Research Article

Oral Methylated N-Aryl Chitosan Derivatives for Inducing Immune Responses to Ovalbumin

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Abstract

Purpose: To investigate different structures of modified chitosan containing different chain lengths and aromatic moieties for vaccine delivery capacity.

Methods: The characteristics of the modified chitosan, namely, methylated N-(4-N,N-dimethylaminobenzyl) chitosan (TM-Bz-CS), methylated N-(4-N,N-dimethylaminocinnamyl) chitosan (TM-CM-CS) and methylated N-(4-pyridinylmethyl) chitosan (TM-Py-CS), with Eqiva degree (equivalent degree) were studied by in vitro absorption enhancement on the transepithelial electrical resistance (TEER) in Caco-2 cell monolayers as well as by in vivo adjuvant activity against ovalbumin (OVA), a model antigen, via oral administration to BALB/c mice.

Results: At the same concentration and pH (0.1 mg/ml, pH 7.4), TM₆₅CS₅₀ exhibited the highest in vitro enhancing paracellular permeability and also the highest in vivo adjuvant activity following oral administration to mice. OVA-specific serum immunoglobulin G (IgG) antibody levels of mice that received OVA in TM₆₅CS₅₀ were significantly (p < 0.05) higher than those that received OVA in TM₅₆CS₄₂, TM₅₃Bz₄₀CS and TM₅₃Py₄₀CS. On the other hand, TM₅₆CS and TM₅₃Bz₄₀CS exhibited in vitro enhancing paracellular permeability but showed no immune responses, while TM₅₃Py₄₀CS failed to enhance paracellular permeability and did not elicit immune responses as well.

Conclusion: This study demonstrates that addition of hydrophobic moiety (dimethylaminocinnamyl) to CS backbone can increase both its absorption enhancing property and adjuvant activity. The chemical structure and the positive charge location play an important role for binding affinity, absorption enhancement and immune responses.

Keywords: Chitosan derivatives; Absorption enhancement; Oral vaccine delivery; Immunoadjuvants; Ovalbumin.

Received: 8 April 2012 Revised accepted: 5 November 2012

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INTRODUCTION

In recent years, considerable research has been focused on non-invasive delivery of vaccines. Among non-invasive routes, vaccination by the oral route remains the preferred route for non-injectable vaccination due to its convenience for both patients and practitioners. However, poor immunogenicity and impaired antigen delivery still remain to be improved. Diverse strategies have been developed to improve the bioavailability of vaccines. Some focused on adjuvants that boost the potency and longevity of a specific immune response to antigens, causing only minimal toxicity or long-lasting immune effects on their own. The mechanism of action of an adjuvant is mainly either as an immunostimulant or as a delivery system [1].

Chitosan (CS) [(1→4)-2-amino-2-deoxy-β-D-glucan] is a copolymer of N-acetyl glucosamine (GlcNac) and glucosamine (GlcN). It is a deacetylated chitin that is now of great interest as a functional material of great potential in various areas, such as the biomedical field. CS has been extensively studied for delivery of therapeutic proteins and antigens particularly via mucosal routes because of their excellent mucoadhesive and absorption enhancing properties [2]. Both properties aid to stimulate the absorption of protein/antigen. In addition, CS has been shown to induce both cellular and humoral responses when administered via parenteral, mucosal, or transcutaneous routes [3]. Various studies have demonstrated the activation of the dendritic cells, macrophages, and lymphocytes by CS [4]. However, the main drawback of CS is its water-insoluble property at physiological pH. CS is readily soluble in dilute acidic solutions below pH 6.0. With increasing pH, the amino groups become deprotonated and the polymer loses its charge and become insoluble. Versatility in the physicochemical properties of CS allows the formulator an excellent opportunity to engineer antigen-specific adjuvant/delivery systems. Many CS derivatives have been synthesized to enhance its solubility, mucoadhesiveness and/or its immunostimulatory properties.

Recently, our research group successfully synthesized modified chitosans, viz, methylated N-(4-N,N-dimethylaminobenzyl) chitosan (TM-Bz-CS), methylated N-(4-N,N-dimethylamino-cinnamyl) chitosan (TM-CM-CS) and methylated N-(4-pyridiniumethyl) chitosan (TM-Py-CS), which showed mucoadhesive properties [5], and in vitro absorption enhancement properties [6,7]. Based on these results, the aim of the present study was to further investigate the feasibility of applying these modified CS as an adjuvant for inducing immune responses to ovalbumin (OVA), as model antigen, via the oral route. The degree of quaternization (DQ) and extent of N-substitution (ES) of the modified CS used in this study are based on our previous report [8].

EXPERIMENTAL

Materials

Chitosan, with an average molecular weight (Mw) of 276 kDa (94 % degree of deacetylation), was purchased from Seafresh Chitosan (Lab) Co, Ltd, Thailand. 4-Dimethylaminobenzaldehyde, 4-Dimethylaminocinnamaldehyde and 4-pyridine-carboxaldehyde were purchased from Fluka (Deisenhofen, Germany). Sodium cyanoborohydride, iodomethane, and 1-methyl-2-pyrrolidone were purchased from Acros Organics (Geel, Belgium). Sodium iodide was purchased from Carlo Erba Reagent (Italy). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma-Chemical Co. (St. Louis, MO, USA). The Caco-2 cell line was obtained from American Type Culture Collection (Rockville, MD, USA). Transwell (12-well plates) cell culture chambers with a 3.0 µm pore size were purchased from Corning Life Sciences (Massachusetts, USA). For determination of immune responses, aluminum hydroxide gel (alum), albumin from chicken egg white (Ovalbumin-OVA, Grade
V, MW=44 kDa) and monoclonal anti-chicken egg albumin (mAb to OVA) were purchased from Sigma Aldrich (St. Louis, USA). HRP-rabbit anti-mouse (Gamma), and 3,3′,5,5′-tetramethylbenzidine (TMB) were supplied by Zymed (Invitrogen, San Francisco, USA). All other chemicals were of molecular biology quality.

**Synthesis of the methylated N-aryl chitosan derivatives**

The N-aryl chitosan derivatives were carried out in accordance with the previous reported procedure [9] whereas the methylation of chitosan and N-aryl chitosan derivatives have been carried out by a single treatment with iodomethane in the presence of N-methyl pyrrolidone (NMP) and sodium hydroxide [10]. The chemical structures of methylated N-aryl chitosan derivatives are shown in Fig. 1.

![Chemical structures of methylated N-aryl chitosan derivatives](image)

**Figure 1:** Chemical structures of methylated N-aryl chitosan derivatives: (a) methylated N-(4-N,N-dimethylaminocinnamyl) chitosan (TM-CM-CS), (b) methylated N-(4-N,N-dimethylaminobenzyl) chitosan (TM-Bz-CS), (c) methylated N-(4-pyridylmethyl) chitosan (TM-Py-CS).

**Cell cultures**

Caco-2 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) at a pH of 7.4, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% non-essential amino acid solution and 0.1% penicillin-streptomycin solution in a humidified atmosphere (5% CO₂, 95% air, 37 °C). The cells were grown under standard conditions until 60-70% confluency. Cells from passages 40-50 were used for all of the experiments. The cells were seeded on tissue culture polycarbonate membrane filters (pore size, 3.0 µm) in 12-well Transwell® plates (Costar®, corning Inc., Corning, NY) at a seeding density of 2x10⁴ cells/cm². The culture medium was added to both the donor and the acceptor compartment. Medium was changed every second day. The cells were left to differentiate for 15-21 days after seeding with monitoring of trans-epithelial electrical resistance (TEER) values were more than 600 Ω.cm² using a Millicell® ERS meter (Millipore, Bedford, MA, USA).

**Measurement of the trans-epithelial electrical resistance (TEER)**

The values of TEER were determined by measuring the potential difference between the two sides of the cell monolayer using a Millicell® ERS meter (Millipore, Bedford, MA, USA) connected to a pair of chopstick electrodes. The procedure of TEER measurement was carried out in accordance as described in a previous report [6]. Briefly, on the day of the experiments, the cells were washed twice with phosphate buffered saline (PBS) and pre-equilibrated for 1 h with Hank balanced salt solution (HBSS) buffered at pH 7.4. After removing the medium, the Caco-2 cell monolayers were treated with chitosan derivative solutions (0.1 mg/ml in HBSS at pH 7.4) in the apical compartment. The TEER was measured every 20 min. After 2 h treatment, the cells were carefully washed twice with PBS and incubated with a fresh culture medium. The recovery of TEER values was monitored for 24 h after treatment.

**Evaluation of cytotoxicity**

The cytotoxic effects of CS derivatives were investigated with Caco-2 cells using the MTT
cytotoxicity assay. Cells were seeded at a density of $2 \times 10^4$ cells/well in 96-well cell culture plates. After pre-incubation for 24 h, cells were then treated with CS derivatives at various concentrations ranging from 0.01 to 1 mg/ml in serum-free medium (pH 7.4) and incubated for 24 h. Dilutions of CS derivatives were made using serum-free medium to ensure that the cells did not die from nutrition deficiency. After treatment, CS derivative solutions were removed, fresh medium was added and the cells were incubated for 4 h. Finally, the cells were incubated with 100 μl MTT-containing medium (0.1 mg/mL MTT in serum-free medium) for 4 h. Next, the medium was removed, and the formazan crystal that formed in the living cells was dissolved in 100 μl DMSO per well. The relative viability (%) was calculated based on absorbance at 550 nm using a microplate reader (Universal Microplate Analyzer, Model AOPUS01 and A53601, Packard BioScience, CT, USA). The viability of non-treated control cells was arbitrarily defined as 100 % [11]. The relative cell viability was calculated as in Eq 1, and IC$_{50}$ was obtained as the CS concentration that inhibited the growth of 50 % of the cells relative to non-treated control cells.

Relative cell viability = ($S/C$)100 ............ (1)

where S is the difference between the absorbance of of sample and blank, and C is the difference between the absorbance of control and blank.

**Oral immunization**

Female BALB/c mice, 6 – 8 weeks of age at the beginning of the experiment, were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. The animals were housed at controlled temperature with free access to rodent chow and water. All studies were evaluated and performed in accordance with the Animal Ethics Committee of Silpakorn University, Thailand. Mice were divided into six groups and each group comprised six mice. The mice were immunized with the various formulations as shown in Table 1. Mice in all groups were immunized on days 0 and 14. For group A–D and N, dose volume was 400 μl containing 500 μg of OVA. For the positive control group (group P), the mice were immunized subcutaneously (s.c.) in the neck region with 200 μl of alum containing 100 μg of OVA.

**Table 1:** Immunization formulations for mice groups A – D, P and N

| Mice group | Formulation | Route of administration |
|------------|-------------|-------------------------|
| P          | 100 μg of OVA in PBS, pH 7.4 with Al(OH)$_3$ | s.c. |
| N          | 500 μg of OVA in PBS, pH 7.4 | p.o. |
| A          | 0.1% w/v TM$_5$CS solution | p.o. |
| B          | TM$_5$GM$_5$CS solution | p.o. |
| C          | TM$_5$Bz$_5$CS solution | p.o. |
| D          | TM$_5$Py$_5$CS solution | p.o. |

**Note:** s.c. = subcutaneous; p.o. = peroral

**Sample collection**

On day 0, blood samples (ca. 0.2 ml per animal) were collected from the cut tail tip. However, at the end of the study (day 21), the blood (ca. 0.6 – 1 ml per animal) was collected by cardiac puncture following anesthetizing the mice with diethyl ether. The blood samples were allowed to clot overnight and then centrifuged at 8000 g for 5 min at room temperature. For tail bleeds, serum was collected and pooled for each group of mice. For blood collected by cardiac puncture, serum from each mouse was kept separately.
All serum samples were stored at −20 °C until assayed.

**Determination of immune responses**

Immune responses to OVA dissolved in various formulations were analyzed by Enzyme-Linked Immunosorbent Assay (ELISA) in order to determine the levels of OVA-specific serum immunoglobulin G (IgG) antibody as described by Pitaksuteeppong [12]. The absorbance was measured at a wavelength of 450 nm using a microplate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601, Packard BioScience, CT, USA).

**Statistical analysis**

All experimental measurements were collected in triplicate. Values are expressed as mean ± standard deviation (SD). Significant differences in permeability enhancement and cell viability were examined using one-way analysis of variance (ANOVA) followed by a least significant difference (LSD). Post-hoc test using SPSS software (version 11). The level of significance was set at p < 0.05.

**RESULTS**

**Synthesis of the methylated N-aryl chitosan derivatives**

Table 2 summarized the results of CS with various aromatic aldehydes. The extent of N-substitution (ES) was determined by 1H NMR spectroscopic method as described in previous report [7]. It was found that the ES was in the range of 40 – 50 %. Methylation of N-aryl chitosan derivatives was carried out by single treatment with iodomethane which yielded the corresponding quaternary ammonium CS derivatives. Methylation occurred at both the aromatic substituent and the primary amino groups of CS [11]. Degree of quaternization (DQ) was in the range of 53 – 65 %, calculated by 1H NMR spectroscopy. Besides quaternization, N,N-dimethylation, N-methylation, and O-methylation at the primary amino groups and hydroxyl groups of CS were also observed.

**Effect of methylated N-aryl chitosan derivatives on TEER**

The effect of methylated N-aryl chitosan derivatives with various aromatic aldehydes on TEER of Caco-2 cell monolayers is shown in Fig 2.

The incubation of the monolayers on the apical side with 0.1 mg/ml polymers at pH of 7.4 for 2 h resulted in a significant reduction (p < 0.05) in TEER values compared to the control group (excepted in TM65Py40CS).

TEER value of TM65CM50CS was immediately decreased while TEER values of TM65CS and TM56Bz42CS were gradually decreased. After the polymer solutions were removed, the cells were repeatedly washed and subse-

### Table 2: Methylation of chitosan and N-aryl chitosan derivatives

| Sample       | ES (%) | DQ<sub>Ar</sub> (%) | DQ<sub>CS</sub> (%) | N(CH<sub>3</sub>)<sub>2</sub> (%) | NHCH<sub>3</sub> (%) | Total O-CH<sub>3</sub> (%) |
|--------------|--------|----------------------|----------------------|---------------------------------|----------------------|-----------------------------|
| TM65CS       | -      | -                    | 65                   | 23                              | Trace                 | 35                          |
| TM65CM50CS   | 50     | 50                   | 15                   | 24                              | Trace                 | 15                          |
| TM56Bz42CS   | 42     | 42                   | 14                   | 2                               | 17                    | 5                           |
| TM53Py40CS   | 40     | 40                   | 13                   | 2                               | 7                     | 5                           |

**Note:** ES = extent of N-substitution; DQ<sub>Ar</sub> = degree of quaternization at aromatic substituents; DQ<sub>CS</sub> = degree of quaternization at the primary amino groups of chitosan; DQ<sub>T</sub> = total degree of quaternization (DQ<sub>Ar</sub> + DQ<sub>CS</sub>); N(CH<sub>3</sub>)<sub>2</sub> = N,N-dimethylation; NHCH<sub>3</sub> is N-methylation; Total O-CH<sub>3</sub> = total degree of O-methylation of 3-O and 6-O at 3-hydroxyl and 6-hydroxyl positions of GlcN of chitosan, respectively.
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Fig. 2: The effect of CS derivatives at concentration 0.1 mg/ml, pH 7.4, on (a) the TEER of Caco-2 cell monolayers, and (b) the TEER recovery pattern of the Caco-2 cell monolayers. Key: (■) control, (▲) TM$_{53}$Py$_{40}$CS, (▼) TM$_{56}$Bz$_{42}$CS, (□) TM$_{65}$CM$_{50}$CS; n = 3; * p < 0.05.

Consequently supplied with fresh medium, and an increase in resistance towards the initial values was found in the control and in cells treated with CS derivatives by 24 h (Fig 2b). Nevertheless, the completely TEER recovery was obvious within 24 h when cells were treated with TM$_{55}$CS, TM$_{56}$Bz$_{42}$CS and TM$_{53}$Py$_{40}$CS. On the other hand, TM$_{65}$CM$_{50}$CS were reversible only at 50% of TEER recovery.

Cytotoxicity of the methylated N-aryl chitosan derivatives

The effect of CS derivatives on cytotoxicity was determined as cationic polymers that have been known to be cytotoxic materials. The results showed that all CS derivatives tested showed concentration-dependent cytotoxicity in Caco-2 incubated for 24 h at pH 7.4. IC$_{50}$ values of CS derivatives were in the following order: TM$_{53}$Py$_{40}$CS (0.69 ± 0.08 mg/ml), TM$_{56}$Bz$_{42}$CS (0.34 ± 0.01 mg/ml), TM$_{65}$CM$_{50}$CS (0.03 ± 0.01 mg/ml) and TM$_{55}$CS (0.03 ± 0.01 mg/ml). These results suggest that addition of trimethyl groups on the cinnamyl moiety showed high cytotoxicity as well as the methylated CS, whereas addition of trimethyl and methyl groups on the benzyl and pyridyl moieties in the polymer structure could reduce cytotoxicity in Caco-2 cells as shown by the increase in IC$_{50}$ value at 24 h.

Adjuvant activity of the methylated N-aryl chitosan derivatives

Adjuvant activity of the methylated N-aryl chitosan derivatives was determined by measurement OVA-specific serum immunoglobulin G (IgG) antibody. IgG titers at day 0 were very low baseline but the IgG titers following the second booster were significantly increased (Fig 3). The results showed that, on day 21, significant difference of IgG levels was observed in group P and group B compared with group N. OVA in CS derivative solutions induced higher immune responses than OVA in PBS solution but less than OVA in alum injected s.c. Comparing the results of CS derivatives, IgG levels of mice which have received OVA in TM$_{65}$CM$_{50}$CS were significantly higher than those that received OVA in TM$_{65}$CS, TM$_{56}$Bz$_{42}$CS and TM$_{53}$Py$_{40}$CS. These results indicated that at the equal concentration of CS derivatives (0.1 mg/ml), OVA in TM$_{65}$CM$_{50}$CS could elicit higher IgG responses than OVA in the other CS derivatives. Most significantly sera collected from the TM$_{65}$CM$_{50}$CS immunized mice showed greater IgG responses than those collected sera from TM$_{65}$CS (16 times), TM$_{56}$Bz$_{42}$CS (10 times) and TM$_{53}$Py$_{40}$CS (7 times).

DISCUSSION

It is well known that CS solutions cause a significant and dose-dependent decrease of
TEER of the Caco-2 cell monolayers by acting on negatively charged sites at the cell surfaces and tight junctions, and it has been shown that CS is able to induce changes in F-actin distribution [13]. The interaction of CS with the cell membrane results in a structural reorganization of tight junction-associated proteins, followed by enhanced transport through the paracellular pathway. Therefore, binding of CS to Caco-2 cells precedes absorption enhancement, and this increase in absorption is mediated by the positive charges on the polymer [14]. However, cationic polymers have been known to be cytotoxic materials. As a result, CS derivatives containing quaternary ammonium functionality in addition to different hydrophobic substitutions were excellent candidates for novel absorption enhancers. Because of the amphiphilic nature of the cell membrane, an increase in the interaction between the cell membrane and the CS derivative could be favored and safer. Recently, our research group successfully synthesized modified chitosans, methylated N-(4-N,N-dimethylaminobenzyl) chitosan (TM-Bz-CS), methylated N-(4-N,N-dimethylaminocinnamyl) chitosan (TM-CM-CS) and methylated N-(4-pyridinylmethyl) chitosan (TM-Py-CS). These modified CS were synthesized by the covalent bond formation between the primary amino groups of CS and N-aryl group to provide a hydrophobic moiety. Thereafter, the methylation of the CS molecule containing hydrophobic moieties was carried out using iodomethane to render CS soluble. They showed in vitro absorption enhancing properties to hydrophilic macromolecules on tight junction permeability at pH 7.4 in a dose-dependent effect. Our studies demonstrated that these modified CS have the potential to be used as an absorption enhancer of therapeutic macromolecules, and the chemical structure and the positive charge location play an important role for absorption enhancement [6-8].

Therefore, further investigation was carried out by evaluating the in vivo characteristics of three methylated CS containing different aromatic moieties for vaccine delivery. The adjuvant activity of these modified CS was also explored. It has been shown that CS solutions could enhance the immunoadjuvant properties of cytokines when co-administered subcutaneously. CS could also enhance the antigen-presenting capability of dendritic cells and induced greater allogeneic T-cell proliferation. Moreover, CS and its derivatives, TMC, exhibit immunoadjuvants and antigen delivery systems for mucosal vaccinations [15].

Fig 2a shows that incubation of the monolayers on the apical side with appropriate concentration of polymers (0.1 mg/ml) at pH of 7.4 for 2 h resulted in significant reduction in TEER values compared to the control group (excepted in TM65Py40CS), and the TEER of monolayers could recover within 24 h. Our previous study reported that incubation with TM65CM50CS and TM65Bz42CS had greater effect on increasing fluorescein isothiocyanate dextran 4,400 (FD-4) transports than TM65CS and TM53Py40CS. The cumulative amounts transported up to 4 h after incubation with FD-4 were in the following order: TM65CM50CS (20.2 ± 1.6 µg) > TM65Bz42CS

**Fig. 3:** Serum IgG antibody titers at day 0 (white bars) and at day 21 (black bars) obtained from mice following oral immunization with OVA in PBS (group N), TM65CS (group A), TM65CM50CS (group B), TM65Bz42CS (group C), TM65Py40CS (group D), and in alum (group P) administered subcutaneously (positive control); n = 6; * p < 0.05

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(15.8 ± 0.7 µg) > TM$_{53}$Py$_{40}$CS (4.0 ± 2.2 µg) ~ TM$_{65}$CS (3.3 ± 1.2 µg) [8]. The results demonstrate that CS containing aromatic functionality, N-dimethylaminobenzyl and N-dimethylaminocinnamyl groups, affected the decrease of TEER values and FD-4 transport. These results correlate with adjuvant activity of CS derivatives in that IgG antibody titer of TM$_{65}$CM$_{50}$CS group was the highest compared with other CS derivatives groups applied via oral route. However, IgG antibody titer of TM$_{56}$Bz$_{42}$CS group was not a significant difference from the control group as well as TM$_{53}$Py$_{40}$CS and TM$_{65}$CS groups. This finding could be explained that TM$_{65}$CM$_{50}$CS appeared to be more toxic than those modified CS. Hence, at the same concentration of polymers, TM$_{65}$CM$_{50}$CS had a great effect on tight junction permeability (Fig 2a).

Due to the different chain length between CS backbone and quaternary ammonium moieties of the TM-Bz-CS and TM-CM-CS, it was postulated that TM-CM-CS would tightly bind to negatively charged sites more than those of TM-Bz-CS at the cell surfaces and tight junctions followed by enhanced transport through the paracellular pathway. From these results of in vitro studies, it could be explained why TM$_{65}$CM$_{50}$CS had a better effect on adjuvant activity in vivo study than TM$_{56}$Bz$_{42}$CS. In case of TM$_{53}$Py$_{40}$CS which was not observed on tight junction permeability and was not significantly different on immune response after oral administration, it could be possible that the steric hindrance of the N-pyridylmethyl group shielded the positive charges of the quaternary ammonium group on the GlcN of CS, and resulted in hindering the binding of the polymers to negatively charged sites at cell surfaces and tight junctions. Moreover, the positive charge in the pyridine ring could be delocalized by resonance effect, while the positive charges in methylated chitosan derivatives were fixed. The adjuvant activity of TM$_{65}$CS was the lowest. This result was in agreement with the observations of a previous study which showed that TMC with 60 % of DQ showed a low immune response and no significant difference in comparison to control and lower DQ of TMC groups [15].

In addition, although cytotoxicity of TM$_{65}$CS was similar to TM$_{65}$CM$_{50}$CS, its absorption enhancing property and adjuvant activity were lower. The mechanism of these CS derivatives for enhancement of immunogenicity via oral route may be caused from the interaction between CS and the cell membrane, resulting in a structural reorganization of tight junction-associated proteins, followed by enhancing the transport through the paracellular pathway and increasing the antigen absorption. The ability of soluble CS to adjuvant activity is also related to its mucoadhesive property, which increases interpenetration of the mucoadhesive molecules into the mucus glycoproteins [14].

In our previous study, we found that these CS derivatives (TMCS and TMCMCS) showed the mucoadhesive property, depending on the DQ and polymer structure. When the DQ was higher than 65%, the TM$_{65}$CM$_{50}$CS had a similar mucoadhesive property to TM$_{65}$CS [5]. Moreover, TM$_{65}$CM$_{50}$CS could protect the degradation of bovine serum albumin (BSA) when it was co-administered with BSA, and incubated with simulated intestinal fluid containing 1% w/v pancreatin porcine pancreas. These studies indicated that the adjuvant effect of these CS derivatives might be from the combination of the protection of antigen degradation from the gastrointestinal tract fluid [17], the induction of mucoadhesive effects [5], and the enhancement of paracellular transport.

In general, there are two distinctive pathways to allow the transport of antigen into the lymphoid tissue, depending on the nature of antigen. Soluble antigen may be able to penetrate the intestinal epithelium into the lamina propria (LP), and may interact with the antigen presenting cells (APCs) such as macrophages and dendritic cells. The APCs migrate to the lymph node where the antigen
is presented to the T cells as a start of the activation of the IgG immune response cascade. In contrast, antigen in particulate form is largely taken up by M-cells for transportation to gut-associated lymphoid tissue (GALT), and is subsequently transferred to underlying APCs for the initiation of antigen-specific mucosal sIgA and IgG responses [18,19].

Moreover, Seferian et al inoculated BALB/c mice with chitosan plus β- human chorionic gonadotropin, and found that the mixed immune response to IgG1, IgG2a and IgG2b antibodies could be observed in the groups with chitosan emulsion as adjuvant by intraperitoneal injection [20]. Bivas-Benita et al immunized mice with oral *Toxoplasma gondii* GRA1 protein and DNA vaccine-loaded chitosan particles, and successfully induced specialized anti-GRA1 IgG1 and IgG2a, indicating that it can enhance immune response to Th1 and Th2 [21]. Xie et al revealed that *H. pylori* with chitosan solution as an adjuvant can protect against *H. pylori* infection and induce both Th1 and Th2 type immune response by oral [22]. Therefore, the type of immune response of these CS derivatives requires further investigation.

**CONCLUSION**

The water-soluble chitosan derivatives, TM-Bz-CS, TM-CM-CS and TM-Py-CS, have been successfully synthesized. The findings of this study indicate that addition of hydrophobic moiety to CS backbone enhanced its absorption enhancing property and adjuvant activity.

**ACKNOWLEDGMENT**

The authors wish to thank the Silpakorn University Research and Development Institute for financial support.

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