A Dissertation

for the Degree of Doctor of Philosophy

Development of Polymeric Carriers Having M Cell Homing Peptide for Targeted Oral Vaccine Delivery

표적형 경구 백신 전달을 위한 M 세포 표적형 펩타이드 작용기 도입 고분자 전달체의 개발

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농 학 박 사 학 위 논 문

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Summary

Compared to other administrative route, oral delivery of vaccine has been received considerable attraction due to providing patient compliances, easy handling, low cost of production, eliminating the injection pain and infection risks, and exerting both systemic and local immune responses. However, low oral bioavailability, degradation in acidic stomach and by gastric enzymes limit the applications of orally delivered drugs including vaccine and probiotics. Polymeric delivery carriers offer potential benefits to limit afore-mentioned problems by protecting the incorporated bioactive agents from the harsh gastric environments.

In the first approach, M cells, the key players of the mucosal immunity induction, are one of the intestinal factors for the efficient delivery of vaccines to mucosal immune tissues. To get M cell targeting, we have developed an efficient oral vaccine carrier that constitutes poly (lactic-co-glycolic acid) (PLGA) microparticle coated with M cell targeting peptide. In this study, a membrane protein B of *Brachyspira hyodysenteriae* (BmpB) as a model vaccine against swine dysentery was loaded into porous PLGA microparticles (MPs). The PLGA MPs was further coated with the water-soluble chitosan (WSC) conjugated with M cell homing peptide

(CKS9) to prepare BmpB-CKS9-WSC-PLGA MPs. Oral immunization of BmpB vaccine with CKS9-WSC-PLGA MPs in mice showed elevated secretory IgA (sIgA) responses in the mucosal tissues and systemic IgG antibody responses, providing an efficient immune response. Specifically, the immunization with these MPs demonstrated to induce both Th1- and Th2-type responses based on elevated IgG1 and IgG2a titers. The elevated immune responses were attributed to the enhanced M cell targeting and transcytosis ability of CKS9-WSC-PLGA MPs to Peyer's patch regions. The high binding affinity of CKS9-WSC-PLGA MPs with the M cells to enter into the Peyer's patch regions of mouse small intestine was obtained by closed ileal loop assay and it was further confirmed by confocal laser scanning microscopy. These results suggest that the M cell targeting approach used in this study is a promising tool for targeted oral vaccine delivery.

In the second approach, oral administration of live probiotics as antigen delivery vectors was studied as a promising approach in vaccine development. However, the low survival of probiotics in the gastrointestinal tract limits this approach. Therefore, the aim of this study was the encapsulation of probiotic expressing vaccine into alginate/chitosan/alginate (ACA) microcapsules (MCs) for efficient oral

vaccine delivery. Here, recombinant *Lactobacillus plantarum* 25 (LP25) expressing M cell homing peptide fused BmpB protein was used as a model probiotic. The viability of LP25 in ACA MCs was more than 65% in simulated gastric fluid (SGF, pH 2.0) and 75% in simulated small intestinal fluid (SIF, pH 7.2) up to 2 h. Encapsulated LP25 were completely released from ACA MCs in SIF within 12 h. When stored at room temperature (RT) or 4°C, the viability of LP25 in ACA MCs was higher than free LP25. Interestingly, the viability of LP25 in ACA MCs at 4°C for 5 weeks was above 58%, whereas viability of free LP25 stored at RT for 5 weeks was zero. After 4 weeks from the first immunization, LP25-M-BmpB-loaded ACA MCs induced a stronger BmpB-specific IgG and IgA production in mice. Collectively, these findings suggest that encapsulation of probiotic by ACA MCs is a promising delivery system for oral administration of probiotic expressing vaccine.

Keywords: Oral delivery; M cell homing peptide; PLGA microparticles; BmpB; Chitosan; Alginate; Microencapsulation; *Lactobacillus plantarum* 25; Probiotics

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List of Abbreviations

ACA: alginate/chitosan/alginate

BmpB: Brachyspira membrane protein B

BSA: bovine serum albumin

CAP: cellulose acetate phthalate

CE: cecum

cfu: colony-forming units

CKS9: M cell homing peptide

CLSM: confocal laser scanning microscopy

CM-HAS: carboxymethyl high amylase starch

DAPI: 4′, 6-diamidino-2-phenylindole dihydrochloride

DI: down portion of small intestine

EDC: 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride

ELISA: enzyme-linked immune sorbent assays

FAE: follicle-associated epithelium

FE-SEM: field-emission scanning electron microscope

FITC: fluorescein isothiocyanate

FT-IR: fourier transform infrared spectrometer

GALT: gut-associated lymphoid tissues

GI: gastrointestinal

GS: Genome shuffled

HPMCAS: hydroxypropyl methylcellulose acetate succinate

HPMCP 55: hydroxyproyl methylcellulose phthalate 55

HRP: horseradish peroxidase

KP: kilopascal

LP25: Lactobacillus plantarum25

LPS: lipopolysaccharide

M-BmpB: M cell homing peptide conjugated BmpB

MCs: microcapsules

mGM-CSF: recombinant mouse granulocyte macrophage colony

stimulating factor

MI: middle portion of small intestine

MPs: microparticles

MS: muscular stomach

NHS: *N*-hydroxysuccinimide

NMR: nuclear magnetic resonance

PA: Pediococcusacidilactia

PBS: phosphate buffered saline

PLGA: poly(lactic-co-glycolic acid)

PVA: poly(vinyl alcohol)

RT: room temperature

SD: swine dysentery

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SGF: simulated gastric fluid

SIF: simulated intestinal fluid

sIgA: secretory IgA

TMB: 3, 3', 5, 5'-tetramethyl benzidine

TRITC: tetramethylrhodamine isothiocyanate

UEA-I: Ulex Europaeus Agglutinin I lectin

UI: up portion of small intestine

WSC: water-soluble chitosan

WT: wild type

Introduction

Oral vaccination offers several advantages over parenteral vaccination, including needle-free delivery, easy and comfortable administration, and the possibility of self-delivery. Most importantly, oral vaccination can induce both mucosal and systemic immunity, leading to the double layers of protective immune responses (Kunisawa J, 2007). In contrast, parenteral immunization primarily yields a systemic immune response. Therefore, effective oral vaccination could establish a first line of immunological defense in the intestinal tract, a major site of pathogen entry, as well as promote immune surveillance perhaps at other mucosal and systemic sites. One of the major strategies of oral vaccine is the induction of pathogen- or toxin-specific secretory IgA (sIgA).

The hostile environment of the gastrointestinal tract (low pH, presence of digestive enzymes, and the detergent activity of bile salts) often makes it difficult to induce protective immune responses by oral vaccination with antigen alone. Additionally, effective oral delivery of antigen to the induction site of the mucosal immune system is made difficult by the significant dilution and dispersion of antigen that occurs in the lumen since

a total interior area of the intestinal wall is thought to be equivalent to over one tennis coat surface. Further, physical barriers, such as mucus and the tight junctions between the epithelial cells prevent the effective delivery of vaccine antigen. To overcome these obstacles, effort has focused on development of effective antigen delivery systems. The use of polymeric carrier presents a great opportunity to solve these problems.

Poly(lactide-co-glycolide acid) (PLGA) as the U.S. Food and Drug Administration (FDA) and European Medicine Agency approved, biodegradable and biocompatible polymer has been commonly used for protection of drug from degradation and controlled release devices (Table 1). One of the most frequently employed techniques for the encapsulation of proteins into PLGA microparticles (MPs) is the double emulsion water-in-oil-in-water ($W_1/O/W_2$) solvent evaporation procedure as proteins tend to be hydrophilic macromolecules. However, the encapsulation of proteins into PLGA MPs presents some challenges due to aggregation of proteins (van de Weert et al., 2000) because the first emulsion of polymer/protein mixture is added dropwise to water and the organic solvent is evaporated.

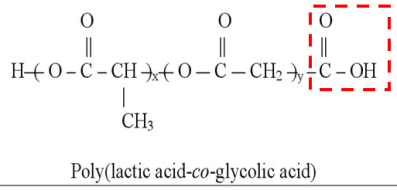
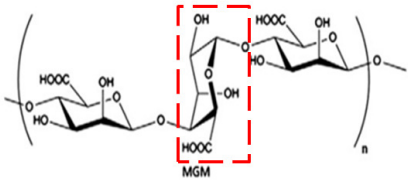
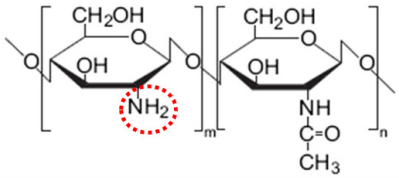
Alginates are natural polysaccharides derived from brown algae

comprising a linear chain of 1-4 linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues (Table 1) (Kuo and Ma, 2001). These M and G residues exist within the chain as alternating (MG/GM) or homopolymeric (GG and MM) regions. By electrostatic interaction with calcium ions, the G homopolymeric blocks within alginate form an "egg-box" structure and cross-link the polymer to form a hydrogel monolith. This property that has led to the alginates being commonly used as an encapsulation material by the external gelation technique (Smidsrød, 1990; George and Abraham, 2006). These alginate microcapsules (MCs) can be used for the microencapsulation of probiotic bacteria, showing improved viability under simulated gastric conditions (Chandramouli et al., 2004). Although a "burst release" of large molecules at intestinal pH has been reported with alginate encapsulation alone, this could be reduced by coating with chitosan (Zhou et al., 2001).

Chitosan is the *N*-deacetylated form of chitin, a mucopolysaccharide derived from various crustaceans and insects (Table 1) (Ravi Kumar, 2000). The chitosan has a 1-4 linked glucosamine structure (Shahidi et al., 1999). The amine residues in chitosan are mostly protonated below pH ~6.5, making chitosan a polycation (Sorlier et al., 2001). The biodegradability

and biocompatibility of chitosan protect the drug from degradation and allow controlled release.

Table 1. Characteristics of used polymers.

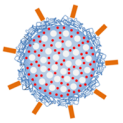
Polymer	Structural	Characteristics
PLGA	 Poly(lactic acid-*co*-glycolic acid)	✓ FDA approved ✓ Nontoxicity ✓ Biodegradable ✓ Biocompatible ✓ Controlled drug release
Alginate		✓ Nontoxicity ✓ Biocompatible ✓ Simple gelation with Ca^{2+}
Chitosan		✓ Nontoxicity ✓ Mucoadhesive ✓ Biodegradable ✓ Biocompatible ✓ Functional groups (-OH and $-NH_2$)

In chapter I, a protein vaccine-BmpB was loaded into porous PLGA MPs coated with M cell homing peptide-coupled chitosan to investigate the in vitro and in vivo efficacy of the MPs for targeted oral delivery of BmpB as a SD vaccine (Table 2).

In chapter II, a probiotic strain was developed through recombinant DNA technology that expresses and produces BmpB antigen fused with M cell

homing peptide. The probiotic was encapsulated into alginate/chitosan/alginate (ACA) microcapsules (MCs) and later delivered through oral route to evaluate the efficiency of vaccine delivery and immune responses (Table 2).

Table 2. Challenges and strategies for oral delivery of vaccine

<table>
<tr><th rowspan="2">Vaccine</th><th rowspan="2">Challenge</th><th colspan="4">Strategy</th></tr>
<tr><th>Types</th><th>Polymers</th><th colspan="2">Characteristics</th></tr>
<tr><td rowspan="2">Protein vaccine</td><td rowspan="4">Low pH
Protease
Barrier function</td><td rowspan="2"> 
Microparticles</td><td rowspan="2">PLGA
Chitosan</td><td>Advantage</td><td>Protection of proteins from degradation
High level of biocompatibility
Biodegradable
M cell homing peptide</td></tr>
<tr><td>Disadvantage</td><td>Usage of organic solvent</td></tr>
<tr><td rowspan="2">Probiotics vaccine</td><td rowspan="2"> 
Microcapsules</td><td rowspan="2">Alginate
Chitosan</td><td>Advantage</td><td>Protection of probiotics from degradation
Biodegradable
Non-usage of organic solvent
M cell homing peptide</td></tr>
<tr><td>Disadvantage</td><td>Poor storage stability</td></tr>
</table>

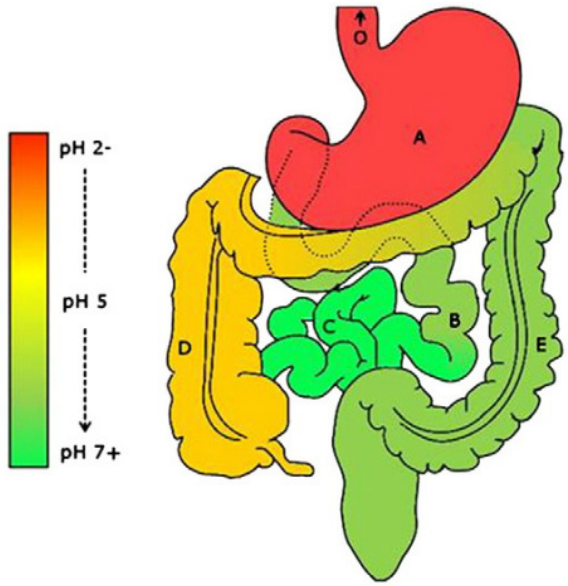
Review of Literature

1. Characteristics of the gastrointestinal tract

When designing probiotics and vaccines for oral delivery systems, it is necessary to consider the complex physiology of the gastrointestinal (GI) tract (Figure 1). Typically the release of the probiotics and vaccines will be triggered by degradation, disintegration or dissolution of the formulations (Gombotz and Wee, 2012). Along the GI tract there are various possible methods based on pH, time, peristaltic pressures and bacterial fermentation for delivery.

After ingestion, probiotics and vaccines will pass quickly through the oesophagus around 10-14 s (Aulton and Taylor, 2013) and reach the stomach; this is the point at which the greatest viability loss of probiotics and vaccines is considered due to high levels of acid. The pH values of the stomach have been reported to lie within pH 1-2.5 for uncontrolled groups (Evans et al., 1988) whereas it may be as high as pH 5 for fed patients (Fordtran and Walsh, 1973). Gastric emptying time is also highly variable (McConnell et al., 2008), and is often reported as between 5 min and 2 hours; however, the half gastric emptying time using ^{13}C breath tests has been estimated to be around 80.5 min (Hellmig et al., 2006). The emptying of stomach contents is usually a result of peristaltic action known as the

migrating myoelectric complex (Janssens et al., 1983), whilst the pyloric sphincter ensures retention of large, insufficiently digested contents (Aulton and Taylor, 2013). As a result, smaller particles will be retained for a short time. The stomach has a fluid capacity of up to approximately 1.5 L but usually contains only 50 ml in a fasted state (Aulton and Taylor, 2013). In addition to acid, the stomach also contains pepsin, a proteolytic enzyme, which breaks down proteins. Pepsin is produced by the auto-catalytic cleavage of its precursor zymogen, pepsinogen, at the low pH of the stomach.



Letter	Region	pH	Transit Time
O	Oesophagus	~7.0	10-14 seconds
A	Stomach	1-2.5 (up to 5 fed)	Half emptying: ~80.5 mins
B	Proximal Small Intestine	6.15-7.35	3.2 ±1.6 hrs (combined)
C	Distal Small Intestine	6.80-7.88	
D	Ascending Colon	5.26-6.72	Highly variable, dependent on bowel evacuations
E	Descending Colon	5.20-7.02	

Figure 1. Characteristics of the GI tract, showing the pH at the different parts (Cook et al., 2012).

After passage through the stomach, the probiotics and vaccines will enter the small intestine, which has been reported in one study to be 3.2±1.6 h (Davis et al., 1986) and in another ranging from 0.5 to 9.5 h (Coupe et al., 1991). Significant variation in small intestinal transit time were observed upon repeated experimentation for a single patient (McConnell et al., 2008), indicating a large variability within individual subjects. The pH of the small intestine has been estimated to lie in the range of pH 6.15-7.35 in the proximal region, rising to pH 6.80-7.88 in the distal small intestine. After passage through the small intestine, the probiotics and vaccines will reach the large intestine at which point the pH lowers slightly to pH 5.26-6.72 in the ascending colon, and pH 5.20-7.02 in the descending colon (Press et al., 1998). The transit time of pharmaceuticals in the large intestine is highly variable, with a range of 6-32 h typically reported (Coupe et al., 1991), but longer time also have been noted (Sathyan et al., 2000). As the probiotics and vaccines descend down the GI tract, the relative quantity of liquid present will decrease as the contents are compacted into feces (McConnell et al., 2008).

2. Characteristics of the intestinal M cell

M cells are specialised epithelial cells mainly found in the follicle-associated epithelium (FAE) overlying Peyer's patches (PPs) and other lymphoid aggregates (Figure 2) (Clark et al., 2001). These cells are distinguished from surrounding epithelial cells by the absence of the surface microvilli that are characteristic of intestinal epithelial cells. Instead, the apical membrane of the M cell has a microfold (or membranous) topography, hence named as M cell. Although M cells also form tight junctions with adjacent cells to maintain the epithelial barrier, they have a deeply invaginated basolateral membrane which contains infiltrating lymphocytes (Owen and Jones, 1974). Consequently, once antigens are transcytosed by the M cell, they can quickly move to the basolateral membrane to reach and interact with underlying lymphoid aggregates (Owen, 1977).

The M cell may be the preferred cell type for mucosally targeted vaccine delivery, but it should be mentioned that other cell types at mucosal surfaces have some antigen sampling ability. In addition, dendritic cells, the professional antigen-presenting cells, can directly sample antigens from

mucosal surfaces (Kaiserlian and Etchart, 1999). However, the M cell is still most attractive for targeted vaccine delivery because of its high transcytotic activity at the FAE. Although villous M cells located outside the FAE have also been identified (Jang et al., 2004), the M cells of the FAE remain the primary vehicles for antigen sampling and subsequent induction of mucosal immune responses (Man et al., 2004). As such, the FAE has lower amounts of secretory IgA (sIgA) and mucus and the M cells of the FAE have a much thinner surface glycocalyx as compared to other intestinal epithelial sites, allowing for easier antigen sampling (Frey et al., 1996; Neutra et al., 1996).

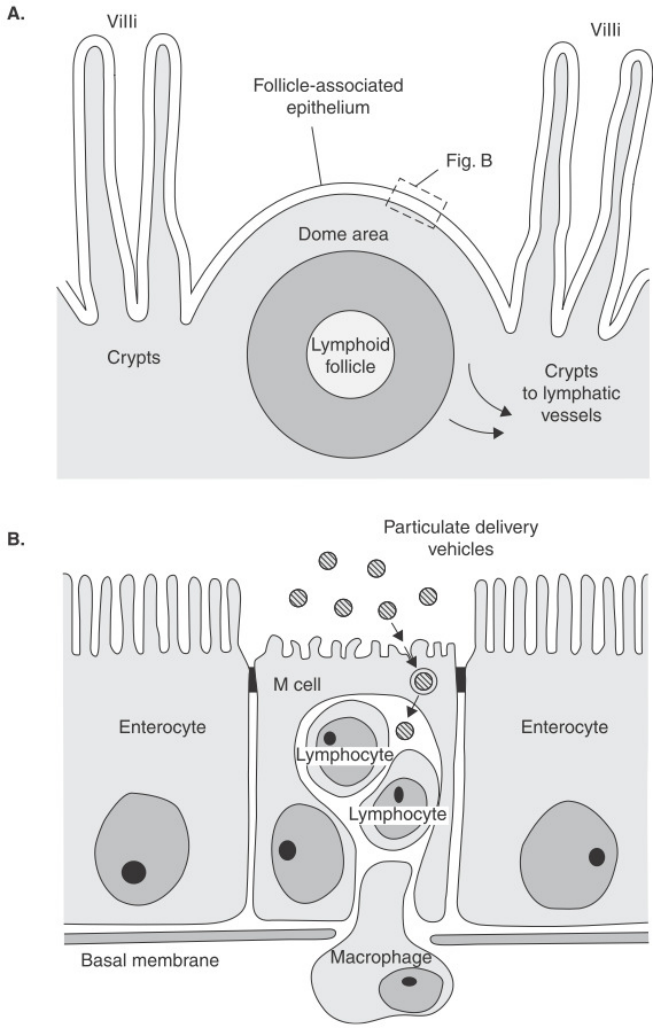


Figure 2. Schematic transverse sections of a PPs lymphoid follicle and overlying FAE, depicting M cell transport of particulate delivery vehicles. The general structure of intestinal organised mucosa-associated lymphoid tissues is represented by the schematic transverse section of a PPs lymphoid follicle and associated structures in (A). The lymphoid follicle is situated beneath a dome area which protrudes into the gut lumen between villi and which is covered by the FAE. This epithelium is characterised by the presence of specialised antigen sampling M cells (depicted in B) (Clark et al., 2001).

3. Oral delivery of vaccine

Vaccination is the best and most effective strategy to make defense against many diseases (Faulk and Dolinoy, 2011) and have been known for hundreds of years in various forms. For example, during the period of Middle Ages, in China it was found that inhaling a powder made from smallpox scabs could protect from future infection and provided immune responses. Historically, the first clear example of vaccination, which remains among the most dramatic ever recorded, was Edward Jenner's successful vaccination against smallpox. Jenner who was an English physician observed that the milkmaids never contracted the more serious smallpox who had recovered from cowpox. On this standpoint of observation, he injected the material from a cowpox pustule into the arm of an 8-year old boy. After then he intentionally infected the boy with smallpox and found that the disease did not develop. This revolutionary invention by Jenner on vaccination was published in 1798. The concept received the widespread acceptances for inducing immunity to infectious diseases. From that time, therefore, vaccination considers the most effective way for preventing infection (Abbas et al., 1994).

Almost all of the vaccines are administered parentally by intravenous,

intramuscular or subcutaneous injections (Mitragotri, 2005; Giudice and Campbell, 2006). Parenteral administration of vaccine is associated with several problems such as the injection is painful, need of high patient compliances, have injection risk factors, need of trained personnel to administer and most importantly, it provides short half-lives of drugs at the target site of application (Quan et al., 2007). To avoid these-mentioned problems associated with parenteral administration of vaccine, scientists have been extensively worked for many years to develop a suitable delivery system for effective vaccination.

Oral vaccination is the most attractive and acceptable administrative route of vaccine delivery (Ahire et al., 2007). Oral delivery of vaccine is easy to administer, provides patient compliances, avoids the risk factors associated with parenteral administration and able to induce both local and systemic immune responses. Despite of having various advantages associated with oral vaccination, however, it is the most challenging and difficult field in the research arena due to several important factors such as degradation of antigen in stomach acidic environment, presence of degradative enzymes and low uptake by the gut-associated lymphoid tissues (GALT) which is the most important target in terms of oral vaccination.

Hence, due to afore-mentioned problems, there is a considerable interest in the development of novel delivery systems for oral administration of vaccine which can be used to package and deliver a range of antigen against important pathogens. Because of the safety talk, it will be desirable if the delivery system is based on non-living carrier systems rather than modified bacterial or viral vectors (O'Hagan, 1998).

An orally administered antigen induces immune responses at GALT which have a largest portion consists of the PPs (Lavelle et al., 1995). The PPs are the main target for the oral vaccines which are present in lower ileum (Van der Lubben et al., 2001). The polymeric microparticles (MPs) offer significant potential to develop suitable carrier systems for orally administered vaccines. A wide range of polymers can be used to prepare the polymeric MPs which can be designed to protect the entrapped vaccines against degradation and subsequently target the vaccines into the GALT for uptake by the PPs. The antigen incorporated into polymeric microparticulate system induces antigen-specific immune response to a greater extent than an antigen in the soluble form. The polymeric particles incorporated with antigens can protect them from degradation by the acidic environment of the stomach, proteolytic activity of degradative enzymes

and bile salts in the gut and subsequently enhances the antigen uptake via the phagocytic M cells present in PPs (Tabata et al., 1996). When the MPs transport and reach to the dome of the PPs, they are degraded and the incorporated vaccines are released into the lymphoid tissues. Stimulation induced by the antigen in PPs which is then presented to B and T cells. The presentation of antigen-specific stimulation to B and T cells accelerates B and T cells proliferation and these cells subsequently leave the PPs via efferent lymphatics and participate in the systemic circulation through the thoracic duct (O'Hagan et al., 1989; O'hagan et al., 1991). Along with the systemic antibody responses following oral administration of polymeric MPs incorporated with antigen, it has also been reported that they can also effectively induce mucosal antibody responses which is essential for oral vaccine delivery (Chang and Prakash, 1998; Young, 2008).

4. Oral delivery of probiotics

Enormous commercial and scientific research interest have been paid to lactic acid bacteria because of their tremendous health benefits in the gastrointestinal (GI) tract. They are considered good and friendly bacteria which can inhibit and reduce numbers of potentially harmful bacteria from

the intestine (Prakash and Chang, 1996). The use of lactic acid bacteria in therapeutic purpose have been received considerable interest for treating several important diseases like cancer, kidney failure uremia, inflammatory bowel diseases (IBD), hypercholesteremia and many others (Anderson and Gilliland, 1999; McIntosh et al., 1999; Lin et al., 2008). Lactic acid bacteria also play very important role for the production of various food products such as yogurt, cheese, fermented milk which also have beneficial health effects. Some lactic acid bacterial strains have shown that they can suppress intestinal tumors induced by chemical mutagens. They also have facilitated several other beneficial effects such as lowering cholesterol, immunomodulatory, anticarcinogenic and antimicrobial action (Reddy et al., 1973; Singh et al., 1997; Fuller and Perdigon, 2000). In 1973, Reddy et al. reported that the feeding of yogurt inhibited the growth of Ehrlich ascites tumor in the peritoneal cavity (Reddy et al., 1973). Perdigon et al. have also extensively investigated the lactic acid bacteria for their therapeutic applications (Fuller and Perdigon, 2000). They demonstrated that feeding of lactic acid bacteria and yogurt augmented systemic as well as local immune responses. They further reported that the feeding of *L. casei* and *L. acidophilus* fermented milk inhibited the GI infection occurred by

Salmonella typhimurium and *Escherichia coli*. They suggested that these biological activities were a result of the immunomodulating abilities of the lactic acid bacteria and their fermented food products.

It is important to know that the live bacterial cells must be delivered to the intestine alive through oral administration to exert the beneficial health effects. From various investigations, there are considerable evidences to support the fact that oral administration is essential for lactobacillus delivery in order to induce therapeutic effects; however, several obstacles have made this novel approach as a challenging field in the scientific area. When live bacterial cells are intended to deliver orally to the target site of intestine, they must be protected from the stomach acidic environment, bile acids and the degradative enzymes; otherwise they will be denatured or degraded (Siuta-Cruce, 2001). All these factors cause their poor survival and stability before their arrival to the target site alive. Therefore, an effective carrier system is necessary for their oral delivery to induce proper therapeutic applications. Microencapsulation technique provides suitable carrier system for live bacterial cells and has been received considerable attention for protecting them from afore-mentioned unfavorable environments and to exert their potential therapeutic applications.

Chapter 1. Targeted oral delivery of BmpB vaccine using porous PLGA microparticles coated with M cell homing peptide-coupled chitosan

1. Introduction

Oral vaccines offer significant advantages over needle-based vaccines, such as easy handling, high patient compliance, low cost of production and induction of mucosal immunity (Islam et al., 2012), yet oral vaccination has several defects, such as degradation of the vaccine in the gastrointestinal (GI) tract due to the low pH and enzymes in the stomach, the impermeable GI epithelium as a physical barrier and inefficient targeting to the action site, resulting in a low bioavailability (Renukuntla et al., 2013). Therefore, the physicochemical properties and biological restrictions of the vaccine need to be considered for the development of an effective delivery system for oral vaccines (Mahato et al., 2003).

Moreover, the vaccine should reach M cells to stimulate adequate immune responses after oral delivery. The M cells, specifically located on the follicle-associated epithelium (FAE) in Peyer's patch (PP) of the gut, possess a unique property to take up and deliver vaccines from the enteric

environment into the PP via transcytosis (Yoo et al., 2010). Vaccines delivered through the M cells in the PP, referred to as gut-associated lymphoid tissues (GALT), facilitate the induction of vaccine-specific immune responses by activating antigen-presenting cells and lymphocytes (Neutra et al., 1996; Ann Clark et al., 2001).

Undoubtedly, the receptor-mediated targeting of M cells would be an approach of oral vaccination (Foxwell et al., 2007), but the lack of differentiation of M cell receptors from the adjacent enterocytes hindered the M cell targeting oral delivery (Tyrer et al., 2006). Recently, we identified a CKSTHPLSC (CKS9) peptide by phage display which showed high affinity towards M cells (Yoo et al., 2010). The peptide facilitated the transport of chitosan nanoparticles across the M cell to enter the FAE in the PP demonstrating CKS9 as a potential M cell-targeting ligand.

In recent years, the use of porous materials has been greatly extended in bio-related fields (Ariga et al., 2012; Vilela et al., 2012; Hartmann and Kostrov, 2013). Particularly in drug delivery system, the porous biodegradable polymeric microparticles (MPs) are applied as protein and peptide carriers for oral delivery (Hanes et al., 1997; Mi et al., 2003; Jaklenec et al., 2008; Lee and Yoo, 2008). These porous MPs have attracted

much attention because of their protection of proteins from degradation and their satisfactory releasing behavior (Edwards, 1997). While the pore sizes in MPs can be adjusted to control the release of the molecules, chemical modifications of the surfaces may indeed offer targeted delivery.

Swine dysentery (SD) is a contagious mucohemorrhagic disease of pigs that is caused by pathogenic intestinal spirochete *Brachyspira hyodysenteriae* (Edwards, 1997). Several attempts have been made to use either attenuated or genetically modified live avirulent vaccines for SD (Stanton et al., 1999). But, a recent approach to prevent pigs from SD used an outer envelope lipoprotein of *B. hyodysenteriae* (BmpB) and confirmed the protection of pigs from SD by vaccination with the recombinant BmpB (Lee et al., 2000).

In this study, we loaded BmpB into porous poly(lactic-co-glycolic acid) (PLGA) MPs coated with M cell homing peptide-coupled chitosan and investigated the in vitro and in vivo efficacy of the MPs for targeted oral delivery of BmpB as a SD vaccine.

2. Materials and Methods

1). Materials

Carboxyl acid-terminated PLGA (lactic acid/glycolic acid: 50/50; Mw = 17kDa) was purchased from Purac Biochem (Gorinchem, Holland). Sodium oleate was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). CKS9 (CKSTHPLSC) was chemically synthesized by Anygen (Gwangju, Korea). Water-soluble chitosan (WSC, MW = 9,600; degree of deacetylation = 91.8%) was kindly provided by Prof. Nah (Sunchon National University, Korea). Difco^{TM} LB broth was purchased from BD (Sparks, MD, USA). Rabbit anti-BmpB antibody was purchased from AbClon (Seoul, Korea). 3,3',5,5'-tetramethyl benzidine (TMB); horseradish peroxidase (HRP)-conjugated goat anti-mouse IgA, IgG, IgG1 and IgG2a; and HRP-conjugated goat anti-rabbit IgG antibody were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Bovine serum albumin (BSA), coumarin 6, poly(vinylalcohol) (PVA), N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), tetramethylrhodamine isothiocyanate (TRITC)-conjugated Ulex Europaeus

Agglutinin I lectin (UEA-I), and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2). Preparation of BmpB protein

E. coli harboring the gene encoding for the BmpB protein was inoculated into LB broth and incubated at 37°C with shaking at 100rpm for 4 h. The culture was induced with IPTG (1 mM) and incubated for an additional 12 h. E. coli cells were collected by centrifugation and washed twice with phosphate buffered saline (PBS). The cells were suspended in His-binding buffer for cell lysis by sonication to collect the expressed His-tagged protein. The protein was separated from the cell debris by centrifugation at 12,000 rpm for 30 min. The protein was finally purified according to the manufacturer's instructions (Clontech). The purity of the protein was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The collected protein was dialyzed overnight against water (pH 7.9) at 4°C. The purified protein was freeze dried and maintained at -20°C until use.

3). Preparation of CKS9-conjugated chitosan

CKS9-conjugated water-soluble chitosan (CKS9-WSC) was prepared by

the conjugation of WSC with CKS9 using NHS/EDC coupling agents following a previously described method (Yoo et al., 2010). The synthetic scheme is shown in Figure 1. The composition of the CKS9-WSC was determined by ^{1}H NMR spectroscopy (Avance 600, Bruker, Germany) and Fourier transform infrared spectrometer (FT-IR; Nicolet 6700, Thermo Fisher Scientific Inc., Waltham, MA, USA).

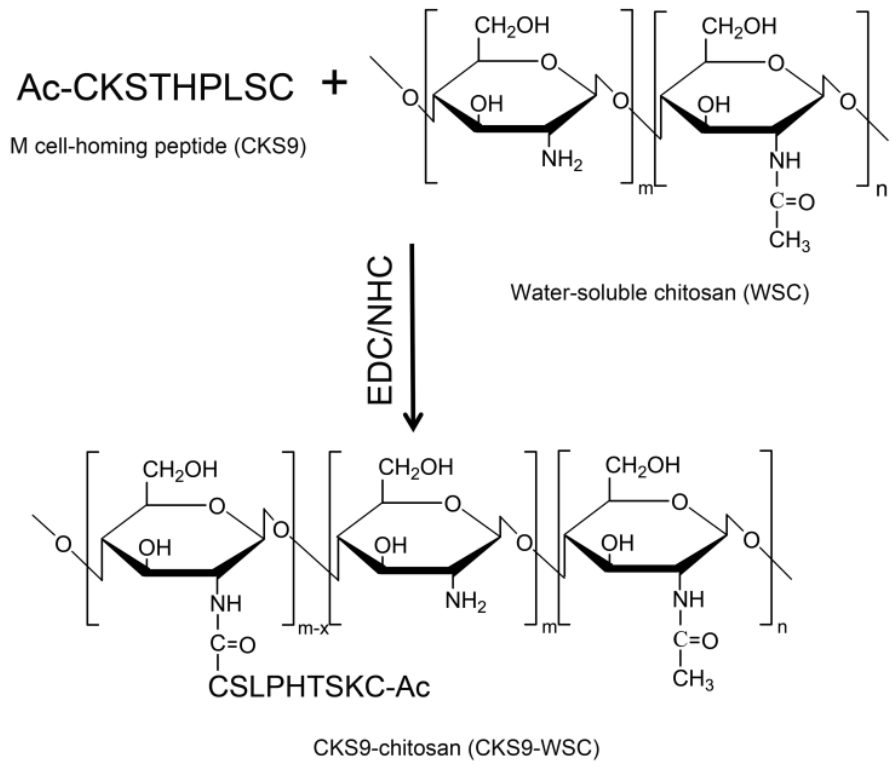


Figure 1. Schematic diagram for the synthesis of CKS9-WSC.

4). Preparation of porous PLGA MPs

4-1). Preparation of BmpB-loaded porous PLGA MPs (BmpB-PLGA MPs)

The BmpB-PLGA MPs were prepared using a water-in-oil-in-water ($W_1/O/W_2$) emulsion solvent evaporation method, as previously described (Sun et al., 2009) with some modifications. First, 7.5 mg of BmpB was dissolved in 100 μl of distilled water, and a sodium oleate solution was prepared in 200 μl of distilled water, which was used as the W_1 phase. PLGA (0.12 g) was dissolved in 2 ml methylene chloride (O phase) and emulsified with the W_1 phase by sonication on ice to form the stable initial emulsion (W_1/O). Second, the resultant emulsion was added drop wise into 40 ml of a 5% (w/v) PVA solution and emulsified for 2 h at a predetermined speed using a direct driven digital stirrer, resulting in the formation of the $W_1/O/W_2$ emulsion. Third, 50 ml of a 6% (v/v) isopropanol solution was poured into the double emulsion to extract the organic solvent and then stirred for approximately 4 h in a fuming cupboard. After the evaporation of the organic solvent, the hardened MPs were collected by centrifugation. The resultant MPs were rinsed with distilled water and collected by

centrifugation. The MPs were dried by freeze drying. Drug free PLGA MPs were also prepared as a control using the same procedure.

4-2). Preparation of coumarin-6-loaded porous PLGA MPs (coumarin-6-PLGA MPs)

In case ofcoumarin-6-PLGA MPs, a sodium oleate solution prepared in 200 μl of distilled water (W_1 phase) was emulsified with 0.12 g PLGA along with 7.5 mg of coumarin-6 dissolved in 2 ml of methylene chloride (O phase) to form the stable initial emulsion (W_1/O). Further processes were followed similar to the preparation of the BmpB-PLGA MPs, as described above.

4-3). Preparation of CKS9-WSC and WSC coated BmpB- or coumarin-6-PLGA MPs

The surface of the PLGA MPs was coated with CKS9-WSC or WSC. Briefly, 10 mg of BmpB- or coumarin-6-PLGA MPs was dispersed into 2 ml of 0.05 M MES buffer (pH 5.6) containing 1% (w/v) WSC or CKS9-WSC with stirring for approximately about 3 h. MPs were collected by centrifugation and freeze drying.

5). Morphology observations and particle size measurements

The surface and cross-section morphologies of the MPs were observed using a field-emission scanning electron microscope (FE-SEM; Supra 55VP; Carl Zeiss, Oberkochen, Germany). Pore size was manually calculated from the SEM images. In addition, the internal porosity of PLGA MPs was approximately obtained from manual calculation, based on the pore-occupied area over the entire cross-sectional area of PLGA MPs as previously described (Bae et al., 2009). The particle sizes were measured using DLS-7000 (Otsuka Electronics. Ltd., Tokyo, Japan).

6). Determination of loading content and loading efficiency

Loading content was determined as follows. The MPs were dispersed into 3 ml of 0.1 M NaOH containing 5% (w/v) SDS (Corrigan and Li, 2009). The suspension was incubated in a water bath at 60 °C for 1 h. Following centrifugation, 1 ml of the supernatant was withdrawn for micro-BCA analysis. The loading efficiency of the BmpB was determined using the ratio of the actual amount of loaded BmpB over the total amount of BmpB used for the preparation of the MPs.

7). Release of BmpB from the BmpB-PLGA or BmpB-WSC-PLGA MPs

The in vitro release of BmpB from BmpB-PLGA or BmpB-WSC-PLGA MPs was determined as follows. The BmpB-loaded MPs, separated from a 1 ml suspension by centrifugation, were placed into 1.5 ml Eppendorf tubes with 1 ml of PBS (pH 7.4 or pH 1.2) for 48 h at 100 rpm (37 °C) in a shaking incubator. A 1 ml aliquot was withdrawn and replaced with an equal volume of release medium at a predetermined time, and the amount of BmpB released was measured using the micro-BCA assay method.

8). Structural integrity of the BmpB antigen

The integrity of protein antigens before and after encapsulation in MPs was assessed by SDS-PAGE and Western blot as previously described (Florindo et al., 2009) with some modifications. While rabbit serum anti-BmpB antibody was used as primary antibody, HRP-conjugated goat anti-rabbit IgG antibody was used as secondary antibody.

9). Validation of targeting property of CKS9-WSC-PLGA MPs

To validate the targeting property of the CKS9-WSC-PLGA MPs to the

PP, the closed ileal loop assay was conducted and assessed using confocal laser scanning microscopy (CLSM; Carl Zeiss LSM710, Carl Zeiss, Inc.) as previously described (Yoo et al., 2010) with slight modifications. The coumarin-6-loaded MPs (1 mg/ml) were injected into the closed ileal loops. After 1 h of retention, the closed ileal loop was excised and washed with cold PBS three times. The tissue samples were then fixed with 10% (v/v) formalin and freeze-sectioned. Sections of the samples were stained using TRITC-conjugated UEA-1 and DAPI.

10). Oral immunization

Female BALB/c mice were obtained from Samtako, Co. Ltd. (Osan, Korea), and used at 8 weeks of age. The animals were maintained under standard pathogen-free conditions and provided with free access to food and water during the experiments. All animals were maintained and used in accordance with the guidelines for the care and use of laboratory animals (Seoul National University). The mice were divided into 5 cohorts (5 mice per cohort). Each mouse was given an oral gavage of 200 μl PBS containing an amount equivalent to 200 μg of BmpB either in BmpB solution, BmpB-PLGA, BmpB-WSC-PLGA, BmpB-CKS9-WSC-PLGA MPs or

control (PBS). All groups received a total of 6 doses of the vaccines (or control) on days 0, 1, 7, 8, 14 and 15 (Figure 2). To assess immune responses, serum tail vein blood and feces samples were taken at days 0, 14, 21 and 28 (prior to the first immunization). The blood samples were centrifuged for 10 min at 5,000 rpm, and the serum was collected and stored at -70°C until analysis for IgG, IgG1 and IgG2a titers. Five fecal pellets, selected randomly from each mouse, were vortexed with 500 µl of PBS. After 30 min of incubation at room temperature, the fecal samples were centrifuged at 6,000 rpm for 15 min. The supernatants were collected and analyzed for the presence of antigen-specific IgA as previously described (Enioutina et al., 1999). Finally, the small intestines (3 cm) of the mice were immediately collected after sacrifice. The intestines were homogenized in 500 µl of ice-cold PBS. Tissues were removed by centrifugation (10,000 rpm, 10 min and 4 °C), and the supernatants were collected and stored at -70 °C until analysis for IgA titers.

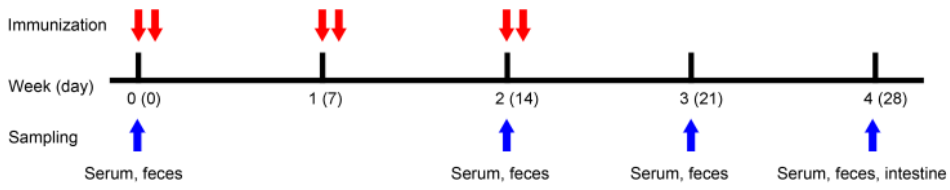


Figure 2. Schematic representation of the oral immunization protocol.

11). Evaluation of antibody production

The induction of BmpB-specific antibodies was measured by enzyme-linked immunosorbent assays (ELISA). ELISA plates were coated with BmpB (25 μg/ml) and incubated overnight (4 °C). The plates were then washed and blocked with 1% (w/v) bovine serum albumin. Serially diluted samples (100 μl/well) were then added, and the mixtures were incubated for 2 h. Following washes with PBS containing 0.1% Tween 20, each sample was appropriately treated with HRP-conjugated goat anti-mouse IgG, IgG1 and IgG2a antibody (1:5,000) or goat anti-mouse IgA antibody (1:2,000). After 1 h of incubation and subsequent washings, the samples were treated with TMB substrate buffer, and the reaction was stopped after 15 min with 50 μl of 2 M H_2SO_4. The optical densities (OD) of the samples were measured at 450 nm using an Infinite 200 PRO multimode reader (Tecan, Switzerland).

12). Statistical analyses

All results are expressed as the mean ± SD. Differences between means were tested for statistical significance using a one-way analysis of variance (ANOVA) and post hoc least significance tests. SPSS 13 program (SPSS®, Chicago, USA) was used for all statistical analyses.

3. Results and Discussion

1). Characterization of porous PLGA MPs coated with M cell homing peptide-coupled chitosan

To prepare M cell homing peptide-coupled chitosan, first, the CKS9 peptide was coupled with WSC through an amide linkage between the carboxylic acid in the C-terminal of the CKS9 peptide and the amino groups in the WSC using EDC/NHS as activation agents as shown in Figure 1. The introduction of the CKS9 peptide to WSC was confirmed by ^{1}H NMR (Figure 3) and FT-IR (Figure 4). The characteristic peaks at 7.06 and 7.78 ppm in ^{1}H NMR were assigned to the two protons of the imidazole ring in the histidine of the CKS9 peptide. However, the other proton peaks of the CKS9 peptide were not distinguished due to the overlap of WSC peaks in the spectra. The degree of substitution of the CKS9 in the CKS9-WSC was estimated by calculating the integral ratio between the two protons of imidazole ring of histidine in CKS9 and three protons of the acetyl group of WSC appeared at 2.1 ppm. The composition of the CKS9 in the CKS9-coupled WSC was 6.37 mol%.

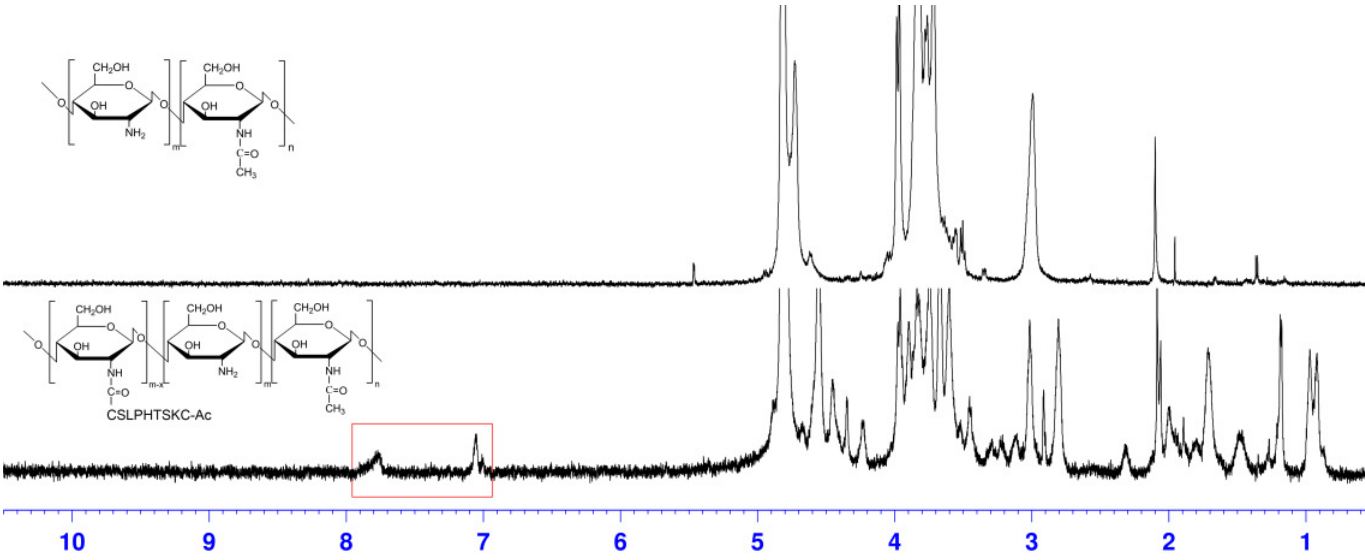


Figure 3. ^{1}H NMR spectra of WSC and CKS9-WSC.

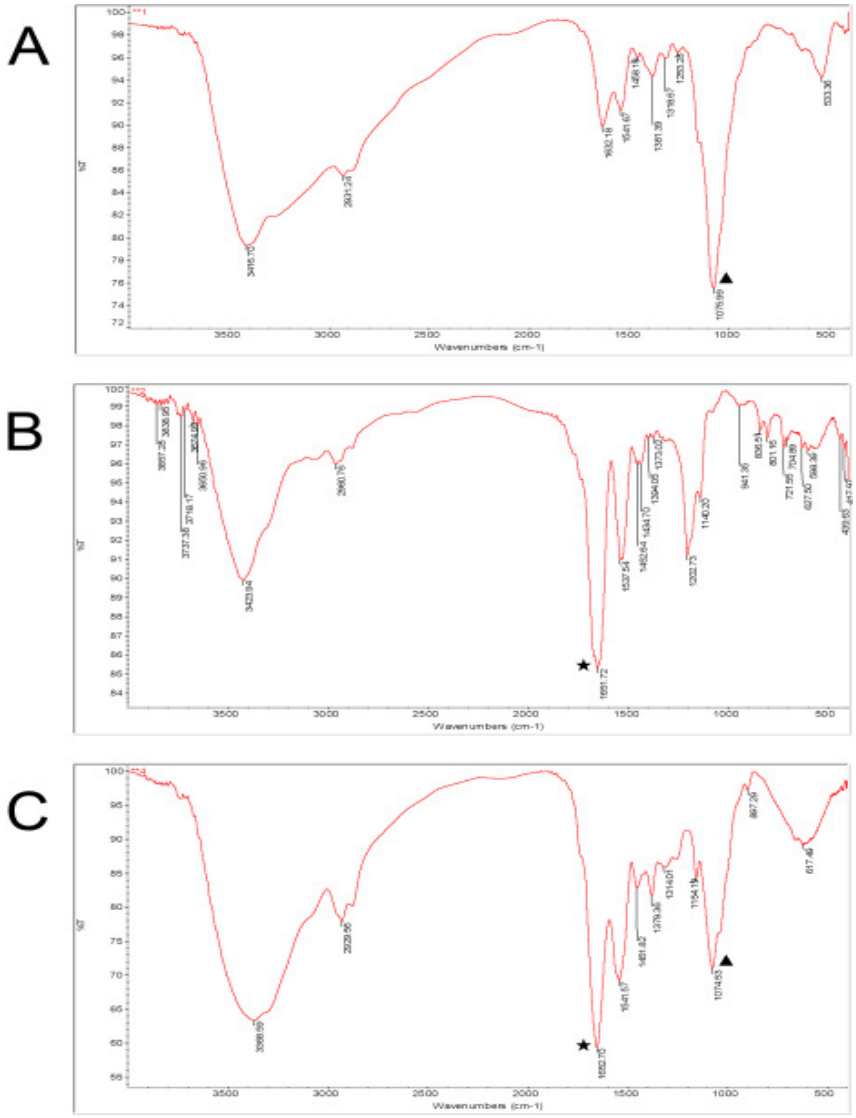


Figure 4. FT-IR spectra of water-soluble chitosan (WSC) (A), CKS9 (B), and CKS9-WSC (C). The FT-IR spectrum of WSC shows some characteristic absorption bands at about 3437 cm-1 (νN-H and νO-H) 1632 (ν-CONH-) and 1060 cm-1 (νC-O-C). While the characteristic peak of chitosan at 1060 cm-1 (νC-O-C) is also observed in the spectrum of CKS9-WSC,the characteristic peak of CKS9 peptide at 1632 (ν-CONH-) is present in the spectrum of CKS9-WSC.

Porous PLGA MPs were prepared using the conventional double emulsion method with sodium oleate as a porogen. As shown in Figure 5, the PLGA MPs have a smooth surface and spherical shape with a slight porosity at the surface and larger inner pores. The morphologies of the PLGA MPs were not significantly altered after loading BmpB into the MPs. The porous PLGA MPs were coated with CKS9-coupled WSC through an ionic interaction between the carboxylic groups of the outer PLGA MPs and the amino groups of the CKS9-coupled WSC. In addition, the morphologies and porosities of the PLGA MPs were not significantly altered after coating with WSC. The pores were well interconnected inside the foam structure, thus enhancing the surface area to volume ratio available for drug diffusion and polymer degradation. The pore size varied from about 200 nm to 1000 nm. PLGA MPs contains about 70% internal porosity as observed by manual calculation, based on the pore-occupied area over the entire cross-sectional area of MPs.

The particle size distributions of the PLGA MPs (A), BmpB-loaded PLGA MPs (BmpB-PLGA MPs) (B), BmpB-loaded WSC-coated PLGA MPs (BmpB-WSC- PLGA MPs) (C), and BmpB-loaded M cell homing peptide-coupled WSC-coated PLGA MPs (BmpB-CKS9-WSC-PLGA MPs)

(D) are shown in Figure 6. The particle size of the PLGA was approximately 3.07 μm with a unimodal particle distribution, but slightly increased after loading BmpB into the PLGA and WSC-PLGA MPs. The particle shape and size can be manipulated to maximize interactions at the cellular level. Phagocytotic cells such as macrophages and M cells are able to ingest micron-sized particles with diameters between 1 and 5 μm, and therefore, targeting these cells in the intestinal or respiratory tract allows the use of larger particles (van der Lubben et al., 2001; Bhavsar and Amiji, 2007)

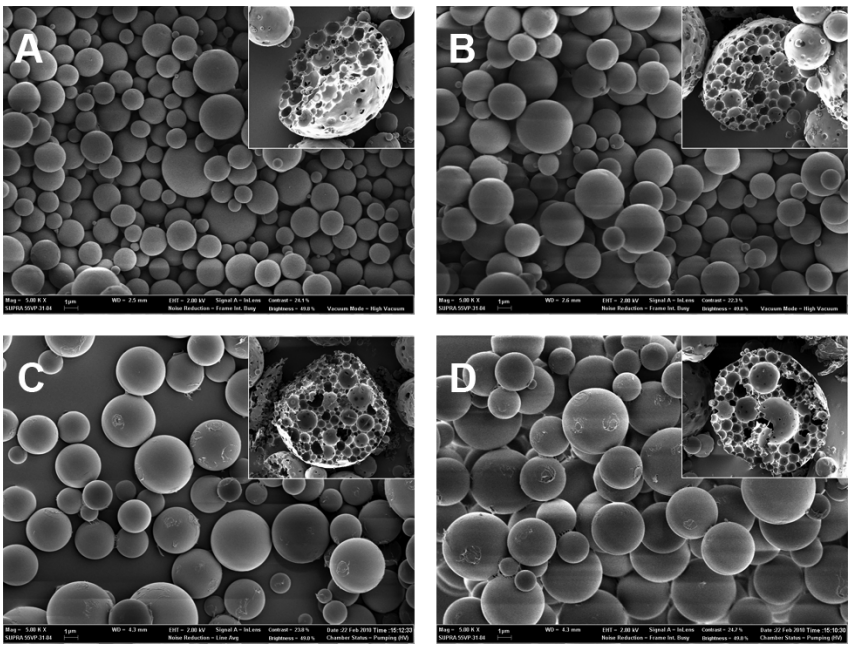


Figure 5. FE-SEM image of the surface and internal morphologies of the PLGA MPs (A), WSC-PLGA MPs (B), BmpB-PLGA MPs (C) and BmpB-WSC-PLGA MPs (D).

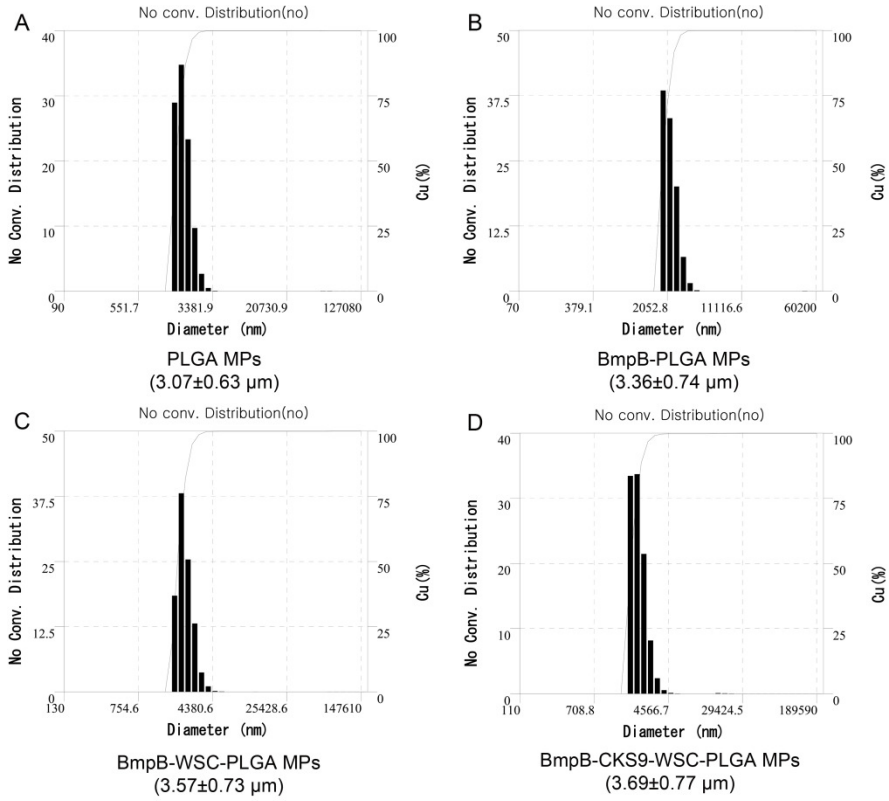


Figure 6. Particle size distributions of the PLGA MPs (A), WSC-PLGA MPs (B), BmpB-PLGA MPs (C) and BmpB-WSC-PLGA MPs (D).

2). BmpB loading into PLGA and WSC-PLGA MPs

The BmpB content and loading efficiency of BmpB in the BmpB-PLGA and BmpB-WSC-PLGA MPs are shown in Table 1. The loading content and loading efficiency of BmpB in the BmpB-PLGA MPs were 12.3% and 60.9% by weight respectively, whereas the loading content and loading efficiency

of BmpB in the BmpB-WSC-PLGA MPs were 10.4% and 57.6% by weight respectively, suggesting that there were not significant differences in the loading content and loading efficiency of BmpB between the PLGA and WSC-PLGA MPs. Porous PLGA MPs typically display a low drug loading efficiency and relatively rapid release profile due to their large surface area (Arnold et al., 2007; Giovagnoli et al., 2007; Bae et al., 2009; Lee et al., 2010; Kim et al., 2012).

Table 1. Loading content and loading efficiency of BmpB loaded PLGA MPs

MPs	Loading content (%)	Loading efficiency (%)
BmpB-PLGA MPs	12.28 ± 2.45	60.93 ± 2.31
BmpB-WSC-PLGA MPs	10.36 ± 1.77	57.64 ± 3.13
BmpB-CKS9-WSC-PLGA MPs	10.13 ± 2.08	56.59 ± 3.07

3). BmpB release from the BmpB-PLGA and BmpB-WSC-PLGA MPs

In vitro BmpB release from the BmpB-loaded MPs was performed in PBS incubated at 37°C with 100 rpm and the amount of released BmpB was evaluated by micro-BCA assay. The release of BmpB from the BmpB-PLGA and BmpB-WSC- PLGA MPs at pH 1.2 and pH 7.2 are

shown in Figure 7. The results indicated that the release of BmpB from the BmpB-PLGA and BmpB-WSC-PLGA MPs was higher at pH 7.2 compared to pH 1.2, likely due to the greater swelling of the PLGA MPs at pH 7.2 compared to pH 1.2. Notably, the burst effect of BmpB release from the BmpB-PLGA MPs at pH 7.2 was obtained within 30 min. In addition, the release of BmpB from the BmpB-PLGA MPs at pH 1.2 and pH 7.2 decreased after coating the PLGA MPs with WSC.

Unlike low molecular weight drugs, proteins possess secondary, tertiary, and even quaternary structures with labile bonds between the side chains of chemically reactive groups. Disruption of these structures or modifications of their side chains can lead to loss of protein activity (Kammona and Kiparissides, 2012). During the loading of vaccine into the MPs, the vaccine's stability is very important for retaining its immune activity. Therefore, the structural integrity of the BmpB released from the BmpB-PLGA MPs was evaluated by SDS-PAGE (Figure 8 A) and Western blot (Figure 8 B) to evaluate BmpB stability. As shown in Figure 8 (A and B), the BmpB release from the BmpB-PLGA, BmpB-WSC-PLGA and BmpB-CKS-WSC-PLGA MPs was similar to native BmpB, indicating that the BmpB was not structurally altered during the loading of the BmpB into

the MPs.

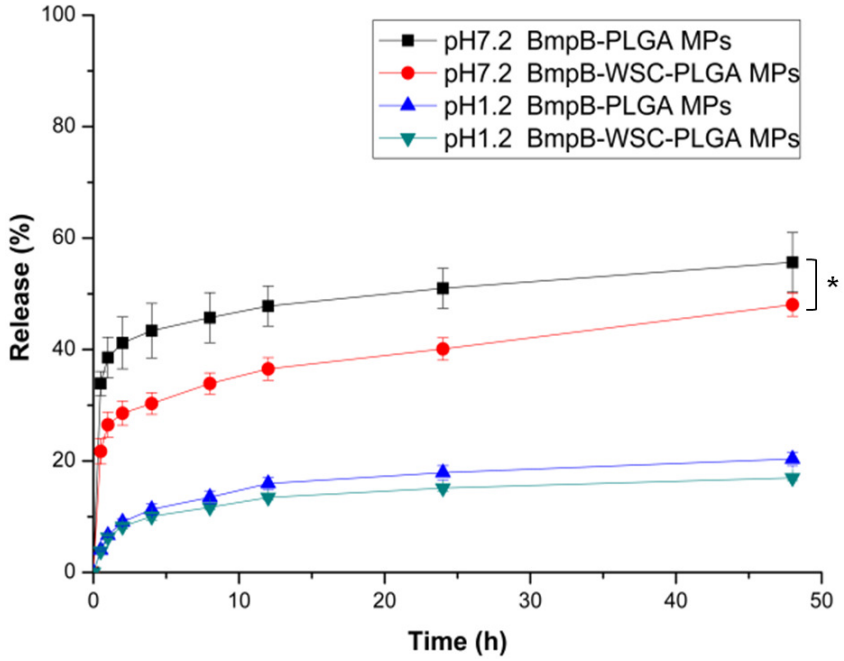


Figure 7. BmpB release profiles from the PLGA and WSC-PLGA MPs at different incubation time points at pH 1.2 and pH 7.2. (means±SD, n=3, *$p < 0.05$).

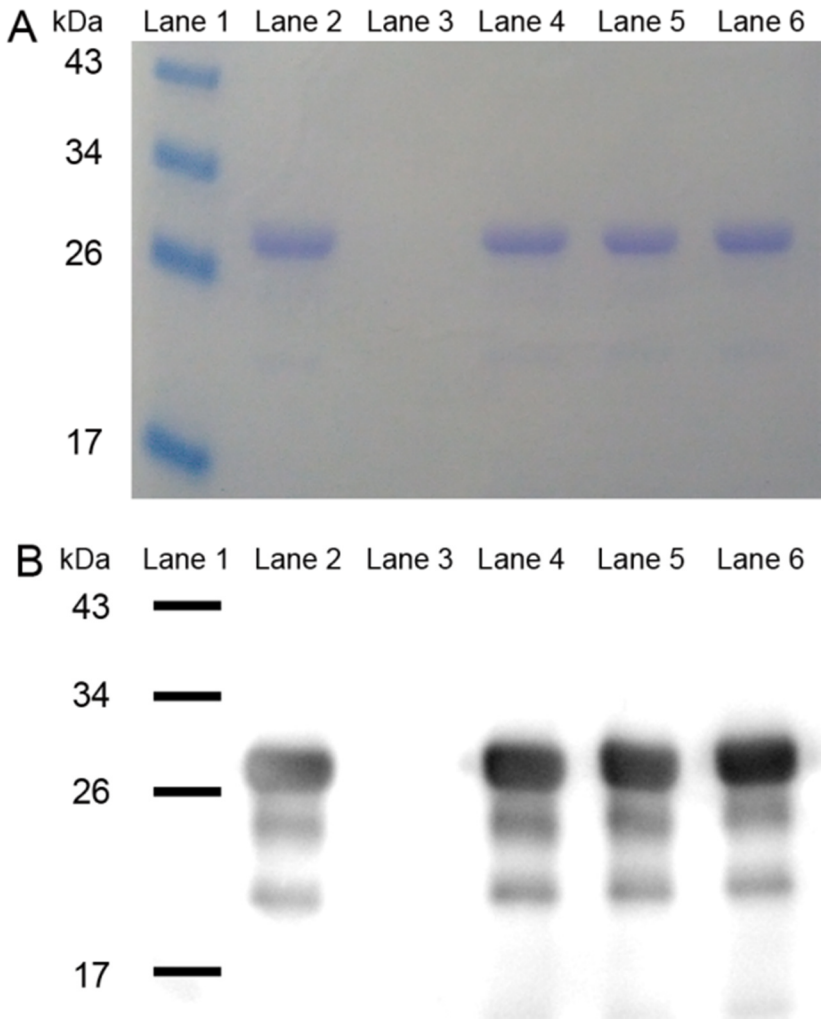


Figure 8. Evaluation of the structural integrity of BmpB released from the BmpB-PLGA MPs by SDS-PAGE (A) and Western blot (B). Lane 1: marker, Lane 2: native BmpB, Lane 3: drug-free PLGA MPs, Lane 4: BmpB release from PLGA MPs, Lane 5: BmpB release from WSC-PLGA MPs and Lane 6: BmpB release from CKS9-WSC-PLGA MPs.

4). Targeting properties of the CKS9-WSC-PLGA MPs

The low potency of the oral vaccine might be due to several challenges, including antigen degradation by proteolylic enzymes, the low dose of antigen absorbed and not actively directing antigens to M cells (Azizi et al., 2010). To overcome these problems, an oral delivery system that would protect antigens and specifically target M cells is necessary. To evaluate the targeting ability of the MPs to PPs in vivo, the coumarin-6 loaded MPs, namely coumarin-6-loaded PLGA (coumarin-6-PLGA), coumarin-6- loaded WSC-PLGA (coumarin-6-WSC-PLGA), and coumarin-6-loaded CKS9-WSC- PLGA (coumarin-6- CKS9-WSC-PLGA) MPs were injected into the closed ileal loops and the in vivo co-localization of the MPs in the PP region were monitored 1 h later by CLSM (Figure 9). While there were no green signals in the PBS group, other PLGA MPs showed significant green signals in the PP region. The figure clearly showed the co-localization of the coumarin-6-CKS9-WSC-PLGA MPs in the PP was the highest among the MPs used. These results strongly suggested that the introduction of CKS9 into the WSC increased the affinity of PLGA MPs with the M cells to enter to the PP in the mouse small intestinal tract compared with the

PLGA and WSC-PLGA MPs. Considering the effect of micro- or nanocarrier surface charges on its cellular uptake rate, it has been postulated that a positive zeta potential is beneficial for M cell transport because the M cell membrane is negatively charged. Therefore, positively charged chitosan was used to coat the PLGA MPs. However, mucus and epithelial cells carry a negative charge as well, making electrostatic interactions with the micro- or nanocarriers very unspecific (Slutter et al., 2008). To overcome the unspecific interactions, the PLGA MPs were decorated with M cell targeting peptide conjugated chitosan. As a consequence, the CKS9-WSC-PLGA MPs (the green signals) were more localized within the FAE layer of the PP (the red signals) on which the M cells were abundant (Figure 9 d) compared to the PLGA (Figure 9 b) or WSC-PLGA MPs (Figure 9 c).

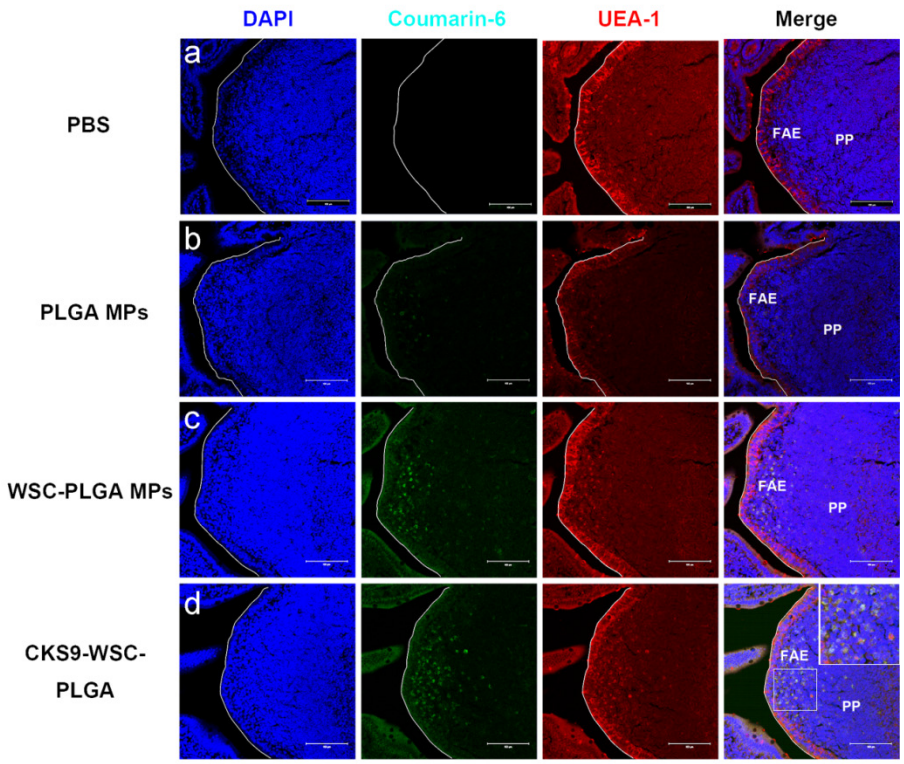


Figure 9. *In vivo* localization of fluorescent CKS9-WSC-PLGA MPs on the mice Peyer's patch region 1 h after an injection into closed ileal loops; (a) PBS, (b) PLGA MPs, (c) WSC-PLGA MPs and (d) CKS9-WSC-PLGA MPs.

5). Immune responses in vivo

The induction of mucosal and systemic immune responses after oral delivery initiates from mucosal sites. At the intestinal mucosal sites, particulate antigens (BmpB) are transported by M cells and delivered to sub-mucosa. Subsequently, antigen-presenting cells process and present the

antigen to T-cells in the adjacent mucosal lymph nodes. Next, activated T-cells that are stimulated by antigen-presenting cells induce IgG and IgA-committed B-cell development in the germinal center of the mucosal-associated lymphoid tissue. Finally, activated B-cells will migrate via the draining lymph nodes and the thoracic duct to the systemic circulation and other mucosal effector sites, where IgG and IgA-committed B-cells differentiate to antibody secreting plasma cells, which produce IgG and IgA antibodies as illustrated in the Figure 10.

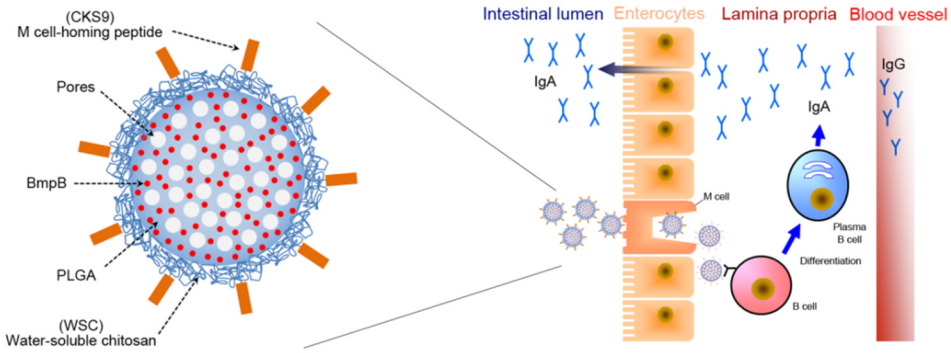


Figure 10. Schematic representation of induction of both mucosal and systemic immune responses at the mucosal-associated lymphoid tissue in the intestine by oral delivery of BmpB-CKS9-WSC-PLGA MPs.

The main goal of this work was to investigate the immune responses of the BmpB released from the BmpB-CKS9-WSC-PLGA MPs after oral delivery. The local production of sIgA would establish a first defense line at the mucosal surface, blocking the pathogen adhesion. To assess the success of the mucosal immunization, the local sIgA levels present in the intestine and excreta were determined. The mice treated with BmpB-WSC-PLGA showed higher titers of anti-BmpB sIgA antibody responses in feces compared with those treated with the BmpB solution or BmpB-PLGA MPs (Figure 11 A). In particular, mice treated with the BmpB-CKS9-WSC-PLGA MPs 4 weeks after their first immunization displayed 5.7- and 7.9-fold higher titers compared to those treated with the BmpB solution or BmpB-PLGA MPs, respectively. The anti-BmpB sIgA antibody responses in the intestine after the immunization are shown in Figure 11 B. The mice treated with the BmpB-WSC- PLGA MPs displayed higher titers compared to those treated with the BmpB solution or BmpB-PLGA MPs, showing a similar tendency as in feces. Four weeks after the first immunization, the sIgA titers of the mice treated with the BmpB-CKS9-WSC- PLGA MPs were 24.7- and 18.5-fold higher compared to those treated with the BmpB solution and BmpB-PLGA MPs, respectively. Humoral immunity at the

mucosal surface is principally mediated by sIgA antibodies, which are the predominant immunoglobulin class in external human secretions. Although sIgA is the predominant humoral defense mechanism at mucosal surfaces, locally produced IgG and serum-derived IgG can also significantly contribute to immune defense (Holmgren and Czerkinsky, 2005). The anti-BmpB IgG antibody responses in serum after immunization are shown in Figure 11 C. The mice treated with BmpB-WSC- PLGA displayed higher titers of anti-BmpB IgG compared to those treated with the BmpB or BmpB-PLGA MPs. In addition, the anti-BmpB IgG titers of the mice treated with the BmpB-CKS9-WSC-PLGA MPs in serum 4 weeks after their first immunization were 4.9- and 4.2-fold higher compared to those treated with the BmpB and BmpB-PLGA MPs, respectively.

The serum isotype levels of IgG1 and IgG2a were determined to investigate the type of immune response. While the production of the IgG2a isotype is associated with a Th1-type response, the IgG1 isotype isassociated with a Th2-type response. The ratios of IgG2a/IgG1 are used to indicate the Th1 or Th2 bias of the generated immune response (Romagnani, 2000; Maassen et al., 2003). The anti-BmpB IgG1 and IgG2a antibody responses in serum after immunization are shown in Figure 11 D. The mice

treated with BmpB-WSC-PLGA showed higher titers of antibody responses compared to those treated with the BmpB or BmpB-PLGA MPs. In addition, the anti-BmpB IgG1 titers of mice treated with the BmpB-CKS9-WSC-PLGA MPs in serum 4 weeks after the first immunization were 6.1- and 8.3-fold higher compared to those treated with the BmpB and BmpB-PLGA MPs, respectively. Furthermore, the anti-BmpB IgG2a titers of the mice treated with BmpB-CKS9-WSC-PLGA MPs in serum 4 weeks after the first immunization were 11.8- and 6.6-fold higher compared to those treated with the BmpB and BmpB-PLGA MPs, respectively. Because the BmpB- CKS9-WSC-PLGA MPs displayed elevated IgG1 and IgG2a titers, these MPs appear to induce both Th1and Th2 immune responses *in vivo*.

The results of this study demonstrated that the oral immunization of mice with BmpB-WSC-PLGA MPs elevated immune responses due to mucoadhesive property of the WSC (Illum et al., 2001; Amidi et al., 2010), that enhanced the retention of BmpB in intestine, maximizing the interactions with M cells. Interestingly, the oral immunization of mice with the BmpB-CKS9-WSC-PLGA MPs dramatically increased sIgA responses in the mucosal tissues as well as systemic IgG antibody responses due to the

enhanced M cell targeting and transcytosis ability of the WSC-PLGA MPs to PP regions.

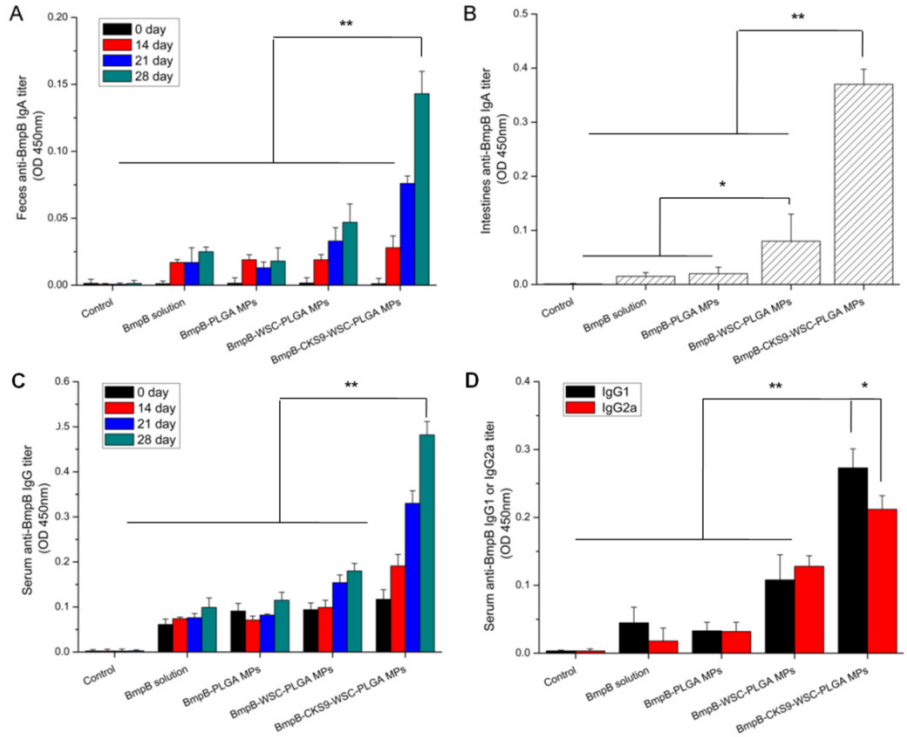


Figure 11. BmpB-specific immune response after oral administration. Anti-BmpB IgA levels in feces (A) and intestine (B), anti-BmpB IgG levels in serum (C) and anti-BmpB IgG subclass antibody (IgG1 and IgG2a) (D) levels were measured using ELISA. (means±SD, n=5 mice, *$p < 0.05$, **$p < 0.01$).

4. Conclusions

Our results indicate that porous PLGA MPs coated with M cell homing peptide-coupled chitosan is a promising approach for the oral delivery of BmpB vaccine with successful stimulation of specific IgG in serum and sIgA in feces and intestine. Moreover, BmpB-CKS9-WSC-PLGA MPs demonstrated to induce both Th1-and Th2-type responses based on elevated IgG1 and IgG2a titers. Based on our present study, we believe that this approach would be applicable for swine dysentery vaccine delivery to induce both mucosal and systemic immune responses in piglets.

Chapter 2. Oral delivery of probiotic expressing M cell homing peptide conjugated BmpB vaccine encapsulated into alginate/chitosan/alginate microcapsules

1. Introduction

Antibiotics have long been used for treating and preventing animal diseases, and improving feed efficiency of live stocks (Allen et al., 2013). But, due to the development of resistant pathogens associated with human and animal diseases, a comprehensive ban on the use of all antibiotics for growth promotion has been implemented in developed countries (Stanton, 2013). Therefore, alternatives to antibiotics are urgently needed in food-producing animals. But, it is not easy to find them due to complexity of the gastrointestinal (GI) ecosystem (Allen et al., 2013).

As an alternative, probiotics have received great attention due to their wide use in fermented foods and dairy products. Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host by improving its intestinal microbial balance. Where antibiotics kill not only the bad bacteria that can cause infection but also destroy the good bacteria that help fight infection, probiotics, on the

other hand, re-populate the intestinal flora with healthy bacteria that can help kill the bad bacteria and fight infection.

Most importantly, probiotics can induce antigen-specific and non-specific IgA antibody responses at mucosal surfaces to prevent invasion by pathogenic microorganisms (Perdigón et al., 2001; Wells and Mercenier, 2008b). Similarly, oral administration of probiotics regulate the Th1/Th2 balance (Torii et al., 2007). It is now well-documented that probiotics stimulate human or murine dendritic cells to produce increased levels of pro-inflammatory cytokines (IL-2, IL-12, TNF-α) (Chiba et al., 2010; Weiss et al., 2010). Such immune stimulating effects are the characteristics of adjuvants. Therefore, probiotic adjuvants when orally administered in conjugation with vaccine repeatedly enhance the immunogenicity of vaccines. Altogether, probiotics modulate both mucosal and systemic immune responses and function as adjuvants by promoting pro-inflammatory cytokine production.

Due to these advantages and no side effects, the probiotics are “generally regarded as safe” (GRAS), and they can manage to survive passage through the acidic conditions of the stomach when administered orally. Besides, these probiotic strains can be manipulated to express targeting molecules

and adjuvants by recombinant DNA techniques (Wells and Mercenier, 2008a). Several attempts have also been made to use combinations of probiotics with other additives (e.g., vaccines or adjuvants) with the goal of increasing the effect of the probiotics synergistically (Bomba et al., 2002; Musa et al., 2009). Therefore, a better understanding of the effects and mechanisms of action of the combinations will allow to design more rational potentiated probiotics (Allen et al., 2013).

Mucosal delivery of vaccines have several advantages such as elimination of the risk of transmission of blood-borne diseases, easy administration by personnel with less medical training and elicitation of both mucosal and humoral immunity (Cutting et al., 2009). However, oral vaccine delivery has to overcome problems associated with poor absorption and degradation of the vaccine within the digestive system. Besides, mucosal vaccine adjuvants in current use do not confer the required efficacy and safety for clinical application. Therefore, the combination of the intrinsic advantages of probiotics, not only as a live vaccine delivery vehicle but also an immune enhancer, with other nano- or micro carrier system would be an approach to develop a synergistic vaccine delivery system.

Recently, an M cell homing peptide, screened by phage display technique, have shown high affinity to M cells to target Peyer's patch regions (Yoo et al., 2010). For illustration, oral immunization of M cell homing peptide coated poly(lactic-co-glycolic) acid (PLGA) microparticles harboring BmpB (*Brachyspira* membrane protein B), a model antigen for swine dysentery (SD), showed high immune responses in the mucosal tissues (Jiang et al., 2014). In this study, we developed a probiotic strain through recombinant DNA technology that expresses and produces BmpB antigen fused with M cell homing peptide. The probiotic was encapsulated into alginate/chitosan/alginate (ACA) microcapsules (MCs) and later delivered through oral route to evaluate the efficiency of vaccine delivery and immune responses.

2. Materials and methods

1). Materials

Sodium alginate (low viscosity), L-glutamine, glycerol, ribonucleosides, pepsin, bile salt (B8756), deoxyribonucleosides, bovine serum albumin (BSA), fluorescein isothiocyanate (FITC), 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant mouse granulocyte macrophage colony stimulating factor (mGM-CSF) was purchased from Peprotech (Rocky Hill, NJ, USA). Chitosan (MW 150,000; degree of deacetylation = 91.8%) was kindly provided by Jakwang Co. Ltd. (Ansung, Korea). Difco^{TM} *Lactobacilli* MRS broth and *Lactobacilli* MRS agar were purchased from BD (Sparks, MD, USA). Fetal bovine serum (FBS), mouse TNF-α ELISA and mouse IL-6 ELISA were purchased from Thermo Scientific (Rockford, IL, USA). 3,3',5,5'-tetramethyl benzidine (TMB), horseradish peroxidase (HRP)-conjugated goat anti-mouse IgA, IgG, IgG1 and IgG2a antibody were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). All other chemicals were of analytical

reagent grade.

2). Probiotic strains

Lactobacillus plantarum 25-wild type (LP25-WT) was used as a model probiotic strain (Yun et al., 2009). The recombinant LP25 expressing BmpB or M cell homing peptide conjugated BmpB (M-BmpB) were constructed by our research group (Table 1).

Table 1. Formulations used in this study.

Cohort	Formulation	Description
1	Control	Saline (0.85%)
2	ACA MCs	Alginate-Chitosan-Alginate Microcapsules
3	LP25-WT	*L. plantarum* 25 wild type
4	LP25-BmpB	*L. plantarum* 25 expressing BmpB
5	LP25-M-BmpB	*L. plantarum* 25 expressing M cell homing peptide conjugated BmpB
6	LP25-WT-ACA MCs	*L. plantarum* 25 encapsulated into ACA MCs
7	LP25-BmpB-ACA MCs	*L. plantarum* 25 expressing BmpB encapsulated into ACA MCs
8	LP25-M-BmpB-ACA MCs	*L. plantarum* 25 expressing M-BmpB encapsulated into ACA MCs

3). Synthesis of FITC-labeled chitosan

FITC-labeled chitosan (FITC-C) was prepared as follows. Chitosan solution (5% w/v in distilled water, 4 ml) was prepared, followed by the addition of FITC (2 mg/ml in DMSO, 1 ml). The reaction was carried out in dark at RT with stirring for 24 h. To remove unreacted FITC, the solutions were dialyzed against distilled water at 4°C. The dialyzed product was then freeze-dried.

4). Synthesis of DAPI-labeled alginate

DAPI-labeled alginate (DAPI-A) was prepared by conjugation of alginate with DAPI using NHS/EDC coupling agents. Briefly, alginate (100 mg) was dissolved in 2 ml distilled water and activated with NHS (131 mg) and EDC (218 mg) at RT for 3 h. The resultant solution was added into the DAPI solution (0.26 mg was dissolved in 1 ml distilled water) and stirred in dark at RT for 24 h. The reaction product was dialyzed for 2 days against distilled water and then freeze-dried.

5). Microencapsulation of probiotic strains

Wild type or recombinant LP25 cultured in MRS broth was centrifuged at

2000×g at 4 °C for 10 min. The probiotic pellets were washed and suspended in 5 ml of normal saline. MCs were prepared by centrifugal extrusion method as previously described (Bajracharya et al., 2012; Jiang et al., 2013) with slight modification. Briefly, the mixture of sodium alginate with LP25 (or recombinant LP25) and 15% (v/v) glycerol was dropped into 0.1M $CaCl_2$ by passing through cannula-like syringe in the presence of N_2 gas pressure. Gelation of the alginate droplets by calcium ions immediately formed alginate MCs, the final concentration of sodium alginate being 1.5 % (w/v). Alginate MCs thus formed were allowed to harden by incubation with 0.1M $CaCl_2$ solution for 30 min and they were washed with 0.85% saline to remove unreacted $CaCl_2$. Then, the alginate MCs were coated with 0.8% (w/v) chitosan solution for 30 min followed by two times washing by 0.85% saline. Chitosan-coated alginate MCs were further coated with 0.1% (w/v) sodium alginate for 10 min followed by washing. They were stored at -70 °C for 6 h and lyophilized. ACA MCs without probiotics were prepared in the same way following the same protocol as described above. The process of MCs preparation is shown in Figure 1.

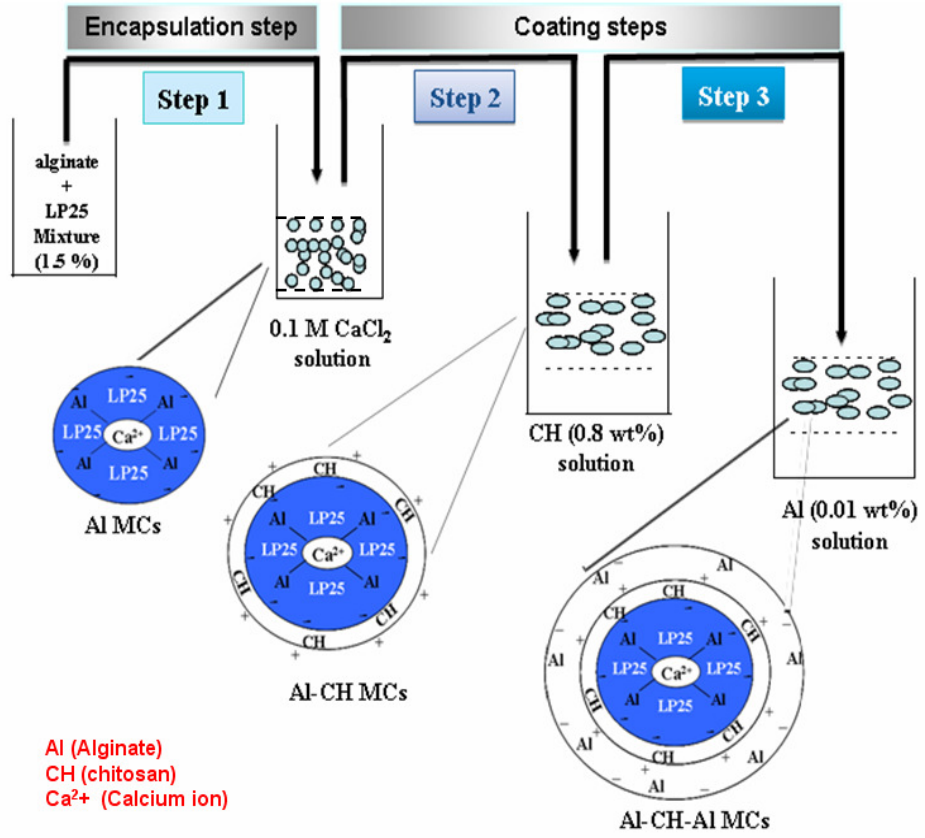


Figure 1. Process of microcapsules preparation.

6). Detection of coatings by confocal microscopy

Alginate MCs were prepared by gelation of alginate droplets by calcium ions as described above. The alginate MCs were placed in 0.8% (w/v) FITC-C solution for 30 min followed by two times washing by 0.85% saline. Chitosan-coated alginate MCs were further coated with 0.1% (w/v)

DAPI-A solution for 10 min followed by washing. The MCs were examined on confocal laser scanning microscope (CLSM; Leica TCS SP8X Gated STED, Germany).

7). Morphology observation and size measurement of MCs

Prior to observation of the morphology of MCs, the MCs were coated with gold using coating chamber (CT 1500 HF, Oxford Instruments Oxford shire, UK). The coated MCs were observed through field-emission scanning electron microscope (FE-SEM) (Supra 55VP; Carl Zeiss, Oberkochen, Germany). The settings of SEM images are as follows: (Mag = 100 X; WD = 6.6 mm; EHT = 7.0 kV). The average size of the MCs was calculated using a hemocytometer (Tiefe Depth Profondeur 0.100 mm, Paul Marienfeld, Germany).

8). Loading and releasing behavior of probiotics in MCs

The encapsulation efficiency (EE) was expressed as the percentage of the probiotics amount found in the MCs to the total amount used to prepare the MCs. The loading content (LC) was expressed as the number of live bacteria (cfu: colony-forming units) found in the MCs, as described in a

previous report (Jiang et al., 2013). Briefly, ACA MCs are incubated in MRS broth at 37°C shaking with 100 rpm for a given interval of time. To test the release of probiotics from ACA MCs, an aliquot (10 µl) of the broth is dropped into the MRS agar plate and incubated at 37°C to count the colonies of the probiotics.

Simulated gastric fluid (SGF) and simulated small intestinal fluid (SIF) were prepared by adjusting the pH of MRS broth medium to 2 and 7.2 respectively (Kim et al., 2008). Survivability of free and encapsulated probiotics in SGF with or without pepsin (1000 units/ml) was calculated by cfu counting of probiotics after incubating at 37°C at 100 rpm against time. Similarly, survivability of free and encapsulated probiotics in SIF with final bile salt concentrations (1.2 % w/v) was checked by following the same method. Encapsulated probiotics were incubated in 10 ml of SIF at 37 °C with 100 rpm shaking. At a given time interval, 0.5 ml aliquot was collected and replaced by an equal volume of fresh medium. The release of probiotics was then assayed by counting cfu as described before (Lee et al., 2004).

Freeze dried LP25-encapsulated ACA MCs were stored at 4°C and RT. Survivability of LP25-encapsulated ACA MCs was monitored as described above for 8 consecutive weeks.

9). Measurement of cytokine inductionfrommacrophagesor de ndritic cells by probiotics

The murine macrophages, RAW 264.7, were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) FBS. The murine dendritic cells, JAWSII, were maintained in α-minimum essential medium containing 20% (v/v) FBS, ribonucleosides and deoxyribonucleosides, 4 mM L-glutamine, 5 ng/ml mGM-CSF. Both cells were plated in 10 cm dish with the cell density of $1x10^7$ cells per dish. To measure the amount of TNF-α and IL-6 cytokines secreted from RAW 264.7 and JAWSII cells respectively, the cells were stimulated with the probiotics following the previously described method (Chon et al., 2010). The cells were incubated with free LP25 ($1x10^8$ cfu/dish), LP25-loaded ACA microcapsules ($1x10^8$ cfu/capsule/dish), unloaded ACA microcapsules (1 capsule/dish).

10). Oral immunization

Female BALB/c mice of 6 weeks of age were obtained from Samtako, Co. Ltd. (Osan, Korea). Animals were kept under standard pathogen-free conditions and supplied with free access to water and food during the

experiments. The animals were maintained and used in accordance with the guidelines for the care and use of laboratory animals (Seoul National University). The mice were divided into 8 cohorts (5 mice per cohorts). Peroral immunization with mice were performed with a dose of probiotics containing 10^9 cfu or freeze-dried MCs (10 MCs per mouse containing 10^9 cfu) resuspended in the appropriate volume of 0.85% saline (200 ul) administered by intra-gastric inoculation using a disposable feeding needle. All cohorts received a total of six doses of the probiotics (or control) on days 0, 1, 7, 8, 14 and 15 (Figure 2).

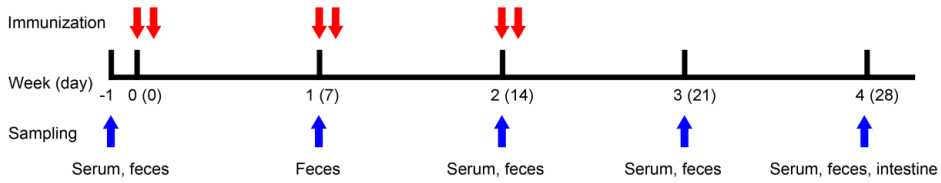


Figure 2. Scheme of oral immunization.

To investigate immune responses, tail vein blood and feces samples were taken at 0, 7, 14, 21 and 28 days prior to immunization where appropriate. The blood samples were centrifuged for10 min at 3,000×g, and the collected serum was stored at -70°C until analysis for IgG, IgG1 and IgG2a

levels. To analyze the IgA level from fecal samples, five fecal pellets of each mouse selected randomly were prepared according to the method described previously (Jiang et al., 2014). Briefly, pellets in 1 ml phosphate buffered saline (PBS) were incubated for 15 min on ice and followed by mashing it with a blunt needle. Samples were centrifuged and IgA was analyzed as previously described (Enioutina et al., 1999). Finally, small intestines (3 cm) with Peyer's patches of the mice were collected after sacrifice. The intestines were homogenized in 500 μl of ice-cold PBS. After removing tissues by centrifugation (10,000×g, 10 min and 4 °C), the supernatants were collected and stored at -70 °C until analysis for IgA level.

11). Evaluation of antibody induction

Induction of BmpB-specific antibodies was assayed using enzyme-linked immunosorbent assays (ELISA) as described before (Jiang et al., 2014). ELISA plates coated with BmpB (25 μg/ml), prepared in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6), were incubated at 4°C overnight. The plates were then washed with washing solution (PBS containing 0.05% (v/v) Tween 20) and blocked with blocking solution (PBS containing 1% (w/v) BSA). Serially diluted samples (100 μl/well) in

blocking solution were then added, and the mixtures were incubated for 2 h. After washing for three times, each sample was treated with HRP-conjugated goat anti-mouse IgG, IgG1 and IgG2a antibody (1:5000) or goat anti-mouse IgA antibody (1:2000). After 1 h incubation and subsequent washing, the samples were treated with TMB substrate buffer and the reaction was stopped after 15 min with 50 μl of 2 M H_2SO_4. The optical densities (OD) of the samples were measured at 450 nm by an Infinite 200 PRO multimode reader (Tecan, Switzerland).

12). Statistical analysis

All results are expressed as mean ± SD. Differences between means were tested for statistical significance using a one-way analysis of variance (ANOVA) and post hoc least significance tests. Statistical significance is denoted by * for $P<0.05$ and by ** for $P < 0.01$.

3. Results and Discussion

1). Characterization of probiotic-loaded ACA MCs

Capsules of non-labeled alginate core treated with FITC-C and DAPI-A were prepared and examined through CLSM as shown in Figure 3. The images indicated that the surface of the MCs is homogeneously coated with chitosan and alginate due to the interactions between oppositely charged polymers (Bartkowiak and Hunkeler, 2000; Cook et al., 2011).

Figure 4 shows SEM images of ACA MCs loaded with probiotics. The MCs were generally spherical with wrinkled surface that is probably due to the loss of water content during the freeze-drying process (Kwok et al., 1991). It was observed that there was only a slight difference in size (around 1.11 mm) and shape of MCs among ACA MCs and probiotic-loaded ACA MCs, indicating that loading of probiotics or probiotics expressing vaccine did not affect size and shape of ACA MCs. Consistently, the size of the MCs does not affect the morphological characteristic of the capsule membrane.

Loading characteristics of probiotics into ACA MCs are shown in table 2. The results indicated that sizes of ACA MCs increased from around 1 mm

to 2 mm with the increase of loading content of probiotics into MCs. However, encapsulation efficiency of MCs did not change with the loading content.

Table 2. Characterization of prepared MCs.

ACA-MCs	Size (mm)	Loading content (cfu/capsule)	Encapsulation efficiency (%)
Unloaded	0.71±0.10	-	-
LP25-WT	1.06±0.08	$1.11\pm0.03\times10^{7}$	98.30 ± 2.31
	1.11±0.22	$2.08\pm0.09\times10^{8}$	98.56 ± 1.33
	2.09±0.26	$1.81\pm0.05\times10^{9}$	97.36 ± 2.37
LP25-BmpB	1.12±0.27	$1.32\pm0.10\times10^{7}$	97.20 ± 3.13
	1.19±0.14	$1.65\pm0.08\times10^{8}$	98.10 ± 2.23
	2.20±0.30	$1.81\pm0.11\times10^{9}$	97.56 ± 1.33
LP25-M-BmpB	1.18±0.13	$0.96\pm0.04\times10^{7}$	97.42 ± 3.75
	1.09±0.27	$1.87\pm0.09\times10^{8}$	97.82 ± 2.07
	2.33±0.24	$2.10\pm0.11\times10^{9}$	96.66 ± 0.83

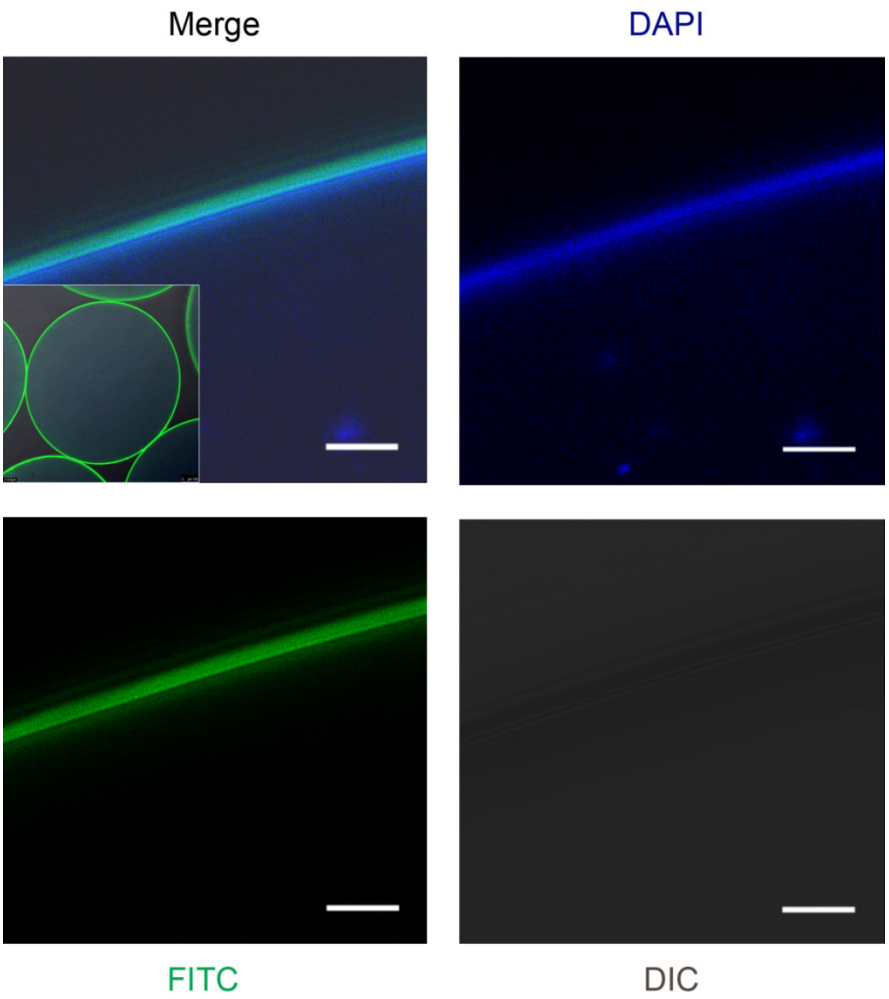


Figure 3. Confocal microscopy image showing layer of chitosan (green) and alginate (blue) on the surface of the microcapsule. Scale Bars: 10 µm.

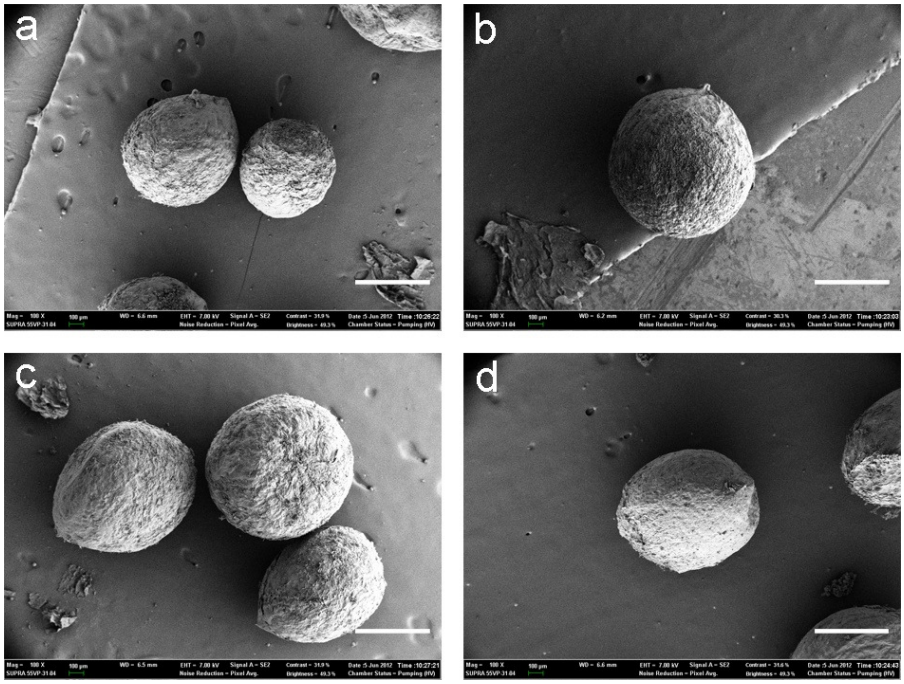


Figure 4. Morphologies of ACA MCs (a), LP25-WT-ACA MCs (b), LP25-BmpB-ACA MCs (c) and LP25-M-BmpB-ACA MCs (d) by field emission sca nning electron microscope. Scale Bars: 500 μm.

2). Survivability of LP25 encapsulated into ACA MCs

Encapsulation of probiotics significantly improves survival, compared to free cells, while drying improves stability of the encapsulated culture during prolonged storage (Solanki et al., 2013). Therefore, we performed all the experiments using freeze-dried MCs. The freeze-dried formulations are easy to handle and less susceptible to breakage. Drying also create

mechanically stable MCs which are desirable property for oral delivery. Therefore, LP25 encapsulated into ACA MCs were freeze-dried to evaluate the storage stability at different temperature and conditions. Figure 5a shows the survivability of probiotics against time in SGF condition (pH 2.0) at 37 °C. The survivability of probiotics, both wild type and recombinants, drastically decreased within 2 h and the survivability was almost zero within 60 min in the presence of pepsin in SGF condition at 37 °C (Figure 5b). The expression of recombinant proteins, BmpB or M-BmpB, did not affect the survivability of the probiotics. On the other hand, the survivability of probiotics increased after encapsulation into ACA MCs (Figure 5c) due to the protection by the capsules at the harsh gastric condition despite the presence of pepsin (Figure 5d).

Figure 5e shows survivability of LP25 against time in SIF (pH 7.2) at 37 °C in the presence of bile salt. The survivability of the LP25 drastically decreased within 24 h and decreased with an increase of bile salt. However, the survivability of the LP25 encapsulated into ACA MCs (Figure 5f) increased due to the protection by the capsules.

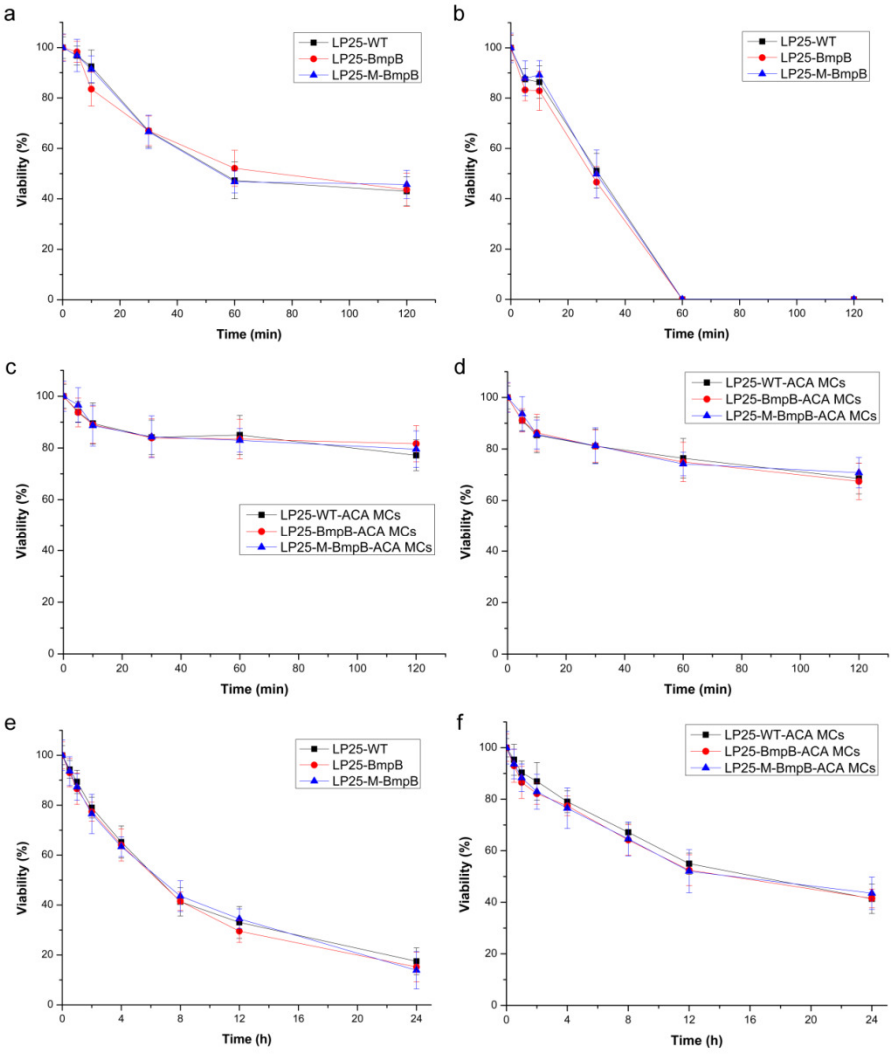


Figure 5. Viability of free and encapsulated probiotics during exposure to SGF (pH 2.0) and SIF (pH 7.2). SGF without pepsin (a, c); SGF with pepsin (b, d); SIF with bile salts (e, f). Viability represents the percentage of cells surviving relative to the initial population (mean±SD, n=3).

The final challenge related to the development of probiotics encapsulation into ACA MCs is represented by their stability during storage. Figure 6a shows the survivability of probiotics against storage time at RT and 4°C. The results indicated that the viability of free probiotics after 5 weeks of storage at 25°C was null whereas encapsulated probiotics had much better stability at 25°C with 30% survivability until 8 weeks. On the other hand, the survivability of free probiotics at 4°C was around 25% at 8 weeks whereas the survivability of probiotics encapsulated into ACA MCs at 4°C was around 60% at 8 weeks (Figure 6b). Taken together, the viability of encapsulated probiotics stored at 4°C was 50 % higher than the storage at 25°C.

3). In vitro release of probiotics encapsulated into ACA MCs

Beyond the protection of probiotics by MCs, they need to withstand the low pH of the stomach during oral delivery, and disintegrate in the gut to release the probiotics. Hence, in vitro release of probiotics encapsulated into ACA MCs was performed at pH 7.2 at 37 °C. The results demonstrated that around 75% of probiotics released within 1 h from the ACA MCs before freeze drying (Figure 7a), suggesting the rapid release due to hydrophilic property of the ACA MCs in SIF. Almost 100% of probiotics

released within 12 h from the ACA MCs before freeze drying. After freeze drying, only 50% of probiotics released during the initial 1 h from the ACA MCs (Figure 7b). Although the initial release of the probiotics retarded after freeze drying of MCs, the release reached almost 100% within 12 h similar to ACA MCs before freeze drying. It is likely that freeze-dried MCs takes longer time for rehydration to release the probiotics.

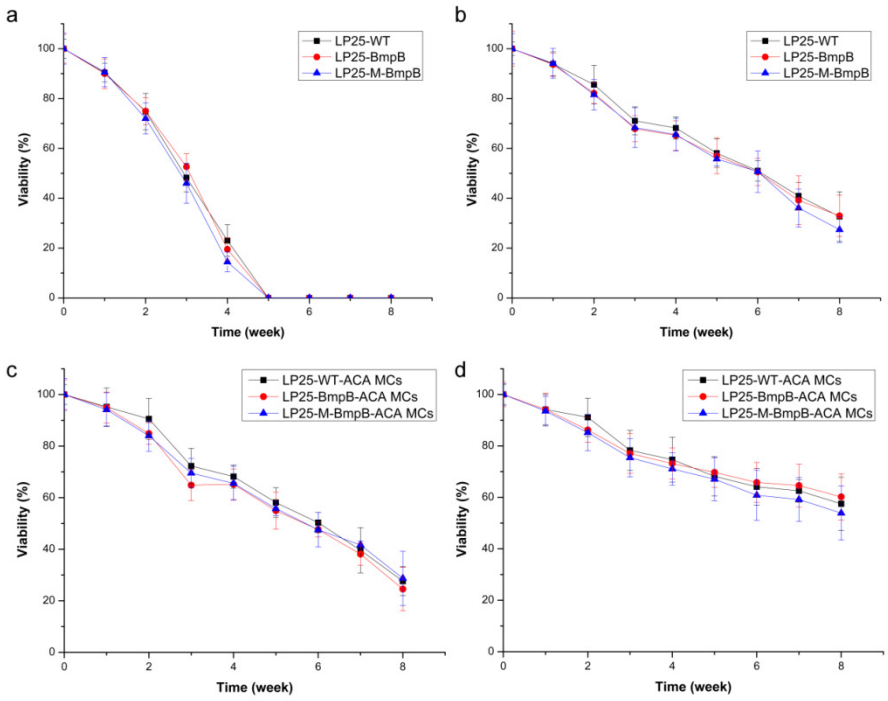


Figure 6. Viability of free and encapsulated probiotics during 8 weeks of storage at RT (a and c) and 4°C (b and d). Viability represents the percentage of cells surviving relative to the initial population (means±SD, n=3).

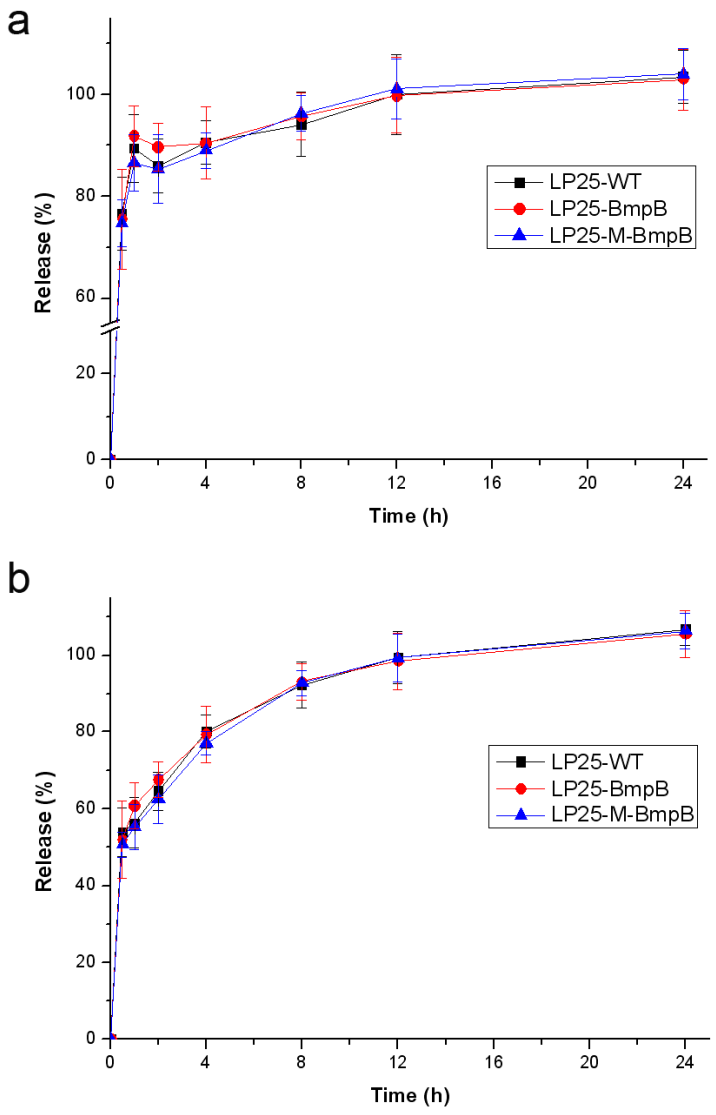


Figure 7. Release of probiotics from ACA MCs in vitro against at pH 7.2 at 37°C. (a) Wet ACA MCs, (b) Freeze-dried ACA MCs (mean±SD, n=3).

4). Measurement of cytokine induction

A vaccine can work most efficiently, if it is able to elicit both humoral and cellular immune responses; however, conventional vaccines often induce antibody responses with only limited cellular immunity (Chadwick et al., 2010). M cells serve as a specific gate for antigens and have the ability to transcytose the antigenic material into the M cell pocket located below follicle-associated epithelium of the Peyer's patch facilitating delivery to antigen-presenting cells including dendritic cells and macrophages (Russell-Jones, 2000). The ability of probiotic bacteria to modulate immune responses are well recognized (Fujiwara et al., 2004; Elmadfa et al., 2010). It is also well-documented that IL-6 is a B cell differentiation factor that induces B cell proliferation leading to humoral immune responses whereas TNF-α induces T cell proliferation that increases the capacity of macrophages to phagocytose and kill the pathogens through cellular immune responses (Koppolu and Zaharoff, 2013). In addition, induction of Th1 mediated immune response (associated with IgG2a antibody response) supports cellular immunity (van Ginkel et al., 2000). These responses help identify correlates of protective cellular

immunity by oral immunization with probiotics. Therefore, induction of cytokines (particularly, IL-6 and TNF-α) from macrophage cell line RAW 264.7 after treating with the probiotics was assayed by ELISA. From ELISA test, it was observed that the free probiotics induced significantly higher level of cytokines production, both IL-6 (Figure 8a) and TNF-α (Figure 8b), from RAW 264.7 cells than probiotics-loaded ACA MCs. The consequence is due to controlled release of probiotics from probiotic-loaded ACA MCs. Importantly, the observed cytokine production from probiotic-loaded ACA MCs was due to the released probiotics, not due to microcapsule itself as it induced only negligible amount of cytokine production. Similar results of cytokine production were obtained in the JAWSII cell line (Figure 8c and d).

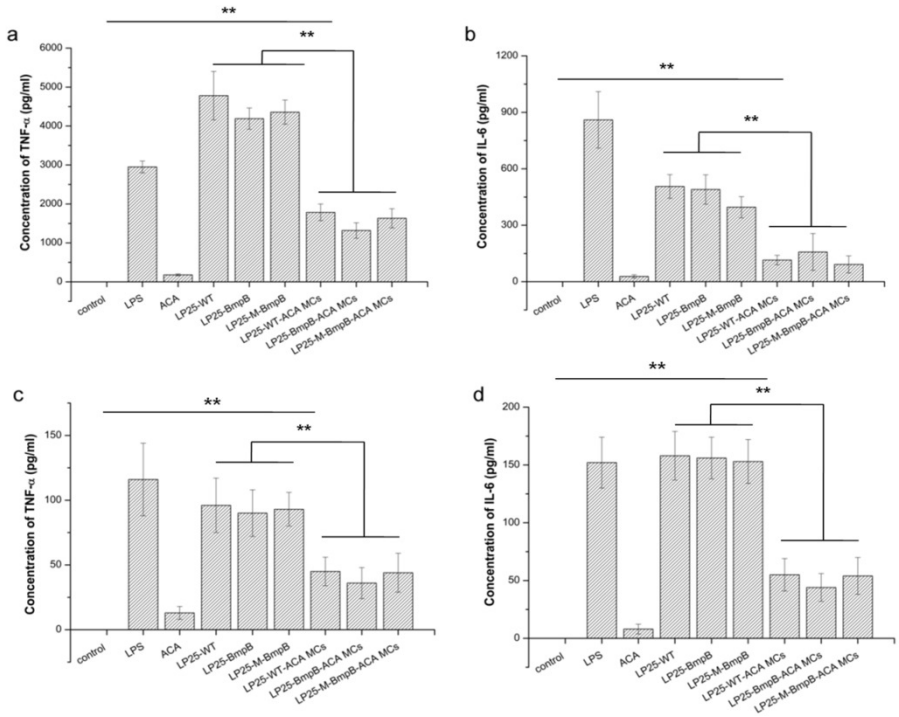


Figure 8. Secretion of TNF-a and IL-6 from RAW 264.7 (a, b) and JAWSII (c, d) stimulated with free and encapsulated probiotics (means±SD, n=3, **p < 0.01).

5). In vivo immune responses

Specific antibodies that inhibit pathogen adherence to receptors on the intestinal cells are important for the protection against animal diarrhea (Winner et al., 1991; Michetti et al., 1992; Zhang et al., 2002; Haesebrouck et al., 2004). For protection against diseases in young piglets, vaccines that stimulate mucosal immunity are necessary (Remer et al., 2009). Therefore,

the main goal of this work was to investigate the protective effect by ACA encapsulation for gastro-intestinal delivery of model vaccine (BmpB or M-BmpB) producing probiotics against harsh digestive conditions in the gastro-intestinal tract. In order to do this, we measured the antibody induction levels from mucosal and systemic immune responses compared to controls as showed in Figure 9.

When the local sIgA levels present in the feces and intestinal were checked, the mice treated with LP25-BmpB-ACA MCs showed the highest level of anti-BmpB IgA in feces among LP25-WT, LP25-BmpB, LP25-M-BmpB and LP25-WT-ACA MCs as shown in Figure 10a. Particularly, the mice treated with LP25-M-BmpB-ACA MCs at 4 weeks after the first immunization showed 3.3- and 2.9-fold higher level of anti-BmpB IgA production than the mice treated with LP25-BmpB and LP25-M-BmpB, respectively, indicating that BmpB expressed in LP25 act as apotent antigen. The consequence was particularly due the protection of LP25 at gastric condition by ACA MCs while M cell homing peptide fused with BmpB enhanced high accessibility of vaccine molecules to gut associated lymphoid tissues (GALT) and Peyer's patch regions through M cells. Due to their transcytotic capability, intestinal M cells represent an

efficient potential route for vaccine delivery (Sansonetti and Phalipon, 1999; Ann Clark et al., 2001).

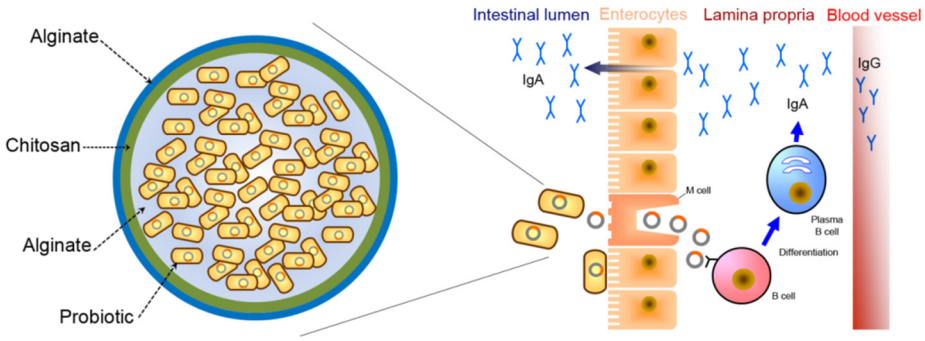


Figure 9. Schematic representation of induction of both mucosal and systemic immune responses at the mucosal-associated lymphoid tissue in the intestine by oral delivery of LP25-M-BmpB-ACA MCs.

The anti-BmpB IgA antibody production in the intestine at 4 weeks after the first immunization is shown in Figure 10b. The mice treated with the LP25-M-BmpB-ACA MCs showed higher level of anti-BmpB IgA production showing similar tendency of IgA production in feces. Particularly, the level of IgA production in the mice treated with the LP25-M-BmpB-ACA MCs was 5.4- and 2.1-fold higher than those treated with the LP25-BmpB and LP25-M-BmpB, respectively. Although humoral immunity at the mucosal area is mainly mediated by IgA antibody as the

predominant immunoglobulin, locally produced IgG and serum-derived IgG also contribute to immune defense (Azizi et al., 2010). Therefore, the anti-BmpB IgG antibody responses in serum after immunization are also evaluated. As shown in Figure 10c, the mice treated with LP25-M-BmpB-ACA MCs displayed higher level of anti-BmpB IgG than those treated with the LP25-WT and LP25-BmpB. However, there was no prominent difference of anti-BmpB IgG between LP25-BmpB and LP25-BmpB-ACA MCs at 3 weeks. In particular, the anti-BmpB IgG level of the mice treated with LP25-M-BmpB-ACA MCs in serum at 4 weeks after the first immunization were 2- and 1.7-fold higher than those treated with the LP25-BmpB and LP25-M-BmpB, respectively.

The serum isotype levels of IgG1 and IgG2a are assayed to check the type of immune response as the production of the IgG2a isotype is related with a Th1-type response whereas the IgG1 isotype is related with a Th2-type response. Generally, the ratio of IgG2a/IgG1 indicate the Th1 or Th2-type bias of the generated immune response (Romagnani, 2000; Maassen et al., 2003). The anti-BmpB IgG1 and IgG2a antibody production in serum at 4 weeks after the first immunization is shown in Figure 10d. It was found that the mice treated with LP25-M-BmpB displayed higher level

of antibody responses than those treated with the LP25-WT and LP25-BmpB although there is not much difference of anti-BmpB IgG1 and IgG2a between LP25-BmpB and LP25-BmpB-ACA MCs. Particularly, the level of anti-BmpB IgG1 in mice treated with the LP25-M-BmpB-ACA MCs in serum at 4 weeks after the first immunization were 2- and 1.4-fold higher than those treated with LP25-BmpB and LP25-M-BmpB, respectively, suggesting that the LP25-M-BmpB-ACA MCs appears to induce both Th1 and Th2 immune responses in vivo due to the simultaneous elevation of IgG1 and IgG2a levels. The results of this research indicated that oral immunization of mice with LP25-BmpB-ACA MCs elevated immune responses due to expression and production of recombinant BmpB in LP25, protection of antigen by the ACA MCs and interactions of M cell homing peptide fused to antigen with M cells in the gut. Moreover, the oral immunization of mice with LP25-M-BmpB-ACA MCs drastically increased IgA responses in the mucosal tissue as well as systemic IgG antibody responses.

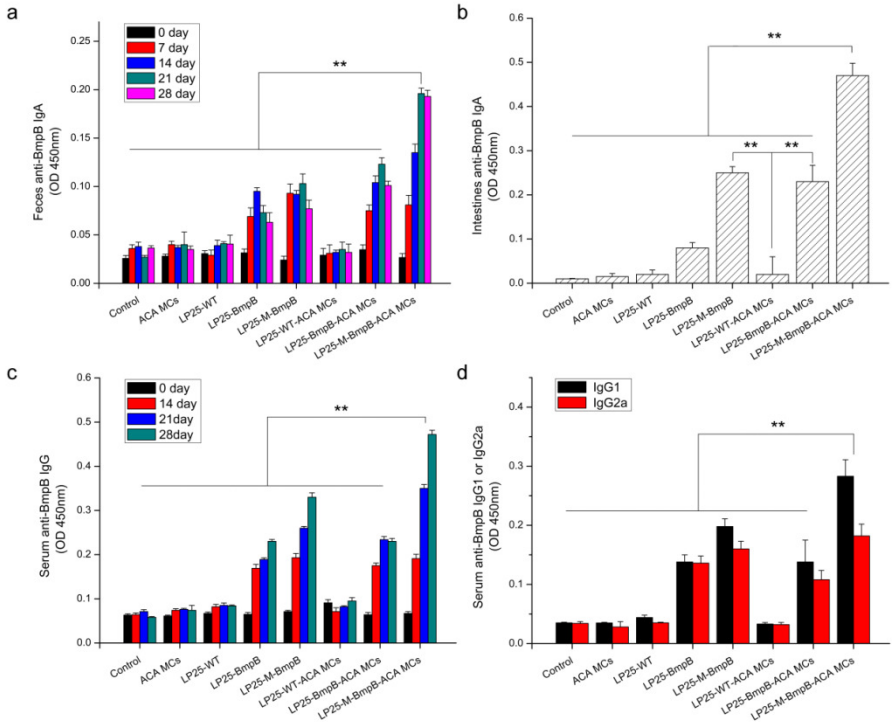


Figure 10. BmpB-specific immune response after oral administration. Anti-BmpB IgA levels in feces (a) and intestine (b), anti-BmpB IgG levels in serum (c and anti-BmpB IgG subclass antibody (IgG1 and IgG2a) (d) levels were measured using ELISA. (means±SD, n=5 mice, **p < 0.01).

4. Conclusions

In present study, the use of ACA MCs to deliver probiotic expressing M cell homing peptide fused BmpB antigen showed successful stimulation of specific IgG in serum and IgA in feces and intestine of mice. Future efficacy studies are needed to evaluate whether and/or how ACA MCs confer protection against swine infection.

Concluding Remarks

A successful drug delivery application depends on the effectiveness and suitability of the delivery system. The participation of a variety of natural as well as synthetic polymers and their applications in various drug delivery systems is a revolution. The versatility and diversity of polymer chemistry greatly offer to design suitable polymeric carriers with desired properties to establish effective drug delivery systems. Oral administration is the most attractive route for drug delivery compared to other systems where polymeric carriers have effective applications to limit the demerits associated with oral administration of drug.

In Chapter I, we have developed an efficient oral vaccine carrier that constitutes PLGA MPs coated with M cell targeting peptide to overcome the some barrier, such as degradation of the vaccine in the gastrointestinal (GI) tract due to the low pH and enzymes in the stomach, the impermeable GI epithelium as a physical barrier and inefficient targeting to the action site, resulting in a low bioavailability. In this study, BmpB as a model vaccine against swine dysentery was loaded into porous PLGA MPs. The PLGA MPs was further coated with the water-soluble chitosan (WSC) conjugated

with M cell homing peptide (CKS9) to prepare BmpB-CKS9-WSC-PLGA MPs. Oral immunization of BmpB vaccine with CKS9-WSC-PLGA MPs in mice showed elevated sIgA responses in the mucosal tissues and systemic IgG antibody responses, providing a complete immune response. Specifically, the immunization with these MPs demonstrated to induce both Th1- and Th2-type responses based on elevated IgG1 and IgG2a titers. The elevated immune responses were attributed to the enhanced M cell targeting and transcytosis ability of CKS9-WSC-PLGA MPs to Peyer's patch regions. The high binding affinity of CKS9-WSC-PLGA MPs with the M cells to enter into the Peyer's patch regions of mouse small intestine was investigated by closed ileal loop assay and it was further confirmed by confocal laser scanning microscopy. These results suggest that the M cell targeting approach used in this study is a promising tool for targeted oral vaccine delivery.

In Chapter II, we have developed an efficient oral vaccine carrier that the encapsulation of vaccine-expressed probiotics into ACA MCs for efficient oral vaccine delivery, to overcome the some barrier, such as the low survival of probiotics in the gastrointestinal tract limits and inefficient targeting to the action site, resulting in a low bioavailability. Here,

recombinant Lactobacillus plantarum 25 (LP25) expressing M cell homing peptide fused BmpB protein was used as a model strain. The viability of LP25 in ACA MCs was more than 65% in simulated gastric fluid (SGF, pH 2.0) and 75% in simulated small intestinal fluid (SIF, pH 7.2) up to 2 h. Encapsulated LP25 were completely released from ACA MCs in SIF within 12 h. When stored at room temperature or 4°C, the viability of LP25 in ACA MCs was higher than free LP25. Interestingly, the viability of LP25 in ACA MCs at 4°C for 5 weeks was above 58%, whereas viability of free LP25 stored at room temperature up to 5 weeks was zero. After 4 weeks from the first immunization, LP25-M-BmpB-loaded ACA MCs induced a stronger BmpB-specific IgG and IgA production in mice. These findings suggest that encapsulation of LP25 by ACA MCs is a promising delivery system for oral administration of vaccine-expressed probiotic.

The currently designed and evaluated polymeric carriers provided effective delivery systems in mice for oral administration of afore-mentioned vaccines and probiotics. Additional investigations should be performed in pigs for future advancements of these novel polymeric carriers as various vaccines and probiotics delivery systems.

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Appendix

Oral delivery of probiotics in poultry using pH-sensitive tablets

1. Introduction

Recently, many developed countries have banned on the use of antibiotics for treating and preventing animal diseases, and growth-promoting of live stocks (Allen et al., 2013) due to the appearance of resistant pathogens related with human and animal diseases (Stanton, 2013). Although alternatives to antibiotics in live stocks are urgently required, it is very hard to find them due to the complexity of the gastrointestinal (GI) ecosystem (Allen et al., 2013).

As a substitute, probiotics may replace the antibiotics as they can inhibit colonization of pathogens on the intestinal receptor. Usually, probiotics confer a health benefit on the host by improving their intestinal microbial balance that can help kill the bad bacteria and fight infection. As a consequence, probiotics are generally regarded as safe, and therefore, they are extensively used in food products. Moreover, they can manage to survive under the acidic conditions of stomach when administered orally although the rate of survival is strain-dependent (Wells and Mercenier,

2008). Among probiotics, *Pediococcusacidilactia* (PA) has been reported to confer antimicrobial activity against *Salmonella gallinarum*, a major pathogen of poultry (Lee et al., 2007).

Oral delivery of probiotics, as one of therapeutics in animals, is highly promising due to easy handling, reduced time, low cost and reduced labor. However, oral delivery of probiotics has to overcome several problems associated with the instability of the probiotics at acidic pH condition of stomach and abundant enzymes in intestine, and poor absorption of probiotics in intestine (Klayraung et al., 2009). Therefore, pH-sensitive polymers in the form of tablets using hydroxypropyl methylcellulose phthalate (HPMCP) (Klayraung et al., 2009), hydroxypropyl methylcellulose acetate succinate (HPMCAS) (Stadler and Viernstein, 2003), cellulose acetate phthalate (CAP) (Silva et al., 2013) and carboxymethyl high amylase starch (CM-HAS) (Calinescu et al., 2005) have been used to deliver probiotics. Due to pH sensitive property of the polymers, the probiotics in the pH-sensitive tablets can be protected from gastric pH and harsh conditions found in the duodenum (Wang and Zhang, 2012). However, pH-sensitive polymers were almost used to deliver the probiotics for human health benefit, not for animals.

In this study, we used pH-sensitive tablets for oral delivery of probiotics in poultry. The probiotic-loaded tablets have several advantages such as simple and convenient production, resistance to environment, easy control of dosage and high patient compliance.

2. Materials and methods

1) Materials

Genome shuffled (GS1) PA was constructed (Song A, 2012) to improve antimicrobial activity of wild-type PA. HPMCP 55 was kindly provided by Shin-Etsu Chemicals Ltd. (Tokyo, Japan). Difco^{TM} lactobacilli MRS broth and lactobacilli MRS agar were purchased from BD (Sparks, MD, USA). Novobiocin, nystatin, vancomycin and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2) Preparation of tablets

Tablets were prepared by a direct compression using a single tablet press at room temperature. An exactly weighed powder mixture (25 mg) of GS1 and HPMCP (weight ratio of GS1 to HPMCP= 1:1) was filled into a die of 4 mm diameter and the tablets were formed under a determined pressure ranging from 3 to 10 kilopascal (KP) with a plane surface.

3) Viability test in tablets

3-1) Viability assay of GS1 inside the tablet

Each tablet was broken and dispersed in 1 ml of phosphate buffer solution (PBS, pH 7.2). The GS1 suspension was then spread onto the pre-dried MRS agar plates. Then, the plates were incubated at 37 ℃ for 24-36 h. Colonies of GS1 were counted and converted to log cfu (colony forming units). The viability of probiotic cells was calculated by the following equation.

Viability (%) = cfu after exposure to the test medium / cfu before equation to the test medium × 100

3-2) Viability assay of GS1-loaded tablets in fluid media

GS1-loaded tablets were transferred into 5 ml of simulated gastric fluid (SGF) (pH 2.0) or PBS (pH 7.2). After the end of the incubation period in the incubator (100 rpm at 37 ℃), the viable cells in the medium and non-disintegrated tested tablets were determined by the same method described above.

4) Disintegration time of GS1-loaded tablets in PBS against compression force

The test tablets prepared by different compression force were transferred into 5 ml of PBS (pH 7.2) and complete disintegration time of GS1-loaded

tablets was measured.

5) Stability of GS1-loaded tablets

For stability test during storage of the tablets, the GS1-loaded tablets were kept in tight light resistant containers at 4 ℃ and room temperature up to 6 months. The stability of GS1 in terms of cell viability in the tablet was monitored as described above for 6 consecutive months.

6) Oral administration in chicken

Eleven day-old broiler chickens (Ross 308, mixed sex) were used for oral administration. The chickens were provided with free access to water and feeds during experiments. The chickens were used in accordance with the guide lines for the care and use of laboratory animals (Seoul National University). The chickens were divided into 3 cohorts (6 chickens per cohort). Per-oral administration in chickens were performed with a dose of probiotics containing 2×10^8 cfu suspended in the appropriate volume of pediococci selective medium (Leuschner et al., 2003) of 1 ml administered by intragastrical inoculation using a feeding needle or one tablet containing 2×10^8 cfu. Only PBS was used as a control. All cohorts received a total of 5 doses of the probiotics (or control) on hours 0, 4, 24, 28, and 48 (Figure 1). To investigate viable cells, the contents of the muscular stomach, small

intestines and cecum of the chicken were immediately collected after sacrifice. The contents were homogenized inappropriate volume of ice-cold PBS. The viable cells in the medium were determined by the same method described above.

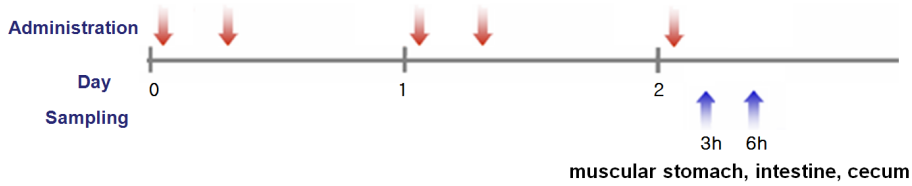

Figure 1. Scheme of oral administration in chicken.

3. Results

1) Effects of compression force and tablet properties on cell viability

The effect of compression force during tabletting on viability of GS1 was investigated. It was found that viability of GS1 decreased slightly with an increase of compression force during tabletting as shown in Figure 2. The change in the morphology of tablet (compression force: 10 KP) was investigated by immersing the tablet in SGF (Figure 3). It was observed that the tablets showed no disintegration within 2 h in the gastric mimicking acidic medium. The viability of GS1 inside the non-disintegrated tablets after SGF immersion was then determined. The results showed that the viability of GS1 in the tablets decreased with the increase of immersion

time and increased with the increase of compression force as shown Figure 4.

Effect of compression force on disintegration time of GS1-loaded tablets in PBS (pH 6.8) was also investigated. As shown in Figure 5, disintegration time of the GS1-loaded tablets was increased with the increase of compression force. While the GS1-loaded tablets prepared with the highest compression force of 10 KP was hardly disintegrated within 1 h, those prepared from the compression force of 3 KP was disintegrated within 30 min.

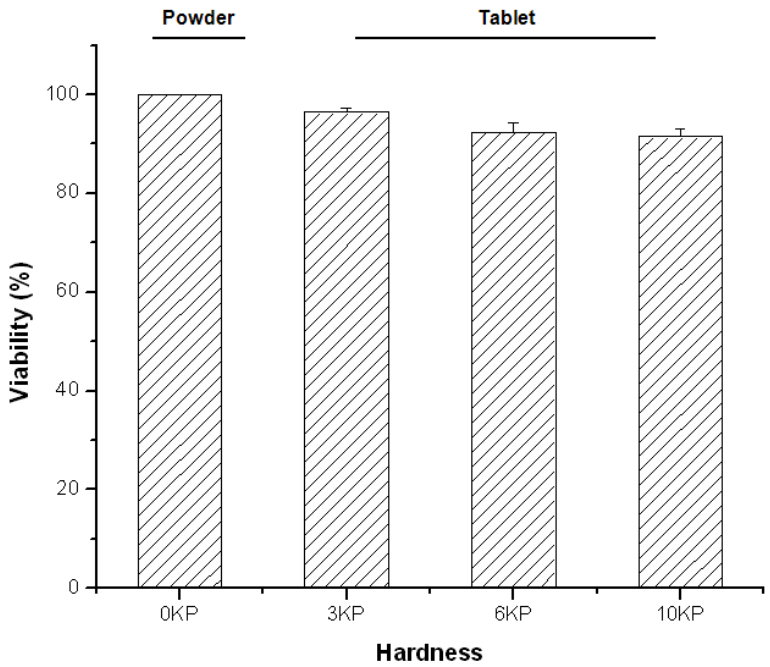

Figure 2. Viability during tabletting (n=3).

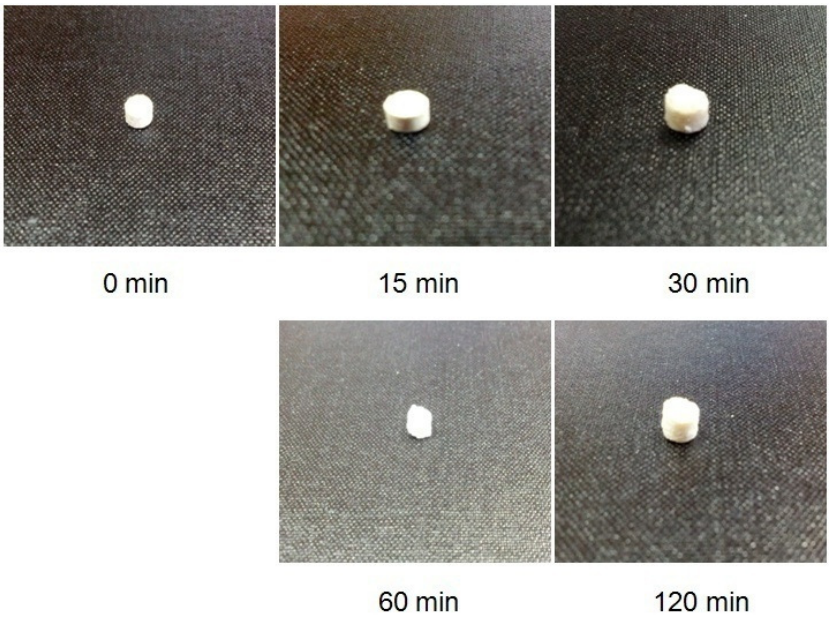

Figure 3. Shape change of tablet incubated in SGF against time.

2) Release of GS1 from GS1-loaded tablets in SGF and SIF

Release test was done by immersing the GS1-loaded tablets for 1.5-2 h in SIF and the released probiotics were calculated. As shown in Figure 6, initially, after 1 h in SGF, no viable released probiotics from the GS1-loaded tablets were found in the gastric medium. The GS1-loaded tablets partially liberated probiotics during the first 30 min in SIF. The higher compression force during tabletting provided delayed liberation of the probiotics from GS1-loaded tablets. Figure 7 shows cell viability of the GS1 in the GS1-loaded tablets after immersing in fluid media of SGF and SIF. The results indicated that cell viability inside the GS1-loaded tablets in

SGF was slowly decreased with time and the cell viability in SIF was slightly changed after 7 h, indicating that the HPMCP 55 does not affect cell viability of the released probiotics from the GS1-loaded tablets in SIF.

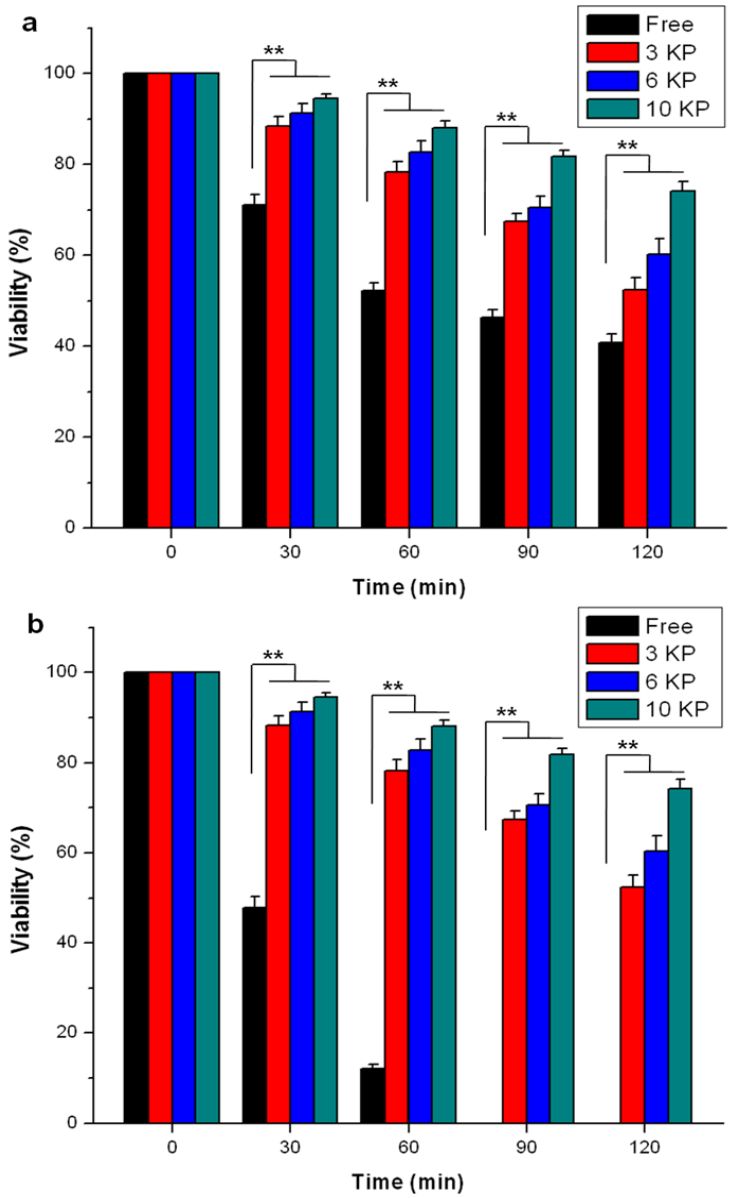

Figure 4. Survivability of GS1 in tablet at SGF (pH 2.0) without pepsin (a) with pepsin (b) (means±SD, n=3, **p < 0.01).

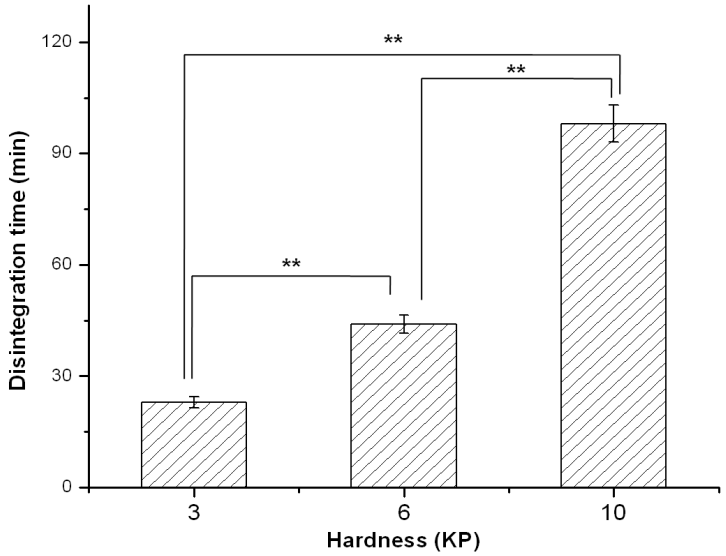

Figure 5. Disintegration study of tablet in PBS (pH 6.8) (means±SD, n=3, **p < 0.01)

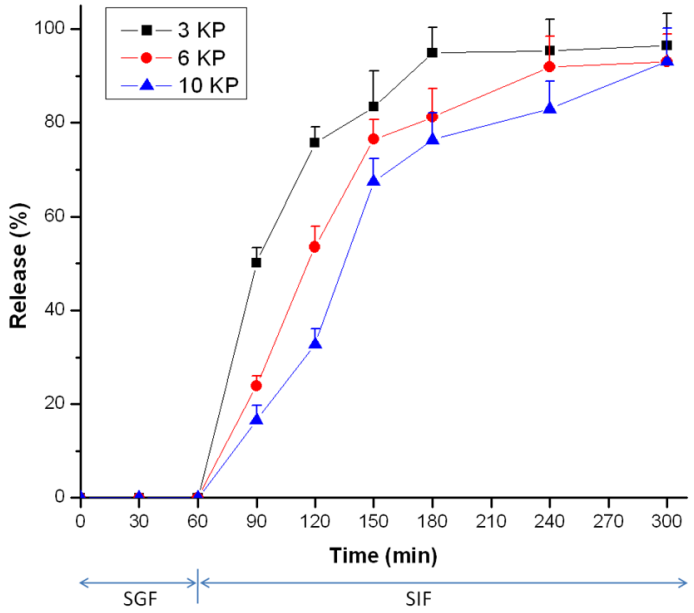

Figure 6. Release of live bacteria from tablets during sequential exposure to SGF and SIF.

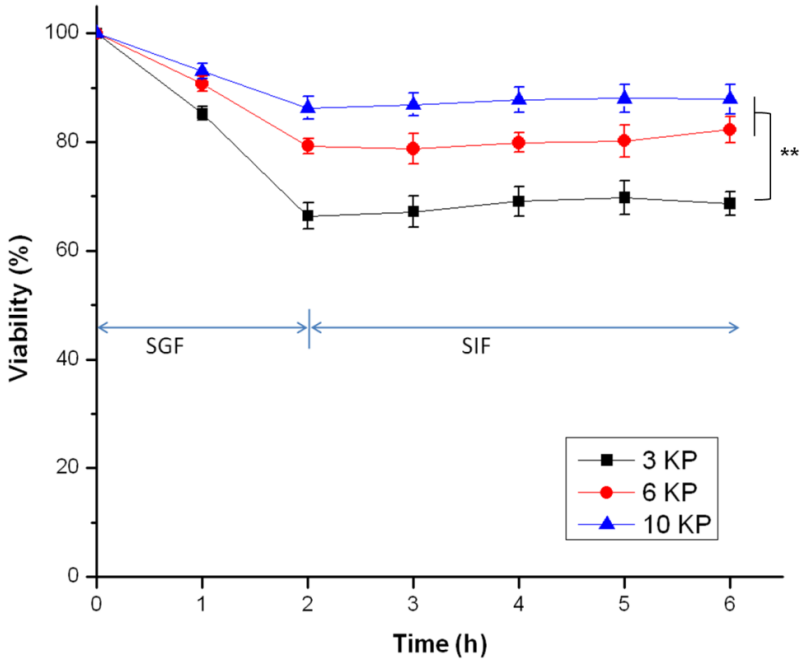

Figure 7. Survivability of GS1 in tablet at SIF (pH 6.8) (means±SD, n=3, **p < 0.01).

3) Stability of the GS1-loaded tablets

Stability of probiotics in terms of cell viability is one of the major indexes which indicates the possibility of poultry excipients and dosage forms to protect the probiotics with long shelf life. Two different temperatures (4 ℃ and room temperature) were selected to evaluate the stability of the GS1-loaded tablets because the selected temperatures represent the common cool storage as in a household refrigerator and ambient room temperature, respectively. Figure 8 shows cell viability inside GS1-loaded tablets after storage at 4 ℃ and room temperature. The results

indicated that storage at room temperature caused more significant decreases in cell viability than storage at 4 ℃. After 6 months of storage at 4 ℃, the loss of cell viability in the GS1-loaded tablets obtained at compression force of 10 KP was observed to be less than 1 log unit, indicating that storage temperature affects the cell stability.

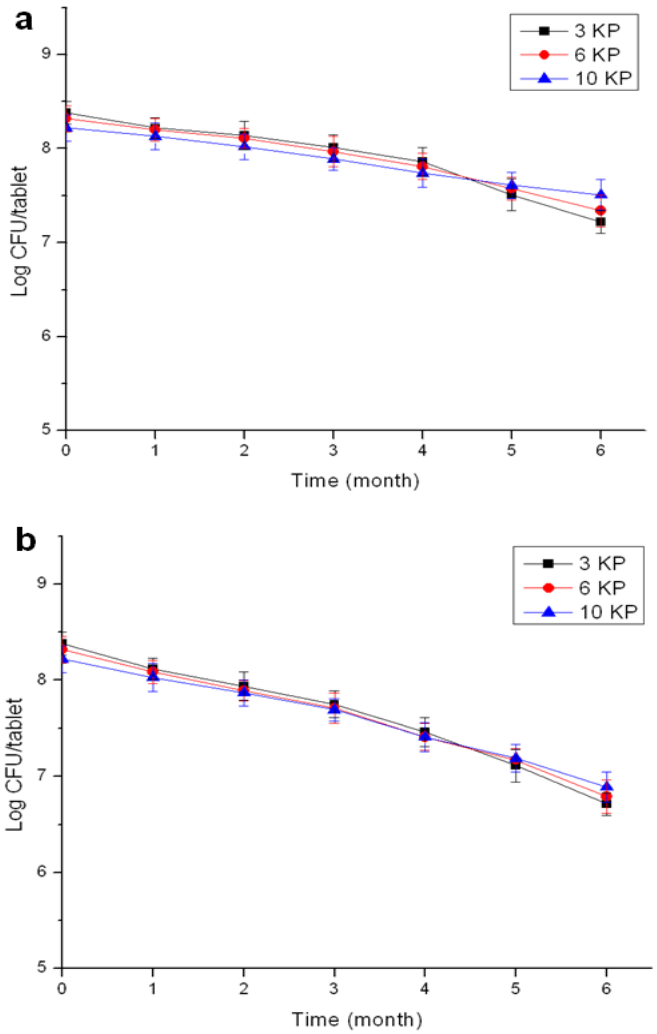

Figure 8. Viability of tablets during 6 months of storage at 4°C (a) and RT (b).

4) Oral administration in chicken

Since the probiotics were orally given to chicken as either tablet or solution form, the viability of probiotics in 6 digestive areas such as muscular stomach (MS), up portion of small intestine (UI), middle portion of small intestine (MI), down portion of small intestine (DI) and cecum (CE) at 3 and 6 h after oral administration were investigated. As shown in Figure 9 (a), more viable cells were observed by oral administration of the tablets than solution inseveral digestive areas of chicken at 3 h after oral administration, suggesting that the tablets could protect probiotics in the digestive areas of chicken. Especially, the survivability of probiotics was 1.3, 1.3, and 1.25 folds higher in MS, MI and DI respectively, by delivering with tablets, than solution. After 6 h of administration, the survivability of probiotics was also higher by using the tablets than solution in several digestive areas except MS of chicken. Especially, the survivability of probiotics was 1.5 fold higher in UI by using tablet than solution.

4. Discussion

The probiotic products available in the market nowadays are mostly in the form of liquid or semisolid formulations which show low cell viability after oral administration, mainly because the bacteria do not survive the harsh conditions in the stomach. The development of suitable dry dosage forms enable higher bacterial survival and consequently is the main aim of the present study. The formulation of probiotics into tablets is an emerging method to reduce cell death during GI passage, as well as an opportunity to control release of these cells across the intestinal tract as a safe and effective oral delivery for feed supplement applications. In this study, the effects on bacterial survival during tabletting were investigated. The results suggested that the used compression force during tabletting does not affect the viability of GS1 which is consistent with the previous result (Calinescu et al., 2005). The viability of GS1-loaded tablets immersed in SGF after 2 h indicated that increasing the compression force to 10 KP improved the tablet efficacy in protecting bacterial cells against acidic challenge. Especially, the survivability of GS1 in GS1-loaded tablets obtained from compression force of 10 KP without pepsin and with pepsin after 2 h was 80% and 75%, respectively, suggesting that the compression force is

considered to be one of the important tablet properties to protect the cells inside the tablet from the contact fluid. The disintegration time of the GS1-loaded tablets indicated that pH-sensitive HPMCP 55 as a tablet excipient with sufficient compression force allowed the preparation of GS1-loaded tablets with suitable properties in terms of long disintegration time and high probiotic cell viability.

The release of the probiotics is closely related to tablet swelling because the HPMCP 55 is insoluble in SGF and soluble in SIF due to the pH-sensitive property. Also, the swelling degree of the HPMCP 55 increased at pH 6.8 than at pH 2.0. The release behavior of GS1 release from GS1-loaded tablets suggested that the release of probiotics from the GS1-loaded tablets can be controlled by the compression force.

The main goal of this work was to evaluate the use of pH-sensitive tablets for efficient oral delivery of probiotics in poultry. Generally, it has been reported that the transient time of the ingested food from the stomach to the intestinal tract for the chicken is about 3 h (Vanbelle M, 1999). Therefore, the tablets should ideally protect probiotics until this time and then should release viable probiotics in the intestinal tract. Here HPMCP 55 tablets prepared with compression force of 10 KP were disintegrated within

2 h in the SIF. Viable cells in GI at 3 and 6 h after oral administration of GS1-loaded tablets in chicken were investigated. It was observed that the survivability of probiotics was higher inseveral digestive areas of chicken at 3 h after oral administration of tablet than solution, suggesting that the tablets can protect and control release of the probiotics in the digestive areas of chicken.

4. Conclusions

This research showed that the extent of cell survival depended on the compression force of the tablets using pH-sensitive HPMCP 55 as a matrix forming material and the probiotics formulated in the tablets were protected at the harsh conditions of stomach in vitro and in digestive areas of chicken. Almost probiotics were preserved as viable cells when the tablets were stored at 4°C for 6 months. These results suggest the possibility of probiotics-loaded tablets as alternatives to antibiotics in poultry.

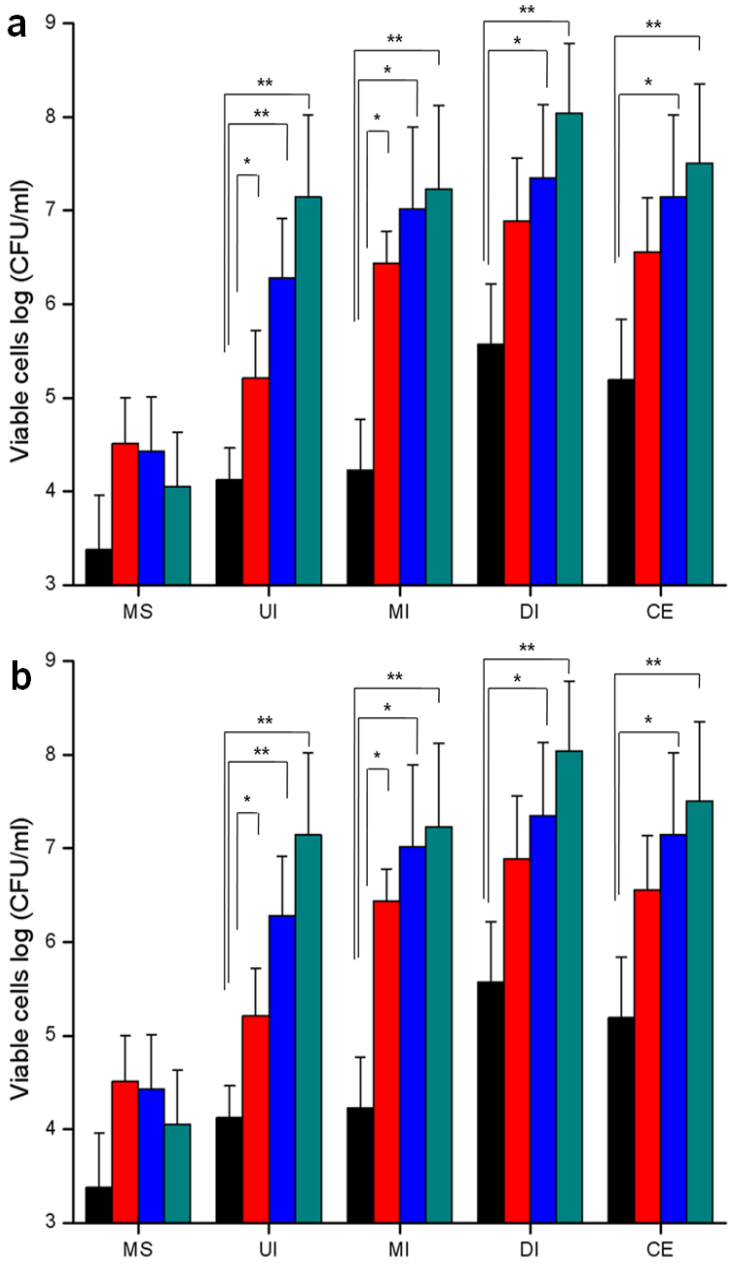

Figure 9. Viable cells of digestive system. UI: up portion of small intestine, MS: muscular stomach, MI: middle portion of small intestine, CE: cecum, DI: down portion of small intestine (means±SD, n=3, *p<0.05, **p < 0.01).

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Summary in Korean

경구를 통한 백신 전달방식은 기타 경로 비해 환자에게 거부감이 없고 스스로 쉽게 조작방법에 따라 투여할 수 있을 뿐 더러 생산적인 측면에서 가격이 저렴하며 또 주사에 따른 고통과 감염의 위험성이 적다. 또한 경구를 통한 백신은 체순환면역반응과 점막면역반응을 동시에 유도할 수 있어서 우수한 백신전달 방법으로 각광을 받고 있다. 그러나 경구백신은 위산과 소화 장관의 효소에 의한 방해로 인해 백신 혹은 생균제를 포함한 경구전달 약물들의 생물학적 이용도가 떨어지는 제한점이 있다. 이러한 문제점들은 조절방출(control release)할 수 있는 폴리머 기반의 전달체를 이용하여 위산과 소화 장관의 효소로부터 전달약물을 보호함으로써 극복할 수 있다.

첫 번째 연구는 poly(lactic-co-glycolic acid)(PLGA) 마이크로 입자에 M 세포 표적형 펩타이드를 연결시킨 경구 백신 전달체의 개발이다. 소장 점막면역 유도에 있어 핵심이 되는 M 세포는 백신을 점막 면역 조직에 효과적으로 전달할 수 있는 한 개의 관문 역할을 한다. 이 연구에서는 *Brachyspira hyodysenteriae* (BmpB)의 외각 단백질을 돼지 설사병에 대한 모델 백신으로 다공성의 PLGA 마이크로 입자(MPs)에 담지시켰다. 또한, BmpB-CKS9-WSC-PLGA MPs 를 구성하기 위해 PLGA MPs 에 수용성의 키토산 (WSC)을 코팅하고 M 세포 표적형 펩타이드 (CKS9)를 접합시킴으로써 M 세포를 표적할 수 있는 능력을 부여하였다. BmpB 백신을 담지한 CKS9-WSC-PGLA MPs 은 경구를 통한 면역을 통해 쥐의 점막 조직에서 IgA 의 분비 반응, 체순환 IgG 항체의 반응을 상승시킴으로써 효과적인 면역 반응을 보였다. 특히, MPs 을 이용한 면역은 Th1 타입과 Th2 타입의 면역반응, 즉 그 지표인 IgG2a 와 IgG1 을 통해 모두 증가하는 것을 확인하였다. 이러한 면역반응은 CKS9-WSC-PLGA MPs 의 M 세포 표적능력으로 인한 peryer's patch 로의 통과(transcytosis)능력이 향상되는 것에 기인한다. 마우스의 소장 peyer's patch 로 들어가기 위한 CKS9-WSC-PLGA MPs 과 M 세포의 높은 결합친화성은 closed ileal loop assay 를 통해 검증하였고 이는 공초점 레이저 현미경에 의해 결과로 제시되었다.

이러한 연구 결과로부터 CKS9-WSC-PLGA MPs 은 효과적인 경구 백신 전달을 위한 유망한 방법임을 검증하였다.

두 번째 연구는 경구백신의 항원 전달체로 생균제를 사용하기 위한 목적으로 생균제를 경구로 투여하기 위한 전달체를 개발하는 것이다. 생균제를 소장으로 전달하기 위한 가장 큰 장애물은 위산의 낮은 pH 로 인한 생균제의 낮은 생존율을 극복하는 것이다. 이 연구는 생균제의 생존율을 높이는 목적으로 백신을 발현하고 있는 생균제를 알긴산/키토산/알긴산 마이크로캡슐에 캡슐화 시켜서 전달함으로써 효과적인 백신 전달체를 개발하는 것이다. 이 연구는 M 세포 표적형 펩타이드가 결합 된 BmpB 단백질을 발현하는 재조합 *Lactobacillus plantarum25*(LP25)을 모델 생균제로 사용하였다. 위의 환경을 모방한 위액 (SGF, pH 2.0)과 소장의 환경을 모방한 소장액 (SIF, pH 7.2)에서 2h 내에 캡슐화 된 LP25 의 생존율은 65%와 70%에 도달하였다. 또한 캡슐화 된 LP25 는 SIF 에서 12h 내에 전 부 방출이 되는 것을 확인하였다. 저장실험결과 실온과 4°C 에서 캡슐화 된 LP25 의 생존율은 캡슐화 시키지 않은 LP25 에 비해 높은 것을 확인하였다. 흥미로운 것은 캡슐화 된 LP25 의 경우 4°C 에서 5 주 후의 생존율은 58%에 달하는 반면 캡슐화 되지 않은 LP25 의 경우 상온에서 5 주 후의 생존율은 0%였다. 마우스 모델을 이용한 경구 면역실험에서 캡슐화 된 항원 생산 LP25 를 경구투여 한 마우스에서 BmpB 특이적인 IgG 와 IgA 가 캡슐화 되지 않은 생균제를 경구 투여한 마우스에 비해 많이 측정되었다. 종합적으로 마이크로캡슐의 백신 생산 생균제의 캡슐화는 소화 장관으로의 백신을 전달 할 수 있는 유망한 전달체임을 검증하였다.

주요어: 경구 전달, M 세포 표적형 펩타이드, PLGA 마이크로 입자, BmpB, 키토산, 알긴산, 마이크로 캡슐화, *Lactobacillus plantarum* 25, 생균제

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