8	FB8

The two formulations with suitable spray dried condition were chosen from FA1-8 and FB1-8 then as a second step the nanoemulsion adjuvant was loaded with maltodextrin by using magnetic stirrer for 15 minutes and then loaded the model antigen BSA 1% w/v or 3% w/v on magnetic stirrer for 15 minutes more.

2. Characterization of adjuvant nanoemulsion

2.1 Particle size and zeta potential measurements

The average particle size, polydispersity index and zeta potential of nanoemulsion was evaluated by a Nano-ZS zetasizer (Malvern Instrument, Malvern, UK) at 25°C with a backscatter detector (173°). The optimized formulations with the smallest particle size were characterized and chosen by this way. For zeta potential, at least 20 sub runs were performed in one measurement. All the measurement of each sample was made in triplicate (Fox, 2009).

2.2 Transmission electron microscope

The morphology of droplets of optimized formulations were visualized by negative staining. The sample were adsorbed on Formvar coated, 300 square mesh copper grid [Electron Microscopy Science, USA] and stained with 1.5% phosphotungstic acid (PTA). It was then dried in a desiccator for 3 hours in order to get rid of water from samples before characterization and observed in FEI TECHNAI T20 G2 (Hatziantoniou et al., 2007).

3. Characterization of spray dried nanoemulsion adjuvant

3.1 Particle size and zeta potential measurement after reconstitution of spray dried powder

Spray dried powder with or without protein were reconstituted with purified water and average particle size, polydispersity index and zeta potential were measured by a Nano-ZS zetasizer (Malvern Instrument, Malvern, UK) at 25°C, equipped with a backscatter detector (173°). For zeta potential, at least 20 sub runs were done. All measurements were done in triplicate. The particle size obtained was used to compare with the nanoemulsion before spray drying and to optimize the formulation condition of spray dried powder for further study (Bouchemal et al., 2004).

3.2 Spray dried powder yield

Spray dried powder yield of each formulation was calculated for optimization step in order to study the effect of parameters such as inlet temperature, concentration of solid content and pump rate. The product yield (PY) was calculated for both spray dried nanoemulsion adjuvant with MD alone and spray dried powder loaded with MD and model antigen BSA (Kusonwiriyawong et al., 2009).

Equation for spray dried powder;

$$PY\ (\%) = \frac{\text{Weight of spray dried powders}}{\text{Total ingredients of formulation without water}} \times 100\ \% \qquad \text{Equation 1}$$

3.3 Spray dried powder particle size measurement by mastersizer

Particle size of spray dried powder with optimized condition was determined for both formulation with or without BSA by laser light

diffraction. The equipment consisted of a Malvern Mastersizer 2000 (Malvern Instruments, UK) including a Scirocco 2000 module for dry measurement system was used. The measurement was operated at 3.0 bar air pressure for sufficient dispersion and feed rate of 50%. Each sample was measured in triplicate to get the average particle size (Rajniak et al., 2008).

3.4 Scanning electron microscope (SEM) analysis

The morphology of spray dried nanoemulsion adjuvant alone or with BSA were mounted on double-sided adhesive tape, which was attached on a sample stub. The samples were sputtered with gold and viewed under the scanning electron microscope (Joel model JSM 5410 LV, Tokyo, Japan) at 15 kV. The photomicrographs of the particles were taken with a suitable magnification. The diameter of particle size was also determined by using SEM images with the scale system.

3.5 Differential scanning calorimetry (DSC) analysis

DSC is an effective method to study either the pure solid content or mixture. Different effects either physical or chemical changes can be observed as a function of temperature or time as the sample in a pan was heated in a uniform rate.

The thermotropic phase behavior of optimized spray dried powder with or without BSA, MD untreated, native BSA, and physical mixture were determined by DSC 2000A, Mettler TA. The machine was calibrated with indium before the analysis. Approximately 5 mg of sample was weighted carefully into 40 µl pinholed Aluminium pans. The sample were heated from 20°C to 250°C at 10°C/min (Cardona et al., 1997). Nitrogen flow of 60 ml/min was used. Thermograms of all samples were done in triplicate.

3.6 Powder crystallinity analysis

The X-ray diffraction was used to determine the powder crystallinity and to confirm the results of DSC. Samples were packed on a glass sample plate and the equipment was operated at room temperature using CuKα radiation at 30mA and 40kV, with an angular increment of 0.05°/s, count time of 2s. The X-ray diffraction patterns were evaluated for (i) MD, (ii) native BSA, (iii) spray dried powder adjuvant blank, and (iv) spray dried powder adjuvant with model antigen BSA.

3.7 Fourier transform infrared spectroscopy (FTIR)

FTIR was used to investigate any possible interaction as well as chemical changes within the spray dried particles and protein by studying the position of peaks compared to the native BSA. The FTIR spectra were carried out for (i) MD (ii) native BSA (iii) spray dried adjuvant blank (iv) spray dried adjuvant with BSA by using potassium bromide disc (KBr) method in the range of 4000-400 cm^{-1} (Carrasquillo et al., 2001).

4. Protein content determination

Ten milligrams of spray dried powder was weighed into 15 ml centrifuge tube and 1 ml of dichloromethane was added to dissolve the particles. Then, 10 ml of phosphate buffer saline (PBS) (pH 7.4) was then added into to the tube to extract BSA by vortexing for 5 min. The tube was transferred to centrifuge machine in order to separate the aqueous and organic phase of nanoemulsion into two layers by centrifugation at 7500 rpm for 20 min. The aqueous supernatant phase was collected to determine the protein content by micro bicinchoninic acid (BCA) protein assay kit analysis. A set of standard BSA was prepared by serial dilution method to obtain a standard curve. The working reagent was prepared as 50 parts: 48 parts: 1 part as indicated by the manufacturer. 1:1 ratio of working reagent and test solution

or standard BSA was added into each well of 96 well plates. The reaction was run at 37°C for 2 hours. Optical density of each well was analyzed at 562 nm on spectrophotometer (Elversson and Millqvist-Fureby, 2006). However, very low amount of Tween80 is present in the formulation, it can interfere with micro BCA assay. For this reason, spray dried powder blank without BSA was run as a control. Furthermore, different batches of BSA containing spray dried adjuvant were also analyzed to evaluate the consistency of BSA concentration in each batch. %BSA loading was then calculated as follows:

$$\% \text{ BSA loading} = \frac{\text{Calculated weight of BSA}}{\text{Weight of spray dried powder}} \times 100 \qquad \text{Equation 3}$$

Phosphate buffer: 1 liter stock of 10x PBS was prepared by dissolving 80 g of NaCl, 2 g of KCl, 14.4 g of Na_2HPO_4 and 2.4 g of KH_2PO_4 in 800 ml of water. The solution was then make up to volume with water to 1 liter. Adjust the pH to 7.4 by adding NaOH or HCl. The buffer solution was then sterilized by filter sterilization and autoclaving at 121°C for 15 min. The solution was stored at room temperature.

Standard curve for protein content determination

A set protein standard solution with a serial dilution was prepared as follows:

Table 6 Standard solutions of BSA

Tube	Diluent (PBS)	Source of BSA stock	Final BSA conc:
A	4.5 ml	0.5 ml	200 µg/ml
B	1 ml	4 ml of tube A	160 µg/ml
C	2 ml	2 ml of tube B	80 µg/ml
D	2 ml	2 ml of tube C	40 µg/ml
E	2 ml	2 ml of tube D	20 µg/ml
F	2 ml	2 ml of tube E	10 µg/ml
G	2 ml	2 ml of tube F	5 µg/ml
H	2 ml	2 ml of tube G	2.5 µg/ml
I	2 ml	2 ml of tube H	1.25 µg/ml
J	2 ml	2 ml of tube I	0.625 µg/ml
K	4 ml	0 ml	0 µg/ml

5. In vitro release study

Ten milligrams of spray dried powders were placed in 2 ml of PBS (pH7.4) and was put in a shaking incubator at 160 rpm, 37°C. At predetermine time intervals of 0.25, 0.5, 1, 2, 4, 8, 14, 24 hours, 1 ml of the sample was taken out and subjected to centrifugation at 7500 rpm for 15 minutes with the refrigerator condition of 5°C. After taking out 1 ml of sample, the same amount of fresh medium PBS (pH 7.4) was added back to the tube in a shaking incubator. The supernatant was separated after centrifugation and the concentration of BSA released was determined by micro BCA assay. A standard curve was prepared as showed in Table 7. Different batches of spray

dried powder containing BSA 1% w/v and 3% w/v were exposed to this experiment as well as the blank formulation without BSA as a control. Each assay was replicated three times (Elversson and Millqvist-Fureby, 2005, Elversson and Millqvist-Fureby, 2006, Elversson et al., 2003).

6. Integrity of model antigen BSA

6.1 Circular Dichroism

This method is excellent and widely used way to determine the secondary structure of proteins. Native BSA 0.1mg/ml and 0.2 mg/ml were prepared. Spray dried adjuvant with BSA 1% w/v and 3% w/v were also prepared by extracting the protein as shown in protein content determination method and evaluated by spectropolarimeter with a quartz cell of 0.1 cm path length. The ellipticity of a solution was recorded in far UV range of 200 to 250 nm. CD spectra were obtained by plotting molar ellipticity against wavelength. Each measurement was done in triplicate (Kusonwiriyawong et al., 2009, Takashima et al., 2007). PBS and spray dried powder adjuvant without BSA were also analyzed as a control. Molar ellipticity [θ], deg cm^2 $decimol^{-1}$ was calculated as follows:

$$[\theta] = \frac{\theta.Mp}{10000.\ n.\ C'.\ l} \qquad \text{Equation 4}$$

Where, Mp= molecular weight of BSA (66430 Da),
n is number of amino acid residues of BSA (583 residues),
C' is concentration of BSA in sample solution (g/ml) and
l is path length of cell (0.1cm).

6.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE has been used to investigate the integrity of protein with response to circular dichroism. Firstly, PBS, untreated maltodextrin dissolved in PBS, native BSA 0.2 mg/ml, spray dried adjuvant alone, two different batches of spray dried adjuvant with 1% w/v BSA concentration, spray dried adjuvant with 3% w/v BSA and MF59 nanoemulsion diluted sample were prepared. The stock solution containing 2.5 µl of sample buffer, 1.5 µl of reducing agent and 3 µl of ultrapure was prepared by calculating the amount needed for each sample. Then, 9 µl of sample and 7 µl of reagent from the stock was mixed and heated at 70-80°C for 10 minutes. The loading amount of the protein in each well was around 700 ng in all sample containing BSA. After heating, 10 µl of each sample was loaded onto 4-12% Bis-Tris polyacrylamide gel and subjected to electrophoresis in Tris-Glycine SDS running buffer at 200V, 700 mA for 30 min. The gel was then separated carefully from its case without getting slit and stained with SImplyBlueTM SafeStain for 1 h. The gel was destained several times with distilled water to visualize protein bands (Kusonwiriyawong et al., 2013).

7. Stability evaluation

7.1 Stability testing of spray dried powders

It important to evaluate the particle size during storage as it can affect the uptake of formulation after administration. Glass vials containing spray dried powders with or without BSA as well as spray dried powder reconstitutions obtained from the same batches were stored at 4°C and at ambient temperature. At given time points (0, 1, 2, and 3 months), samples in both powder form and reconstituted one were collected and analyzed the particle size, polydispersity and zeta potential as mention in section 3.1 (Pohlmann et al., 2002, Soottitantawat et al., 2005).

7.2 Stability testing of liquid nanoemulsion adjuvant

Stability testing of optimized nanoemulsion was performed by keeping it in a refrigerator condition at 4°C and in the oven to maintain the ambient temperature. This study was carried out for 3 months (Qian et al., 2012). At predetermined time intervals of 0, 1, 2 and 3 months, droplet size of nanoemulsion as well as zeta potential and polydispersity was determined as mention in section 2.1.

8. In vitro cell culture

8.1 Cell culture of human monocyte macrophage cell line (CRL-9855)

In this study, human monocyte macrophage cell line (SC ATCC® CRL-9855^{TM}), suspension cells was used. The cells were cultured in 25 cm^2 flasks. Isocove's modified Dulbecco's medium with 4mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, supplemented with 0.05mM 2-mercaptoethanol, 0.1 mM hypoxanthine and 0.016 mM thymidine, 90% fetal bovine serum, 10% were used as complete growth medium for cells. Cell culture was incubated at 37°C in 5% CO_2 air atmosphere. The addition of medium or splitting of cells to other flask was done in every 2 or 3 days depending on cell density. Then, the appropriate cell concentrations was calculated by using hemocytometer and used for further study.

8.2 Cell culture of human nasal epithelial cell line (CCL-30)

RPMI 2650 (ATCC® CCL-30^{TM}) is originally from a human nasal septum, derived from metastatic site: pleural effusion. The cells were cultivated in 25 cm^2 culture flask at first. The cells were maintained in Eagle's minimum essential medium (EMEM), 90% in the presence of sodium pyruvate, non-essential amino acids as complete growth medium and fetal bovine serum to a final concentration of 10%. The cells were maintained at 37°C in 5% CO_2 atmosphere.

9. Cell viability study

9.1 Cell viability study by human monocyte macrophage cell line (CRL-9855)

Cell viability of spray dried powders in human monocyte macrophage cell line (CRL-9855) was examined by Alamar Blue assay. Briefly, different concentrations of cells were appointed first and seeded in 96 well plates. $1/10^{th}$ of Alamar blue reagent was added and the cells were incubated at 37°C in 5% CO_2 air atmosphere. At different time points (5 h, 8 h, 20 h) , the fluorescence intensity were measured at the excitation wavelength of 560 nm and the emission wavelength of 590 nm. The standard curve was drawn from the obtained results, which indicated the optimum concentration of cells and optimum incubation time with Alamar blue for human monocyte macrophage cell line.

From these results, the assay was carried out by seeding the optimum concentration of cells and treated with reconstituted spray dried powder (100 µl) at different concentration of BSA (140, 70, 35 µg/ml). The cells were then incubated for 24 h with the formulation. Then, $1/10^{th}$ of Alamar blue reagent was added and incubated with the optimum incubation time obtained from the standard curve. The percentage of cell viability was then calculated from the fluorescence intensity at the excitation wavelength of 560nm and the emission wavelength of 590 nm. Spray dried nanoemulsion blank was also determined. Cells co-incubated with PBS (pH 7.4) was also analyzed as negative control and was considered as 100% viability (D'Souza et al., 2012, Kusonwiriyawong et al., 2013, Ubale et al., 2013).

9.2 Cell viability study by human nasal epithelial cells (CCL-30)

The cell viability of CCL-30 was also determined by using Alamar blue assay. Cell concentrations of $2x10^4$ were seeded in 96-well plates and allowed to attach for one day. After 24 hours, the cells were treated with the

different concentrations of formulation FB5-BSA1 and FB5-BL control. The cell co-incubated with PBS (as a negative control) was considered as 100% cell viability. The cell incubation time with the formulations was 24 hours after which $1/10^{th}$ volume of Alamar blue reagent was added back to each well. Onwards, the plate was incubated at 37°C for 4 hours and the absorbance was measured at the excitation wavelength of 560 nm and the emission wavelength of 590 nm (Wong et al., 2007).

10. Uptake study by using confocal laser scanning microscope (CLSM)

10.1 Uptake study using CRL-9855

FB5 was in cooperated with FITC-BSA 1% and spray dried to observe the uptake process by confocal laser scanning microscope (CLSM). Glass slips were pre-coated with 0.01% of poly-L-lysine and kept it in a well plate before cell seeding. The FB5-FITCBSA1 was then incubated with human monocyte macrophage cells for 1h. The cells were then fixed with 4% paraformaldehyde (Kusonwiriyawong et al., 2013, Takashima et al., 2007, Yue et al., 2011). After 15-20 min, the cover slips were washed with PBS for three times and the cell membrane were dyed with wheat germ agglutinin. It was then incubated for 10 min and then the cover slips were again wash with PBS for three times. Two or three drops of DAPI was placed on the specimen and allowed to incubate for 10 min. Then, the cover slips were inverted on the slides to analyze under the confocal laser scanning microscope.

CHAPTER IV
RESULTS AND DISCUSSION

1. Preliminary study of nanoemulsion

The stable nanoemulsion with the smallest particle size was achieved by varying the compositions of oil, surfactant and process parameters. The highest wheat germ oil composition (5%) with 0.5% Tween80 and 0.5% Span85 while fixed parameters of 3 min ultraturrax processing time and 40°C during high pressure homogenization at 1000 bar for 6 cycles gave the largest particle size of 211 nm, zeta potential 1.38 mV and polydispersity 0.238. When the oil compositions were reduced to 1%, the particle size decreases gradually. The particle size variation in different formulations could be due to the interfacial tension of oil and emulsifier solutions (Mao et al., 2009). The smallest particle size of 130 nm was obtained with the formulation A1 (0.5%) containing 1% of wheat germ oil with the same surfactant compositions and constant parameters. Higher negative values of zeta potential were observed with decreased in oil compositions and that made the nanoemulsion more stable. Thus, A1 (0.5%) was chosen as the optimized one from the table 7.

Table 7 Particle size, zeta potential and polydispersity of A1, A2, A3, A4, A5 containing Tween80 0.5%, Span85 0.5% with 3 min of ultraturrax homogenizer and 40°C of high pressure homogenization

	A1 (0.5%)	A2 (0.5%)	A3 (0.5%)	A4 (0.5%)	A5 (0.5%)
Particle size (nm)	**133± 0.88**	161± 1.07	172± 1.02	196± 3.02	211± 1.01
Zeta potential (mV)	**-20.0± 0.7**	-18.8± 0.7	-14.5± 0.5	-3.3± 0.9	1.3± 1.7
Polydispersity (PDI)	**0.268**	0.212	0.223	0.242	0.291

On the other side, two types of oil, WGO and Vitamin E acetate in different concentrations (1:0.05, 0.5:0.5, 0.9:0.1, respectively) were prepared with fixed 0.5% Tween80 and 0.5% Span85 and constant parameters of 3 min ultraturrax processing time and 40°C of high pressure homogenization. The particle size, zeta potential and polydispersity are shown in Table. 9.

Table 8 Particle size, zeta potential and polydispersity of B1, B2, and B3 with fixed parameters of 3 min ultraturrax homogenizer and 40°C of high pressure homogenization

	B1 (0.5%)	B2 (0.5%)	B3 (0.5%)
Particle size (nm)	196± 4.43	155± 6.47	130 ± 0.506
Zeta potential (mV)	-22.6 ±1.01	-20±0.9	-19.4±0.65
Polydispersity (PDI)	0.252	0.254	0.260

Formulation B3 (0.5%) was chosen from table 8 as it had the smallest particle size compared to B1 and B2. The density of vitamin E acetate (0.953 g/ml) is higher than density of WGO (0.93 g/ml). Thus, higher concentration of vitamin E acetate containing formulation B2 gave the larger particle size compared to B3. Formulation B1 has highest total oil composition, which gave the bigger particle size in comparison with B2 and B3. Zeta potential was -19.4 mV which means nanoemulsion was quiet stable with the suitable polydispersity of 0.260.

Thus, to study the effect of surfactant concentrations, 1% of Tween80 and 1% of Span85 were used and applied on formulation A1 (0.5%) and B3. The resulted particle were compared with the lower amount of surfactants, Tween80 (0.5%) and Span85 (0.5%) as shown in table 9.

Table 9 Particle size, zeta potential and polydispersity obtained after increasing the surfactant concentration compared with the smaller amount of surfactant concentration

Formulation code	Particle size (nm)	Zeta potential (mV)	Polydispersity (PDI)
A1 (0.5%)	**130 ± 0.88**	**-20±0.7**	**0.268**
B3 (0.5%)	137 ± 0.506	-19.4±0.65	0.260
A1 (1%)	133± 0.98	-21±0.57	0.258
B3 (1%)	**130 ± 1.21**	**-26±0.77**	**0.278**

From Table 9, A1 (0.5%) and B3 (1%) was chosen as the optimized nanoemulsion formulation. There is not much difference in particle size for formulation A1 when the surfactant concentrations were increased. Thus, the lower amount of surfactant concentrations of Tween80 (0.5%) and Span85 (0.5%) were chosen for A1

(0.5%). For formulation B3, slight decrease in particle size was observed when surfactant concentrations were increased. While comparing the zeta potential of B3 (0.5%) and B3 (1%), increased surfactant concentrations B3 (1%) possess higher negative values of zeta potential. Thus, it can be concluded that B3 (1%) was more stable than B3 (0.5%) and even surfactant concentrations (1%) is not enough to cover the surface of the droplets. Negative zeta potential values were observed in all nanoemulsion which could be possibly due the presence of relatively high levels of 55% polyunsaturated fatty acids, especially linoleic fatty acid in WGO.

As the last step in optimizing nanoemulsion, various parameters were appointed and applied on formulation A1 (0.5%) and B3 (0.5%).

Table 10 Effect of parameters on formulation A1 (0.5%)

Ultraturrax	High pressure homogenizer	Particle size (nm)	Zeta potential (mV)	Polydispersity
3 min	40°C	130±0.88	-20±0.7	0.268
	50°C	135±0.32	-24±1.24	0.293
	60°C	127±1.87	-26±1.83	0.295
5 min	40°C	129±2.2	-25±1.5	0.265
	50°C	121±1.5	-27±2.9	0.285
	60°C	120±1.2	-23±0.9	0.277
7 min	40°C	112±1.9	-22±2.5	0.303
	50°C	**110± 0.4**	**-29±0.7**	**0.275**
	60°C	127±1.15	-24±1.9	0.288

From Table 10, the particle size of the formulations were decreased with increased in processing time of ultraturrax homogenizer and temperature during high pressure homogenization. However, zeta potential and polydispersity were slightly changed in each parameter sets. Increasing the temperature up to 60°C has increased the particle size up to 127nm. Zeta potential with high negative values provides good stability but higher polydispersity index can lead to instability. Hence, formulation A1 with the ultraturrax processing time of 7 min and 50°C during high-pressure homogenization was chosen as the optimized nanoemulsion as it has higher zeta potential negative values and lower polydispersity. Hence, this formulation was coded as FA.

Table 11 Effect of parameter on formulation B3 (1%)

Ultraturrax	High pressure homogenizer	Particle size (nm)	Zeta potential (mV)	Polydispersity
3 min	40°C	130±1.21	-23±0.77	0.282
	50°C	140±1.4	-27±1.8	0.305
	60°C	148±0.5	-26±0.84	0.257
5 min	40°C	125±0.9	-17±1.15	0.287
	50°C	121±1.67	-27±0.97	0.285
	60°C	121±1.3	-25±1.27	0.273
7 min	40°C	120±0.99	-22±1.89	0.313
	50°C	121±1.29	-21±0.64	0.330
	60°C	**100±0.5**	**-24±0.65**	**0.289**

Table 11 has the similar results compared as Table 10 except that temperature during high pressure homogenizer at 60°C had reduced the particle size. The smallest particle size was 100 nm, zeta potential of -24 and polydispersity 0.289 at 7 min of ultraturrax processing time and 60°C during high pressure homogenization. This formulation was chosen as the optimized one and coded as formulation FB.

2. Preliminary study of spray dried nanoemulsion with the carrier maltodextrin

Optimized spray drying condition was investigated by varying different concentrations of maltodextrin as well as process parameters such as inlet temperature and pump rate. The resulted powder was reconstituted with ultrapure water in order to obtain the same concentration as before spray drying and measured the particle size, zeta potential and polydispersity. The smallest difference in particle size between initial nanoemulsion before spray drying and reconstituted spray dried powder was chosen as the optimized condition for spray drying. Moreover, spray dried powder yield was also observed.

Table 12 Effect of maltodextrin concentration and process parameters on formulation FA

	FA1	FA2	FA3	FA4	FA5	FA6	FA7	FA8
MD (% w/v)	3	5	3	5	3	5	3	5
Inlet Temp: (°C)	110	110	130	130	110	110	130	130
Pump rate (%)	20	20	20	20	30	30	30	30
Particle size (nm)	160± 1.2	216± 2.13	201± 2.89	227± 1.5	149± 0.7	160±1. 85	185± 5.8	191± 1.66
Zeta potential (mV)	-15± 0.37	-16± 0.51	-16± 0.1	-19± 0.64	-15± 0.11	-9± 0.33	-18± 0.15	-8± 0.48
PDI	0.22	0.302	0.296	0.266	0.173	0.263	0.329	0.236
Yield (%)	34.2	46.0	34.6	44.5	31.8	49.2	26.0	41.2

In Table 12, it has been observed that increasing the maltodextrin concentration had increased the particle size but no effect was observed on percent yield. Increasing the inlet temperature leads to the larger particle size up to 227 nm. The spray dried powder yield considerably varied from 41% to 57%. The pump rate of 20 % with maltodextrin concentration of 3% gave the higher percent yield. The higher pump rate 30% did not affect the percent yield. Low spray dried powder

yield could be due to adhesion of powder to the spray dryer wall or the removal of small particles by the aspirator. FA1 and FA3 had the highest spray dried powder yield with the larger particle size of 160 nm and 201 nm, respectively. Generally, the lower concentration of maltodextrin was likely to give the smaller particle size (Sinsuebpol et al., 2013). The smallest particle size of 148 nm was obtained with FA5, by using the inlet temperature 110°C, pump rate 30% and 3% of maltodextrin concentration. Zeta potential of all formulations decreases compared to original nanoemulsions, which could be due to maltodextrin covering up the surface of the particles during spray drying.

Table 13 Effect of maltodextrin concentration and process parameters on formulation FB

	FB1	FB2	FB3	FB4	FB5	FB6	FB7	FB8
MD (% w/v)	3	5	3	5	3	5	3	5
Inlet Temp: (°C)	110	110	130	130	110	110	130	130
Pump rate (%)	20	20	20	20	30	30	30	30
Particle size (nm)	163± 2.02	189± 2.56	206± 5.8	178± 2.17	153± 0.6	185± 2.16	187± 5.54	160± 2.2
Zeta potential (mV)	-17± 2.02	-10± 2.56	-16± 0.41	-11± 0.17	-23± 0.12	-11 ±0.5	-13± 0.8	-14± 0.2
PDI	0.268	0.309	0.553	0.257	0.260	0.336	0.351	0.309
Yield (%)	35.0	55.8	30.8	36.6	34.7	37.5	22.5	41.6

*FB5 – particle size was consistent when repeated three times.

Table 13 has the similar results with Table 12 except that the concentration of maltodextrin had no effect on particle size. From spray drying of FB, it can be concluded that spray drying condition of FB5 was the most suitable one in response to particle size, zeta potential, polydispersity and spray dried powder yield. FB1 had the highest spray dried powder yield but the particle size was slightly larger than

FB5. As all the formulations were prepared in triplicates, the particle size consistency in each batch was observed to get the optimum spray drying conditions and FB5 was the most consistent one in terms of particle size. As both FA5 from Table.12 and FB5 from Table.13 with the optimum particle size has the same spray drying condition. Thus, it can be concluded that inlet temperature 110°C, pump rate 30% and maltodextrin concentration 3% is the best condition that met the requirement. Hence, as the last step in preliminary study, both FA5 and FB5 was spray dried by including different concentrations of BSA (1% w/v and 3% w/v) as a model antigen to determine the particle size, zeta potential, polydispersity, spray dried powder yield as well as consistency in different batches of spray drying.

Table 14 Particle size, zeta potential, and spray dried yield after loading BSA of different concentration

Formulation	MD (%w/v)	BSA (%w/v)	Particle size (nm)	Zeta potential (mV)	PDI	Yield (%)
FA5	3	1	185±2.8	-10±0.2	0.184	47
		3	200±4	-12±0.32	0.222	49
FB5	3	1	154±0.8	-12±0.66	0.253	58
		3	188±2.1	-6±0.23	0.239	41

Each spray dried formulation FA5 and FB5 loaded with BSA were prepared in three different batches in order to determine the particle size consistency. The particle size of FA5-BSA formulation increases inconsistently in every batch. On the other side, FB5-BSA has the same consistent particle size in every batch. Therefore, in the preliminary study, FB5 was chosen as the optimized formulation in corresponding to the consistency results between each batch. FB5 with BSA

concentration of 1% gave the optimum particle size 154nm and zeta potential of -12 mV. The original nanoemulsion prior to spray drying and FB5 spray dried reconstituted one has not much difference in particle size with narrow size distribution could be observed. FB5 blank without BSA (FB5-BL) has the zeta potential of -22 mV, which slightly decreases after loading with BSA. It may be due to BSA having positive charge that mask the surface of the particles. FB5 was appointed for further investigation and effect of protein concentration between 3% and 1% containing spray dried formulation was compared in some experiments.

Incorporation of BSA with maltodextrin resulted in a white powder and maltodextrin acting as amorphous diluent. It could be concluded from the preliminary study that the percentage yield obtained from all these formulations in cyclone part was higher than that in the collector part of spray dryer. The particles adhered on the wall of cyclone might be due to the glass transition temperature (Tg) of maltodextrin. The droplet surface would be sticky if the surface layer Tg is lower than droplet temperature (Sansone et al., 2011). While increasing the maltodextrin concentration and inlet temperature to 130°C, larger amounts of powder were found in collector part and the adhesion of particles on cyclone wall was reduced.

3. Characterization of nanoemulsion by transmission electron microscope

TEM analysis was performed to investigate the morphology of nanoemulsion adjuvant droplets. Fig. 16 and 17. The optimized nanoemulsion FA and FB were characterized. Both the optimized nanoemulsions has the spherical droplets shape but some are deformed and the diameter of particle size determined under the microscope is in a good agreement with the size obtained from Zetasizer (Deng et al., 2014).

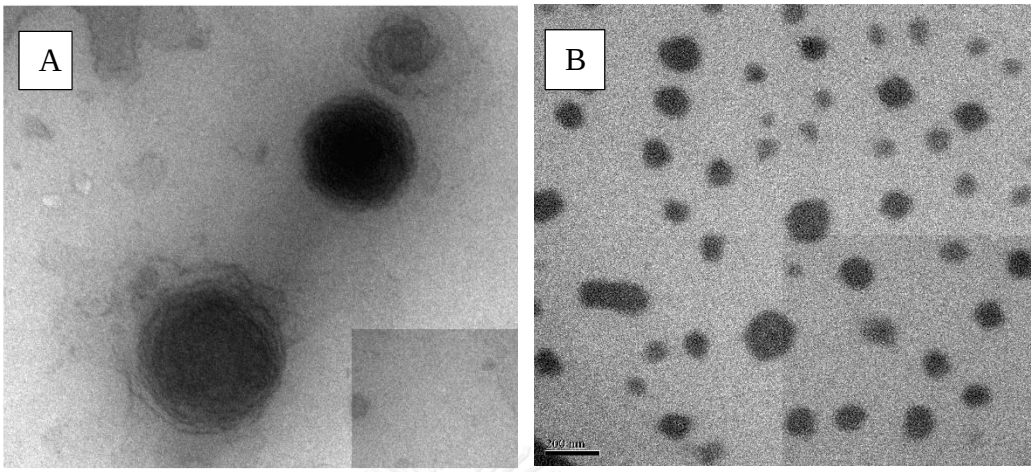


Figure 16 TEM images of FA, (A) with the magnification of 9900x and (B) magnification of 11500X

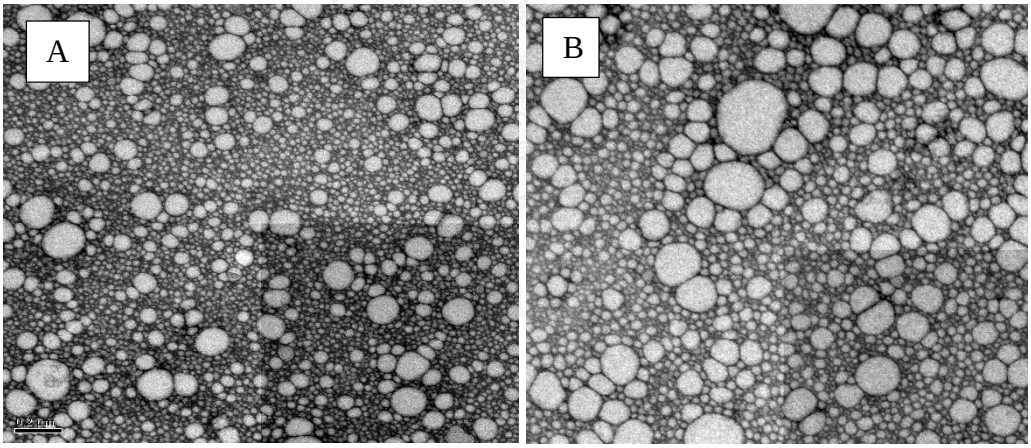


Figure 17 Fig. 17. TEM images of formulation FB, changes in contrast of the picture, (A) magnification of 9000x, (B) magnification of 9000x.

4. Characterization of spray dried powders
   a. Powder morphology by scanning electron microscope (SEM)

SEM micrographs of FA5 and FB5 spray dried powder with or without BSA were determined. The particles of both formulations were spherical shape in general with various sizes and porous like surface. FA5 without BSA (FA5-BL) had smooth

distorted surface but after loading with BSA, the particles become completely spherical with small pores. The similar results was observed in FB5 formulation. The smoother surface was seen in FB5-BL in comparison with FB5-BSA1, which gave a porous like surface. Fig. 18. When loading of the BSA concentration was increased, more compact and dense pores with smaller particle size was observed on the surface of the particles in FB5-BSA3. Fig. 19, 20 and 21. Some small particles were found to be fused onto larger ones. (Ståhl et al., 2002) had shown that the composition of protein formulation is very important and addition of more surfactant or carrier could give a smoother surface. Another researcher had showed that a spherical morphology would favor higher uptake by macrophages and other cells in the immune system (Ubale et al., 2013). Generally, all the powders showed a quiet homogeneous size distribution.

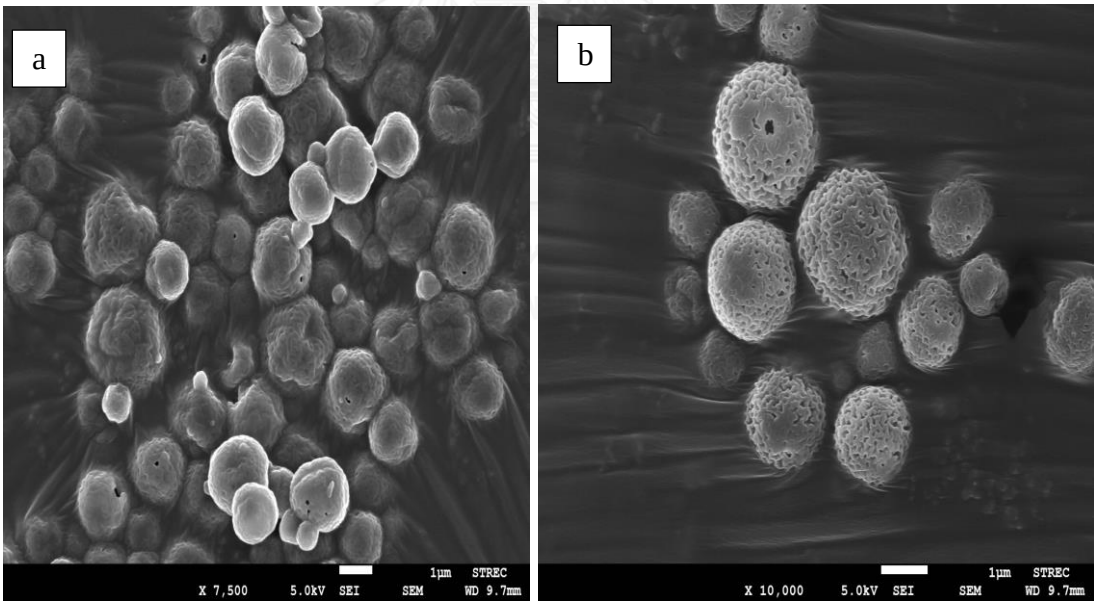


Figure 18 SEM images of (a) FA5-BL, magnification of 7,500x (b) FA5-BSA1, magnification of 10,000x

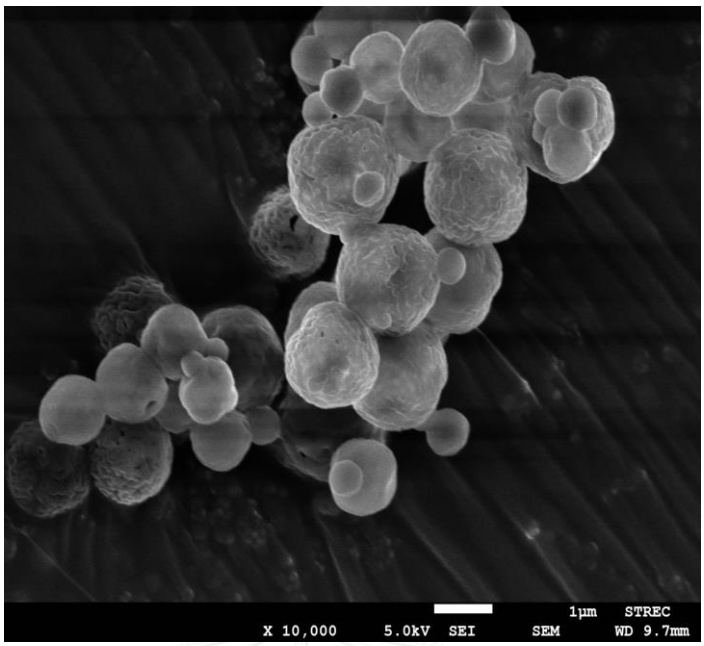


Figure 19 SEM image of FB5-BL, magnification of 10,000x

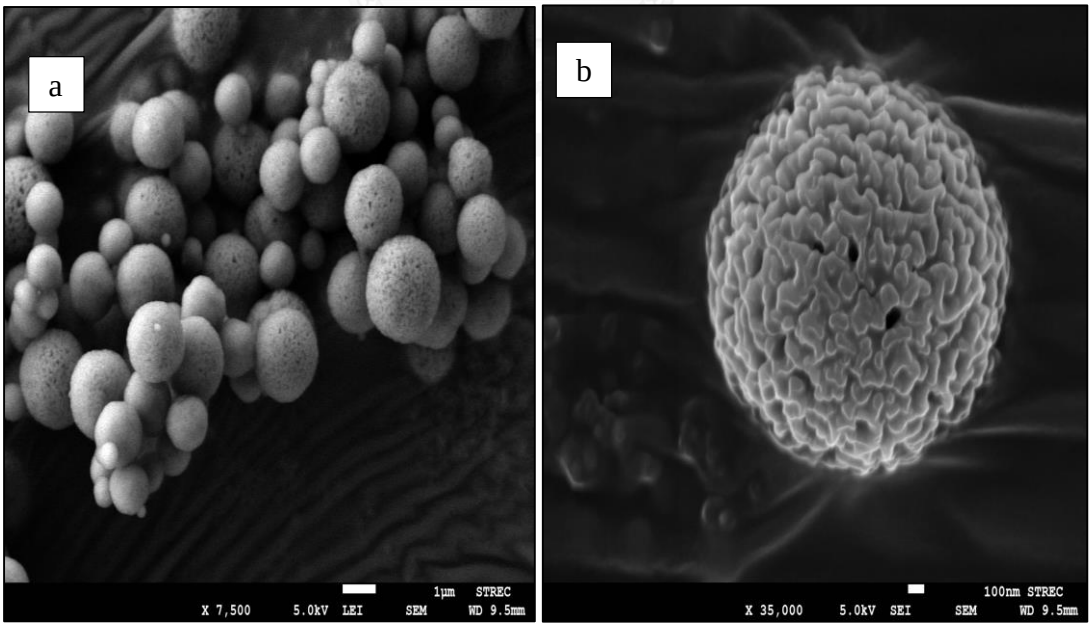


Figure 20 SEM images of FB5BSA1 (a) magnification of 7,500x, (b) magnification 35,000x

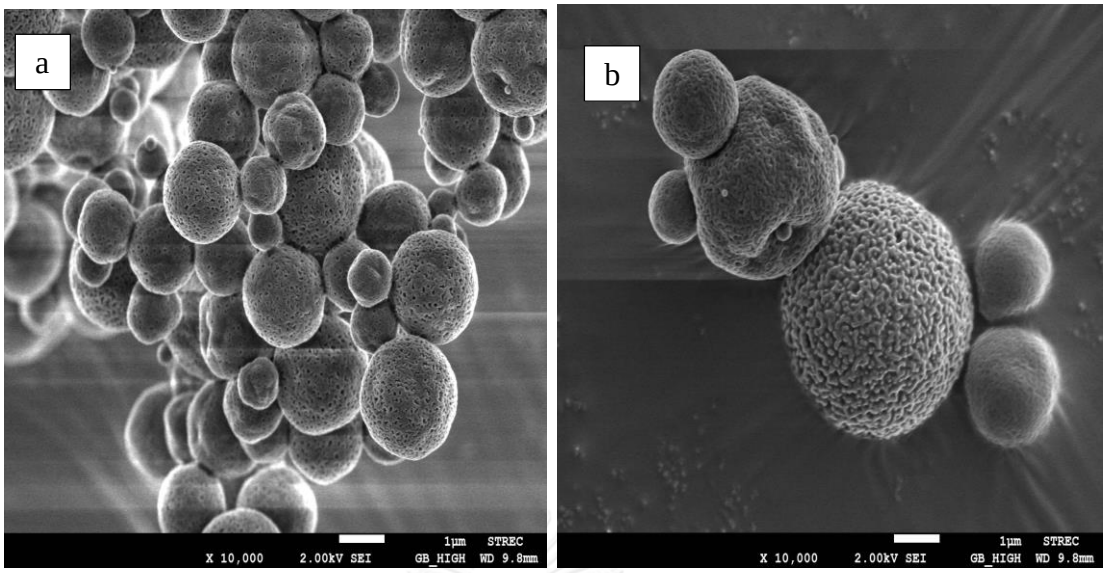


Figure 21 SEM images of FB5BSA3 (a) magnification of 10,000x, (b) magnification 10,000x

b. Particle size determination by mastersizer

Table 15 . Particle size of spray dried powder

Formulation	Particle size (µm)	Span
FA5-BL	5.225± 0.4	12.479
FA5-BSA1	7.997± 0.62	8.550
FB5-BL	6.882± 0.27	5.704
FB5-BSA1	4.852± 0.83	5.203
FB5-BSA3	3.087± 0.04	8.55

The particle sizes of FA5-BL and FB5-BL have the range of 5-6 µm while BSA incorporated formulation FA5-BSA1, FB5-BSA1 and FB5-BSA3 have the size range between 3-8 µm. The higher BSA concentration of 3% in FB5-BSA3 gave the smaller particle size than FB5-BSA1. While comparing the FB5-BL with FB-BSA1 and FB5-BSA3, it can be determined that the concentrations of BSA had effect on particle size but not on uniformity (Kusonwiriyawong et al., 2009). All

the formulations have a wide size distribution with Span (= 5-12). FB5-BSA1 and FB5-BSA3 has a particle size of < 5μm and this particle size is usually considered as within the respirable range (Lucas et al., 1998).

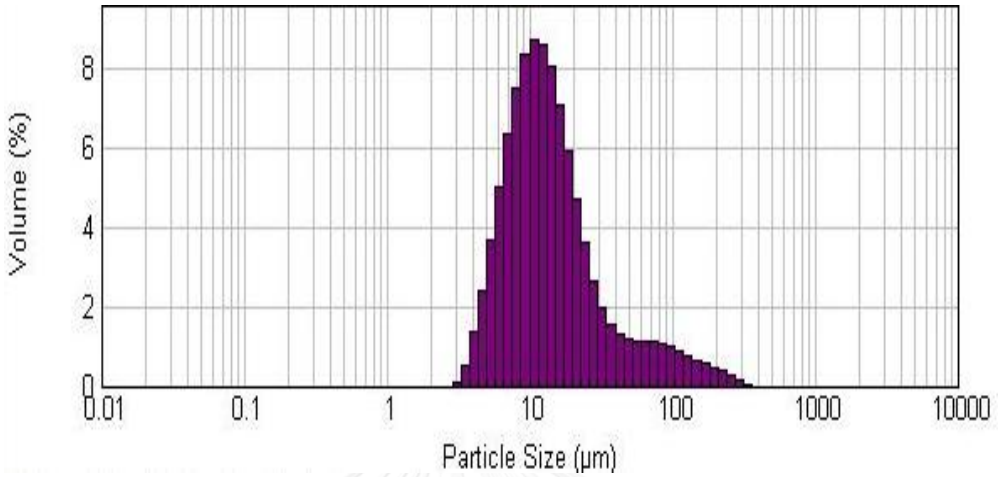


Figure 22 Particle size distribution of FB5-BL spray dried powder

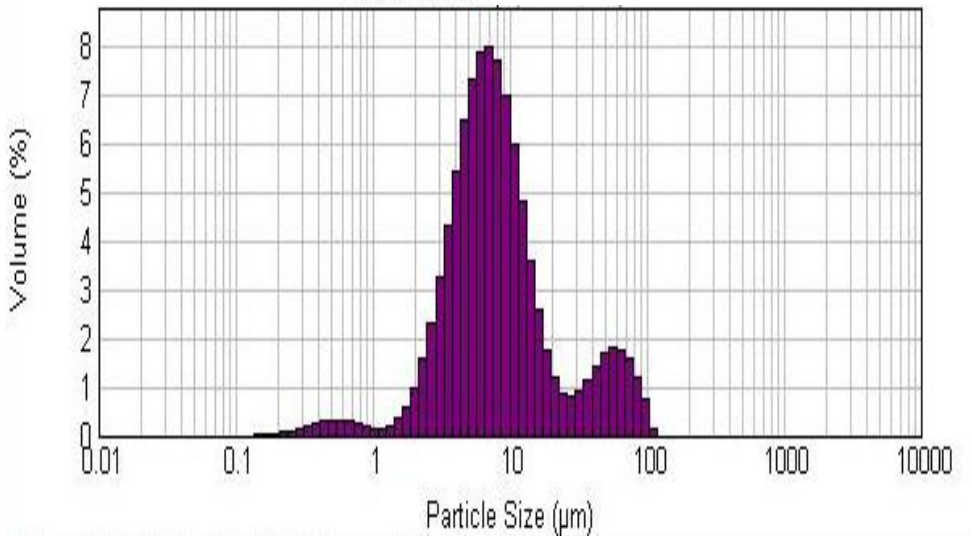


Figure 23 Particle size distribution of FB5-BSA1 spray dried powder

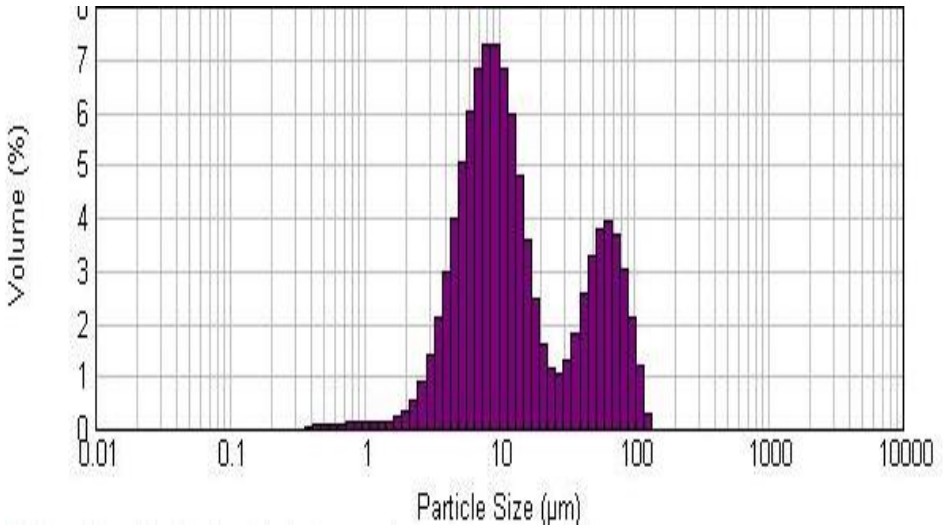


Figure 24 Particle size distribution of FB5-BSA3 spray dried powder

5. Differential scanning calorimetry (DSC) analysis of spray dried powder

DSC thermograms of spray dried formulations as well as native BSA and MD untreated is shown in Fig.25. It should be noted that different DSC thermograms could be obtained with various grades of protein or maltodextrin. Low intensity broad peak was observed between 40°C and 140°C followed by the intense exothermic peak around 230°C in untreated MD. The glass transition temperature of maltodextrin was found to be around 122°C. The first broad peak could be due to the elution of loosely bound water and the second peak 220°C was the melting point of maltodextrin. However, thermogram of FB5-BL did not show any specific low broad intensity peak compared to untreated MD and it might be due to spray drying. FB5-BSA1 shows a broad peak around 200°C and 240°C, it could be the combination of the nanoemulsion formulations with BSA and MD. DSC results interfered that no specific interaction between maltodextrin and native BSA was observed. To confirm this, further investigation was done by XRD (Luppi et al., 2005, Mi et al., 2003, Elnaggar et al., 2010, Elversson and Millqvist-Fureby, 2005).

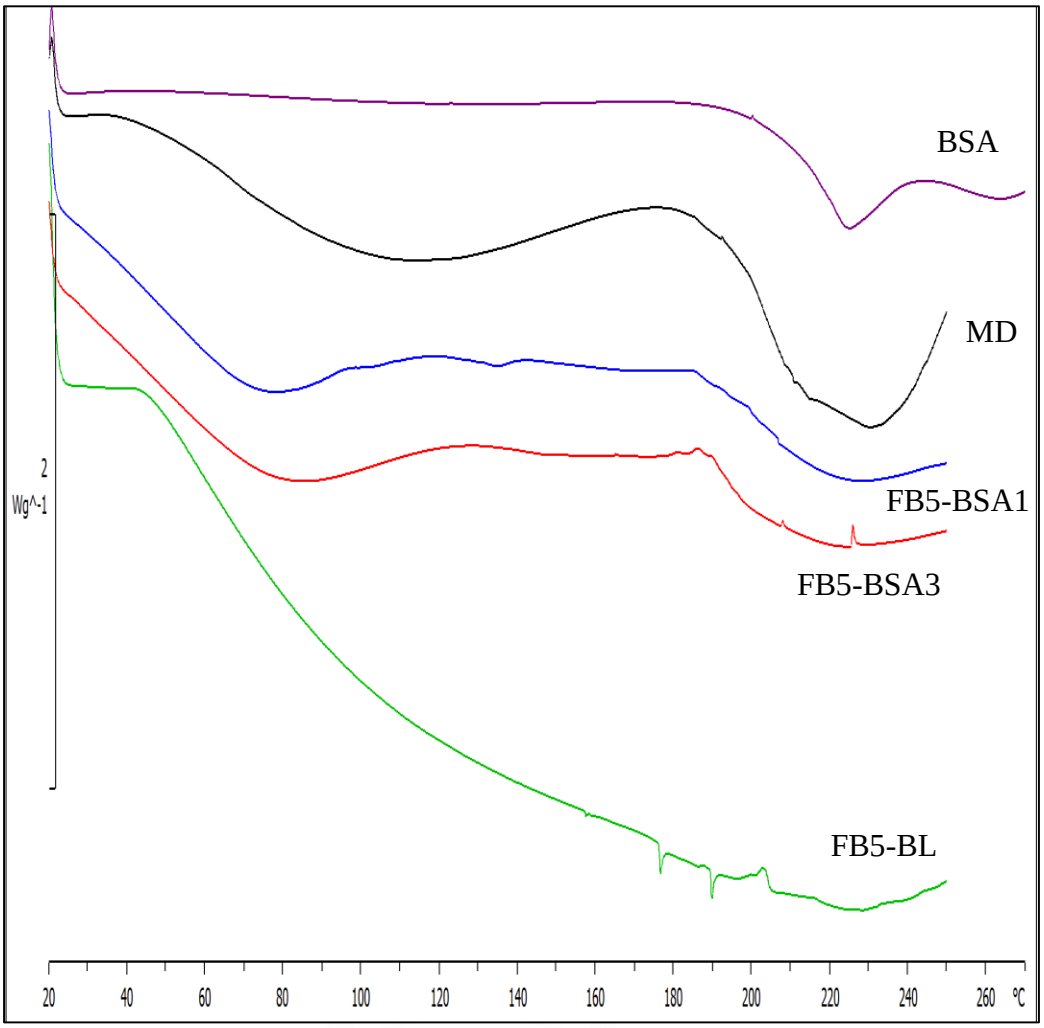


Figure 25 DSC thermograms of MD untreated, native BSA, FB5-BSA1, FB5-BSA3, FB5-BL

6. Powder crystallinity analysis by XRD

The crystallinity of FB5-BL, FB5-BSA1, untreated MD and native BSA was investigated by using X-ray powder diffractometer is shown in Fig. 26 and 27. Amorphous materials did not produce sharp diffraction peaks (Lo et al., 2004). The peak observed in all XRD patterns were not the sharp one, they were broad peaks. Thus, all the powders were amorphous in shape. In XRD pattern of untreated MD, 2θ 18° and 35° showed a broad peak. On the other side, FB5-BL, which was the spray dried nanoemulsion with maltodextrin also showed a broad peak shifted toward 2θ 20° compared with MD untreated and disappearance of 2θ 35° peak was observed. Even after spray drying, the formulation was in an amorphous form. In native BSA XRD pattern, two broad intense peak was identified around 2θ 8° and 18°. It did not show a significant crystalline character as well. On comparison between, XRD patterns of FB5-BL and FB5-BSA1, even though there was no significant new peak was observed. On careful observation, it was noted that a slightly low intense broad peak of 2θ 10° was observed. It could be due to inclusion of both between MD and BSA in which it had been confirmed that BSA is totally incorporated with MD (Bai and Shi, 2011, Maa et al., 1997, Rogers et al., 2003).

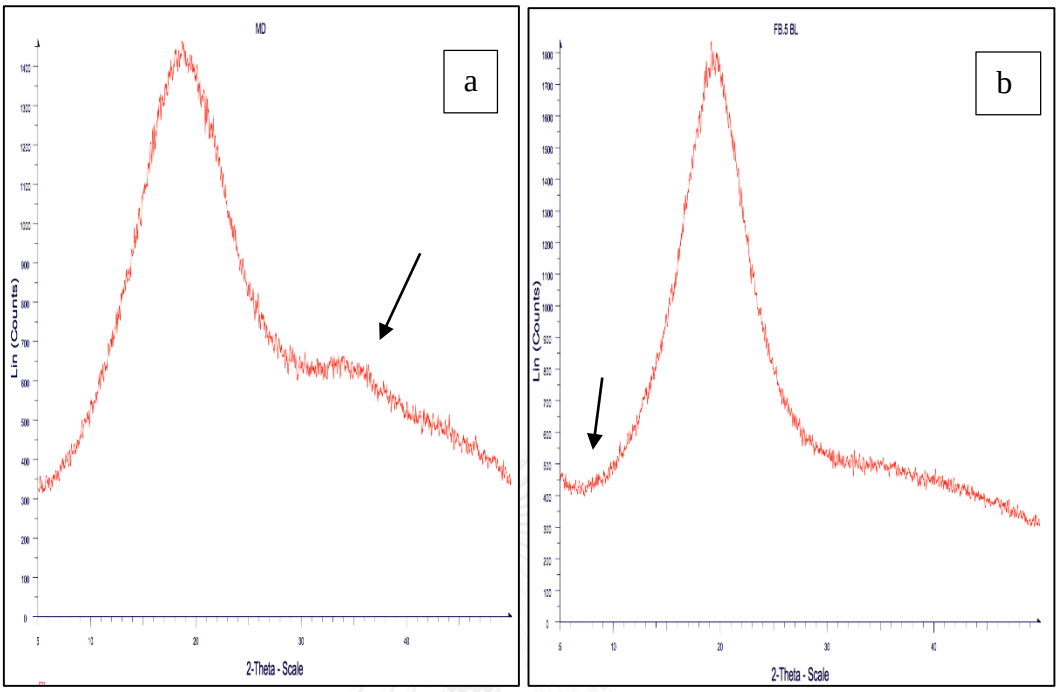


Figure 26 XRD pattern of (a) MD untreated and (b) FB5-BL

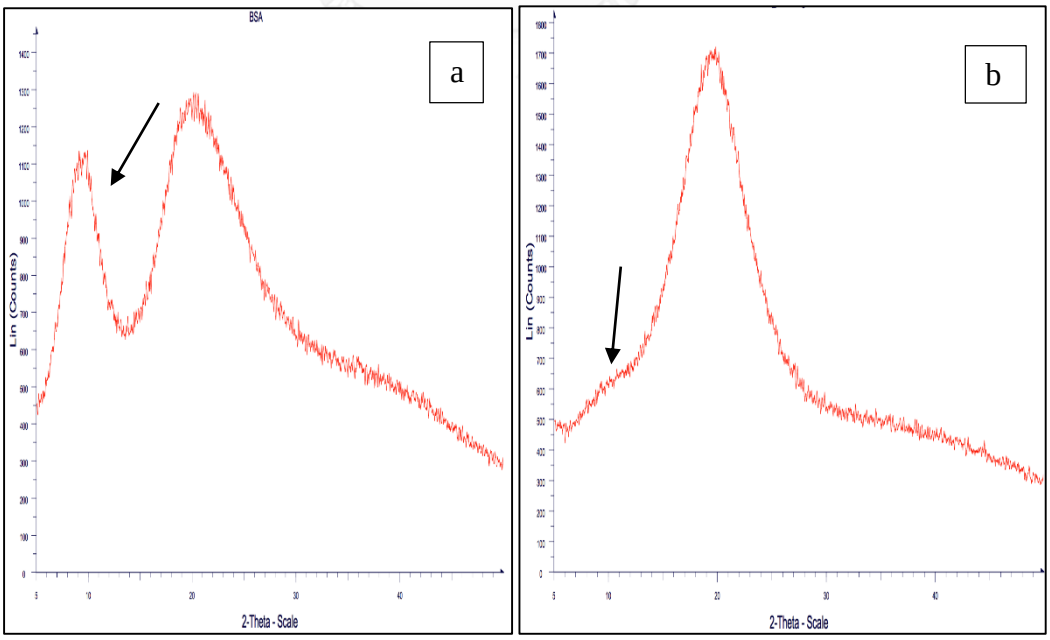


Figure 27 XRD pattern of (a) native BSA and (b) FB5-BSA1

7. Fourier transform infrared spectroscopy (FTIR)

FTIR is generally used to determine the presence of possible interaction within the formulations. FTIR spectra of MD, native BSA, FB5-BL and FB5-BSA1 in stack comparison are shown in Fig 28 and 29.

The band around 3300 cm^{-1} is normally belongs to OH stretching vibration, which is observed in all spectrum. The C-H stretching region is around (3000-2800 cm^{-1}) containing the asymmetric and symmetric lipid CH_2 stretching vibration bands, significantly visible in FB5-BL and FB5-BSA1 at 2855 cm^{-1}. The amide I vibration modes represents between 1700 cm^{-1} and 1660 cm^{-1} , which are responsible for protein structure. The region below 1500 cm^{-1} is generally called the fingerprint region and bands from this region are difficult to define however, they are the characteristics of the sample (Oldenhof et al., 2005). On spray drying, a new peak was observed at 1744 cm^{-1} in both formulation FB5-BL and FB5-BSA1 when comparing with untreated MD. It might be the inclusion of the nanoemulsion formulation. In native BSA spectra, two amide bands corresponding to its structure was observed at 1658cm^{-1} and 1532 cm^{-1}. FB5-BSA1 spray dried powder, which includes both maltodextrin and BSA also showed amide bands at 1659 cm^{-1}, and 1547 cm^{-1}. These two bands were referred to BSA α-helix conformation and slight increase in peak ratio was observed in comparison with native BSA and it is due to the addition of nanoemulsion formulation and MD. These two bands of BSA observed in both native BSA and FB5-BSA1 are due to the C=O stretching band of the carboxyl functional group on the tryptophan moiety as well as carboxyl groups (Sun et al., 2012).

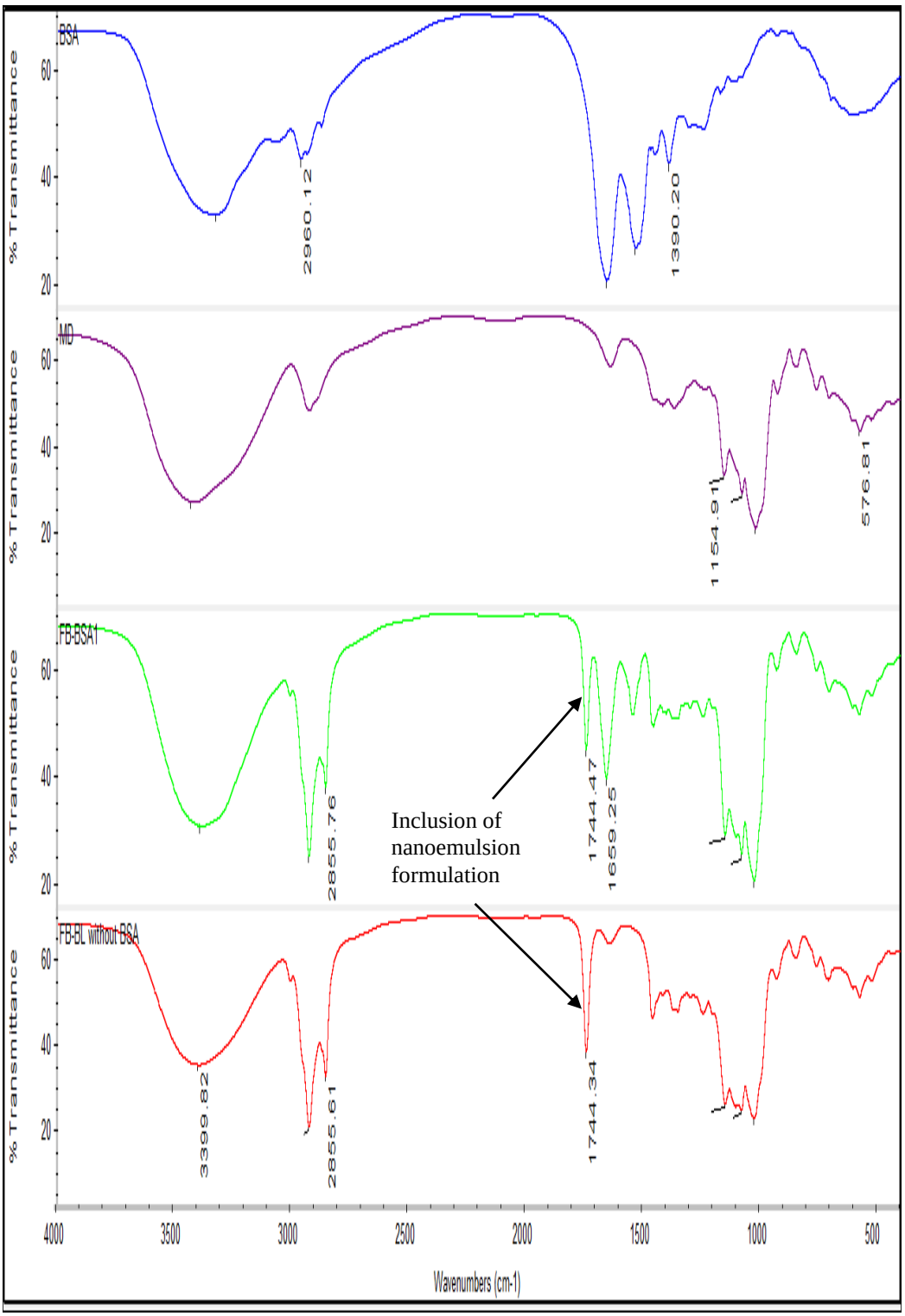


Figure 28 FT-IR spectra stack comparison of (1) Native BSA, (2) MD, (3) FB5-BSA1 and (4) FB5-BL

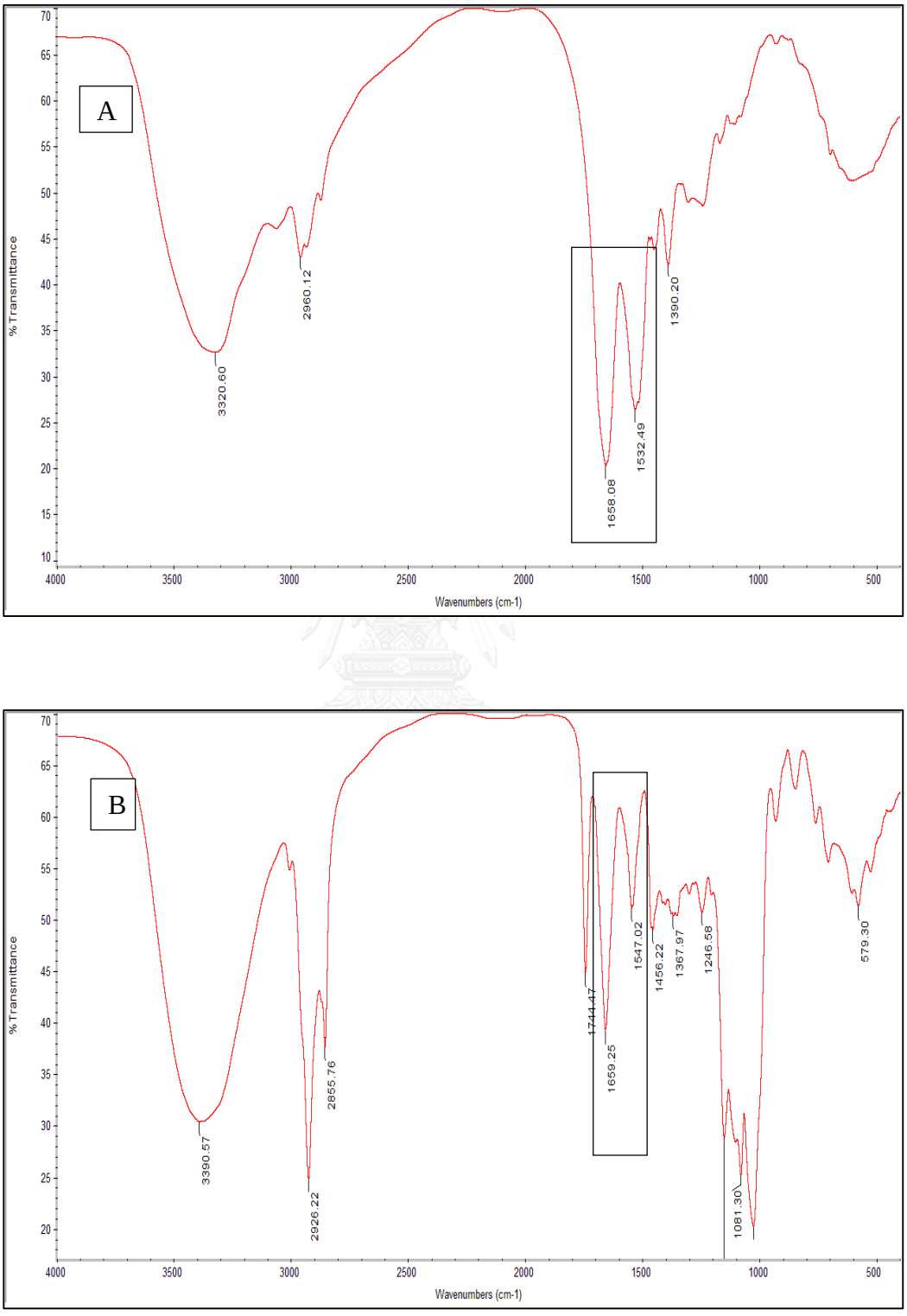


Figure 29 FTIR spectra (A) Native BSA (B) FB5-BSA1

8. Protein content determination

The protein loading of optimized formulations is summarized in Table 16. Protein content of different batches was also investigated. According to micro BCA assay kit instruction, higher concentration >1% of Tween80 could interfere with the absorbance value. After centrifugation, aqueous phase was separated to determine the protein content. As Tween80 was also soluble in water, it could interfere with the absorbance value. Therefore, FB5-BL was run as control and the absorbance obtained was subtracted from protein containing spray dried formulations. The percentage of protein content of FB5-BSA1 was in the range of 13.14 – 14.36 % while FB5-BSA3 was in the range of 23.57- 34. 2 %. FB5-BSA1 had 1% BSA, 3% MD, 1% Tween80, 1% Span85, 1% oil thus total 7% and protein content was 1/7 part (= 0.14*100). Hence, the results obtained from this calculation was correlated with the results obtained from micro BCA assay kit. FB5-BSA3 contained 3% of BSA and protein content was 3/9 part (=0.33*100) but this calculated results was not in correspondence with the results obtained from micro BCA assay kit. It could be possibly due to the higher protein concentration which was not within the standard curve range. Thus, the extracted protein was diluted and included in the assay. The results obtained after dilution was in good agreement with the calculated results.

Table 16 Protein content determination of spray dried formulation

Formulation	Protein loading (%)	Protein content (μg/ml)
FB5-BSA1 (1st batch)	14.05	140.56
FB5-BSA1 (2nd batch)	14.36	143.65
FB5-BSA1 (3rd batch)	13.14	131.45
FB5-BSA3 (1st batch)	23.57	235.73
FB5-BSA3 (2nd batch)	23.91	239.1
FB5-BSA3 (1st batch – 4 fold dilution)	34.2	342

9. In vitro release study

The release of BSA from the spray dried formulation was carried out in vitro at pH 7.4 at 37°C. The profiles of the results were illustrated in fig. 30.

During the time course (24 h) of release studies, the spray dried formulation FB5-BSA1 and FB5-BSA3 released protein slowly and the patterns of release profile for both formulations were the same. On the evaluation of the first 15 min, the protein released was around 7.5 % in FB5-BSA1 and 4.5 % in FB5-BSA3. The BSA burst released occurred after 30 min with 34 % from FB5-BSA1 and 22 % from FB5-BSA3. After 1 h, BSA release from FB5-BSA1 was 61% and FB5-BSA3 released only 43% of BSA. It was noted that with periods of time, FB5-BSA3 released BSA slowly and gradually compared to FB5-BSA1. This could be due to the compact and dense porous structure of FB5-BSA3 corresponding to the SEM images, even though the particle size of FB5-BSA3 was smaller than FB5-BSA1. After 24 h, BSA was released 100 % completely from FB5-BSA1. BSA released between different batches of FB5-BSA1 was consistent as well (Kusonwiriyawong et al., 2013).

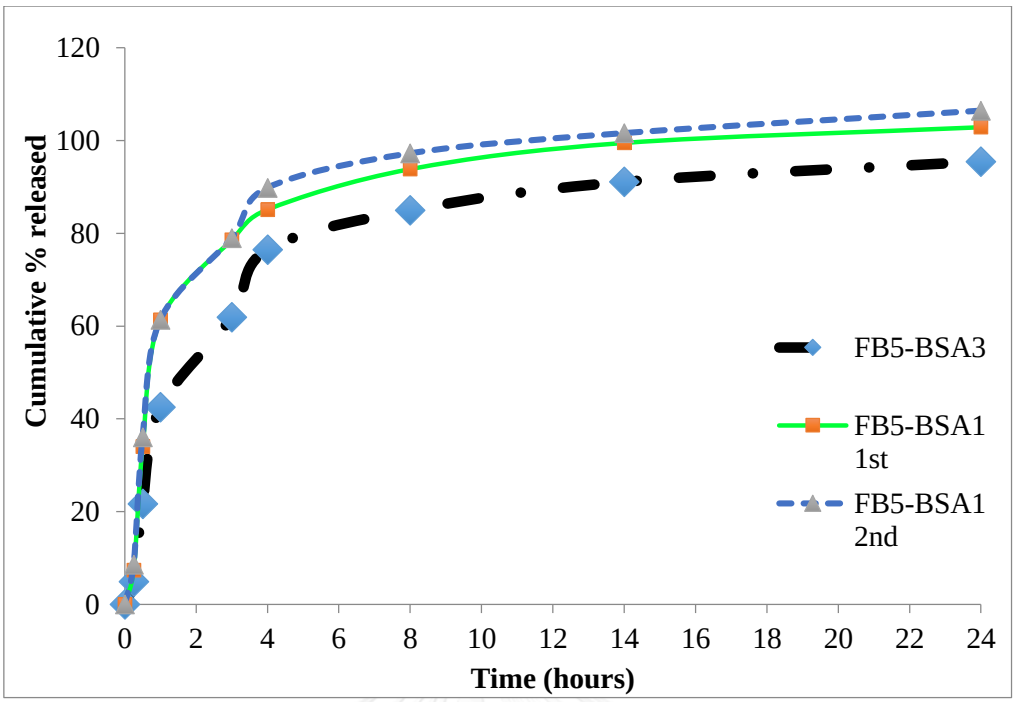


Figure 30 Graph showing cumulative percent released of different batches of FB5-BSA1 and FB5-BSA3

10. Integrity of model antigen BSA

a. Circular Dichroism

Circular dichroism has been widely used to investigate the secondary and tertiary structure of protein after thermal induced processing. CD produced strong far UV range because of peptide groups in helical or antiparallel orientations or purines and pyrimidine bases in helical arrays in nucleic acids. The far UV range is used to investigate the secondary structure of protein. Analysis of tertiary structure of protein was done in near UV range because of the presence of enhanced chirality of aromatic amino acids.

The CD spectra of native BSA, FB5-BL, FB5-BSA1 and FB5-BSA2 were analyzed to determine the secondary structure of protein and shown in Fig. 31 and 32. Native BSA was prepared as the same concentration as FB5-BSA1 and FB5-BSA3 corresponding to the results obtained from protein content determination. It

was observed that the CD spectra of BSA obtained from spray dried formulation either FB5-BSA1 or FB5-BSA3 had not much difference in comparison with native BSA, the commercial one. Native BSA spectra showed the minima at 208 nm and 220 nm indicating the presence of alpha-helical secondary structure predominantly. In FB5-BSA1, the minima slightly shift to 212nm, in FB5-BSA3, slight shift of the peak in β-sheet was observed. Fig. 32. That shift of the peaks could be possibly due to the extraction of the protein with small amount of dichloromethane as shown in section 7, Chapter III. However, no new peak has been observed in spray dried formulations (Xie and Wang, 2007). It was seen that CD spectra of BSA even after spray drying had retained its conformation (Carrasquillo et al., 2001).

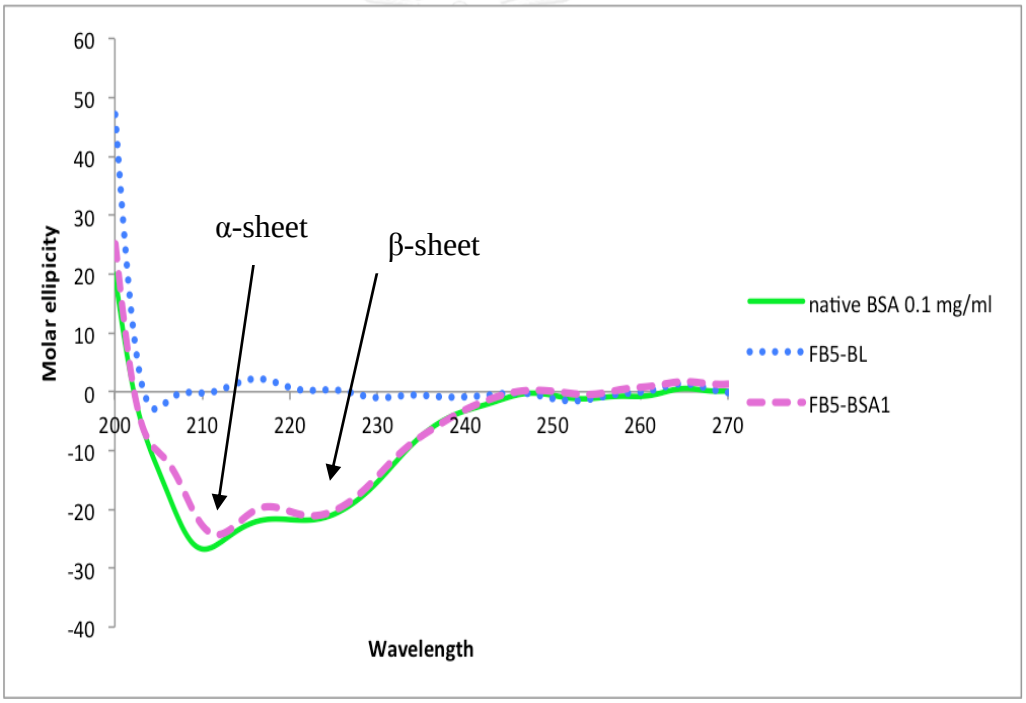


Figure 31 Graph showing the effect of spray drying on the integrity of protein BSA in the formulation FB-BSA1 compared with native BSA.

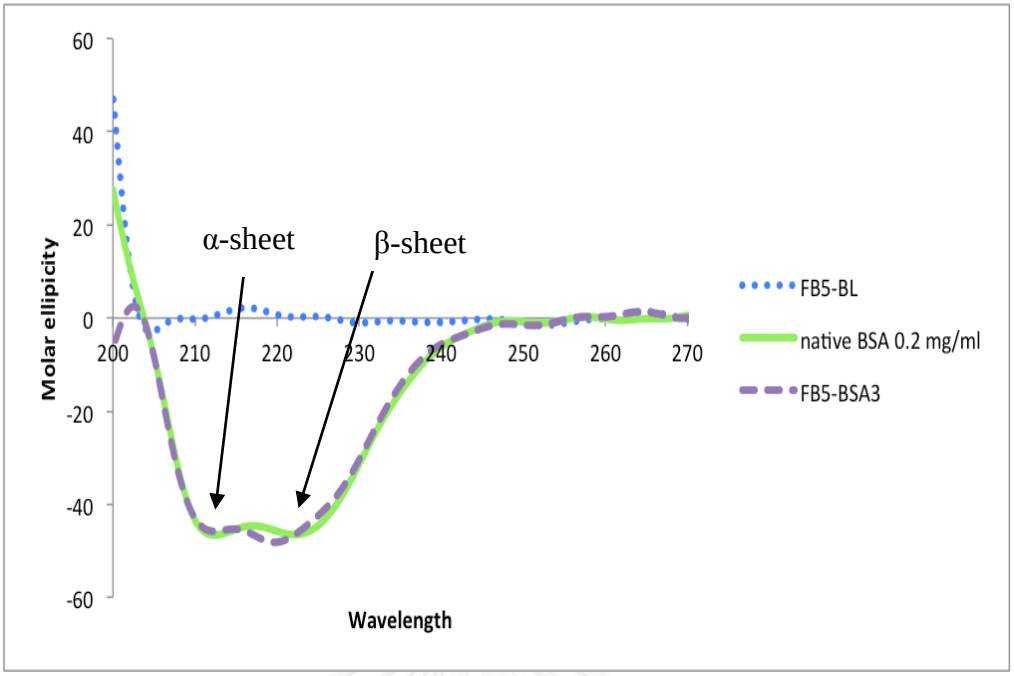


Figure 32 Graph showing the effect of spray drying on the integrity of protein BSA in the formulation FB-BSA3 compared with native BSA.

b. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Potential protein degradation of BSA in spray dried formulations was detected by SDS-PAGE. Fig 33 shows the results obtained from SDS-PAGE of spray dried formulation with BSA comparing with the native BSA and blank formulation. BSA from spray dried formulation were extracted the same way as discussed in protein content determination. β-mercaptoethanol has been used to break any disulfide bonds present in the proteins and disrupts the protein cross-links. These disulfide bonds were also responsible for protein secondary structure and breaking them allow all the sites to be easily accessible for SDS binding (Determan et al., 2004). Then, it imparts a negative charge and helps to migrate the protein through the gel.

The lane 1 represents the protein standard maker, lane 2 represents PBS, lane 3 represents maltodextrin 0.1mg/ml in PBS and lane 4 represents FB5-Bl. The molecular weight of BSA is 66400 Da, which was not observed in any of the lane from 2 to 4. Lane 10 was Addavax, which is the MF59 nanoemulsion without antigen and no protein bands was observed. However, fainted thick bands could be seen at the bottom of gel in all lanes and that was the surfactant in the blank formulation or MD, which was also low molecular weight compound. Lane 5 represents the native BSA, lane 6 and 7 was FB5-BSA1 from different batches, lane 8 was FB5-BSA3 and lane 9 was FB-BSA3 5 fold diluted one, thus in all these lanes, BSA protein bands had been identified at the molecular weight of 66400 Da. Another high molecular weight bands were also observed and that band was also present in lane 5 native BSA, the commercial one. Finally, it could be concluded that it was the impurities or protein aggregates, which did not turned into reduced form on exposure to β-mercaptoethanol. One more possible reason could be that biomaterials like lipids or salts could cause smears and slowed down the rate the migration of protein in gel. Thus, as that band could also be seen in native BSA, it could be concluded that the physicochemical properties of BSA after spray drying had been conserved and no significant irreversible aggregation or degradation of model antigen was observed (Baras et al., 2000a).

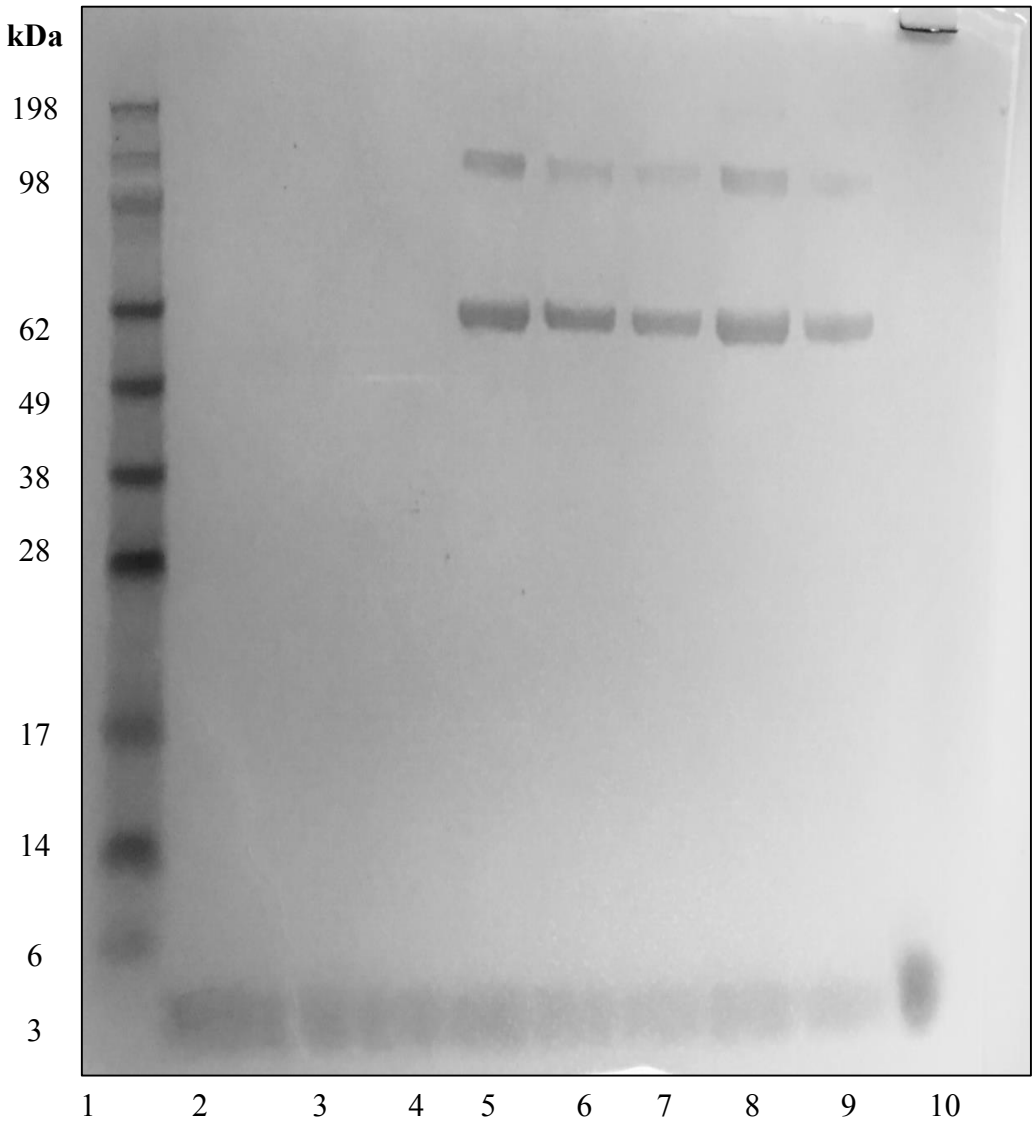


Figure 33 SDS-PAGE of BSA from spray dried formulation in comparison with native BSA. Lane 1: protein standard marker, lane 2: PBS, lane 3: MD 0.1 mg /ml in PBS, lane 4: FB5-BL (no protein), lane 5: Native BSA 0.2mg/ml (commercial one), lane 6: FB5-BSA1, lane 7: FB5-BSA (different batch), lane 8: FB5-BSA3, lane 9: FB5-BSA3 diluted 5 fold, lane 10: Addavax

11. Stability evaluation

The results obtained at different time intervals (0, 1, 2, 3) using Nano-zetasizer provide an insight into stability of nanoemulsion FA and FB as well as it spray dried powder and reconstitutions shown in Table 17-20 and Fig. 34 - 37. Spray dried powder were reconstituted right before the measurement and compare with the particle size of stored reconstituted spray dried powder. The temperature during the storage has high influence on the formulations, thus two temperature conditions of 4°C and ambient temperature was applied.

a. Stability evaluation of spray dried powders and its reconstitution

Stability evaluation of reconstituted powder as well as spray dried powder reconstituting just before the measurement of both FB5-BL and FB5-BSA1 were stable throughout the storage period at 4°C. Fig 34 and 35, Table 17 and 18. The particle size did not increase significantly however decrease in zeta potential was observed. However, storage of spray dried powder at the ambient temperature and reconstituting it just before the measurement showed significant increase in particle size. Fig. 36. This could be due to the aggregation of particles as maltodextrin is a bit sticky and not enough concentration to cover and stabilize the surface of the droplets (Pohlmann et al., 2002). On the other side, storage of reconstituted spray dried powder did not show much increase in particle size. Fig.37, Table 20.

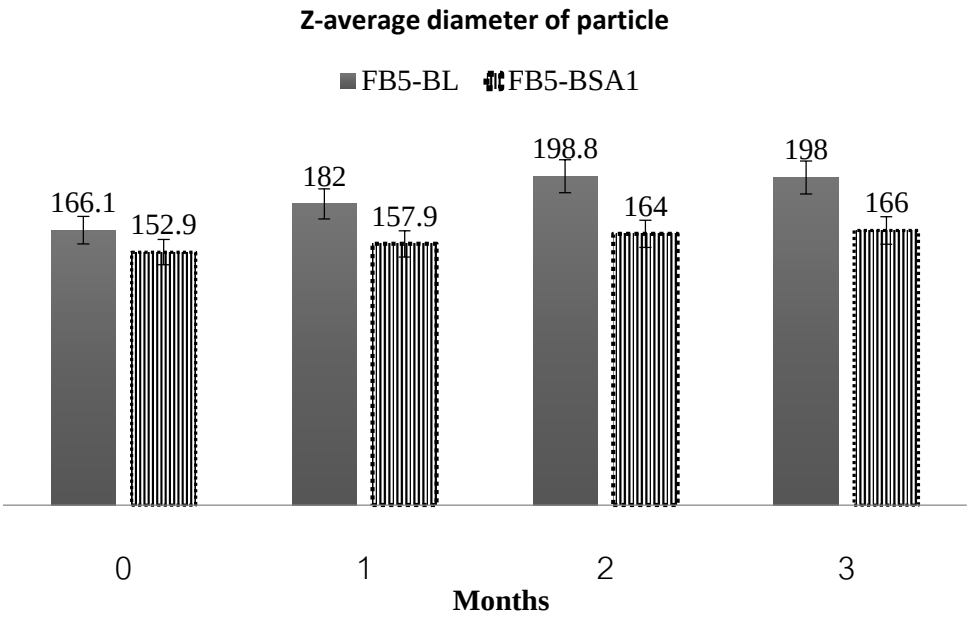


Figure 34 Graph showing particle size results during stability of spray dried FB5-BL and FB5-BSA1, reconstituted just before measurement stored at 4°C

Table 17 Zeta potential and polydispersity results obtained from stability of spray dried FB5-BL and FB5-BSA1, reconstituted just before measurement stored at 4°C

Formulation	Month	Zeta potential (mV)	Polydispersity (PDI)
FB5-BL	0	-18±0.35	0.226
	1	-22±0.41	0.230
	2	-13.1±2.77	0.253
	3	-13±2.7	0.256
FB5-BSA1	0	-11.4±0.25	0.263
	1	-13±0.58	0.249
	2	-13±0.4	0.222
	3	-13±0.43	0.224

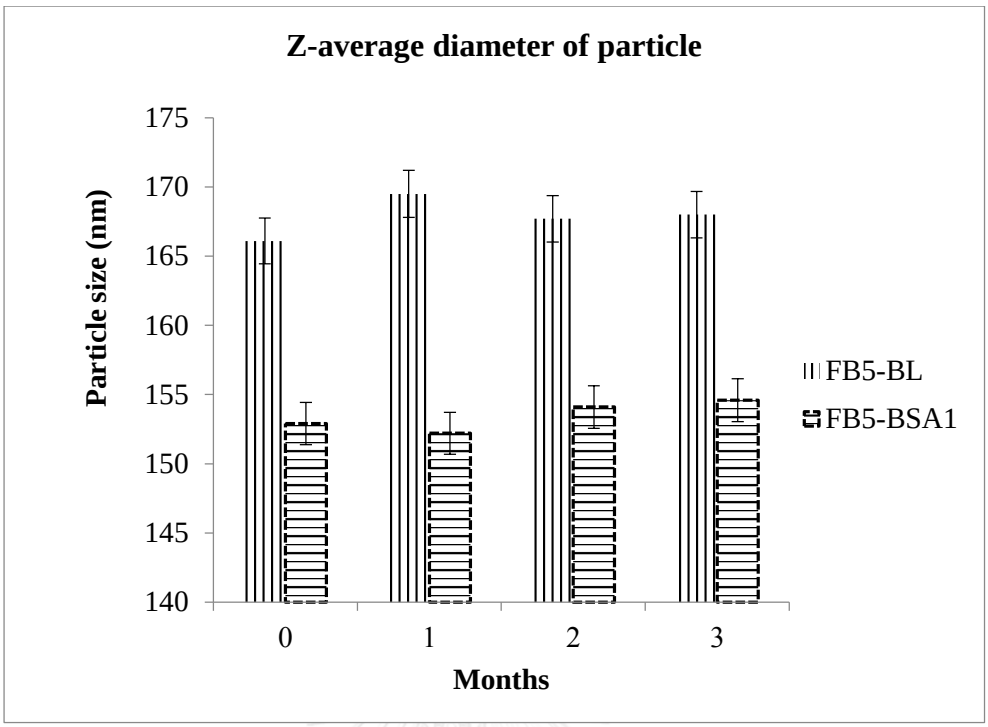


Figure 35 Graph showing particle size results during stability of reconstituted FB5-BL and FB5-BSA1, at 4°C.

Table 18 Zeta potential and polydispersity results obtained from stability of reconstituted FB5-BL and FB5-BSA1, at 4°C

Formulation	Month	Zeta potential (mV)	Polydispersity (PDI)
FB5-BL	0	-18±0.35	0.226
	1	-20±0.75	0.228
	2	-20±0.75	0.208
	3	-20±0.8	0.222
FB5-BSA1	0	-11.4±0.25	0.263
	1	-14.5±0.35	0.246
	2	-12±0.37	0.258
	3	-10±0.45	0.258

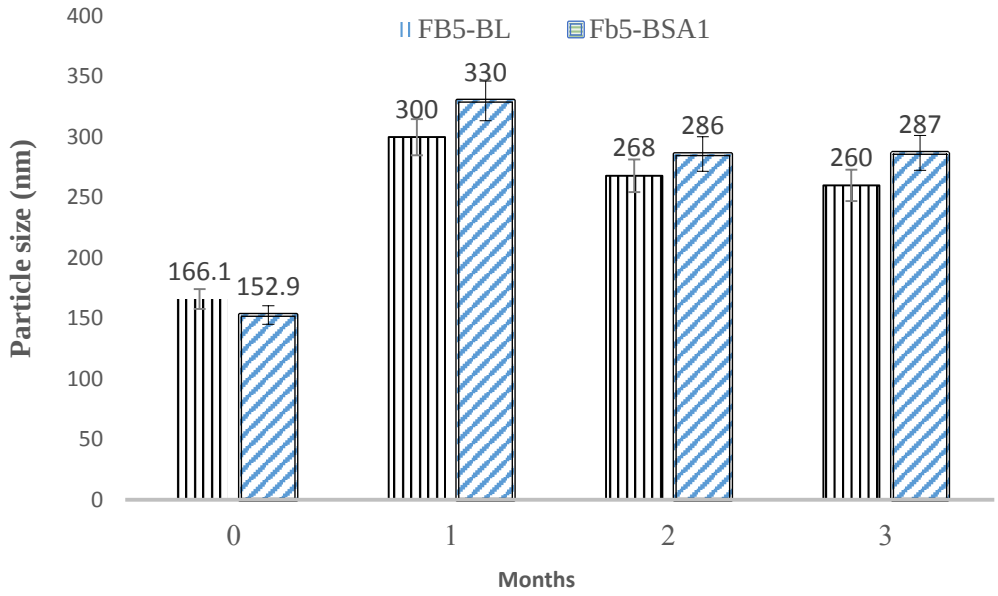
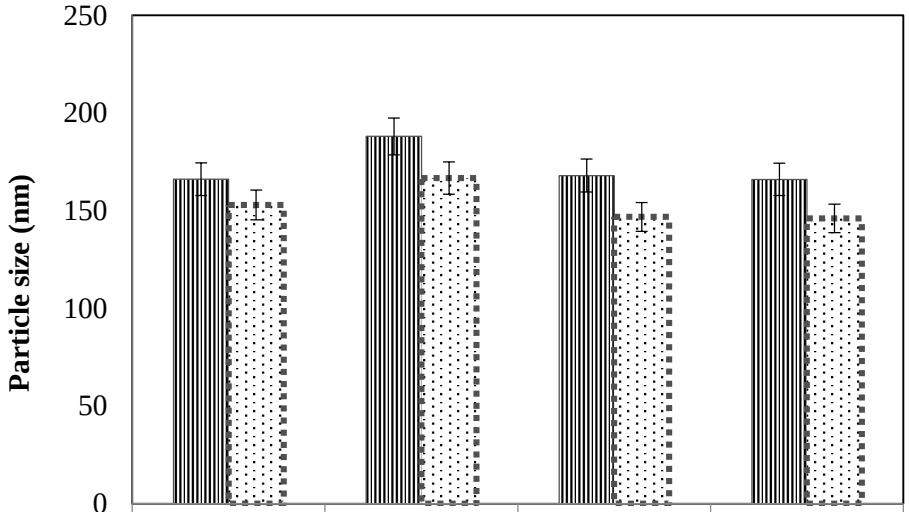


Figure 36 Graph showing particle size results during stability of spray dried FB5-BL and FB5-BSA1, reconstituted just before measurement stored at ambient temperature

Table 19 Zeta potential and polydispersity results obtained from stability of spray dried FB5-BL and FB5-BSA1, reconstituted just before measurement stored at ambient temperature

Formulation	**Month**	**Zeta potential (mV)**	**Polydispersity (PDI)**
FB5-BL	0	-18±0.35	0.226
	1	-18±0.98	0.676
	2	-6±0.66	0.467
	3	-13±0.62	0.388
FB5-BSA1	0	-11.4±0.25	0.263
	1	-13±0.7	0.455
	2	-14±0.15	0.470
	3	-13±0.1	0.474



Months	0	1	2	3
FB5-BL	166.1	188	168	166
FB5-BSA1	152.9	166.7	146.8	146

Figure 37 Graph showing particle size distribution results obtained from stability of reconstituted FB5-BL and FB5-BSA1, stored at ambient temperature

Table 20 Zeta potential and polydispersity results obtained from stability of reconstituted FB5-BL and FB5-BSA1, stored at ambient temperature

Formulation	Month	Zeta potential (mV)	Polydispersity (PDI)
FB5-BL	0	-18±0.35	0.226
	1	-4±0.39	0.318
	2	-6±0.31	0.212
	3	-6±0.35	0.222
	0	-11.4±0.25	0.263
	1	-2.7±0.29	0.340
	2	0.8±31	0.191
	3	0.8±0.33	0.201

b. Stability evaluation of liquid nanoemulsion adjuvant

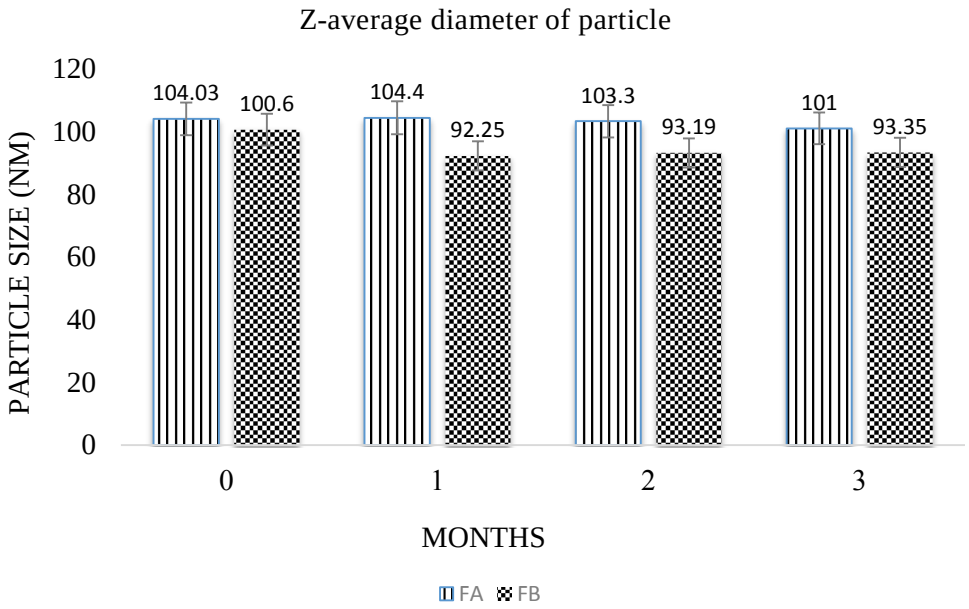


Figure 38 Graph showing particle size distribution results obtained from stability of nanoemulsion adjuvant for 3 months period at 4°C

Table 21 Zeta potential and polydispersity results obtained from stability of nanoemulsion adjuvant for 3 months period at 4°C

Formulation	**Month**	**Zeta potential (mV)**	**Polydispersity (PDI)**
FA	0	-29±0.7	0.275
	1	-18±0.8	0.292
	2	-20±0.6	0.302
	3	-20±1.5	0.301
FB	0	-18±0.64	0.334
	1	-17±1.14	0.310
	2	-16±0.9	0.344
	3	-15±0.8	0.355

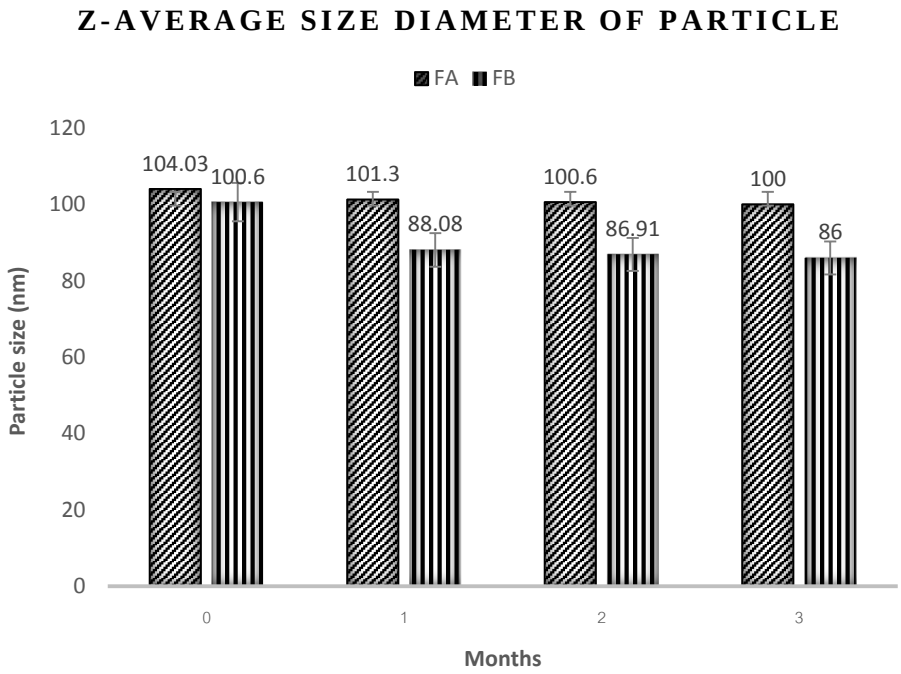


Figure 39 Graph showing particle size distribution obtained from stability nanoemulsion FA and FB stored at ambient temperature

Table 22 Zeta potential and polydispersity results obtained from stability nanoemulsion FA and FB stored at ambient temperature.

Formulation	Month	Zeta potential (mV)	Polydispersity (PDI)
FA	0	-29±0.7	0.275
	1	-4±1.2	0.235
	2	-2±1.8	0.220
	3	-2±1.12	0.229
FB	0	-18±0.64	0.334
	1	-17±0.7	0.297
	2	-2.7±1.1	0.247
	3	-2±0.6	0.262

The particle size of nanoemulsion FA and FB was stable throughout the storage period of 3 months at both 4°C and at ambient temperature. Zeta potential was slightly decreased during the storage at ambient temperature. No creaming or sedimentation was observed.

12. Cell viability study using CRL-9855

Cell viability study have confirmed that both formulation FB5-BL and FB5-BSA1 gave the cell viability of nearly 100%. The concentrations of non-ionic surfactants were quite low to affect the cell viability. The results are shown in Fig 40.

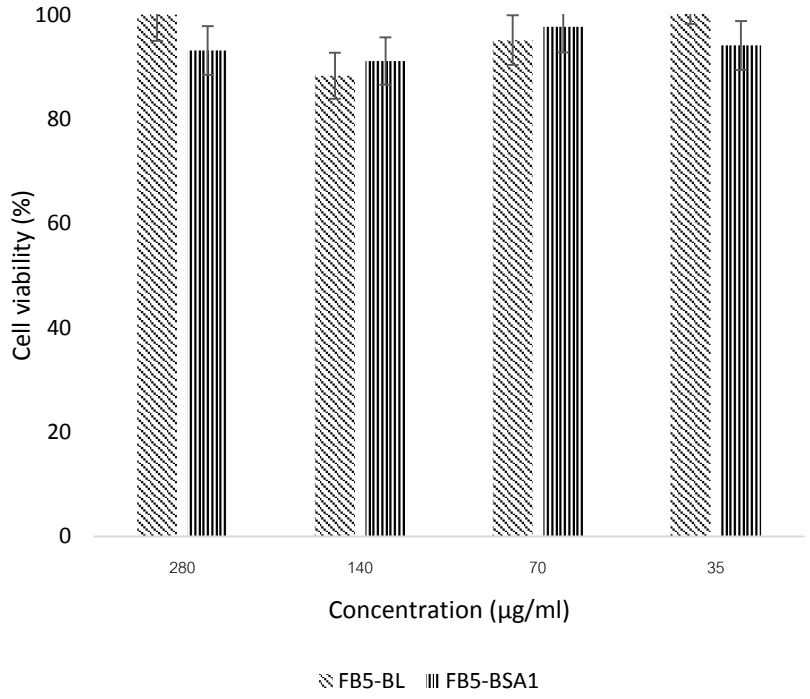


Figure 40 Cell viability graph of FB5-BL and FB5-BSA1

13. Cell viability study using human nasal epithelial cell line

Alamar blue assay was used to study cell viability. Metabolic activity of cells results in the chemical reduction of the blue-colored Alamar blue (AB) dye to fluorescent red form. The concentrations shown in the graph is BSA concentration present in FB5-

BSA1 and for FB5-BL, the same dilution was done as FB5-BSA1 in order to observe the cytotoxicity of surfactant presence in the formulations. After incubation period of 24 h with the formulation, the fluorescence was measured (Pereswetoff-Morath, 1998). While culturing the cells, too low amount of the obtained cells might affect the cell viability results of each concentrations. Spray dried powders of FB5-BL and FB5-BSA1 (35µg) had no effect on human nasal epithelial cells. Thus, lowest concentration of BSA (35 µg/ml) would be the most suitable one as both FB5-BL and FB5-BSA1 gave approximately 100% cell viability.

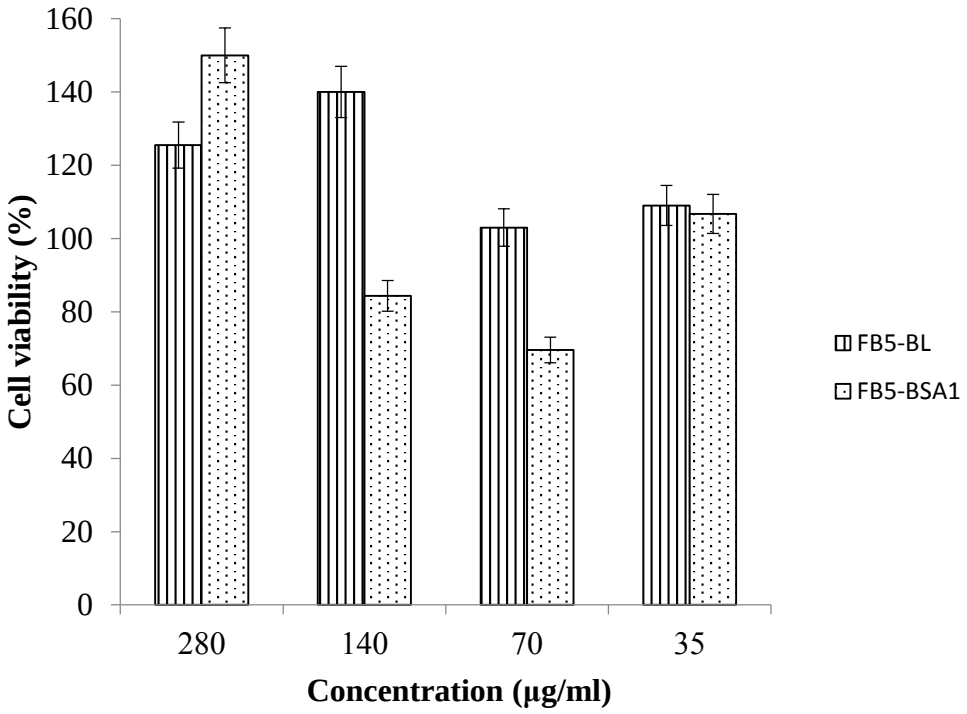


Figure 41 Cell viability study of FB5-BL and FB5-BSA1 using human nasal epithelium cell line

13. Confocal laser scanning microscope (CLSM)

Uptake study was analyzed by using human monocyte macrophage cell line. All the formulations were allowed to incubate with cells for 1h prior to fixation. For the formulation FB5-BL, nanoemulsion was prepared using rhodamine as a dye in the lipid phase. On the other side, FB5-BSA1 was spray dried using FITC-BSA. FITC-

BSA was also dissolved in PBS as the same concentration as FB5-FITCBSA1. Cell treated with FB5-BL (Fig 45) , it can be observed that the formulation was taken up into the cells even without BSA. FB5-BL could be the potent nanoemulsion adjuvant even though further investigation might be needed concern with immunity. FB5-FITCBSA1 treated in cells in Fig 46 also proved that the formulation was successfully uptake by the cells as it could be observed the green fluorescence inside the cells. According to the literature, uptake of the formulation by macrophages occurred after 90 minutes. However, in these figures, uptake was observed after incubation with the formulations for 1h. FITC-BSA in PBS was also uptake into the cells as seen in Fig. 44 and the cell adhesion of FITC-BSA was also observed around the cell membrane. All the formulations were closed in contact with the cell membrane, suggesting the occurrence of cell adhesion (Amidi et al., 2007, Gordon et al., 2010). Present experiment and images have proved that spray dried nanoemulsion adjuvant with BSA can be effectively used in the field of vaccines.

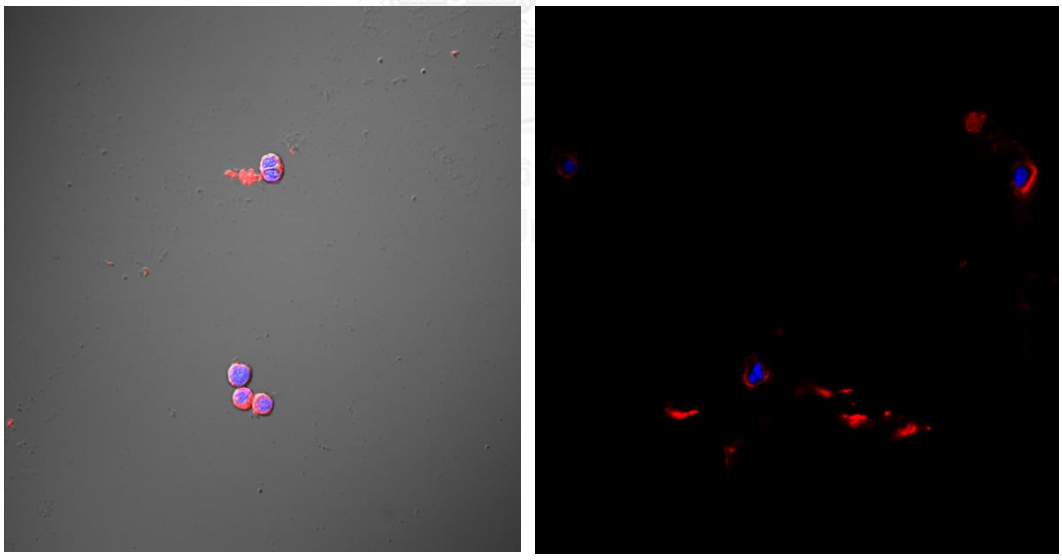


Figure 42 CLSM images showing cell untreated, nuclei showing blue color, cell membrane showing red color (dye with wheat germ agglutinin)

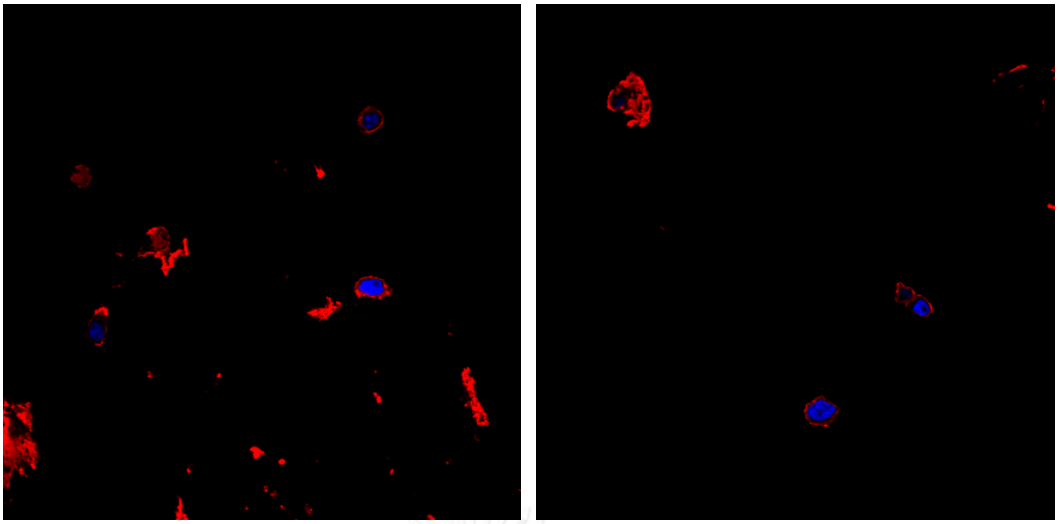


Figure 43 CLSM images showing cells treated with PBS, nuclei showing blue color, cell membrane showing red color (dye with wheat germ agglutinin)

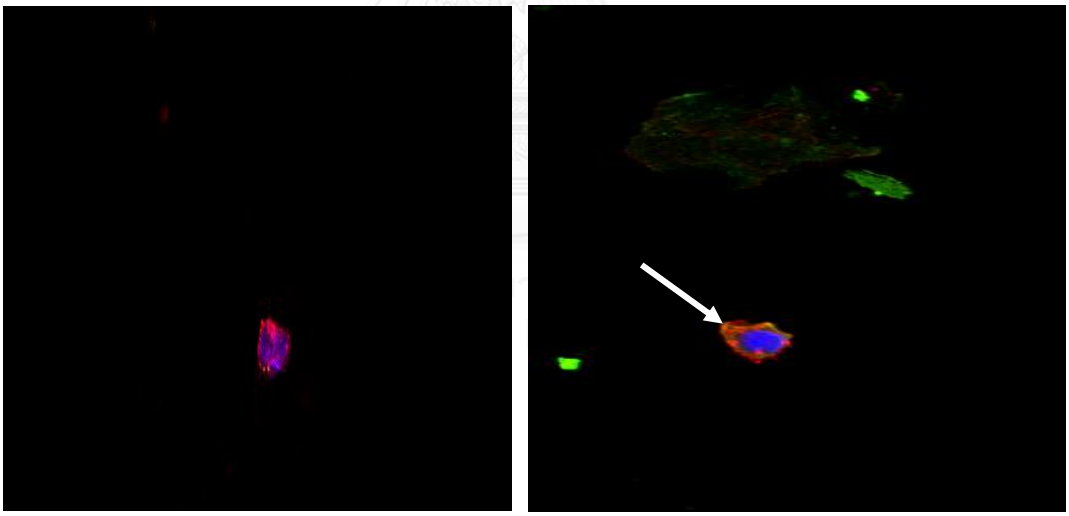


Figure 44 CLSM images showing cells treated with FITC-BSA in PBS, nuclei showing blue color, cell membrane showing red color (stained with wheat germ agglutinin), FTIC-BSA showing green color.

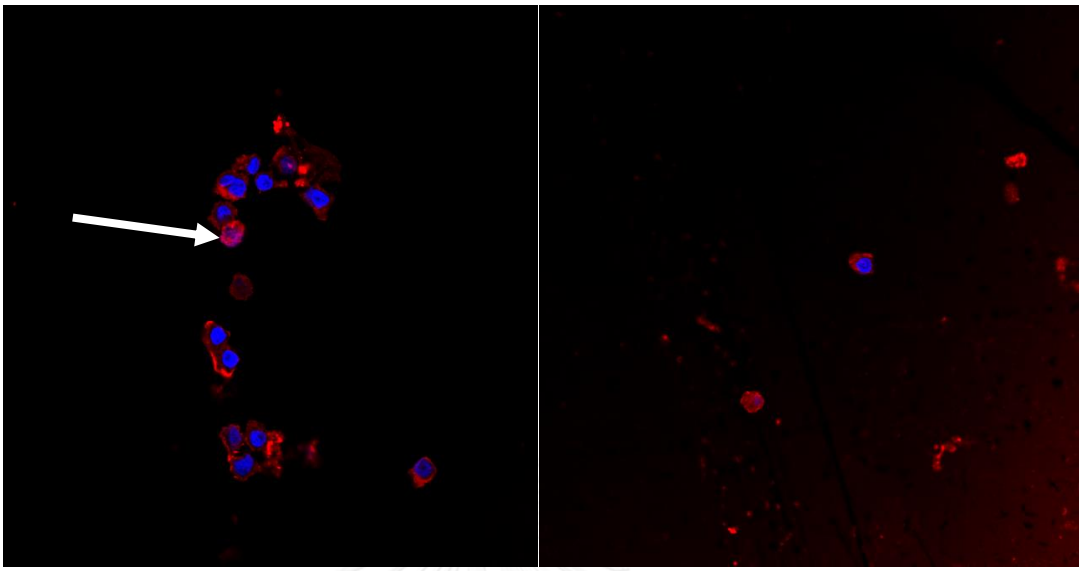


Figure 45 CLSM images showing cells treated with FB5-BL (dyed lipid phase with rhodamine), nuclei dye with blue color (DAPI)

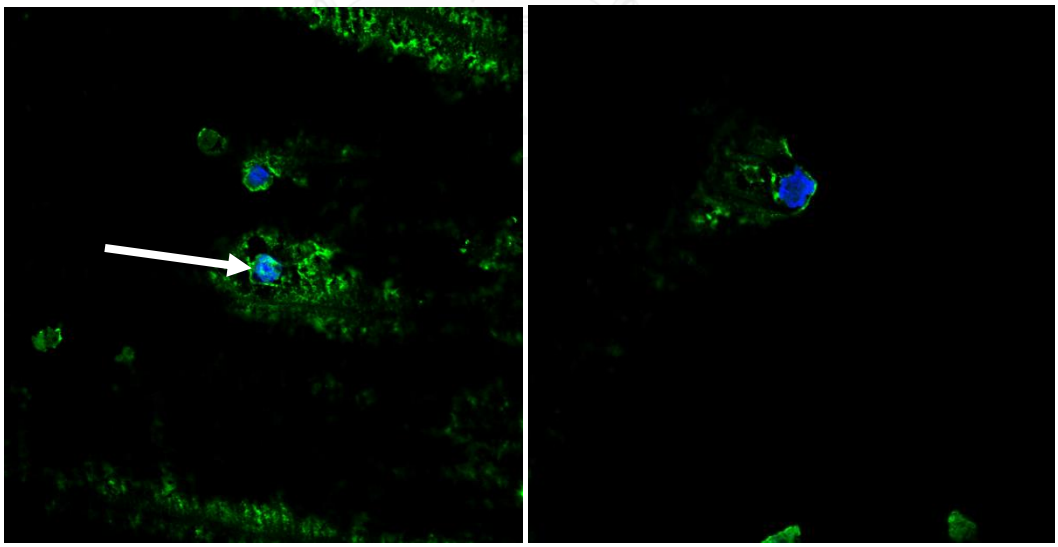


Figure 46 CLSM images showing cells treated with FB5-FITCBSA1, nuclei stained with blue (DAPI) , green color represents FITC-BSA in spray dried formulation

CONCLUSION

Spray dried nanoemulsion adjuvant was successfully developed by using BSA as a model antigen. Particle size was influenced by the composition and process parameters. BSA incorporated into the formulation seemed to be at the surface of the particle as indicated by the zeta potential. All the spray dried formulations and nanoemulsion adjuvant were spherical in shape while characterized by SEM and TEM, respectively. SEM images of FB5-BSA1 showed more porosity than FB5-BSA3. Spray dried powder of DSC thermograms and XRD was performed and all the formulations were amorphous in state. FTIR showed slight shift in BSA protein amide I band but the secondary structure was retained. A new peak of 1744 $cm^{-1}$was observed in FTIR spectra of spray dried formulation together with the protein peak, which could be the combination of formulation, MD and BSA. The protein loading was around 14 % in FB5-BSA1 and 23.9% in FB5-BSA3. The cumulative in vitro release profile of FB5-BSA1 different batches and FB5-BSA3 showed the same pattern. The release of the formulation at the first 30 minutes was slow, and then after 1h burst released of all formulations was observed. FB5-BSA3 release was slower than FB5-BSA1, although the latter one had larger particle size, it could be due to denser and less porosity of FB5BSA3. CD confirmed the integrity of BSA and spray drying conserved BSA conformation with slight changes in α-helix structure but most likely the same as native BSA. During the period of nanoemulsion stability testing no creaming or sedimentation was observed. The particle size showed not much changes over the period 3 months. Stability data of spray dried powder reconstituted just before the measurement at ambient temperature showed an increase in particle size over period of time but storage of reconstituted one showed not much change in particle size. At storage of 4°C, both reconstituted one and spray dried powder storage provides good stability. Cell viability on monocyte cells showed nearly 100% viability profile. Cell viability study on human nasal epithelium showed some toxicity in high concentrations. However, FB5-BSA1 (35 μg) provides good safety profile. Although, the surface charge of the particles were negative in nature, good cell adhesion was observed under the CLSM. FB5-BSA1 as well as FB5-BL without BSA was uptake by the cells. Before cell fixation, the

incubation time of formulation with the cell may need to be longer than one hour. Thus, it could be concluded that the particles of spray dried nanoemulsion adjuvant after reconstitution were in nano size and it had the potential to uptake by the cell even without model antigen.

APPENDICES

Standard Curve prepared for protein content determination

Table 23 Concentrations prepared for standard curve

Concentration (µg/ml)	Absorption
200	2.178
160	1.763
80	0.959
40	0.490
20	0.242
10	0.116
5	0.056
2.5	0.030
1.25	0.011
0.625	0.009
0	0.000

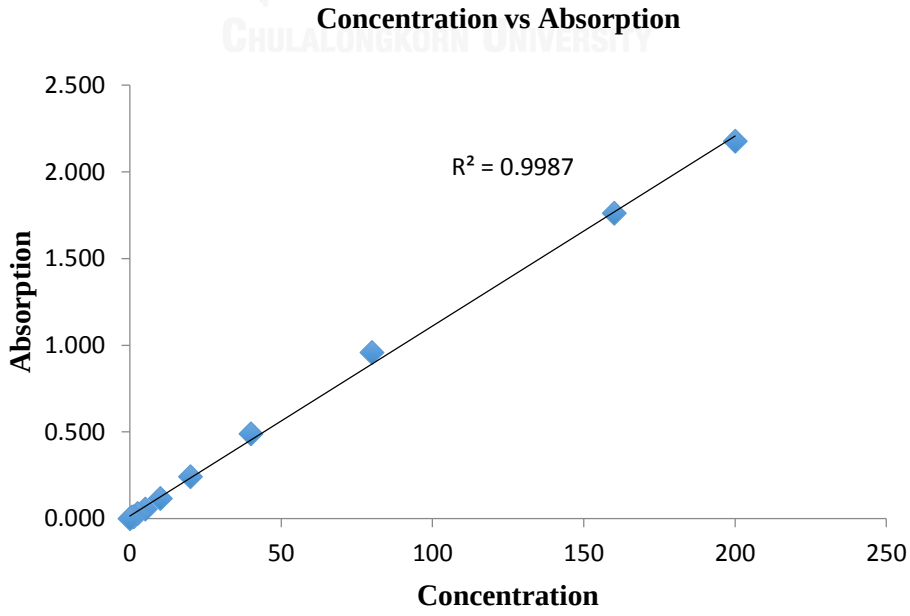


Figure 47 Standard curve used for protein content determination

Standard Curve prepared for in vitro release study

Table 24 Concentrations prepared for standard curve

Concentration (µg/ml)	Absorbance
200	1.809
160	1.361
80	0.703
40	0.37
20	0.171
10	0.072
5	0.058
2.5	0.034
1.25	0.012
0.63	0.009
0	0

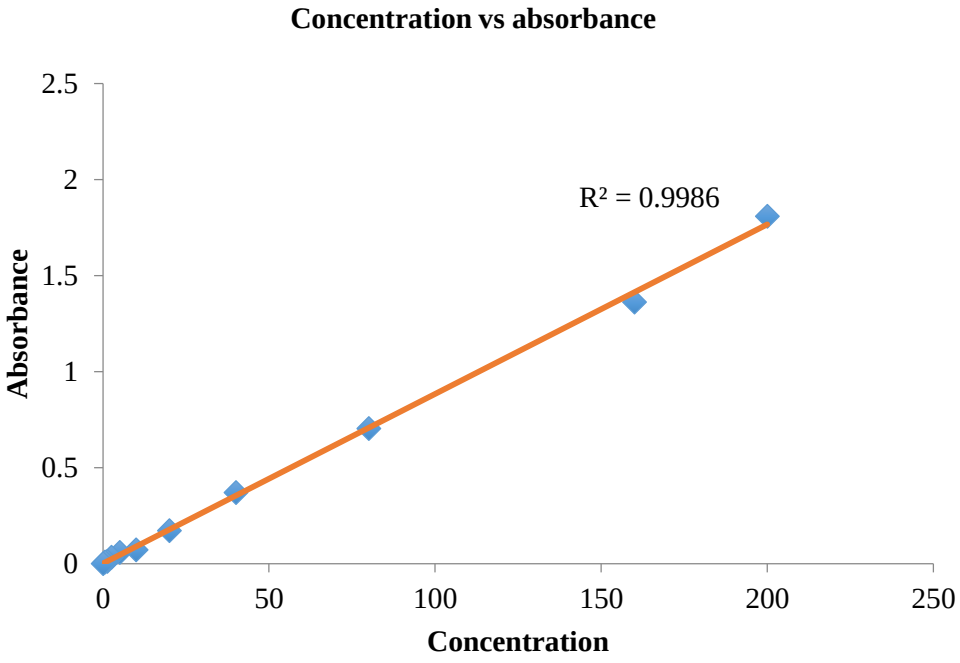


Figure 48 Standard curve for in vitro release study

Table 25 Cumulative drug released percent of FB5-BSA1 1st batch, FB5-BSA1 2nd batch, FB5-BSA3

	FB5-BSA1 1st batch	FB5-BSA1 2nd batch	FB5-BSA3
Time points			
0.25	8.56 %	8.56 %	4.91 %
0.5	36.02 %	36.02 %	21.71 %
1	61.38 %	61.38 %	61.88 %
2	78.98 %	78.98 %	76.47 %
4	89.82 %	89.81 %	84.95 %
8	93.83 %	97.23 %	91.06 %
14	99.58 %	101.58 %	95.44 %
24	102.86 %	101.5 %	96 %

Table 26 Stability results of spray dried powder, FB5-BL and FB5-BSA1 stored at 4°C and reconstituted just before the measurement

Formulation	Months	Particle size (nm)
FB5-BL	0	166.1±1.25
	1	182±2.007
	2	198.8±2.779
	3	198±3.011
FB5-BSA1	0	152.9±1.500
	1	157.9±2.793
	2	164±3.526
	3	166±3.455

Table 27 Stability results of reconstituted FB5-BL and FB5-BSA1 stored at 4°C

Formulation	Months	Particle size (nm)
FB5-BL	0	166.1±1.25
	1	169.5±3.20
	2	167.7±1.973
	3	168±1.988
FB5-BSA1	0	152.9±1.500
	1	152.2±2.02
	2	154.1±2.72
	3	154.6±2.876

Table 28 Stability results of spray dried powder FB5-BL and Fb5-BSA1 stored at ambient temperature and reconstituted just before the measurement

Formulation	Months	Particle size (nm)
FB5-BL	0	166.1±1.25
	1	300±10.55
	2	268±6.51
	3	260±6.77
FB5-BSA1	0	152.9±1.50
	1	330±19.09
	2	286±6.88
	3	287±6.95

Table 29 Stability results of reconstituted FB5-BL and FB5-BSA1, stored at ambient temperature.

Formulation	Months	Particle size (nm)
FB5-BL	0	166.1±1.25
	1	188±2.71
	2	168±2.83
	3	166±2.37
FB5-BSA1	0	152.9±1.50
	1	166.7±2.87
	2	146.8±2.35
	3	146±2.55

Table 30 Stability results of optimized nanoemulsion FA and FB at refrigerator condition of 4°C

Formulation	Months	Particle size (nm)
FA	0	104.03±0.77
	1	104.4±0.65
	2	103.3 ±0.73
	3	101±1.0
FB	0	100.6±0.40
	1	92.25±0.39
	2	93.19±0.44
	3	93.35±0.32

Table 31 Stability results of optimized nanoemulsion, FA and FB obtained at the ambient temperature

Formulation	Months	Particle size (nm)
FA	0	104.03±0.77
	1	101.3±0.43
	2	100.6±0.96
	3	100±1.2
FB	0	100.6±0.40
	1	88.08±0.66
	2	86.91±0.53
	3	86±0.96

Table 32 Standard curve used to determine the optimum cell concentration and optimum incubation time of Alamar blue for cell viability of human monocyte macrophage cell line

Concentration of cells
1.15 x 10^5
5.75 x10^4
2.8 x 10^4
1.4 x 10^4
7.15 x 10^3
Cell medium (blank)

Incubation time
20 h
8 h
5 h

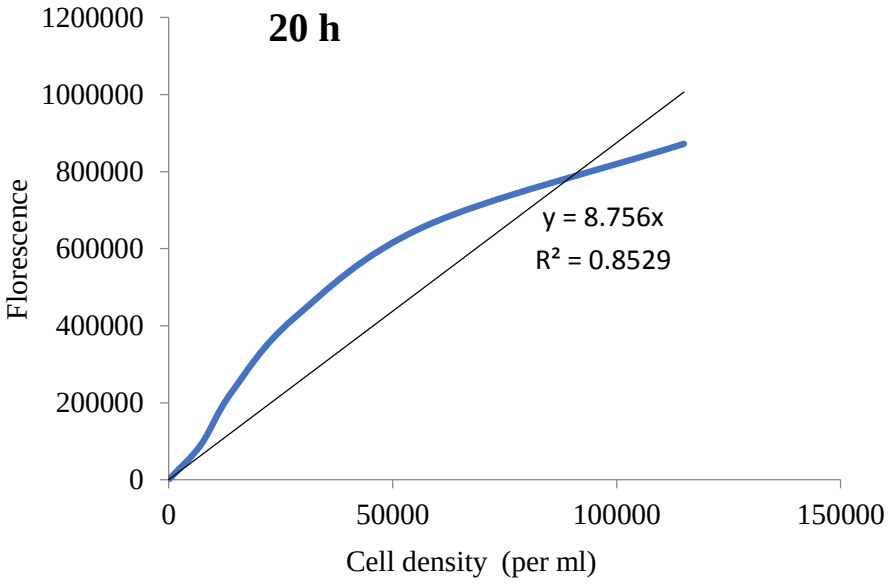


Figure 49 Standard cure after 20 hours of incubation with Alamar blue with various cell concentrations

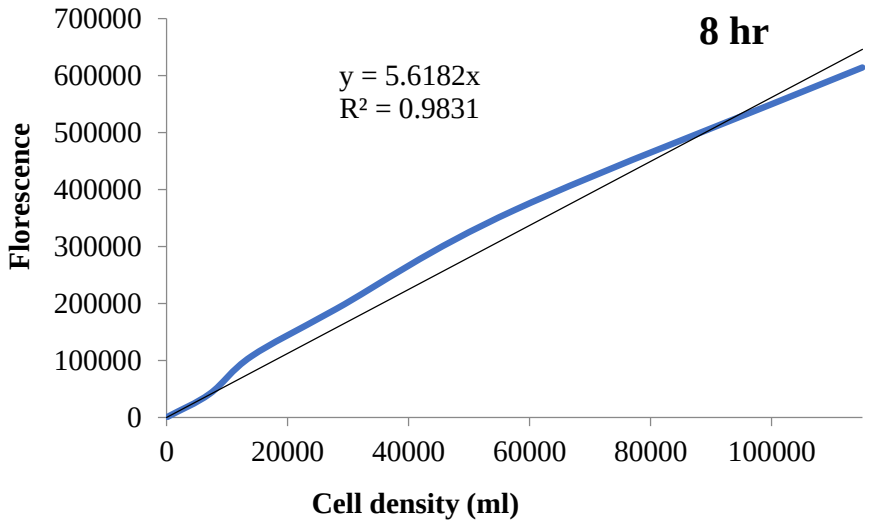


Figure 50 Standard cure after 8 hours of incubation with Alamar blue with various cell concentrations

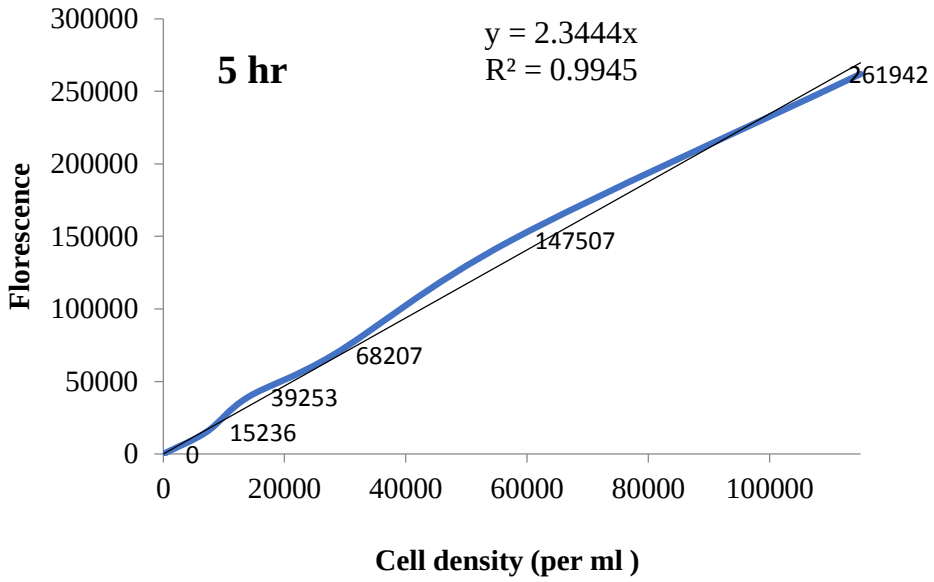


Figure 51 Standard cure after 5 hours of incubation with Alamar blue with various cell concentrations

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