A Dissertation
for the Degree of Master of Science

Development of polymeric carriers for enhanced immune response of foot-and-mouth disease virus subunit vaccine

FMDV 아단위 백신의 면역반응 증진을 위한 고분자 전달체의 개발

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Abstract

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Foot-and-mouth disease (FMD) is a highly contagious disease susceptible to cloven-hoofed animals such as cattle, pigs, goats, *etc*. affecting livestock industry. In this aspect, FMDV subunit vaccines have been developed to prevent the spread of this fatal animal epidemic, because they provide several advantages such as no need for attenuation and serological tests that can differentiate infected animals from vaccinated ones and they can be produced with epitopes and have less side effects than live attenuated/inactivated vaccine although FMDV is continuously evolving and mutating, making it difficult to develop FMDV vaccine (mainly live attenuated or inactivated vaccines) to protect animals from disease.

However, there are several limitations such as for practical application of subunit vaccines the low stability of subunit vaccine, easy degradation by enzyme and physiological environment. In addition, their low immunogenicity compared to live attenuated vaccines limits the efficacy of subunit vaccine. Th erefore, enhancement of stability and immunogenicity of subunit vaccines is main bottleneck in vaccine development.

To overcome these limitations, polymeric adjuvants have been

introduced to enhance the immunogenicity of subunit vaccines because they introduce immunomodulatory properties and provide the flexibility in the route of vaccine delivery depending on the vaccination strategies. Furthermore, immune response can be greatly regulated by single factor or combination of multiple factors by modification of the polymeric adjuvants such as size of polymeric particle, surface charge, hydrophilicity, molecular weight and chemical properties.

In chapter I, pH-sensitive and mucoadhesive thiolated CAP (T-CAP) as a polymeric carrier was developed for efficient delivery of mucosal subunit vaccine M5BT through oral route. In this study, cellulose acetate phthalate (CAP), the pH-sensitive polymer that dissolve at > pH 6.2 was modified by thiolation to introduce mucoadhesive property and to dissolve at ileum pH. FMDV recombinant antigen M5BT was encapsulated into thiolated CAP microparticles (T-CAP MPs) using double emulsion solvent evaporation method. As a result, T-CAP MPs showed sustained release of encapsulated M5BT from the MPs at intestinal pH (pH 7.4), while releasing less M5BT at gastric pH (pH 2) due to its pH-sensitive property. Also, porcine mucosa assay showed 1.4-fold enhanced mucoadhesiveness of T-CAP MPs than non-modified CAP MPs *in vitro* due to the formation of disulfide bond between thiol group in T-CAP and mucin glycoproteins in mucus layer by thiol/disulfide exchange reactions. Finally, M5BT delivered by T-CAP MPs elicited higher IgA production than only M5BT in *in vivo* mouse experiment. Therefore, this study represents an effective mucosal subunit

vaccine delivery through oral route.

In chapter II, mannan-decorated inulin acetate (M-INAC) MPs as an immunostimulatory polymeric carrier were developed for efficient delivery of subunit vaccine M5BT. In this study, inulin was modified by acetylation to introduce hydrophobic moiety. And vaccine FMDV recombinant antigen M5BT was encapsulated into INAC MPs and decorated with mannan using double emulsion solvent evaporation method. As a result, M-INAC MPs showed released more than 90% of loaded antigen for 6 days, while less than 50% of M5BT was released from INAC MPs.

As a result of *in vivo* immunization in murine model, after 4 weeks of immunization, M5BT/M-INAC MPs and M5BT/INAC MPs showed similar level of FMDV serotype O specific antibody with the M5BT group coinjected with conventional adjuvant CFA. And M5BT encapsulated in M-INAC MPs elicited higher IgG titer than M5BT/INAC MPs groups exhibiting similar level of IgG titer with M5BT group coinjected with CFA. It indicates that antigen-loaded INAC MPs can enhance antigen specific immune response comparable to the conventional group implying the potential polymeric adjuvant system for subunit vaccine. Therefore, this study represents an effective subunit vaccine for the better enhancement of adaptive immune response.

Keywords : FMD subunit vaccine, polymeric adjuvant, thiolated CAP, mannan-decoration, inulin acetate, oral delivery

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List of Abbreviations

Ab : antibody

Ag : antigen

APCs : antigen presenting cells

BSA : bovine serum albumin

BCA assay : bicinchoninic acid assay

CAP : cellulose acetate phthalate

CMF HBSS : calcium- and magnesium-free Hank's balanced salt solution

DCs : dendritic cells

DCC : *N,N'*-dicyclohexyl carbodiimide

DCM : dichloromethane

DMF : dimethyl formamide

DMSO : dimethyl sulfoxide

DTNB : 5,5-dithio-bis-(2-nitrobenzoic acid)

E.coli : *Escherichia coli*

EDTA : ethylenediaminetetraacetic acid

ELISA : enzyme-linked immunosorbent assay

FACS : fluorescence-activated cell sorting

FBS : fetal bovine serum

FDA : fluorescein diacetate

FE-SEM : field emission scanning electron microscope

FMDV : foot-and-mouth disease virus

HRP : horseradish peroxidase

IN : inulin

INAC : inulin acetate

IPTG : isopropyl β-D-1-thiogalactopyranoside

LB : Luria-Bertani

M-cells : microfold-cells

MPs : microparticles

NaOAc : sodium acetate

NHS : *N*-hydroxysuccinimide

NMR : nuclear magnetic resonance

PBS : phosphate buffered saline

PMSF : phenylmethanesulfonyl fluoride

PPs : Peyer's patches

PVA : poly vinyl alcohol

RPMI 1640 : Roswell Park Memorial Institute 1640

SDS : sodium dodecyl sulfate

SDS-PAGE : SDS-polyacrylamide gel electrophoresis

T-CAP : thiolated CAP

TMB : tetramethylbenzidine

TLR : toll-like receptor

General Introduction

Foot-and-mouth disease (FMD) is a highly contagious disease susceptible to cloven-hoofed animals affecting livestock industry. FMDV is continuously evolving and mutating, making it difficult to develop FMDV vaccine to protect animals from disease. In this aspect, FMDV subunit vaccines have been developed to prevent the spread of this fatal animal epidemic as they provide several advantages such as serological tests that can differentiate infected animals from vaccinated ones.

However, there are limitations for practical application of subunit vaccines because they can be easily degraded by enzyme and physiological environment, and its low immunogenicity compared to live attenuated vaccines. So, enhancement of stability and immunogenicity of subunit vaccines is main bottleneck in vaccine development.

To improve the efficacy of subunit vaccines, two vaccine delivery strategies were designed based on the adjuvant effect of polymeric carriers. Experimental flow chart of this study was shown in Figure 1.

In this study, FMDV recombinant antigen M5BT was used for model subunit vaccine. M5BT is consisted of multi-epitopes which contain five B-cell epitopes and one T-cell epitope from viral protein 1 (VP1) region of FMDV serotype O as well as M-cell targeting peptide (CKSTHPLSC) in N-terminal region. VP1 region is the main target of subunit vaccine development because FMDV infect the host cells through binding of RGD

(arginine-glycine-aspartate) motif in VP1 region to integrin receptor (Cao, Y. *et al.,* 2012). Providing T-cell epitope with B-cell epitope can induce differentiation of B-cells to plasma cells because T-cell epitope can present the processed antigen from dendritic cells to B-cells. This subunit vaccine can protect various serotype O FMDV because of five B-cell epitopes that are originated from five different serotype O FMDV strains.

In Chapter Ⅰ, FMDV subunit vaccine M5BT was encapsulated in thiolated CAP microparticles (T-CAP MPs) for efficient oral delivery of mucosal subunit vaccine. To overcome low bioavailability and antigen uptake of oral subunit vaccine, the pH sensitive and mucoadhesive T-CAP polymer was used to encapsulate the subunit vaccine. Release of FMDV subunit vaccine to ileum (M-cell rich region), and mucoadhesion to mucus layer may maximize the antigen uptake by M-cells in Payer's patches.

In Chapter Ⅱ, FMDV subunit vaccine M5BT was encapsulated in mannan-decorated inulin acetate microparticles (M-INAC MPs) for enhancing immune response against loaded antigen. Because it is known that INAC can function as a vaccine adjuvant when antigen was encapsulated into INAC particles (DIEGO G SILVA *et al.,* 2014), formation of antigen-loaded INAC particles can enhance immune response about subunit vaccine to overcome its low immunogenicity. In addition, mannan which is also agonist for TLR and MR (mannose receptor) was decorated on the surface of INAC MPs to enhance antigen uptake by immune

cells.

Both particulate vaccine may function as vaccine adjuvants by enhancing the uptake of the antigen by antigen presenting cells including macrophages and dendritic cells and delivering the encapsulated antigen to immune cells resulting in improved immune response (Adams, J. R. *et al.*, 2014).

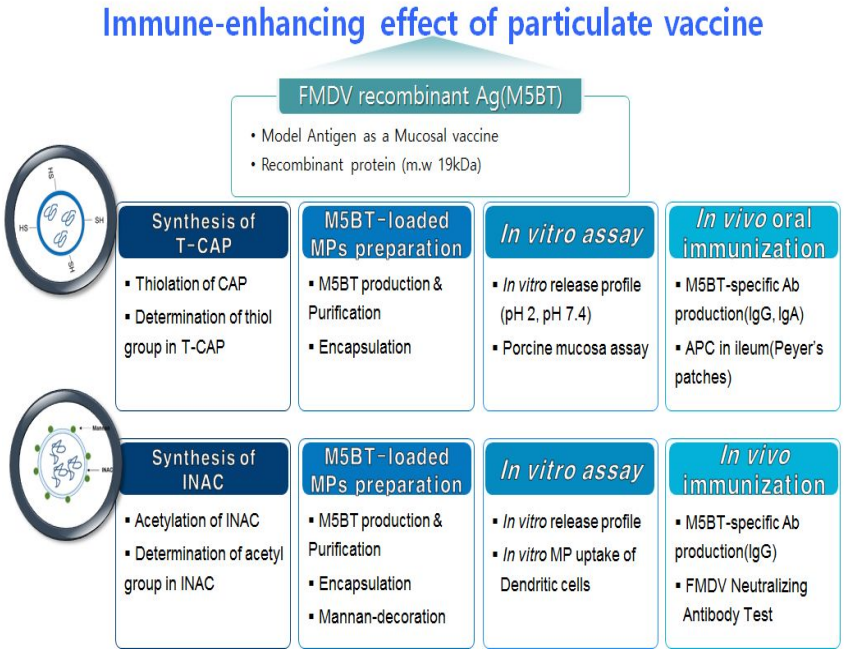


Figure 1. Experimental flow chart of this study.

Review of Literature

1. Foot-and-mouth disease virus subunit vaccine

1) FMD

Foot-and-mouth disease (FMD) is a highly contagious disease susceptible to cloven-hoofed animals including pigs, cattle, goats, and sheep and continuously outbreaks affecting livestock industry. Foot-and-mouth disease is characterized by high fever, blisters in mouth, tongue and hoofs, excessive salivation, and anorexia. Foot-and-mouth disease is caused by an aphthovirus of the family *Picornaviridae*, which is a non-enveloped capsid virus with icosahedral symmetry and single strand positive RNA (Alexandersen *et al.*, 2003). Because of the different serotypes (A, O, C, SAT1, SAT2, SAT3 and Asia1) and its subtypes within each serotypes (E. Domingo *et al,* 2002), FMDV is continuously evolving and mutating, making it difficult to develop FMDV vaccine to protect animals from disease.

2) FMD vaccine

The purpose of vaccination is to generate immune response against infectious agent such as pathogens and viruses and to generate memory response by stimulating innate and adaptive immune response. Vaccines can be categorized as live attenuated vaccines, inactivated vaccines, recombinant vaccine, and subunit vaccine.

To this date, many FMDV vaccines have been developed to

prevent the spread of this fatal animal epidemic. Especially, live attenuated vaccines have been developed and manufactured worldwide.

But, this traditional vaccines have several disadvantages although it showed good protection ability of FMDV in livetstock. The disadvantages include the possibilities of insufficient inactivation of FMDV of vaccines to a virulent form or the risk of the spread of live virus during vaccine production (T.R. Doel *et al.*, 2003), the different virus population resulted from the adaptation step in serial passages to make the virus infect the new host cells such as chicken embryo kidney cell. So, attenuated virus is a similar version of original virus in the wild (so called 'field strain') making it hard to protect field strain virus. In addition, this kind of vaccine cannot quickly response to the rapidly spreading of new virus strain and immunized animals are difficult to be distinguished from infected animals in nature b ecause of the presence of non-structural proteins of FMDV in the vaccine (D.K. Mackay, *et al.*, 1988).

To overcome these limitations of traditional vaccines, it is important to design antigen (Ag) that can elicit neutralizing antibodies with less or no safety concerns. In this context, subunit vaccine is receiving the spotlight. The subunit vaccine is made with a recombinant virus protein by inserting an antigen gene into virus- or bacterial vector to produce recombinant subunit vaccine. Nowadays, subunit vaccine is being explored because they can be consisted of epitopes that can be recognized by antigen presenting cells (APCs) and are immunogenic.

Vaccination with the recombinant virus antigens can protect these problems described above, and these antigens can provide advantages such as no need for large scale culture of virus and attenuations steps by producing antigens synthetically (Roitt's essential immunology 12th, 353p).

Despite of these advantages of subunit vaccine, there are limitations of subunit vaccine in practical uses. Enzymatic degradation in physiological conditions and low immunogenicity severely limit the efficacy of subunit vaccine. To improve the lower immunogenicity of synthetic subunit vaccines compared to traditional vaccines as well as bioavailability, the importance of introducing subunit vaccine carriers is being addressed.

2. Subunit vaccine delivery strategy

Conventional vaccines usually do not require adjuvants because they have enough structures that can be recognized by immune cells as invader. However, the immunogenicity of subunit vaccine which is composed of proteins/polypeptides is poor. So it is necessary to use adjuvant to initiate proper immune response.

1) Conventional adjuvant

Conventional adjuvant includes mineral salts, emulsion, immune-stimulatory complexes (ISCOMs), microorganism-derived adjuvants, virosomes and virus-like particles (VLPs), and cytokines (Adams and Mallapragada, 2014). The action of adjuvants can be categorized by antigen delivery and immunostimulation.

First, adjuvant that acts as an **antigen delivery vehicle** can trap or disperse the antigen inside so that it can deliver and present it to the immune cells. This kind of action is called "depot effect". Due to the depot effect, particulate vaccine can elicit immune response better than soluble vaccine by presenting antigen as a multimeric form (Roitt's essential immunology 12^{th}).

Second, adjuvant that acts as an **immunostimulants** can stimulate immune system resulting in enhanced immune response. Examples are microorganism-derived components (such as TLR agonist, mannan, CpG, LPS, MPLA, CFA), ISCOMs, cytokines. It is important to design immunostimulants in vaccine development because it can activate the dendritic cells (DCs), one of the professional APCs, leading to increased antigen uptake, migration

to lymph nodes followed by induction of adaptive immune response. Many adjuvants including mineral salts (such as alum), emulsions (such as CFA, IFA), liposomes, ISCOMS and polymeric carriers can function as antigen delivery vehicle and immunostimulants at the same time (Roitt's essential immunology 12th).

The most widely used adjuvants are complete freund's adjuvant (CFA). Antigens are emulsified in the CFA, which is composed of inactivated *mycobacterium.* So, the adjuvant effect of CFA resulted from the depot effects and immunostimulatory effects. CFA is used in several FMD vaccines although its increased immune-boosting effect is related to the toxicity of CFA itself. For the safety concerns, its use in human is forbidden by regulatory authorities and its use in animals is regulated by the guidelines.

2) Polymeric adjuvant

Biomaterial-based polymeric adjuvant can function as delivery vehicle in forms of nanoparticles (NPs), microparticles (MPs), micelles, matrices and *etc.* offering more advantages than conventional adjuvants through a number of mechanisms. It is possible to reduce immunization dose due to its depot effects releasing antigens in a sustained manner.

Polymeric adjuvants can also be designed in a number of ways to introduce immunomodulatory properties and to provide the flexibility in the route of vaccine delivery depending on the vaccination strategies. The summary of the multiple ways of

modifying and functionalizing polymers for antigen delivery is shown in Figure 2. For polymeric adjuvant development, immune response can be greatly affected by single factor or combination of multiple factors by modification of the polymeric adjuvant such a size of polymeric particle, surface charge, hydrophilicity, molecular weight and chemical properties as listed in Table 1 (Adams and Mallapragada, 2014).

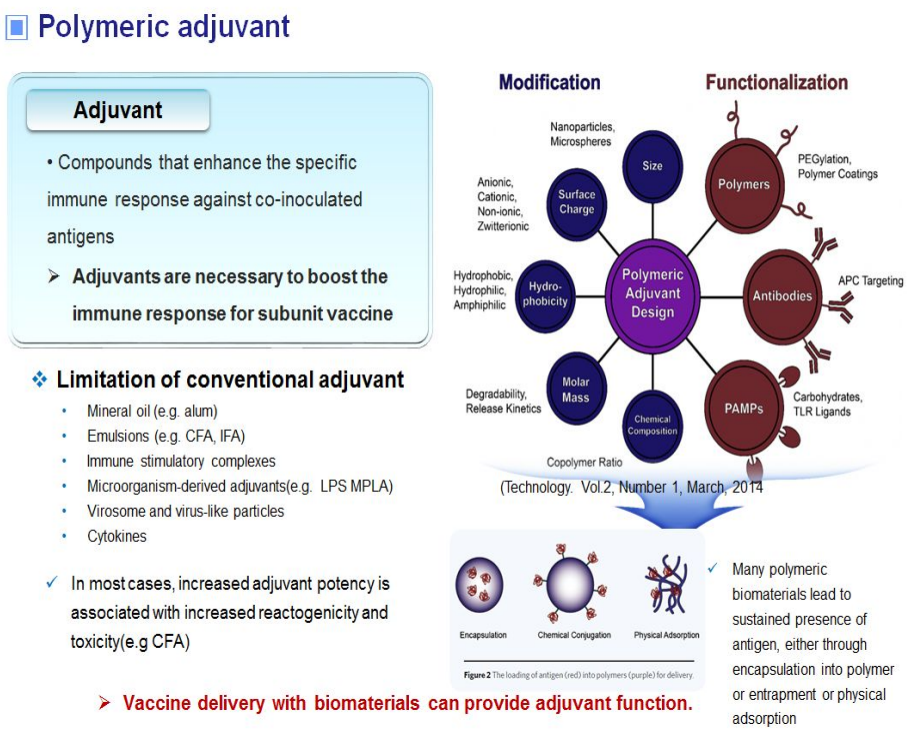


Figure 2. Polymeric adjuvant strategies for substituting conventional adjuvants (This figure was modified from Adams and Mallapragada, 2014).

Table 1. Parameters of polymeric particles affecting immune response (Reference: Adams and Mallapragada, 2014)

Parameters		Related immune response
Modification	Size	Tissue uptake, cellular uptake
	Surface charge	Interaction with cell membrane and mucosal surface
	Hydrophilicity	Hydrophilicity ↓ = identified by the body as foreign, enhance cell adhesion and phagocytosis of macrophages. an increase of adjuvant effect
	Molecular weight	Hydrolysis of polymer Release pattern
	Chemical properties	Various
Functionalization	Modification with other polymers	Stealth effect (ex: PEGylation) Opsonization Blood circulation time (Poloxamer and poloxamine block copolymer) Penetration of mucosal surface (ex: Chitosan)
	Conjugation of antibodies/carbohydrates	Targeting and stimulating antigen presenting cells (ex: DCs, Macrophages)

3. Polymeric adjuvant carrier for FMDV subunit vaccine

1-1) Mucosal immunity and M cells

A number of pathogens gain entry to the body via mucosal surfaces and the induction of immune responses at these surfaces can be crucial in providing the best protection against disease. These may occur by the collections of lymphocytes, plasma cells and phagocytes throughout the lung and the lamina propria (connective tissue) of the intestinal wall, or as organized mucosa-associated lymphoid tissue (MALT) with well-formed follicles.

Gut-associated lymphoid tissue (GALT) is separated from the lumen by epithelium with tight junctions and a mucous layer. This epithelium is scattered with **microfold (M)-cells**; specialized antigen-uptaking cells with short, irregular microvilli on their apical surface that endocytose antigens. The endocytic vesicles carry the antigen to be exocytosed at the basal surface for the attention of intraepithelial lymphocytes, dendritic cells and macrophages. The cells and tissues involved in mucosal immunity form an interconnected secretory system which IgA-producing B-cells may circulate. Foreign material, including bacteria, is taken up by M-cells and passed on to the underlying **Peyer's patch** antigen-presenting cells, which then activate the appropriate lymphocytes. Thus, the Peyer's patches constitute the inductive site for immune responses in the gut. After their activation is induced, the lymphocytes travel via the lymph to the mesenteric lymph nodes where additional activation and

proliferation may occur. A special feature of antigen-presenting cells from Peyer's patches, mesenteric lymph nodes and the lamina propria is that they contain a population of CD103 + dendritic cells.

The "imprinted" T cells then move via the thoracic duct into the bloodstream and finally in to the lamina propria. In this responsive site, they assist the IgA-forming B-cells that protect a wide area of the bowel with protective antibody. T- and B-cells also appear in the lymphoid tissue of the lung and in other mucosal sites guided by the interactions of specific homing receptors with appropriate HEV addressins. It is interesting that mucosal immunization at inductive site can be effective at another mucosal tract generating antibody production

Many of the adjuvants can be used as mucosal adjuvants although there are also a number of molecules that are particularly effective as mucosal adjuvants, most notably cholera toxin (CT) and *E. coli* heat-stable enterotoxin (LT). Modified forms of the toxins and their subunits can powerfully stimulate mucosal responses although their toxicity limits practical application of mucosal adjuvant (Roitt's essential immunology 12^{th}).

1-2) Passive immunization

Although the morbidity of FMD is 100%, the mortality is about 50% in piglets whereas less than 5% in adult animals (Brownlie, 1985). Passive immunization is the key point that gives piglets protection from FMD. The passive immunization can be achieved in the fetus by maternally derived antibodies acquired by

placental transfer and in the newborn by intestinal absorption of colostral immunoglobulin (Roitt's essential immunology 12th). Secretory IgA (sIgA) accounts for the major immunoglobulin in colostral milk and it remains in the intestine instead of the absorption, so that it can protect bacteria or virus at the mucus layers.

Moreover, oral vaccination of mucosal subunit vaccine has several advantages as belows:

1) immune responses at the site of primary colonization of pathogens, 2) mucosal immune response to other sites such as upper respiratory tract, mammary gland, 3) safety, efficacy and high patient compliance rates because the vaccines are administered by the natural route of infection, 4) vaccine formulation of higher stability such as solid tablets is available for oral administration (Brownlie, 1985).

1-3) Mucoadhesive polymeric carrier for oral vaccination

Mucoadhesive polymeric carrier systems are designed to extend the resident time of polymer in targeted region/tissue maximizing the amount of absorbed drug in desired absorption site (oral, nasal, respiratory, gastrointestinal, vaginal, *etc.*) and protecting vaccine from enzymatic degradation.

Generally, mucoadhesive polymers have characteristics such as hydrophilicity, high molecular weight, optimum surface tension, hydrogen bonding capacity, sticky to mucus glycoproteins, non-toxicity and non-allergenicity, chemical inertness and cost-effectiveness.

Mucoadhesive polymers are categorized as synthetic

mucoadhesive polymers and natural mucoadhesive polymers. The synthetic mucoadhesive polymers include Carbopol®, polycarbophil, poly(acrylic acid), polyacrylate, poly (methylvinylether-co-methacrylic acid), polymethacrylate, polyalkylcyanoacrylate, poly(hydroxyethyl methylacrylate), poly(ethylene oxide), poly(vinyl pyrrolidone), poly(vinyl alcohol) and thiol-containing synthetic polymers. The natural mucoadhesive polymers include cellulose derivatives (carboxymethyl cellulose, hydroxyethyl cellulose, hydroxypropyl cellulose, sodium carboxymethyl cellulose, methyl cellulose, methylhydroxyethyl cellulose etc.), alginate, dextran, karaya gum, guar gum, xanthan gum, soluble starch, gelatin, pectin, chitosan, tragacanth, hyaluronic acid, and thiol-containing natural polymers *etc.* Among them, thiol-containing polymers have paid attention so called a new generation of mucoadhesive polymers.

Thiolated polymers, also known as 'thiomers', are modified from existing polymers by introducing free thiol group (-SH) given by thiol group donor such as cysteine, homocysteine, cysteamine, *N*-acetylcysteine, glutathione, thioglycolic acid, mercaptobenzoic acid, 4-aminothiophenol, and mercaptophenylacetic acid (A. Bernkop-Schnurch *et al.*, 1999; S. Bonengel, A. Bernkop-Schnürch, 2014). Unlike other mucoadhesive polymers that adhere to mucus layer by non-covalent bonds like hydrogen bonds, van der Waal's forces and ionic interactions, thiolated polymers are considered as a promising new generation of mucoadhesive polymer in the aspect of formation of strong covalent bond between thiolated polymer

and mucus glycoproteins via thiol/disulfide exchange reaction and an oxidation process (Leitner *et al.*, 2003). Due to the ubiquitous cysteine-rich domain in mucus layer, thiomers mimic the natural mechanism of secreted mucus glycoproteins, which are also covalently anchored in the mucus layer by the formation of disulfide bonds, the most commonly encountered bridging structure in biological systems (A. Bernkop-Schnurch *et al.,* 2000). Thiolated polymers have several advantages: (1) stability enhancement due to the formation of inter- and/or intra-molecular disulfide bonds, (2) acquisition of mucoadhesion property, (3) controlled drug release, and (4) a permeation enhancing effect (Bernkop-Schnurch *et al.,* 2000). Numerous thiolated polymers were developed including thiolated Eudragit (Bijay Singh *et al.*, 2015), thiolated polycarbophil, thiolated carboxymethylcellulose (A. Bernkop-Schnurch *et al.*, 2000b), thiolated poly(acrylic acid) (Leitner *et al.*, 2003), thiolated hydroxypropyl methylcellulose (Singh *et al.*, 2015; Li *et al.*, 2016). Characteristics of thiomers are listed in Table 2.

Thiolated polymers as peptide drug carriers have been developed for oral delivery systems in pharmaceutics, biomedical sciences, and biomaterial sciences and so on. Oral peptide delivery system with thiolated polymers can enhance the uptake of peptide due to the high mucoadhesive and permeation enhancing property resulted in the significantly improved oral bioavailability (A. Bernkop-Schnurch *et al*, 2005; Singh *et al.*, 2015; Li *et al.*, 2016).

2) Immunostimulatory polymeric carrier

An ideal vaccine adjuvant must bridge the gap between innate and adaptive immunity, to not only initially engage the immune system, but also elicit a successful memory response against future infections. Immune cells such as macrophages and dendritic cells recognize invading pathogens through pattern recognition receptors (PRRs) which recognize pathogen-associated molecular patterns (PAMPs) on viruses, bacteria, and other pathogens. Recent data also suggests that the central mechanism of adjuvanticity that was once attributed to antigen adsorption, a process known as the depot effect, may be through the engagement of PRRs. Given the increased knowledge of how pathogens and adjuvants interact with the immune system, it is now possible to design novel vaccines to train the immune system to respond to specific pathogens.

Polymers have been studied for many years as adjuvants as they offer a unique set of advantages over more conventional adjuvants. One of advantages is that the size, molecular weight and chemistry of polymers can be tailored to target various cells of the immune system. Polymers that have been extensively evaluated as vaccine adjuvants include polysaccharides, polyesters and non-ionic block copolymers. These polymers can be further modified with other components including carbohydrates to activate PRRs that may provide an optimal immune response against a given pathogen. So, it is important to design vaccine with polymeric adjuvants so that they can improve the effectiveness vaccines.

Typically, vaccines interact with pathogen recognition receptors (PRRs) to initiate an innate immune response mimicking the infection of pathogen (Roitt's essential immunology 12th). Some polymers like inulin can also activate PRRs.

Inulin acetate (INAC) is refers to acetylated inulin. Typically at least about 90% of available hydroxyl groups of the inulin are acetylated. INAC is insoluble in water even at elevated temperatures, but is soluble in various organic solvents such as acetone, chloroform, dichloromethane, ethyl acetate, *etc*. It is reported that INAC can only exert the function of vaccine adjuvant when antigen was co-injected with INAC, not a mixed form. So antigen should be encapsulated into INAC particles for enhancing immune response against injected antigen. INAC microparticles and nanoparticles may function as vaccine adjuvant by enhancing the uptake of the antigen and delivering the encapsulated antigen to immune cells for activation. INAC is also a novel TLR agonist. So, INAC can work as a vaccine adjuvant by encapsulating antigens as particle forms.

Table 2. Characteristics of thiomers

Polymer	Thiol group content	Method	References
PAA-Cys	402.5 ± 58.2 – 776.0 ± 47.1 umol thiol groups/g PAA-Cys	Ellman's method	Leitner *et al.*, 2003
Chitosan-TBA	1022 umol/g polymer	Ellman′s method	Dünnhaupt *et al.*, 2011
PAA-Cys	955 umol/g polymer		
Chitosan-TBA conjugate 60	59.8 ± 3.1 umol thiol groups/g	Ellman′s method	Bernkop-Schnürch *et al.*, 2003
Chitosan-TBA conjugate 100	95.1 ± 9.0 umol thiol groups/g		
Chitosan-Thiobutylamidine conjugate	264 umol thiol group/g	Ellman′s method	Roldo *et al.*, 2004
PCP-Cys	180-344 umol/g polymer	Iodometric titration	Clausen *et al.*, 2000
PCP-Cys	12.3 umol thiol groups/g	Iodometric titration	Bernkop-Schnürch *et al.*, 2000
CMC-Cys	22.3 umol thiol group/g		
Chitosan-TBA	203.7 ± 40.9 mol/g μ	Ellman′s method	Bernkop-Schnürch *et al.*, 2004
Chitosan-TBA	100 mol/g μ	Ellman′s method	Elhassan Imam *et al.*, 2005
Eudragit L100-Cys	390.3 ± 13.4 mol/g μ	Ellman′s method	Zhang *et al.*, 2012
HMW Chitosan-TBA	213 mol/g	Ellman′s method	Bravo-Osuna *et al.*, 2006
LMW Chitosan-TBA	473 μmol/g μ		
Thiolated Chitosan	320 ± 50 umol/g	Ellman′s method	Mueller *et al.*, 2012
Thiolated hydroxyethylcellulose	131.58 ± 11.17 umol/g	Ellman′s method	Sarti *et al.*, 2010
HA-Cys	201.3 ± 18.7 umol/g	Ellman′s method	Kafedjiiski *et al.*, 2007
PCP-Cys	100 ± 8 umol/g	Iodometric titration	Bernkop-Schnürch *et al.*, 2000
CMC-Cys	1280 ± 84 umol/g	Iodometric titration	

Chapter I. Development of pH-sensitive and mucoadhesive T-CAP MPs for efficient delivery of subunit vaccine M5BT through oral vaccination

1. Introduction

Oral vaccination is considered as a most convenient and easiest way to vaccinate livestock animals although low bioavailability of orally delivered antigen (Ag) in harsh gastrointestinal environment severely limits the wide application of oral vaccine in livestock industry. Vaccine delivery with polymeric carriers can provide advantages such as physical protection of vaccine from physiological pH and enzymes, improved antigen stability, improved immunogenicity, controlled release and functionalization with functional or immune-enhancing materials to overcome these limitations.

Here, we developed a pH-sensitive and mucoadhesive thiolated CAP (T-CAP) as a polymeric carrier for efficient delivery of mucosal subunit vaccine M5BT through oral route. In this study, cellulose acetate phthalate (CAP), the pH-sensitive polymer that dissolve at > pH 6.2 was modified by thiolation to introduce mucoadhesive property and to dissolve at ileum pH. FMDV recombinant antigen M5BT was encapsulated into thiolated CAP microparticles (T-CAP MPs) using double emulsion solvent evaporation method. As a result, T-CAP MPs showed more

release of M5BT from M5BT-loaded MPs at intestinal pH (pH 7.4) than at gastric pH (pH 2) due to its pH-sensitive property. Also, porcine mucosa assay showed 1.4-fold enhanced mucoadhesiveness of T-CAP MPs than non-modified CAP MPs *in vitro* due to the formation of disulfide bond between thiol group in T-CAP and mucin glycoproteins in mucus layer by thiol/disulfide exchange reactions. Finally, M5BT delivered by T-CAP MPs elicited higher IgA production than M5BT itself in *in vivo* mouse experiment. Therefore, this study represents an effective mucosal subunit vaccine delivery through oral route.

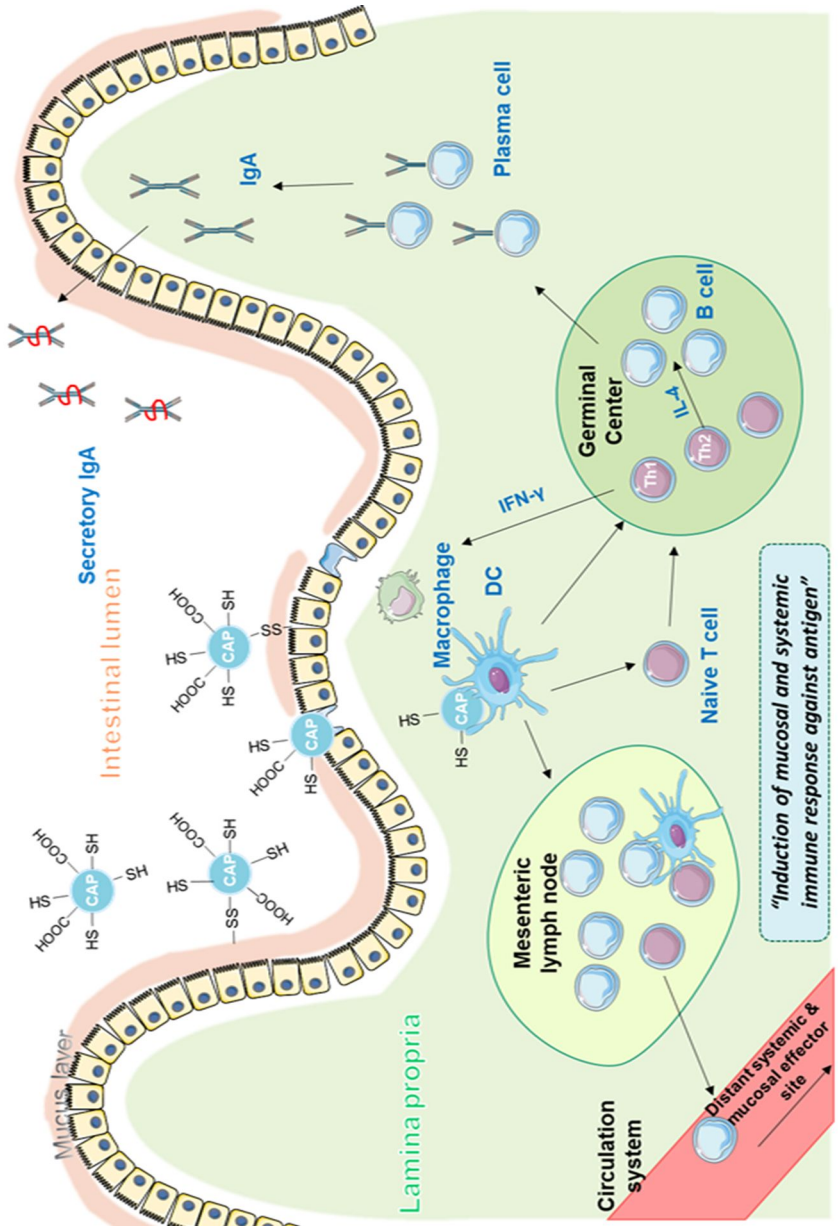


Figure 3. Graphical abstract of chapter I

2. Materials and Methods

1) Synthesis of thiolated CAP

The synthesis of thiolated CAP (T-CAP) was carried out according to the method described previously (Singh, B *et al.*, 2015). Briefly, 4 g of CAP was dissolved in 100 ml of dimethyl sulfoxide (DMSO) and the carboxylic acid moieties of the polymer were activated by *N, N'*-dicyclohexylcarbodiimide (DCC) (4.189 g) and *N*-hydroxyl succinimide (NHS) (2.337 g) with constant stirring at room temperature for 24 h under nitrogenous condition to avoid the oxidation of sulfhydryl groups by atmospheric oxygen. By-products were removed by filtration with buchner funnel (90 mm) and the filtrate was further reacted with L-cysteine hydrochloride monohydrate (0.355 g) for 18 h under similar condition. The reaction mixture was filtered to remove by-products and the filtrate was dialyzed initially against 3 L of DMSO to remove the unbound L-cysteine hydrochloride and then against distilled water several times to remove DMSO. Finally, the polymer solution was lyophilized after dialysis and the product was stored at -20℃ until use. The conjugation of L-cysteine was confirmed by 600MHz ^{1}H NMR spectroscopy (AVANCE 600, Bruker, Germany).

2) Quantification of thiol group content in T-CAP

The degree of thiol group substitution in the T-CAP was determined by Ellman's method according to the manufacturer's instructions. Briefly, 10 gm/ml of T-CAP solution was prepared

and diluted with 0.1 M sodium phosphate buffer (pH 8) containing 1 mM EDTA to prepare different dilutions. 50 ul aliquots of each dilution were added to 500 ul of 0.5 M phosphate buffer (pH 8.0) and 10 ul of Ellman's reagent (0.4 mg/ml of DTNB in 0.5 mol/l phosphate buffer, pH 8.0). Control reactions were carried out with non-modified CAP. The samples were shielded from light and incubated at room temperatures for 15 min. And then, 100 ul of the supernatant was transferred to a micro titration plate and the absorbance was measured at 412 nm using microplate reader (TECAN Infinite 200 PRO). The amount of thiol groups was calculated from the standard curve prepared by measuring the absorbance of L-cysteine hydrochloride monohydrate solution as described above.

3) Preparation of M5BT protein

E.coli BL21(DE3) harboring a gene encoding for M5BT protein was seed in 4 ml of LB medium supplemented with ampicillin and incubated overnight at 37℃ with shaking at 200 rpm. 4 ml of seed culture was inoculated in 800 ml of LB medium supplemented with ampicillin and incubated at 37℃ with shaking at 200 rpm. When the culture reached an optical density (O.D 600) of 0.5-0.6, the culture was induced with 0.5 mM IPTG and incubated at 37 ℃ with shaking at 200 rpm for 4 h. After IPTG induction, the cells were harvested by centrifugation at 6,000 rpm for 10 min, washed twice with ice-cold PBS and pellets were resuspended in 20 ml of his-binding buffer per 200 ml culture volume. Then, the cell suspensions were sonicated (10s pulse on;

5s pulse off) for 8 min with a blunt-end tip. The crude protein was collected after centrifugation at 17,000 rpm for 15 min at 4 ℃.

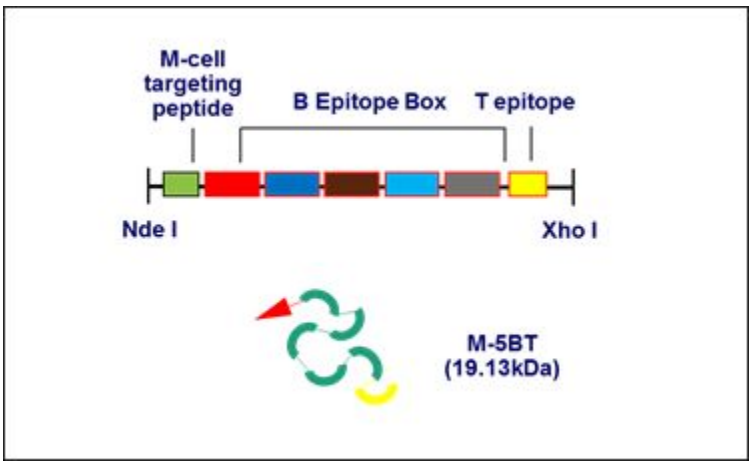


Figure 4. Composition of M5BT protein

The crude protein was purified using histidine-tag (his-tag) affinity chromatography. Before purification, the crude protein was filtrated by 0.45 um syringe filter to remove cell debris. Crude protein solution was loaded onto his-bind resin (6 ml), equilibrated with 3 volume of binding buffer and charged with 5 volume of charging buffer. After washing with 3 volume of binding buffer to remove un-charged nickel ions, column was washed with washing buffers containing different imidazole concentration (5, 40, and 70 mM) to remove non-specific protein. 6 histidine-tag bearing M5BT was eluted with elution buffer. Buffer compositions are listed in Table 3. Each fractions were analyzed by SDS-PAGE to check the purification quality and purity of protein. The purified protein was dialyzed against 5 L of distilled water at 4 ℃ for 24 h with water changes for three

times to remove the salts in elution buffer followed by lyophilization.

Table 3. His-tag affinity chromatography buffer composition

Buffer		Imidazole	Tris-Cl	NaCl	$NiSO_4$	EDTA	pH	Volume	Flow rate (ml/min)
Charging buffer		-	-	-	50 mM	-	-	5	5
Binding buffer		5 mM	20 mM	0.5 M	-	-	7.9	3	5
Washing buffer	1	5 mM						10	1-2
	2	40 mM						15	1-2
	3	70 mM						1	1-2
Elution buffer		1 M						6	1-2
Strip buffer		-				100 mM		3	5

* 1 volume = resin volume

Endotoxin was removed by ToxinEraser™ Endotoxin Removal Kit (GenScript) according to the manufacturer's instructions. Briefly, 1.5 ml of pre-packed column consisted of the matrix of modified polymyxin B (PMB) is activated by adding 5 ml of cold regeneration buffer and let the buffer drain completely at speed of 0.25 ml/min and repeated twice more. And the column was equilibrated by adding 6 ml of equilibration buffer and let the buffer drain completely at speed of 0.5 ml/min and repeated twice more. After applying the sample to the column, endotoxin-removed protein was collected and endotoxin level in protein sample was detected by ToxinSensor.

4) Preparation of T-CAP MPs

4-1) Preparation of M5BT-loaded T-CAP MPs

M5BT-loaded T-CAP MPs were prepared using a

water-in-oil-in-water ($W_1/O/W_2$) double emulsions solvent evaporation method described previously with a little modification (Singh, B *et al.*, 2015). 200 ul of aqueous solution of M5BT (5 mg) was stabilized with 100 ul of 10% Pluronic F-127 solution to form an internal aqueous phase (W_1). 100 mg of each T-CAP and CAP was dissolved in 5 ml of dichloromethane and ethyl acetate: ethanol (1:1) respectively. Organic phase was emulsified with the aqueous phase using an ultrasonic processor (Sonics, Vibra cells™) (4 output watts) on ice for 1 min 30 sec to form 1st W_1/O emulsion. The mixture emulsion was added drop by drop into 50 ml of 1% (w/v) poly(vinyl alcohol) (PVA) solution and then homogenized with Ultra Turrax (T25, IKA, Germany) at 13,000 rpm for 1 min 30 sec to form $W_1/O/W_2$ emulsion. The resulting double emulsion was stirred for 4 h at room temperature to evaporate the organic solvent. After solvent evaporation, the hardened MPs were collected by centrifugation at 6,000 rpm for 10 min, washed with distilled water, and lyophilized under vacuum. M5BT-loaded T-CAP and CAP MPs were stored at - 20 ℃ until use.

4-2) Preparation of FDA-loaded T-CAP MPs (FDA/T-CAP MPs)

Fluorescein diacetate (FDA)-loaded T-CAP and CAP MPs were similarly prepared as above-mentioned M5BT-loaded T-CAP MPs. 5 mg of FDA was dissolved into 200 ul of dichloromethane, then added to T-CAP (100 mg) solution dissolved in 5 ml of dichloromethane, and homogenized with 50

ml of 1 % (w/v) PVA solution using Ultra Turrax (T25, IKA, Germany) at 13,000 rpm for 1.5 min to form O/W emulsion.

5) Morphology by FE-SEM

The surface topography was analyzed by field-emission scanning electron microscope (FE-SEM) using SUPRA 55VP-SEM (Carl Zeiss, Oberkochen, Germany). MPs were mounted on metal stubs with thin adhesive copper tape and coated with platinum under vacuum using coating chamber (CT 1500 HF, Oxford Instruments Oxfordshire, UK).

6) Determination of loading content and loading efficiency

Loading content was determined as follows. The MPs (5 mg) were dispersed into 0.5 ml of 0.1 M NaOH containing 0.5 % (w/v) SDS. The suspension was incubated in a water bath at 60 ℃ for 2 h. Following centrifugation at 14,000 rpm for 5 min, 0.5 ml of the supernatant was withdrawn for BCA assay. The encapsulation efficiency of the M5BT into MPs was determined by measuring the unloaded protein concentration in the supernatant during the double emulsion method steps. The loading content and encapsulation efficiency was calculated using the following equations (Figure 5):

$$* \text{Loading content(\%)(w/w)} = \frac{\text{(Weight of protein in MPs)}}{\text{(Weight of MPs)}} \times 100$$

$$* \text{Encapsulation efficiency(\%)(w/w)} = \frac{\text{(Actual amount of protein loaded in MPs)}}{\text{(Theory amount of protein loaded in MPs)}} \times 100$$

Figure 5. Equation for encapsulation efficiency and loading content

7) *In vitro* release behavior test

The *in vitro* release of M5BT from M5BT/CAP or M5BT/T-CAP MPs was determined as follows. The MPs were placed into 1.5 ml tubes with 0.5 ml of 0.2 M sodium phosphate buffer (pH 7.4) or 0.2 M HCl-KCl buffer (pH 2) for 24 h at 37 ℃ with 100 rpm shaking. A 0.5 ml aliquot was withdrawn and replaced with an equal volume of each buffer at a predetermined time, and the amount of M5BT released was measured at using spectrophotometer (NanoPhotomter™).

8) Structural integrity of the M5BT protein

The integrity of M5BT before and after encapsulation in MPs was assessed by SDS-PAGE.

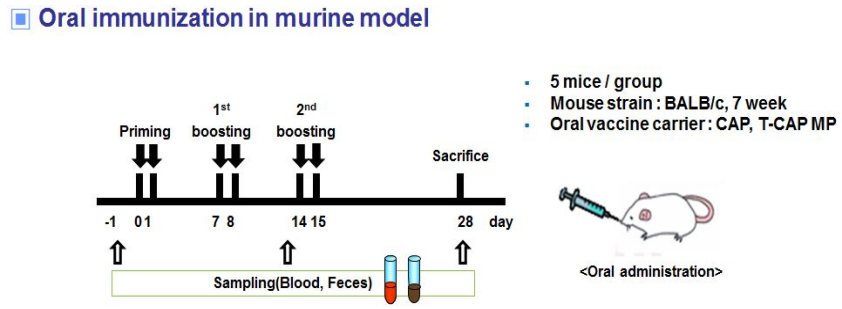
9) *Ex vivo* porcine mucosa assay

Mucoadhesive property of MPs was investigated using porcine intestinal mucosa. 4 mg of each of FDA-loaded MPs was dispersed on a freshly excised porcine intestinal mucosa, and incubated at 37℃ for 1 h with shaking at 100 rpm. The MPs attached on the mucosa were collected, and remaining concentration of each MPs was calculated by measuring the absorbance of FDA at 490nm (n=3).

10) *In vivo* oral immunization in murine model

5 female BALB/c mice of 7 weeks of age were used per group in this study. Mice were purchased from Samtako, Co. Ltd.

(Osan, Korea) and housed in cages providing ad libitum access to feed and water in accordance with the guidelines for the care and use of laboratory animals (Seoul National University). After 1 week of acclimatization, mice were orally immunized by oral gavage of MPs equivalent to 200 ug of M5BT protein suspended in 200 ul of PBS via a 1 ml syringe fitted with an oral zonde for mouse (20 G, 5 cm). All immunization groups received a total of 6 doses of vaccine for 2 priming(day 0, 1) and 4 boosting (day 7, 8, 14, 15) and *in vivo* oral immunization scheme in murine model was shown in Figure 6.



No.	Materials	Mouse	Dose of vaccine	Vaccination route	Total doses of vaccine
1	No Treatment	5	-	-	-
2	M5BT (oral)	5	200 μg	Oral	6
3	M5BT/CAP MP	5	200 μg	Oral	6
4	M5BT/T-CAP MP	5	200 μg	Oral	6

Figure 6. *In vivo* oral immunization scheme in murine model

11) Blood and fecal sampling

Blood samples of immunized mice were collected at 0, 2, and 4 weeks. Each blood collection was conducted before each immunization. The blood samples were collected from tail vein

using BD microtainer followed by isolation of serum from blood by centrifugation at 14,000 rpm for 3 min and stored in -20 ℃, and used for detection of antigen-specific antibodies by ELISA. Fecal pellets were homogenized in 10 volumes of resuspension buffer (PBS containing 1 mM PMSF and 1% BSA) at 4 ℃ overnight, centrifuged at 14,000 rpm for 10 min, supernatants were collected and analyzed for the presence of antigen-specific IgA by ELISA.

12) Anti-M5BT antibody detection by ELISA

Levels of serum M5BT-specific immunoglobulin G (total IgG) and levels of IgA in the fecal samples with specificity to M5BT were determined by ELISA. M5BT protein antigen (1 ug/ml) was diluted in carbonate buffer (pH 9.6) and diluted antigen was used for coating wells (100 ul/well) of 96-well immunoplate (SPL 32096). The plates were incubated at 37 ℃ for 2 h and washed with PBS (200 ul/well) for 3 times and blocked with blocking buffer (PBS containing 1% BSA) (200 ul/well) at room temperature for 1 h. Following blocking at room temperature, mouse sera with a 1:100 dilution in blocking buffer were added to the wells (100 ul/well). For fecal samples, 1:25 diluent were used. Plates were incubated at 37 ℃ for 2 h followed by washing three times with PBST (PBS containing 0.05 % Tween 20, 200 ul/well). For specific antibodies detection, plates were incubated for 1 h at room temperature with appropriately diluted HRP-labeled goat anti-mouse immunoglobulin conjugates specific for IgG (1:5000 dilutions) or IgA (1:5000 dilutions). The plates

were washed three times with PBST and then treated with TMB substrate solution (100 ul/well) for 5 min in the dark followed by the addition of stop solution (0.16 M H_2SO_4; 100 ul/well) in order to stop the enzymatic reaction. The absorbance was measured at 450 nm using microplate reader (TECAN Infinite 200 PRO).

13) Flow cytometric detection of MHC class II-expressing cells in Peyer's patches

After final sampling from the immunized mice, the mice were dissected to collect Peyer's patches from the ileum. Immune cells were further isolated as described earlier (Geem D. *et al.*, J Vis Exp JoVE, 2012). Briefly, a short ileum fragment with Peyer's patch was cut longitudinally and incubated at 37 ℃ in 2 mM EDTA in CMF HBSS buffer for three sequential 15 min incubations to remove the epithelial layer. Tissues were digested with 1.5 mg/ml Type VIII collagenase in CMF HBSS/FBS, and the resulting suspension of cells was passed through a 100 um cell strainer before centrifugation at 1,500 rpm for 5 min at 4 ℃. The cells were washed twice in ice-cold CMF PBS and blocked with 2.4G2 anti-FcγRIII/II in ice-cold staining buffer (CMF PBS + 5 % FBS) for 10 min on ice. Following washing with ice-cold staining buffer, cells were stained with antibody staining cocktail (CD11c and MHC class II) for 20 min on ice in the dark. Finally, cells were washed with ice-cold staining buffer twice and resuspended in 400 ul of ice-cold staining buffer for FACS analysis.

14) Statistical analysis

All results are expressed as mean ± standard deviation (SD). Statistical significance was assessed using t-test and a one-way analysis of variance (ANOVA) and post-hoc Tukey multiple comparison test. All statistical analysis was performed using GraphPad PRISM software (GraphPad Software, Inc.) All statistic al significance is denoted by *P < 0.05, **P < 0.01, and *** P < 0.001.

3. Results and Discussion

1) Preparation and characterization of T-CAP

T-CAP was prepared by conjugation with L-cysteine hydrochloride by DCC/NHS chemistry under nitrogenous condition to prevent the oxidation of sulfhydryl group. The reaction scheme for synthesis of T-CAP is shown in Figure 7. The coupling of cysteine and CAP was confirmed by proton nuclear magnetic resonance (^{1}H NMR) as shown in Figure 8. The thiol group content in T-CAP was 16.04 umole/g polymer as determined by Ellman's method.

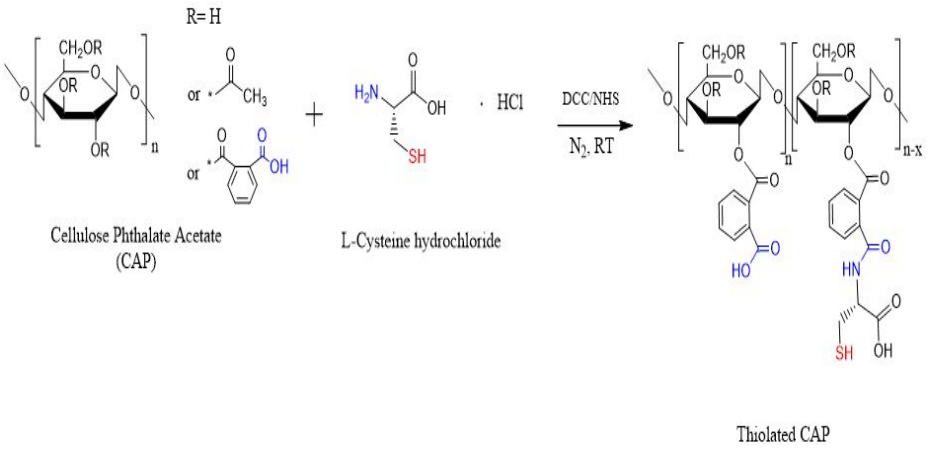


Figure 7. Synthesis scheme of thiolated CAP

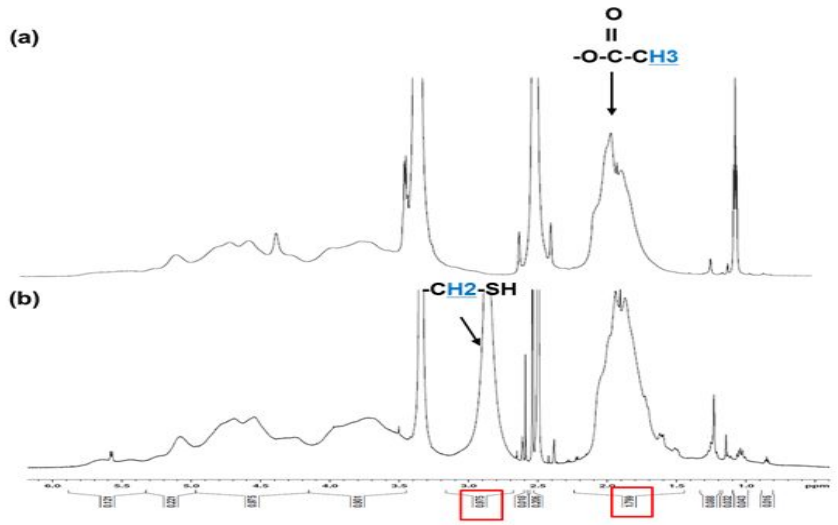


Figure 8. ^{1}H 600MHz NMR spectra of (a) CAP and (b) T-CAP

2) Preparation and characterization of M5BT/T-CAP MPs

2-1) Isolation and purification of M5BT protein

To prepare FMDV recombinant antigen M5BT for model subunit vaccine, M5BT protein was expressed with *E.coli* BL21 (DE3) harboring a gene encoding for M5BT protein. M5BT protein was induced by addition of 0.5 mM of IPTG followed by sonication for isolation of crude protein and purification by his-tag affinity chromatography. Purification quality of purified protein was checked by SDS-PAGE shown as in Figure 9.

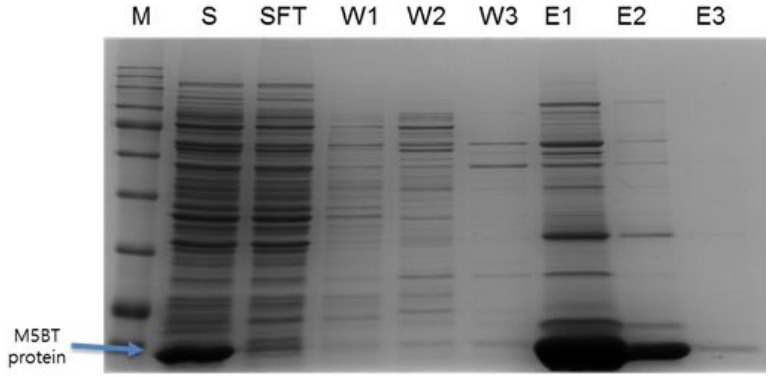


Figure 9. M5BT protein purification by his-tag affinity chromatography. Purified M5BT was identified by SDS-PAGE. (M: Protein marker; S: Crude protein sample; SFT: Sample flowthrough; W1-W3: Washing fraction; E1-E3: Elution fraction)

2-2) Preparation and characterization of M5BT/T-CAP MPs

M5BT-loaded T-CAP MPs were prepared by double emulsion solvent evaporation method (Figure 10). Briefly, M5BT solution (W_1 phase) stabilized with Pluronic F-127 was sonicated with T-CAP solution dissolved in dichloromethane (O phase) to form primary W_1/O emulsion. The primary emulsion was added into 1% PVA solution (W_2 phase) followed by homogenization to form $W_1/O/W_2$ double emulsion. After organic solvent evaporation, hardened MPs were collected by centrifugation and lyophilized.

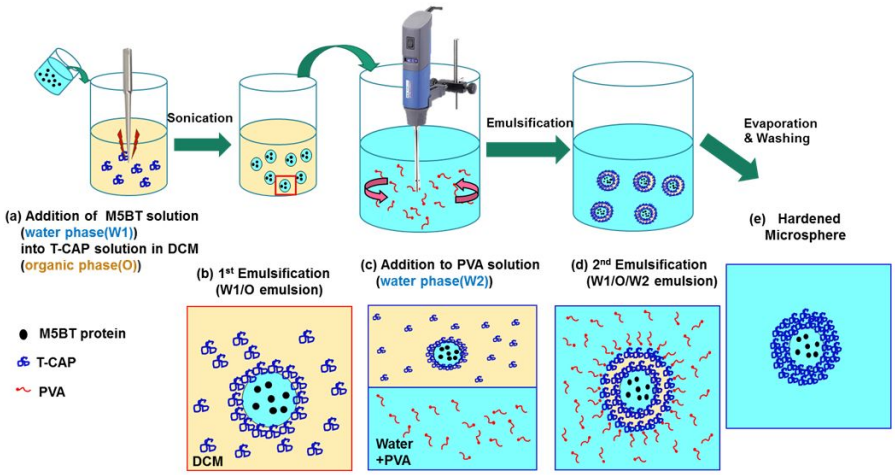


Figure 10. Procedure of M5BT/T-CAP MPs by double emulsion method (Modified from the figure from Sander, Soft matter, 2014)

3) Morphology of M5BT/CAP and M5BT/T-CAP MPs

The morphology of M5BT/CAP and M5BT/T-CAP MPs was observed by FE-SEM. Both MPs had well-formed spherical particles with smooth surfaces (Figure 11). MPs smaller than 10 um can be efficiently taken up by M-cells (antigen uptaking cell) of Peyer's patches in ileum (J.H. Eldridge *et al.*, 1990). Moreover, MPs with this range in diameter can be internalized through phagocytosis by antigen-presenting cells (APCs) playing a crucial role in initiating innate immune response.

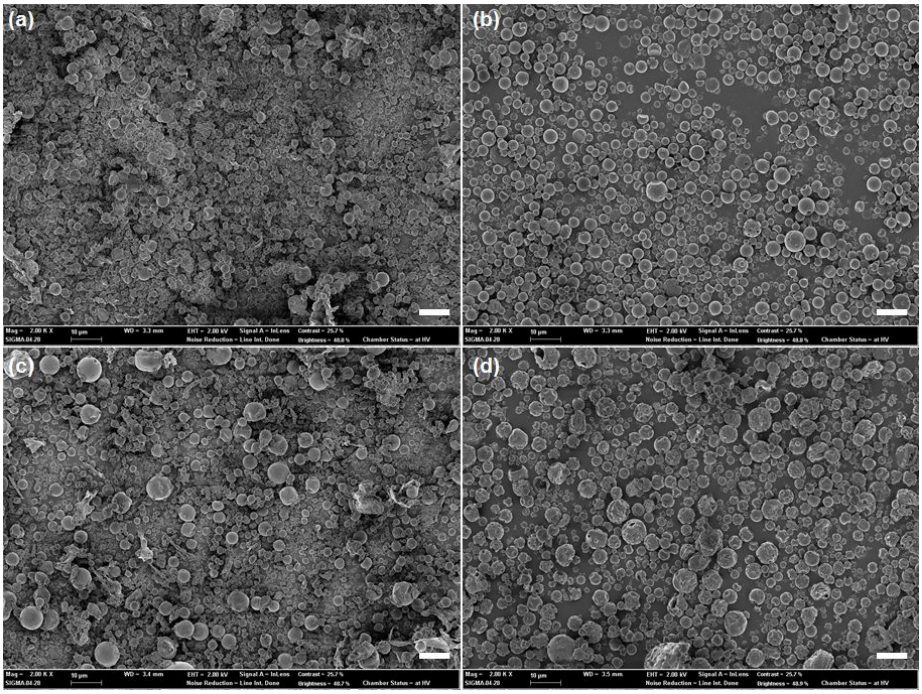


Figure 11. Analysis of morphology of MPs by FE-SEM. (a) CAP MPs; (b) T-CAP MPs; (c) M5BT/CAP MPs; (d) M5BT/T-CAP MPs (Magnification: 2,000 X, scale bar: 10 μm).

4) Loading content and encapsulation efficiency

The loading content and encapsulation efficiencies of M5BT in the T-CAP MPs is shown in Table 4. The loading content of M5BT/CAP MPs and M5BT/T-CAP MPs were 4.62% (w/w) 4.97% (w/w), respectively, showing similar antigen amount per microparticles. And encapsulation efficiency of M5BT/CAP MPs and M5BT/T-CAP MPs were 82.2%(w/w) and 72.1% (w/w), respectively. When it comes to the encapsulation efficiency, different solvent used in microparticle formation may affect the result.

During the loading of vaccine into the MPs, the vaccine's stability is important for retaining its immune activity because B-cell receptor (BCR) recognize antigen as linear or conformational epitopes whereas T-cell receptor (TCR) can only recognize the processed antigen by APCs. Therefore, the structural integrity of the M5BT released from M5BT/T-CAP MPs was evaluated by SDS-PAGE (Figure 12)

Table 4. Loading content and encapsulation efficiency of M5BT-loaded MPs

Microparticles	Loading content (%)(w/w)	Encapsulation efficiency (%)(w/w)
M5BT/CAP MPs	4.62 ± 0.239	82.2 ± 3.27
M5BT/T-CAP MPs	4.97± 0.219	72.1 ±1.36

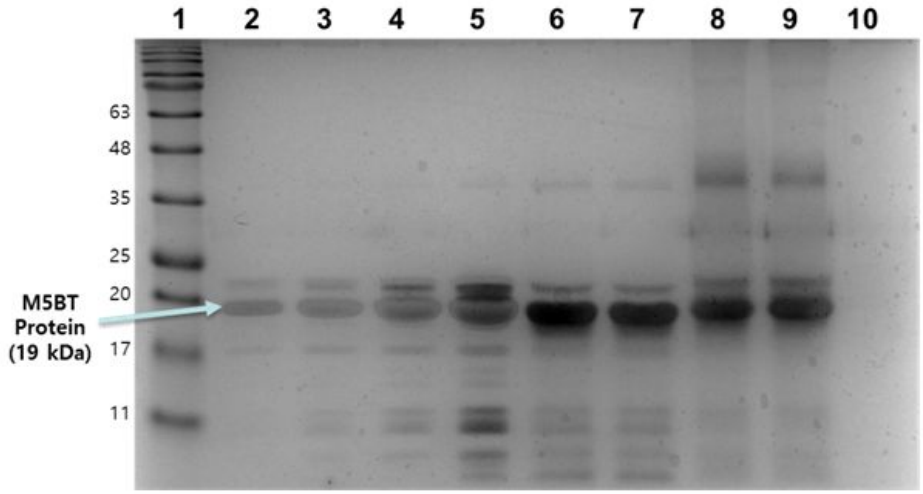


Figure 12. Evaluation of protein structure of M5BT released from M5BT/CAP MPs and M5BT/T-CAP MPs by SDS-PAGE. Lane 1 : protein marker; Lane 2-5 : native M5BT (2.5-20 ug); Lane 6-7 : M5BT released form CAP MPs; Lane 8-9 : M5BT released from T-CAP MPs; Lane 10 : protein-unloaded T-CAP MPs.

5) *In vitro* release behavior of M5BT from CAP and T-CAP MPs

The *in vitro* release profile of M5BT from CAP and T-CAP MPs was investigated at simulated gastric acid (pH 2) and simulated intestinal fluid (pH 7.4) for 24 h and was shown in Figure 13. The release profiles of M5BT from MPs were presented as the percentage of amount of M5BT released from MPs with respect to the amount of M5BT loaded in MPs.

The results indicated that the release of M5BT from M5BT/T-CAP MPs was higher at pH 7.4 compared to pH 2. Thiolation of CAP exhibited gastro-resistant property of CAP at simulated gastric solution (pH 2) with releasing 18.6 ± 1.18 %

and 17.1 ± 1.42 % from MPs at 2 h, respectively. The burst release effect of M5BT/CAP MPs at pH 7.4 was obtained within 1 - 2 h due to the rapid dissolution of CAP above pH 6.2. M5BT release profile of M5BT/T-CAP MPs at simulated ileum pH (pH 7.4) showed 43.5 ± 1.63 %, 56.8 ± 1.49 % at 12 and 24 h respectively while it released 28.8 ± 3.68 %, 33.6 ± 3.92 % at 12 and 24 h respectively at pH 2. The release behavior of both MPs at pH 2 might be resulted from the diffusion of protein inside/outside the MPs.

J.H. Eldridge *et al.* (1990) reported that total number of microparticles within Peyer's patches was increased until day 4 and microparticles less than < 5um accounted for 76-82% of the observed microparticles. This result implies that M5BT/T-CAP MPs can continuously release antigen from MPs after M5BT/T-CAP MPs were internalized into Peyer's patches.

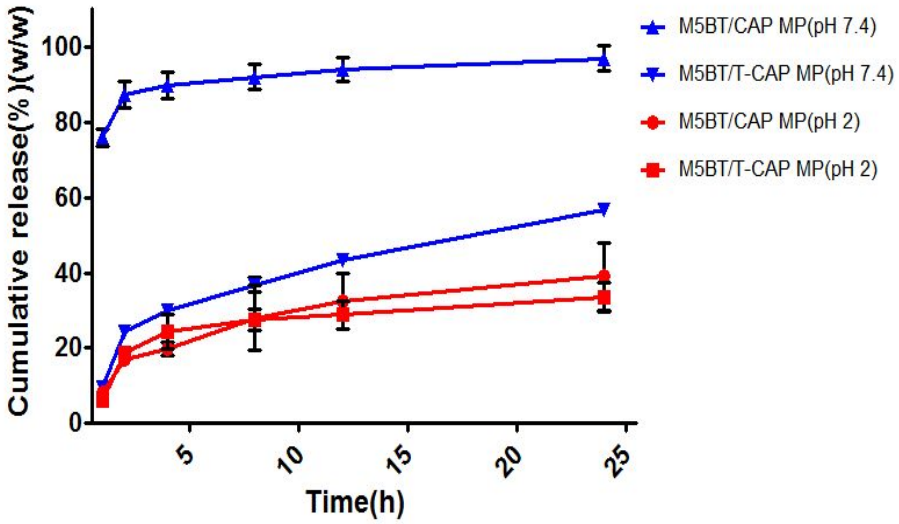


Figure 13. *In vitro* release profile of M5BT protein from M5BT/CAP and M5BT/T-CAP MPs at simulated gastrointestinal pH. MPs (10 mg/ml) were suspended in different pH buffer (pH 2, pH 7.4). Protein concentration was measured by micro BCA assay. All values represent the means ± SD (n=3).

6) Mucoadhesive property of T-CAP MPs

Mucoadhesive property of T-CAP MPs was evaluated by *ex vivo* experiment using freshly excised porcine intestinal mucosa with FDA-loaded MPs as fluorescence marker. The amount of FDA-loaded MPs attached on freshly excised porcine intestine at 37 ℃ is shown is Figure 14. The results revealed that the mucoadhesion of T-CAP MPs was 1.48-fold higher than that of CAP MPs after 1 h of incubation. Due to the enhanced mucoadhesion of T-CAP MPs, it is possible that M5BT/T-CAP MPs continuously release M5BT from T-CAP MPs remaining on the mucus layer.

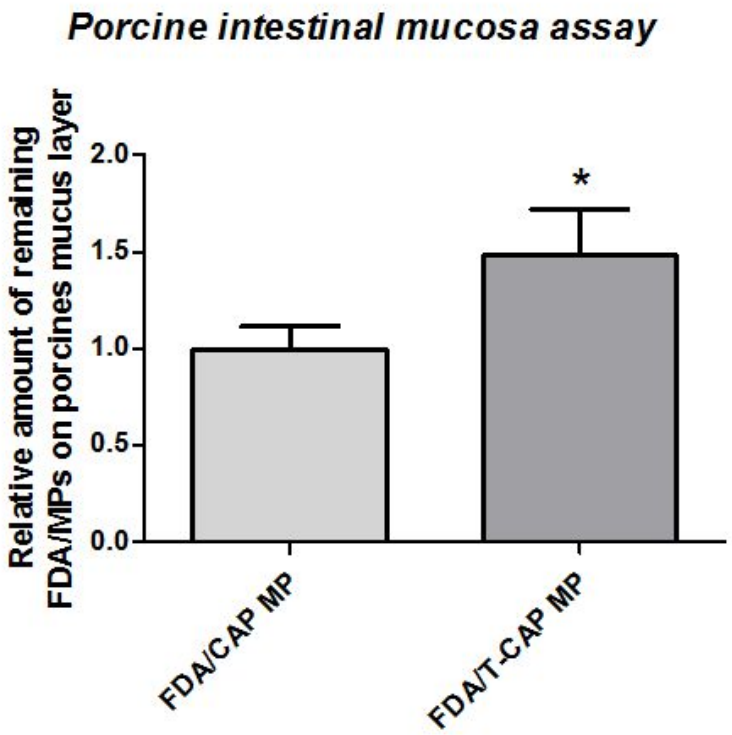


Figure 14. Analysis of mucoadhesive property of MPs in small intestine. 4 mg of each of FDA-loaded MPs was dispersed on a freshly excised porcine intestinal mucosa, and incubated at 37℃ for 1 h with shaking at 100 rpm. The MPs attached on the mucosa were collected, and remaining concentration of each MPs was calculated by measuring the absorbance of FDA at 490nm (n=3). (*$p<0.05$ by t-test)

7) Flow cytometric detection of MHC class II-expressing cells in Peyer's patches in ileum

Immune cells located throughout the intestinal lamina propria, especially in Peyer's patches, play a crucial role in sampling and processing luminal antigen for presentation to B, T cells. To determine the population of antigen-presenting cells (APCs) interacting with the antigen to initiate adaptive immune response *in vivo*, immune cells from Peyer's patches in ileum were isolated and analyzed by flow cytometry (Figure 15). Here, APCs populations in Peyer's patches were analyzed using the MHC class II surface marker. After gating, major immune cell populations expressing MHC class II were identified. The mice fed with M5BT via MPs (M5BT/CAP MPs; 26.1 %, M5BT/T-CAP MPs; 26.87 %) showed increased population of MHC class II-positive cells when compared to that of mice fed with M5BT only (16.23 %) although there is not much difference of MHC class II-positive cells between CAP and T-CAP MPs.

In addition, CD11c-positive cells in Peyer's patches from immunized mice with M5BT/T-CAP MPs were increased in total populations (N.T, M5BT; 0.30%, M5BT/CAP MP; 0.47%, M5BT/T-CAP MPs; 0.6 %).

Increased population of MHC class II-expressing cells (*i.e.* APCs) in Peyer's patches may influence the production of IgA in intestine.

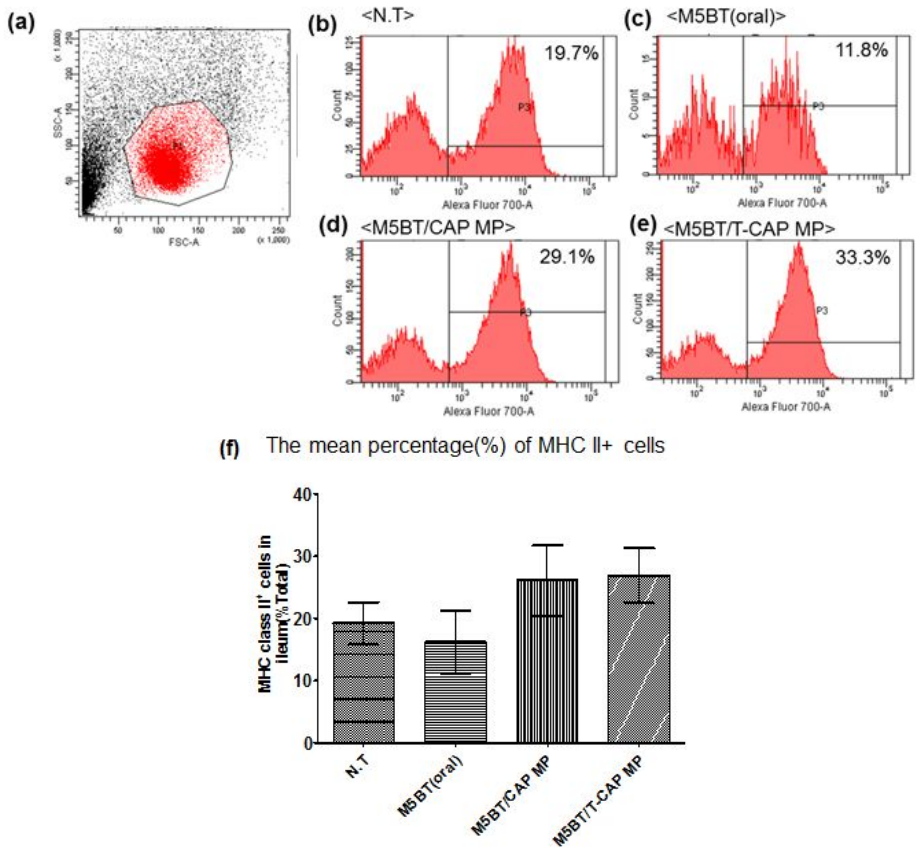


Figure 15. Flow cytometric detection of MHC class II-expressing cells in Peyer's patches from immunized mice. Peyer's patches were collected from the mice immunized with M5BT/CAP or M5BT/T-CAP MPs. Isolated cells were stained with MHC class II markers prior to detection by FACS. The percentage of MHC class II-positive cells is indicated. (a) Gating area; (b-f) MHC class II-positive cells. (b) N.T; (c) M5BT; (d) M5BT/CAP MPs; (e) M5BT/T-CAP MPs. (f) The mean percentage of MHC class II-positive cells from total populations (%total) (n=3).

8) M5BT-specific antibody production after oral immunization with MPs

To evaluate the immune-enhancing effect of M5BT-loaded MPs by oral route, mice were immunized with M5BT alone, M5BT/CAP MPs and M5BT/T-CAP MPs by oral gavage.

Antigen-specific immunoglobulin in serum and fecal samples from immunized with M5BT/T-CAP MPs were analyzed by M5BT-specific ELISA. M5BT-specific ELISA was conducted by coating immunoplate with M5BT recombinant antigen.

To assess the systemic immune response after oral immunization with M5BT/T-CAP MPs, anti-M5BT IgG levels in serum samples from immunized mice were analyzed by ELISA (Figure 16). Among the immunized groups, only mice immunized with M5BT/T-CAP MPs showed significantly higher M5BT-specific IgG levels compared to N.T group.

As a result, anti-M5BT IgA in fecal sample from immunized mice with M5BT-loaded MPs was significantly higher than that of mice immunized with M5BT only without carrier (Figure 17). This result indicate that polymeric carrier can deliver antigen to lymphoid tissue to induce antigen-specific immune response. When it comes to the oral vaccine carrier, T-CAP MPs exhibited more IgA production compared to CAP MPs at 2 and 4 weeks resulted from different pH-sensitive property by modifying CAP with thiolation (Figure 17 a,b). However, difference in fecal sampling method between 2 and 4 weeks resulted in the different final concentration of fecal pellets affecting lower absorbance of fecal samples at 4 weeks. After normalization of each IgA levels

by IgA levels of control groups to compensate the final fecal concentration, M5BT-specific IgA levels were significantly higher at 4 weeks only in mice immunized with M5BT/T-CAP MPs (Figure 17 c).

Because thiolation of CAP altered its pH-sensitive property that can dissolve at above pH 6.2 to dissolve at ileum pH (pH 7.4). Therefore, encapsulated antigen can be released from MPs and resulted in minimizing exposure of antigen to harsh intestinal environment that can denature the antigen. Thus, instead of the exposure of antigen at upper intestine part, proper antigen delivery to distal intestine part where M-cell abundant region in ileum may be important for protecting and delivering the antigen to elicit antigen specific immune response. IgA secreted by plasma cells can protect the host from infection by pathogen or virus at the mucosal site.

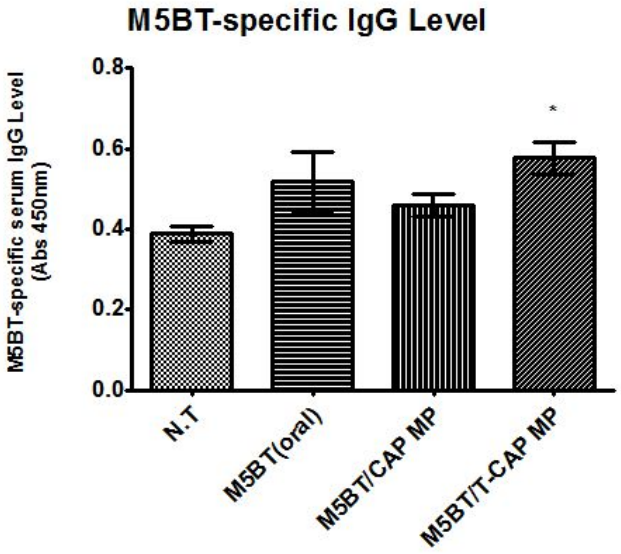


Figure 16. M5BT-specific immune response after oral immunization with MPs. Anti-M5BT serum IgG levels at 4 weeks after immunization were measured using ELISA. All values represents the means ± SD (n=5). (*P<0.05, one-way ANOVA)

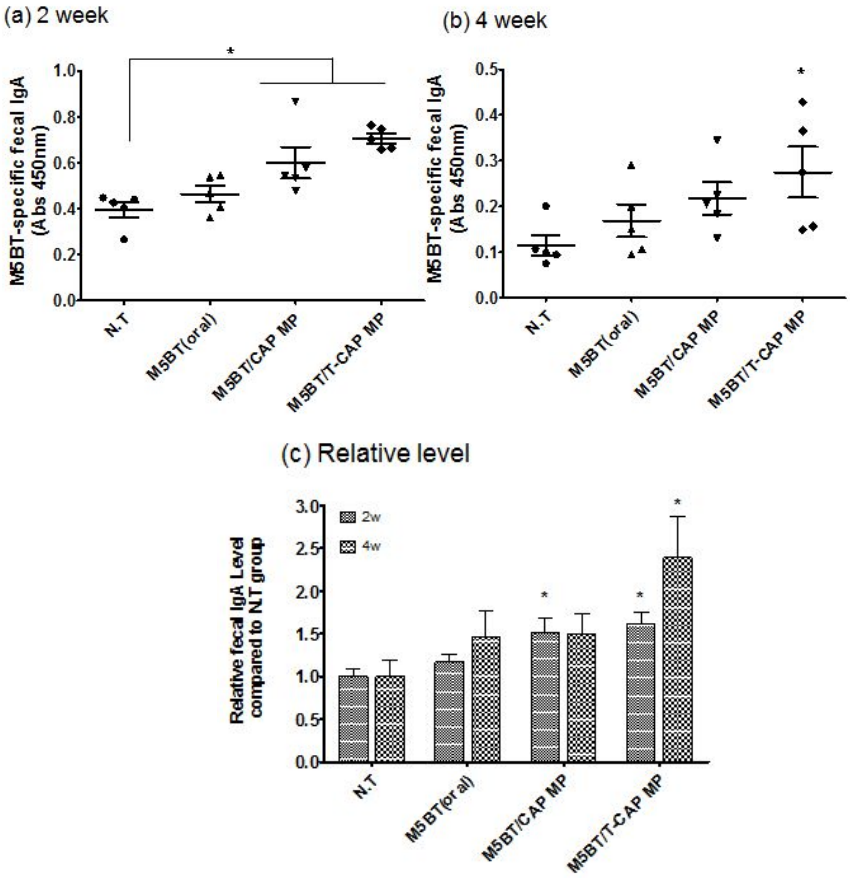


Figure 17. Anti-M5BT IgA level in feces after oral immunization with MPs. Fecal samples were taken from mice at (a) 2 weeks, (b) 4 weeks. (c) Relative anti-M5BT IgA level compared with N.T group. Antibody levels were analyzed by ELISA. (n=5, error bars represent standard deviations; *$p<0.05$, one-way ANOVA)

Summary

In this chapter, pH-sensitive and mucoadhesive T-CAP were prepared for delivering FMD subunit vaccine because oral vaccination of mucosal subunit vaccine has limitation due to its poor immunogenicity and low bioavailability despite of many advantages compared to parenteral administration.

And T-CAP MPs as a mucosal oral vaccine adjuvant producing more IgA, the most important immunoglobulin for preventing FMD at the early stage of infection, was prepared. It was found that T-CAP MPs resulted in the elevated population of MHC class II^{+} cells in Peyer's patches because antigen uptaken by M-cells from lumen can be uptaken and be transported to lymph node by immune cells (DCs, Macrophages, B cells) to active both innate immunity and adaptive immunity. This indicate that oral immunization of mucosal subunit vaccine via T-CAP MPs effectively delivered the vaccine to Peyer's patches eliciting mucosal IgA response. It will make a step forward into promising oral subunit vaccine development in livestock industry.

Chapter II. Development of mannan-decorated inulin microparticles for enhancing the immunogenicity of subunit vaccine

1. Introduction

The goal of vaccination is to provide long-term protection against infection by generating a strong immune response to the administered antigen. Conventional live attenuated vaccines typically do not require adjuvants. However, the immunogenicity of proteins is typically poor and the use of adjuvants is required. Vaccines often require the addition of immune stimulatory agents called adjuvants to boost the specific immune response to antigens. The most widely used adjuvants are complete freund's adjuvant (CFA), which is composed of inactivated *mycobacterium*. CFA is used in several FMD vaccines, although its increased immune-boosting effect is related to the toxicity of CFA itself. To substitute the conventional adjuvant like CFA, development of new vaccine adjuvant is required for FMD vaccine which can enhance the antibody response. An ideal vaccine adjuvant should stimulate both humoral and cellular immune responses against co-injected antigens.

In this study, to cover the limitation of FMD subunit vaccines, inulin acetate microparticles decorated with mannan (M-INAC MPs) were used as a polymeric adjuvant and carrier system.

Inulin is a storage carbohydrate of a number of plants including Jerusalem artichoke, chicory, dahlia, wheat, *etc*. Inulin

exists in several forms depending on its solubility and precipitation method. Recently, it was reported that particle form of inulin can act as an efficient adjuvant for vaccine (Kumar, S., & Tummala, H., 2013). So, particulation of inulin is the main point for developing inulin as an adjuvant for vaccine.

To develop inulin adjuvant for subunit vaccine, INAC was prepared to make particle form. Inulin was acetylated by introducing acetyl group from acety anhydride to inulin. INAC can be particulated in water due to self-assembled mechanism although it is soluble in various organic solvents including dichloromethane. Because it is known that INAC MPs can function as a vaccine adjuvant when antigen was encapsulated into INAC particles, antigen-loaded INAC MPs can enhance immune response of the subunit vaccine by overcoming its low immunogenicity. Particulated adjuvants may function as efficient adjuvants by enhancing the uptake of the antigen by immune cells such as macrophage, dendritic cells and delivering the encapsulated antigen to immune cells for activation. Also, subunit vaccine inside INAC particles can be released in a sustained manner known to induce long-term immune response of the antigen. Besides, it was reported that INAC is a novel TLR-4 agonist (Tummala, H., & Kumar, S., 2013). Through these benefits, INAC can work as vaccine adjuvant and vaccine carrier.

Double emulsion solvent evaporation method was used to develop INAC microparticles to encapsulate subunit vaccine M5BT. In addition, INAC MPs were decorated with mannan, the TLR-4 agonist, as a specific ligand to recognize immune cells.

2. Materials and Methods

1) Synthesis of INAC

The synthesis of inulin acetate was carried out according the method of Wu *et al.* (1999) with a little modification. Briefly, inulin (1 g) was added to 5 ml of dimethyl formamide (DMF) and then 0.2 ml of 5% acetic anhydride was added. Sodium acetate (NaOAc, 5% (w/v)) was used as a catalyst for the reaction. The acetylation reaction was carried at 40 ℃ for 24 h under nitrogen. After 24 h, INAC was dialyzed against DMF for 24 h to remove free acetic acid and against distilled water to remove DMF and unreacted inulin. After dialysis, INAC was lyophilized and stored at -20 ℃ until use. The conjugation of acetyl group was confirmed by 600 MHz ^{1}H NMR spectroscopy (AVANCE 600, Bruker, Germany) and acetyl group content in INAC was quantified.

2) Preparation of M5BT-loaded INAC MPs and M5BT-loaded M-INAC MPs

M5BT-loaded INAC and M5BT-loaded M-INAC MPs were prepared by a double emulsion solvent evaporation method. Briefly, 200 ul of 25 mg/ml M5BT solution was mixed with 100 ul of 10% (w/v) Pluronic F-127 solution as an aqueous phase (W_1). This aqueous phase was emulsified with 5 ml of dichloromethane (DCM) as an oil phase (O) containing 100 mg of INAC by sonication for 1.5 min, resulting in the formation of primary W_1/O emulsion. This primary emulsion was then added

dropwise into another aqueous (W_2) phase (50 ml water) containing 1% (w/v) poly(vinyl alcohol) (PVA) solution as a surfactant, with continuous stirring at 13,000 rpm using Turrax resulting in the formation of double emulsion ($W_1/O/W_2$). In case of M5BT-loaded M-INAC MPs, 0.75% PVA solution containing 0.25% mannan was used. The stirring was continued overnight for complete evaporation of the organic solvent. Resulting MPs were collected via centrifugation at 6,000 rpm for 10 min at 4 ℃. The pelleted M5BT-loaded INAC or M5BT-loaded M-INAC MPs were washed with distilled water and centrifuged. The final MPs were resuspended in 10 ml of distilled water and frozen by liquid nitrogen followed by lyophilization under vacuum.

3) Morphology of MPs

The surface topography was analyzed by field-emission scanning electron microscope (FE-SEM) using SUPRA 55VP-SEM (Carl Zeiss, Oberkochen, Germany). MPs were mounted on stubs with adhesive copper tape and coated with platinum under vacuum using coating chamber (CT 1500 HF, Oxford Instruments Oxfordshire, UK).

4) Confirmation of mannan-decoration into MPs

To confirm mannan-decoration in M-INAC MPs, FITC was conjugated with mannan. Briefly, 100 mg of mannan dissolved in 1 ml of distilled water was slowly mixed with 5 mg of FITC dissolved in 1 ml of DMSO. After stirring for 4 h at room temperature in dark conditions, the reaction product was dropped

into 8 ml of ethanol to remove the unreacted FITC. The precipitated FITC-mannan conjugate was washed with ethanol and collected by centrifugation at 16,000 rpm 3 times for 10 min. FITC-mannan-decorated INAC MPs were visualized by confocal laser scanning microscope (Carl Zeiss LSM710).

5) Determination of loading content and encapsulation efficiency

Loading content was determined as follows. The MPs (5 mg) were dispersed into 0.5 ml of dimethyl sulfoxide (DMSO). The completely dissolved solution was used for measurement of protein concentration spectrophotometer (NanoPhotomter™). The encapsulation efficiency of the M5BT into MPs was determined by measuring the unloaded protein concentration in the supernatant during the double emulsion method steps. The loading content and encapsulation efficiency was calculated using the following equations (Figure 18):

$$* \text{ Encapsulation efficiency(\%)(w/w)} = \frac{\text{(Actual amount of protein loaded in MPs)}}{\text{(Theory amount of protein loaded in MPs)}} \times 100$$

$$* \text{ Loading content(\%)(w/w)} = \frac{\text{(Weight of protein in MPs)}}{\text{(Weight of MPs)}} \times 100$$

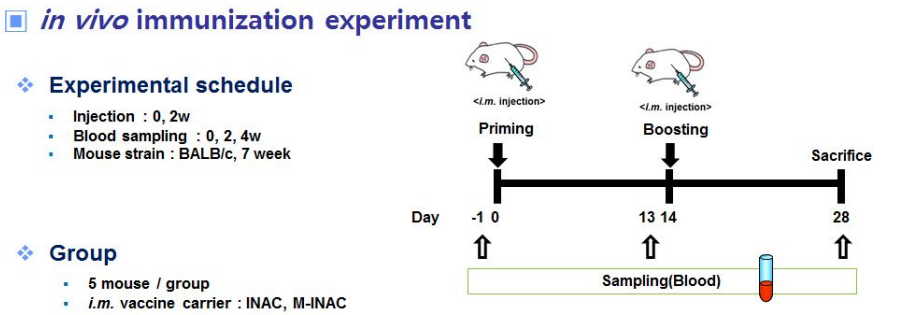
Figure 18. Calculation equation for encapsulation efficiency and loading content

6) *In vitro* release behavior test

The *in vitro* release of M5BT from M5BT-loaded INAC or M5BT-loaded M-INAC MPs was determined as follows. The M5BT-loaded MPs (5 mg) were placed into 1.5 ml tubes with 0.5 ml of PBS (pH 7.4) at 37 ℃ with 100 rpm shaking. A 0.5 ml aliquot was withdrawn and replaced with an equal volume of PBS at a predetermined time, and the amount of M5BT released was measured using spectrophotometer (NanoPhotomter™).

7) *In vivo* immunization in murine model

5 female BALB/c mice of 7 weeks of age were used per group in this study. Mice were purchased from Samtako, Co. Ltd. (Osan, Korea) and housed in cages providing *ad libitum* access to feed and water in accordance with the guidelines for the care and use of laboratory animals (Seoul National University). After 1 week of acclimatization, mice were immunized intramuscularly with MPs in 50 ul of PBS or M5BT resuspended in 50 ul of PBS and CFA (1:1) via a 0.3 ml insulin syringe. CFA was replaced with IFA for boosting. All immunization groups received a total of 2 doses of vaccine for 1 priming (day 0) and 1 boosting (day 14). *In vivo* immunization experiment schedule in murine model was shown in Figure 19.



No.	Materials	Mouse strain	Mice	Dose of vaccine	Administration route	Total dose	Adjuvant
1	PBS	Balb/c	5	-	-	-	
2	iFMDV vaccine	Balb/c	5	50ul (>6PD_{50})	I.M	2	
3	M5BT	Balb/c	5	20ug	I.M	2	
4	M5BT (CFA)	Balb/c	5	20ug	I.M	2	CFA
5	M5BT/INAC MP	Balb/c	5	20ug	I.M.	2	
6	M5BT/M-INAC MP	Balb/c	5	20ug	I.M.	2	

- CFA : immunopotentiator composed of inactivated and dried mycobacteria(*M. tuberculosis*)
- PD50 : 50% protective dose

Figure 19, I*n vivo* immunization experiment schedule in murine model

8) Blood and fecal sampling

Blood samples of immunized mice were collected at 0, 2 and 4 weeks after immunization. Each blood collection was conducted before each immunization. The blood samples were collected from tail vein using BD microtainer followed by isolation of serum from blood by centrifugation at 14,000 rpm for 3 min and stored in -20 ℃, and used for detection of antigen-specific antibodies by ELISA. Fecal pellets were homogenized in 10 volumes of resuspension buffer (PBS containing 1 mM PMSF and 1% BSA) at 4 ℃ overnight, centrifuged at 14,000 rpm for 10 min, supernatants were collected and analyzed for the presence of antigen-specific IgA by ELISA.

9) FMDV serotype O specific antibody production

Levels of FMDV type O specific antibody were measured using PrioCHECK FMDV Type O ELISA Kit according to the manufacturer's instructions. Briefly, levels of FMDV type O specific antibody in serum samples from immunized mice were determined by blocking ELISA with FMDV type O antigen-coated plate. The reaction between FMDV type O antigen and monoclonal antibody was blocked by specific antibodies that are present in the test samples (Figure 20). The vaccination efficacy was presented as percentage inhibition (P.I). P.I value was calculated by following equation: P.I = 100 - $(O.D_{Sample}/O.D_{Negative})*100$.

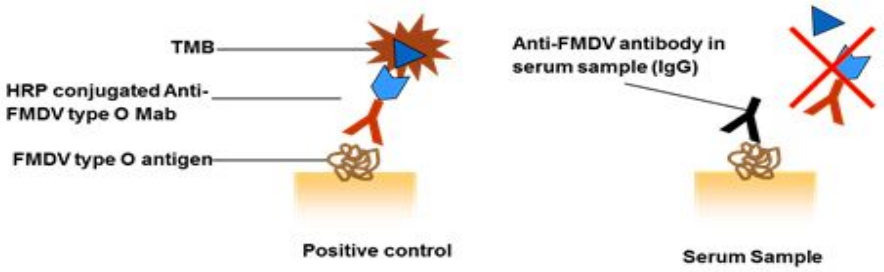


Figure 20. Schematic diagram of FMDV type O antigen blocking ELISA. The reaction between Ag and mAb was blocked by specific antibodies that are present in the test sample.

10) Anti-M5BT antibody detection by ELISA

Levels of serum M5BT-specific immunoglobulin G (total IgG) with specificity to M5BT was determined by ELISA. M5BT protein antigen (1 ug/ml) was diluted in carbonate buffer (pH 9.6) and diluted antigen was used for coating wells (100 ul/well) of 96-well immunoplate. The plates were incubated at 37 ℃ for

2 h and washed with PBS (200 ul/well) 3 times and blocked with blocking buffer (PBS containing 1% BSA) (200 ul/well) at room temperature for 1 h. Following blocking at room temperature, mouse sera with a 1:100 dilution in blocking buffer were added to the wells (100 ul/well). Plates were incubated at 37 ℃ for 2 h followed by washing 3 times with PBST (PBS containing 0.05 % Tween 20, 200 ul/well). For specific antibodies detection, plates were incubated for 1 h at room temperature with appropriately diluted HRP-labeled goat anti-mouse immunoglobulin conjugates specific for IgG (1:5000 dilutions). The plates were washed three times with PBST and then treated with TMB substrate solution (100 ul/well) for 5 min under the dark followed by the addition of stop solution (0.16 M H_2SO_4; 100 ul/well) to stop the enzymatic reaction. Finally, the absorbance was recorded at 450 nm using microplate reader (TECAN Infinite 200 PRO).

11) Statistical analysis

All results are expressed as mean ± standard deviation (SD). Statistical significance was assessed using t-test or a one-way analysis of variance (ANOVA) and post-hoc Tukey multiple comparison test. All statistical analysis was performed using GraphPad PRISM software (GraphPad Software, Inc.) All statistical significance is denoted by $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

3. Results and Discussion

1) Preparation and characterization INAC

Inulin acetate was synthesized by conjugation of acetyl anhydride with inulin at 40℃ for 24 h using sodium acetate as a catalyst as shown Figure 21. The conjugation of acetyl groups with inulin was confirmed and calculated by 600 MHz ^{1}H-NMR spectroscopy (Figure 22). The peak from acetyl groups (COCH3) was identified at 2 ppm in the NMR spectra of INAC whereas there is no peak at 2 ppm in that of IN. And the degree of acetylation was 75 mole%.

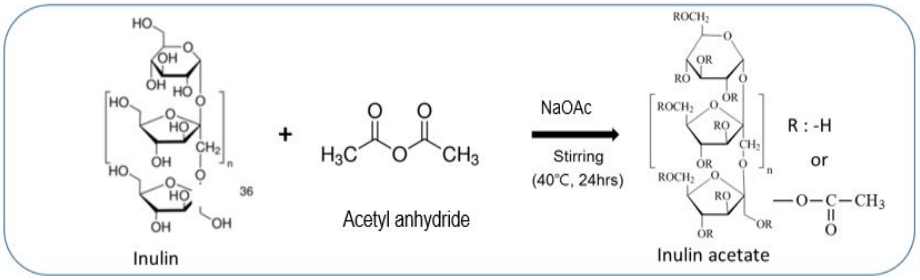


Figure 21. Chemical reaction scheme for synthesis of INAC.

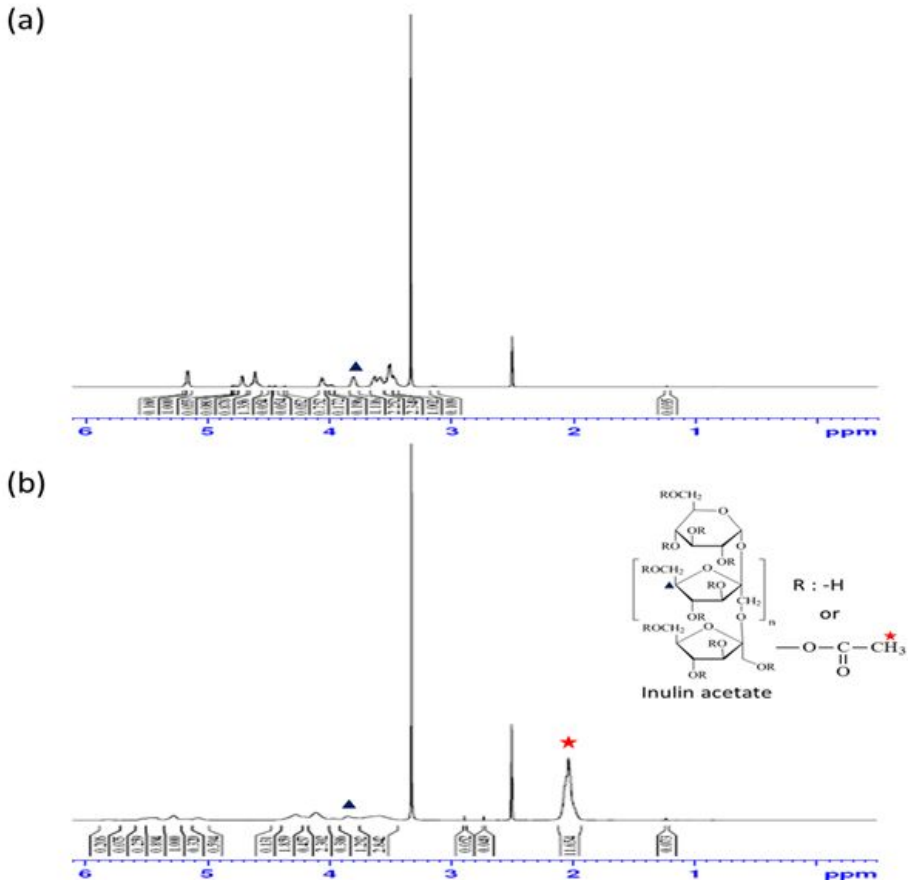


Figure 22. 600 MHz ^{1}H-NMR spectra of (a) inulin and (b) inulin acetate. Samples were measured in DMSO-d6.

2) Preparation and characterization of M5BT-loaded INAC and M5BT-loaded M-INAC MPs

M5BT-loaded INAC and M-INAC MPs were prepared by W/O/W emulsion method (Figure 23). Briefly, the W_1 phase containing M5BT stabilized with Pluronic F-127 was emulsified with organic phase (INAC in DCM) by sonication to form W_1/O emulsion. The mixture was then added into W_2 phase of 1% PVA solution followed by homogenization to form $W_1/O/W_2$ emulsion. After organic solvent evaporation, hardened MPs were collected by centrifugation.

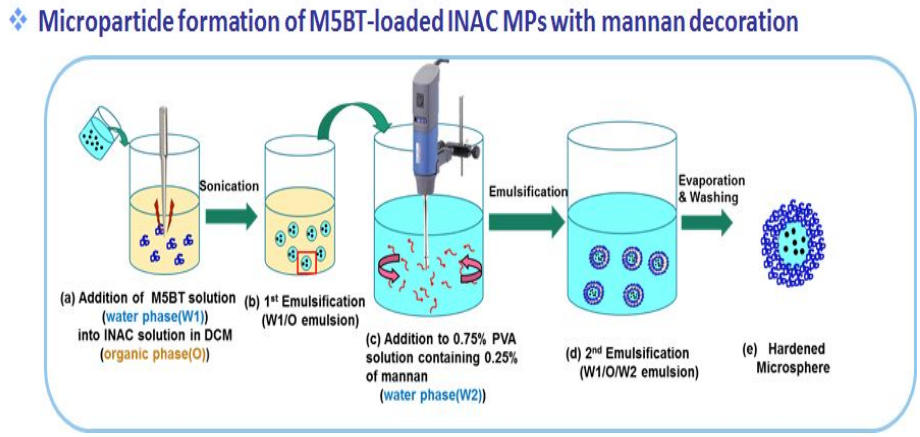


Figure 23. Graphical illustration of microparticle formation protocol of M5BT-loaded M-INAC MPs by double emulsion method.

3) Morphology and size distribution of MPs

The morphology of MPs was observed by FE-SEM. Both MPs had well-formed spherical shapes with smooth surfaces (Figure 24). And mannan-decoration of INAC MPs didn't affect the microparticle formation showing similar microparticle sizes (M5BT-loaded INAC MPs: 2.84 ± 0.022 μm, M5BT-loaded M-INAC MPs: 2.43 ± 0.104 μm). It was reported that only MPs less than 10 μm can be uptaken by Peyer's patches (PPs) in ileum and the transport of MPs to the lymph node by phagocytes are restricted to the MPs less than 5 μm (J.H. Eldridge *et al.*, 1990).

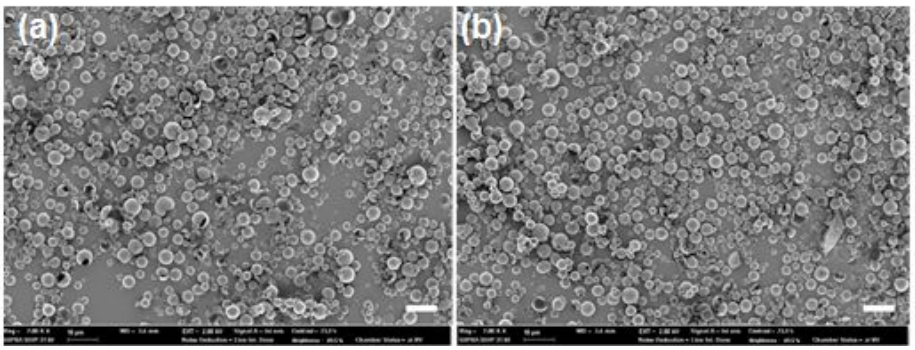


Figure 24. The morphologies of (a) M5BT-loaded INAC MPs and (b) M5BT-loaded M-INAC MPs analyzed by FE-SEM. (Magnification: 2,000X)

4) Confirmation of mannan-decoration in INAC MPs

To confirm the mannan-decoration on the surface of INAC MPs, FITC-mannan was used to decorate INAC MPs and visualized by CLSM (Figure 25). The confocal images indicated that FITC-mannan was decorated on the INAC MPs surface.

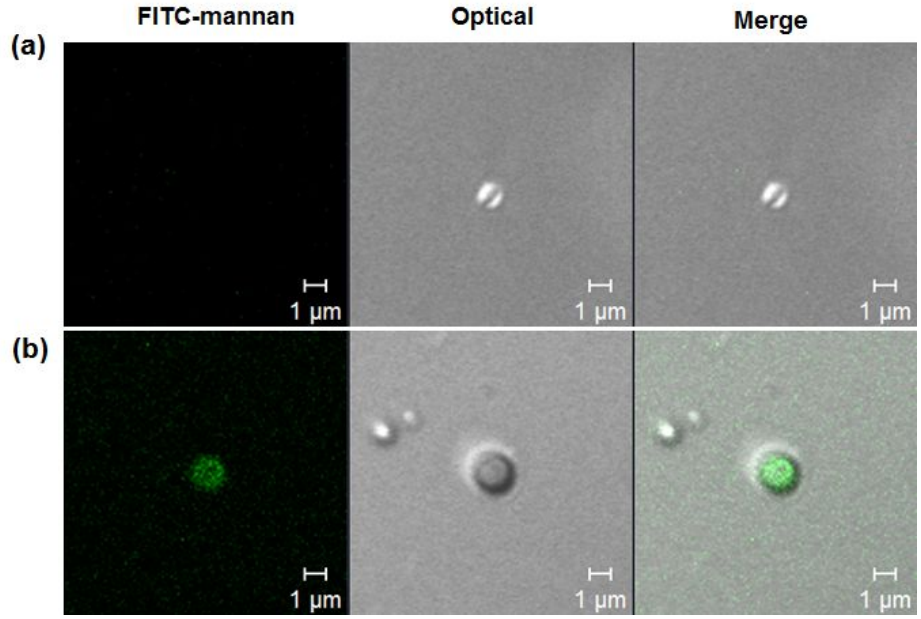


Figure 25. Confirmation of mannan-decoration of INAC MPs by CLSM. FITC-labeled mannan was used to decorate INAC MPs. (a) M-INAC MPs and (b) FITC-M-INAC MPs.

5) Loading content and encapsulation efficiency

The loading efficiencies of M5BT-loaded INAC MPs and M5BT-loaded M-INAC MPs is shown in Table 5.

Table 5. Loading characteristics of MPs.

Microparticles	Loading content (%)(w/w)	Encapsulation efficiency (%)(w/w)
M5BT/INAC MPs	7.30 ± 0.647	61.4 ± 4.23
M5BT/M-INAC MPs	4.59 ± 0.066	56.5 ± 12.44

6) *In vitro* release behavior of M-INAC MPs

M5BT-loaded INAC and M5BT-loaded M-INAC MPs were dispersed in PBS (pH 7.4) and incubated at 37 ℃ with 100 rpm shaking. At predetermined time intervals, tubes were taken and centrifuged at 6,000 rpm for 10 min at 4 ℃.

This *in vitro* release study showed that release of M5BT from M5BT-loaded M-INAC MPs was faster than that of M5BT-loaded INAC MPs. And 35.3 ± 8.03% of M5BT was released from M5BT-loaded M-INAC MPs within 24 h, whereas 8.3 ± 2.11% of M5BT was released from M5BT-loaded INAC MPs (Figure 26). More than 80% of M5BT was released from M-INAC MPs within 5 days, while less than 50% of M5BT was released from INAC MPs. The results might be regarded that ma nnan-decoration affected fast release of M5BT from M5BT-loaded M-INAC MPs due to the hydrophilic property of the mannan.

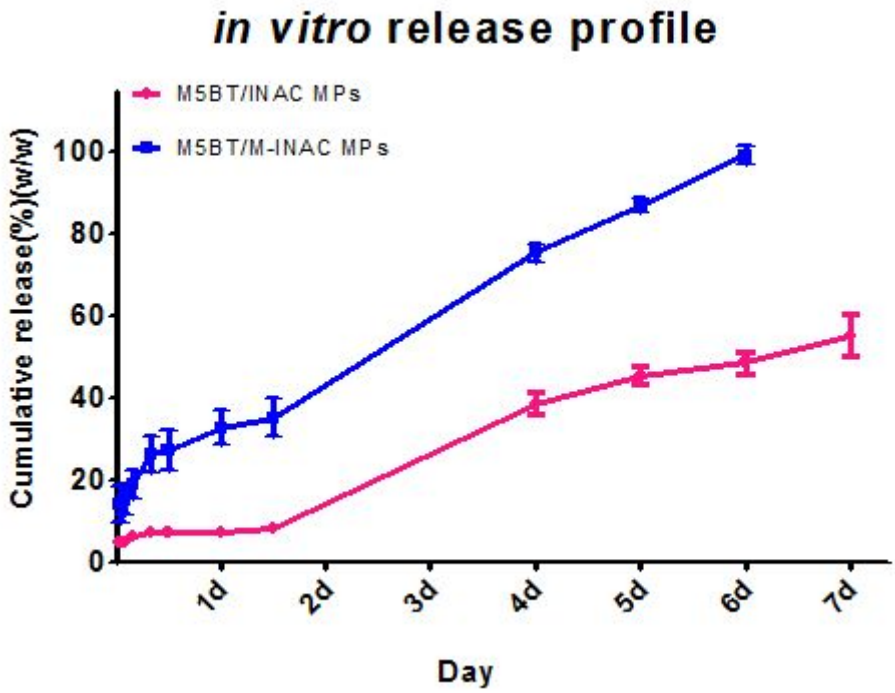


Figure 26. I*n vitro* release profile of M5BT protein from M5BT/INAC or M-INAC MPs at PBS (pH 7.4). MPs (10 mg/ml) were suspended in PBS (pH 7.4) at 37 ℃. All values represents the means ± SD (n=3).

7) FMDV serotype O specific antibody production

To assess the immunization efficacy of MPs, anti-FMDV type O serotype antibody was detected with serum sample. Levels of FMDV type O specific antibody in serum samples from immunized mice was determined by blocking ELISA with FMDV serotype O antigen-coated plate (PrioCHECK). The vaccination efficacy was presented as percentage inhibition titer (P.I).

The results indicate that M5BT delivered by CFA, INAC or M-INAC MPs showed similar level of FMDV serotype O

Ag-specific Ab compared to that of M5BT group (Figure 27). However, among the adjuvant groups, only M5BT-loaded M-INAC MPs showed similar P.I value with iFMDV groups showing positive response (P.I > 50) in 3 and 4 mice, respectively.

It suggests that antigen-loaded M-INAC MPs can enhance anti gen-specific immune response similar with conventional adjuvant implying the potential of alternative polymeric adjuvant and polymeric carrier for subunit vaccine.

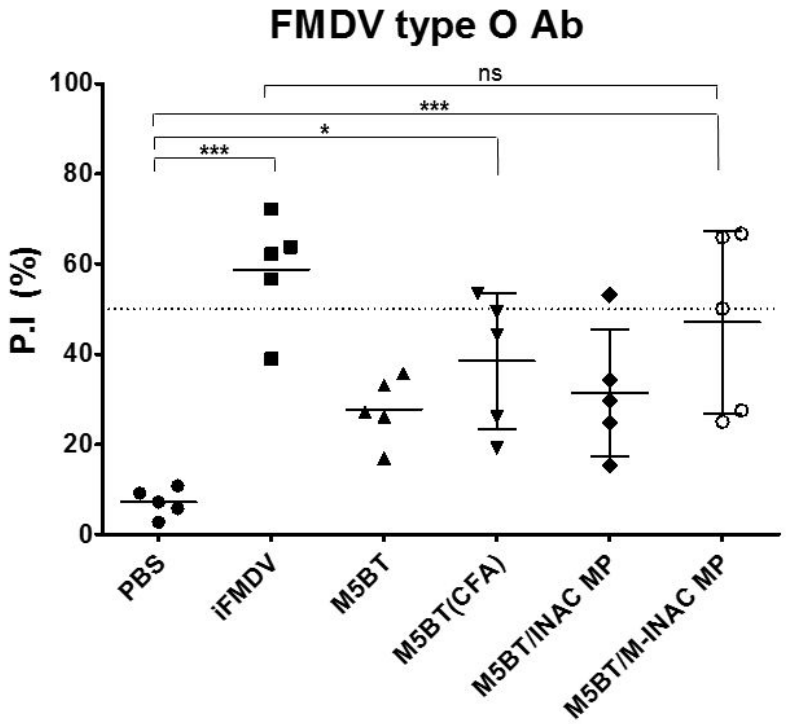


Fig 27. ELISA for *in vitro* detection of antibodies against FMDV serotype in serum from immunized mice. All values represent the means ± SD (n=5).

8) M5BT-specific immune response after immunization with MPs

To evaluate the immune-enhancing effect of M5BT-loaded MPs, mice were immunized with M5BT with CFA, M5BT-loaded INAC MPs and M5BT-loaded M-INAC MPs by intramuscular (*i.m*) injection.

Antigen-specific immunoglobulin in serum samples from immunized mice with M5BT-loaded INAC and M5BT-loaded M-INAC MPs were analyzed by M5BT-specific ELISA. M5BT-specific ELISA was conducted by coating 96-well immunoplate with M5BT recombinant antigen.

To assess the systemic immune response after immunization with M5BT-loaded INAC and M5BT-loaded M-INAC MPs, anti-M5BT IgG levels in serum samples from immunized mice were analyzed by ELISA (Figure 28). Among the immunized groups, anti-M5BT IgG titer of M5BT was improved in adjuvant groups (CFA, INAC MP, M-INAC MPs) compared to that of M5BT group. And M5BT along with the CFA group and M5BT-loaded M-INAC MPs groups elicited similar anti-M5BT IgG titer suggesting the immune cell activating effect of mannan-decoration of INAC MPs compared to the M5BT-loaded INAC MPs. M5BT-specific IgG titer increased by 1 priming and 1 boosting schedule resulting in a similar IgG titer with M5BT along with CFA group due to the polymeric adjuvant effect of M-INAC MPs.

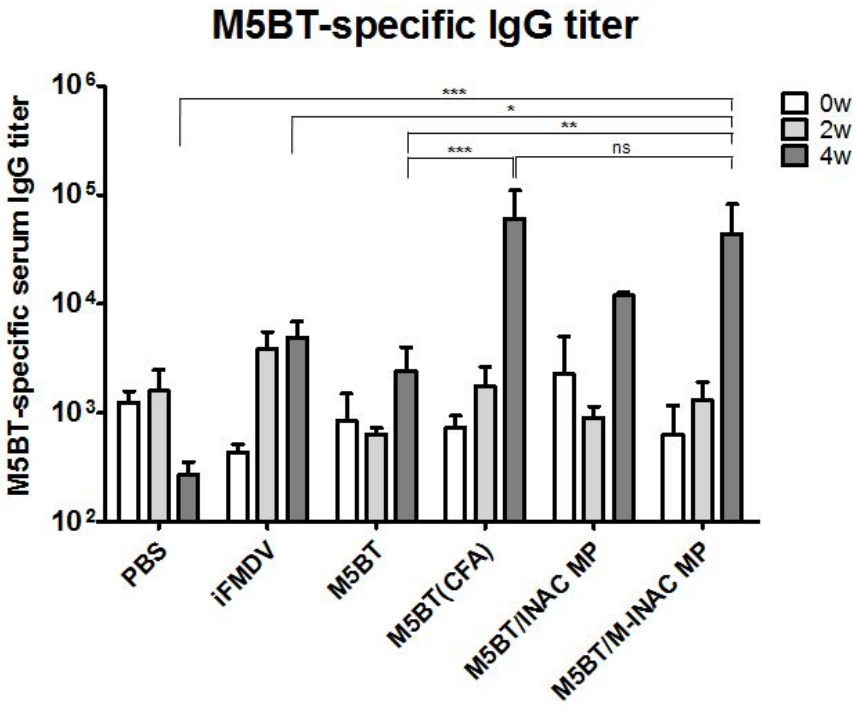


Fig 28. Antigen-specific immune response after immunization with MPs. Anti-M5BT serum IgG levels at 0, 2 and 4 weeks were measured using ELISA. All values represent the means ± SD (n=5).

Summary

Despite of a number of advantages of subunit vaccine as next generation vaccines, many subunit vaccines have difficulties in promoting efficient innate and adaptive immune response against antigen due to the poor immunogenicity and low stability although the use of adjuvant caused side effects by vaccination sometimes.

Here, we developed M-INAC MPs as a new candidate for subunit vaccine adjuvant to substitute conventional adjuvant system with safe and functional polymeric adjuvant system.

In this chapter, M5BT-loaded M-INAC MPs enhanced innate and adaptive immunity eliciting higher antigen specific IgG and FMDV type O specific antibody. This result can suggest a powerful polymeric adjuvant system by the combination of 2 TLR agonists such as inulin acetate and mannan showing comparable immunostimulatory effect to the conventional adjuvant.

Conclusion and Further Prospects

There are many attempts to prevent FMD via vaccination in livestock industry. Despite of remarkable advancement in vaccine development, outbreaks of FMD occurred almost every year having a negative influence in the nation. To stop this vicious circle, there is no doubt that efficient FMD subunit vaccine development is a huge task in livestock industry. Therefore, the aim of this study is to increase vaccine efficacy of FMD subunit vaccine by using polymeric carrier system.

To improve the efficacy of subunit vaccines, we designed two vaccine delivery strategies based on the adjuvant effect of polymeric carriers.

In Chapter Ⅰ, FMDV subunit vaccine M5BT in thiolated CAP microparticles (T-CAP MPs) was developed for efficient oral delivery of mucosal subunit vaccine. T-CAP MPs gave a result that mucoadhesive polymeric particles can induce adaptive immune response, which was proved by production of higher IgA in intestine, by mucoadhsion to mucus layer for longer time maximizing the antigen uptake by immune cells.

In Chapter Ⅱ, FMDV subunit vaccine M5BT in M-INAC MPs was developed for enhancing immune response against loaded antigen. M5BT-loaded INAC MPs elicited antigen-specific antibody production as it is known as immune-boosting adjuvant about Th1 and Th2 immune response (DIEGO G SILVA *et al.,* 2014). With the decoration of mannan on the surface of INAC MPs, it is presumable that they had a synergistic effect on TLR

signaling pathway.

Unlike conventional adjuvant, biomaterial-based polymeric adjuvant have benefits in safety, stability, biocompatibility and *etc*. Two different polymers, INAC and T-CAP used in this study were derived from the naturally occurring carbohydrate. By modificating the solubility in water, each polymeric adjuvant can have advantages such as the depot effect and the enhanced tissue or cellular uptake. Furthermore, particulate vaccine less than 10 μm can be efficiently uptaken by M-cells and immune cells consequently resulting in the recirculation of activated immune cells that recognized antigen. Simply encapsulating subunit vaccine into polymeric carriers gives many opportunities for subunit vaccine development.

Through this study, advanced subunit vaccine delivery system based on polymeric adjuvants provide protective immunity of livestock animals. And it is possible to substitute conventional adjuvant systems for subunit vaccine with polymeric carrier systems with safety. Further investigations on the efficacy of subunit vaccine using polymeric carrier systems should be followed in various livestock animals with subunit vaccines (Figure 29).

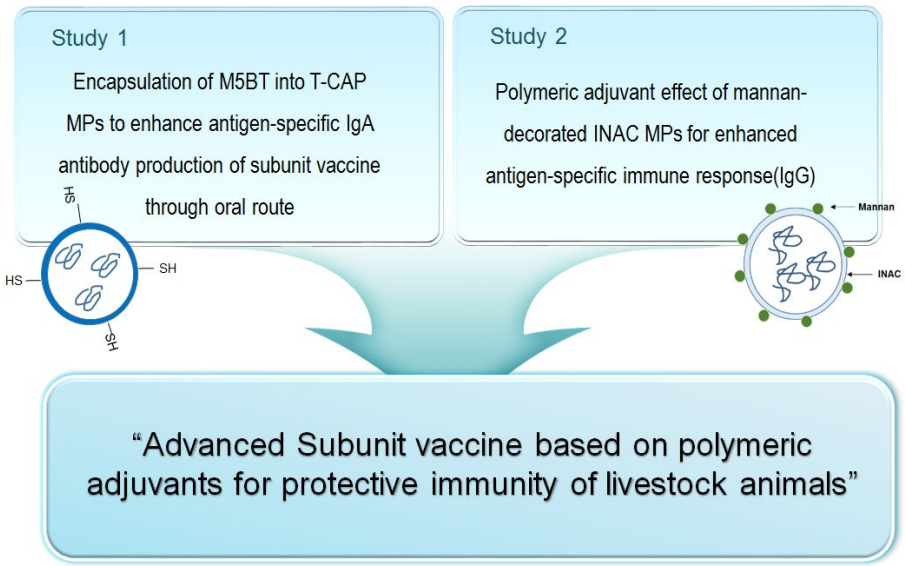


Figure 29. Scheme of the conclusion and further prospect

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Summary in Korean

FMD(Foot-and-Mouth Disease)는 소, 돼지, 염소 등의 우제류에서 발생하는 가축 전염병이다. 본 가축 전염병은 전염성이 매우 강하고 식욕부진, 물집, 유량감소, 어린 가축의 폐사 등 축산업에 막대한 경제적 손실을 끼치고 있다. 이러한 FMD의 발생과 전파를 막기 위하여 FMD 바이러스에 대한 백신의 개발이 활발히 이루어지고 있으나, 바이러스의 다양한 혈청형과 아형의 존재, 돌연변이의 출현으로 인하여 약독화/불활화백신이 주를 이루는 FMD 백신의 효능이 떨어지는 문제점을 지니고 있다.

아단위백신은 기존 백신들이 지니는 생산과정에서의 안전성 문제, 증상 유발 가능성, 약독화 과정 등의 단점을 보완할 수 있어 FMD 아단위 백신이 주목받고 있다. 아단위백신은 항원성을 지닌 부위의 서열만을 이용하여 생산이 가능하고 부작용이 적다는 큰 장점을 지닌다. 하지만, 바이러스의 일부만을 이용하기 때문에 기존 백신에 비해 면역원성과 안정성이 낮아 면역보조제가 필요하다는 점에서 실제 산업에서 응용되기 어렵다는 한계점이 있다.

본 연구에서는 앞서 언급한 아단위백신의 한계점을 극복하기 위하여 고분자 면역보조제를 이용하여 FMD 아단위백신의 면역반응 증진을 꾀하고자 하였다. 고분자 면역보조제는 고분자의 크기, 형태, 분자량, 화학적 특성, 기능성 부여 등의 다양한 조합을 통해 약물/백신의 효능을 증대시킬 뿐만 아니라 약물/백신의 전달체로서도 기능하여 기존 면역보조제를 뛰어넘는 차세대 면역보조제로서의 역할 수행이 가능하다.

먼저 제1장에서는 FMD 아단위백신의 경구투여를 통한 점막면역 활성화를 유도할 수 있도록 경구백신용 T-CAP 미립자(thiolated CAP microparticles)를 개발하고자 하였다. 주사백신과 달리 경구백

신은 점막을 통해 감염되는 병원체의 감염경로를 모방하여 항원특이적 분비형 IgA 항체 생성을 유도할 수 있다. 따라서 점막면역경구백신은 감염의 최전방인 점막에서 FMD 바이러스의 감염을 초도에 방어함으로써 FMD의 효과적인 예방이 가능하다. 특히 면역력이 약해 폐사율이 높은 자돈의 경우, 모돈의 초유에서 얻는 IgA를 통해 수동적 면역화가 중요하다. 하지만 아단위백신은 생체이용률이 낮아 소화장관에서 분해되고, 면역원성이 낮아 면역세포에 잘 인식되기 어렵다.

이러한 한계점을 극복하기 위하여 제1장에서는 기존 pH 민감성 고분자인 CAP를 티올화하여 점막점착성을 부여한 T-CAP 미립자를 경구백신전달체로 이용하여 M5BT(모델 FMD 아단위 백신 항원 단백질)를 담지하였다. T-CAP 미립자의 pH 민감성 및 점막점착성을 통해 항원포집세포인 M 세포가 많이 분포된 회장에 백신을 전달하고 잔류시간을 늘려 생체이용률을 향상시키고, 백신의 미립자화를 통해 M 세포에 많이 포집되어 Peyer's patch 내에서의 점막면역반응을 유도하여 최종적으로 IgA의 생성량을 높이는 것을 목적으로 한다.

제1장에서는, T-CAP 미립자에 이중 에멀젼 방법(W/O/W)을 이용하여 M5BT를 담지하여 M5BT/T-CAP 미립자를 형성하였다. M5BT/T-CAP 미립자는 기존 CAP 미립자와 마찬가지로 위산의 pH인 pH 2에서 약 17-18%의 적은 양의 항원을 방출하며 위산에서 견디는 능력을 보여주었다. 또한, 회장의 pH(pH 7.4)에서 M5BT/CAP 미립자는 급격하게 용해되는 반면 M5BT/T-CAP 미립자는 시간에 따른 지속형 방출 양상을 보이며 24시간동안 최대 56.8%의 항원을 방출하였다. 돼지 소장 점막을 이용한 점막점착성 실험을 실시한 결과, FDA/T-CAP 미립자가 FDA/CAP 미립자 대비 1.48배의 높은 점막점착성을 나타내었다. 최종적으로 T-CAP 미

립자의 점막면역증진효과를 살펴보기 위하여 실험 쥐를 이용하여 4주간에 걸쳐 *in vivo* 경구백신투여실험을 실시하였다. 결과적으로 회장의 Peyer's patch를 FACS 분석을 통해 살펴보았을 때 항원 특이적인 후천성 면역반응에 중요한 항원제시세포들이 공통적으로 발현하는 MHC class II의 발현양이 항원을 단독 투여한 그룹(16.23%)보다 미립자 투여그룹(M5BT/CAP MP; 26.1%, M5BT/T-CAP MPs; 26.87%)에서 높게 나타나는 것으로 관찰되었다. 또한, M5BT/T-CAP 미립자 투여 그룹이 M5BT 특이적 IgG 및 IgA 수준이 가장 높게 나타났다. 이와 같은 면역 실험 결과는 항원의 미립자화가 궁극적으로 M 세포를 통한 포집을 증가시킴으로써 장의 점막면역관장기관인 Peyer's patch 내에 항원을 인식한 면역세포가 증가된 결과에서 기인했을 것이다. 이러한 결과는 효과적인 점막면역백신 면역보조제로서의 T-CAP 미립자의 가능성을 제시하였다.

제2장에서는 기존의 면역보조제를 고분자 면역보조제로 대체할 수 있도록 만난(mannan)이 수식된 INAC 미립자(M-INAC MPs)를 개발하고자 하였다. 앞서 설명한 바와 같이, 아단위백신은 대부분 충분한 면역반응유도를 위해 면역보조제의 사용이 불가피하지만, 면역보조제의 경우 주사부위의 염증, 독성 등의 부작용이 문제가 존재한다.

이를 해결하기 위하여 제2장에서는 1차적으로 면역보조제 기능이 알려진 천연 유래 다당류인 inulin을 응용한 inulin acetate 미립자를 형성하여 아단위백신을 담지하고 2차적으로 APC를 표적화할 수 있는 mannan으로 표면을 수식하였다. 이를 통해 백신의 미립자화 및 mannan 수식을 통해 APC 세포로의 포집 능력을 증진시켜 항원에 대한 면역글로불린의 양을 높이는 것을 목적으로 한다.

제2장에서는, INAC 미립자에 이중 에멀젼 방법(W/O/W)을 이용하여 M5BT를 담지 후 mannan으로 수식하여 M5BT/M-INAC 미

립자를 형성하였다. M5BT/M-INAC 미립자는 *in vitro* 상에서 6일에 걸쳐 항원을 전부 방출하는 양상을 보이며 고분자 면역보조제의 특징 중 하나인 저장소 효과(depot effect)를 보여주었다. 최종적으로 M5BT/M-INAC 미립자의 면역증진효과를 살펴보기 위하여 실험쥐를 이용하여 4주간에 걸쳐 *in vivo* 면역실험 후 혈청 분석을 진행하였다. FMD type O에 대한 백신화 여부를 판단하는 키트를 이용하여 혈액을 분석한 결과, M5BT/INAC 및 M5BT/M-INAC 미립자 그룹이 상용 면역보조제인 CFA를 함께 주입한 M5BT 투여 그룹과 유사한 P.I value를 나타내었다. 또한 M5BT/M-INAC 미립자의 M5BT 특이적 IgG titer가 M5BT/INAC 미립자 그룹보다 높고 CFA를 함께 주입한 M5BT 그룹과 유사하였으며, M5BT/M-INAC MP 그룹에서 iFMDV 그룹과 더불어 가장 많은 FMDV 백신화 양성 반응이 관찰되었다. 이와 같이 mannan-decorated INAC 미립자가 기존의 면역보조제인 CFA에 버금가는 면역반응 유도효과를 보임에 따라 아단위백신에 대한 새로운 면역보조제로서의 가능성을 제시하였다.