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In vitro and *in vivo* study of *N*-trimethyl chitosan nanoparticles for oral protein delivery

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Abstract

In this study, the effects of alginate modification on absorption properties of FITC-BSA loaded TMC nanoparticles were investigated on an *in vitro* model of GI epithelium (Caco-2 cells). The feasibility of applying TMC nanoparticles loaded with a model vaccine urease in oral vaccination was also studied. Alginate modified TMC nanoparticles showed higher FITC-BSA permeate efficiency than non-modified TMC nanoparticles. However, alginate modification barely had any effect on TMC nanoparticles' property of decreasing TEER or enhancing drug paracellular transport. Mice s.c. immunized with urease loaded TMC nanoparticles showed highest systematic immune response (IgG levels) but the lowest mucosal response (secretory IgA levels). In the contrast, mice i.g. immunized with urease loaded TMC nanoparticles showed much higher antibody titers of both IgG and secretory IgA than those with urease solution or urease co-administrated with TMC solution. These results indicated that TMC nanoparticles are potential carriers for oral protein and vaccine delivery.

Keywords: *N*-Trimethyl chitosan; Nanoparticles; Protein carriers; Oral vaccination

1. Introduction

Oral drug delivery is considered as the preferred route of administration because of its non-invasive nature. It reduces the risk of infection, and do not require trained personnel. Moreover, the micro-fold (M)-cells, mainly located within the epithelium of Peyer's patches, called Follicle Associated Epithelium (FAE), are specialized for antigen sampling, which represent a potential portal for oral delivery of peptides and for mucosal vaccination, since they possess a high transcytotic capacity and are able to transport a broad range of materials, including nanoparticles (Frey and Neutra, 1997; Clark et al., 2000). However, the bioavailability of orally administered biotechnology drugs, such as proteins and vaccines, are usually poor because of the hostile gastric and intestinal environments and also the poor gastrointestinal (GI) mucosal permeability.

For the last 10 years, many strategies have been developed to enhance oral protein and vaccine delivery (Wang, 1996; Kompella and Lee, 2001; Steffansen et al., 2004; des Rieux et al., 2006). Among these approaches, nanoparticulate systems have attracted especial interests for the following reasons. First, they can protect drugs from degradation (Lowe and Temple, 1994). Second, they improve drug transmucosal transport (Janes et al., 2001) and transcytosis by M cells (Gullberg et al., 2000; Clark et al., 2001). Third, they can provide controlled release properties for encapsulated drugs (Galindo-Rodriguez et al., 2005).

Among these systems, chitosan nanoparticles have received particular interest because it is a very efficient and non-toxic absorption enhancer for both orally and nasally administered peptide drugs (Fernandez-Urrursuno et al., 1999; van der Lubben et al., 2001). But when put in physiological pH, chitosan will lose this capacity which can only be achieved in its protonated form in acidic environments (Kotzé et al., 1999a). Recently, people turn their attentions to *N*-trimethyl chitosan chloride (TMC), a quaternized chitosan derivative, since it has perfect solubility in water over a wide pH range. It also shows absorption enhancing effects even in neutral and basic-pH environments (van der Merwe et al., 2004). Moreover, recent studies showed that the use of TMC solution and nanoparticles in nasal vaccination (Baudner et al., 2004; Amidi et al., 2007), and TMC

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microspheres in oral vaccination (van der Lubben et al., 2002) could enhance the immunogenicity of the vaccine, and improve the systemic and local immune response compared to free antigen.

The local and systemic immune responses are mainly initiated by delivering antigens to M cells located in peyer's patches. It is generally believed that particles above 1 μm are taken up by M cells and trapped in peyer's patches (Jani et al., 1990), while nanoparticles (particles smaller than 1 μm) are taken up by M cells and delivered in the basal medium (des Rieux et al., 2005), thus the optimal size for particles to be transcytosed by M cells would be below 1 μm (Gullberg et al., 2000; Florence, 2004; Shakweh et al., 2005). Although TMC microspheres had been studied for oral vaccination (van der Lubben et al., 2002), these microspheres had a diameter of around 2 μm. Therefore, it was necessary to study the feasibility of applying TMC nanoparticles in oral vaccination.

Although TMC nanoparticles have many interesting features suitable for protein and vaccine delivery, such as high protein loading capacity, low cytotoxicity, and enhanced nanoparticle internalization (Amidi et al., 2006; Sandri et al., 2007), the release feature showed a high initial burst caused by the adsorption of protein drugs on the particle surface (Amidi et al., 2006; Chen et al., 2007). This may compromise the drug bioavailability, because the attack of the drug by enzymes or acidic substances from the body fluids may occur during the travelling of nanoparticles in GI tract (Borges et al., 2005). In a previous study, we successfully reduced the initial burst by alginate modification, but the incorporation of alginate had also changed particle size and zeta potential (Chen et al., 2007) while these two factors are very important for particle uptake by intestinal cells (des Rieux et al., 2006), and the presence of negatively charged alginate at the surface of TMC nanoparticles may influence their ability to enhance drug permeation through paracellular pathway. Thus it is very important to investigate the effect of alginate modification on the absorption properties of TMC nanoparticles.

Based on the above-mentioned reasons, the aims of the present work were to further investigate the intestinal epithelial absorption properties of TMC nanoparticles, especially the effect of alginate modification on their absorption properties using an *in vitro* model of GI epithelium (Caco-2 cells), and the feasibility of applying TMC nanoparticles in oral vaccination. Fluorescein isothiocyanate labeled bovine serum albumin (FITC-BSA) and Urease, a vaccine protein against *Helicobacter pylori* infection, were used as model protein and vaccine.

2. Materials and methods

2.1. Materials

Chitosan (with 95% degree of deacetylation (DD) and a molecular weight (*M*w) of 200 kDa) used for synthesizing TMC was purchased from BoAo Biochemical Company (Shanghai, China). TMC with a degree of quaternization (DQ) of 37% was synthesized by methylation of chitosan using CH_3I in a strong base (NaOH) and analyzed by ^1H nuclear magnetic resonance (NMR) spectroscopy as previously described (Sieval et al., 1998). The degree of quaternization (DQ) was calculated using the following equation, DQ (%) = [(∫TM/∫H) × 1/9] × 100 (Thanou et al., 2000), where ∫TM is the integral of the trimethyl amino group (quaternary amino group) peak at 3.3 ppm and ∫H is the integral of the ^1H peaks from 4.7 to 5.7 ppm. FITC-BSA was obtained by labeling BSA (BoAo Biochemical Company, China) with FITC (Amresco, USA) according to a standard method (Schauenstein et al., 1978). Urease and Lucifer Yellow (LY) were purchased from Sigma (USA). Goat anti-mouse IgG-horseradish peroxidase conjugate (IgG-HRP) and avidin-horseradish peroxidase conjugate (Avidin-HRP) were purchased from Chengwen Biochemical Company (Beijing, China). Biotinylated goat anti-mouse IgA (IgA-biotin) was purchased from Kirkegaard & Perry Laboratories (USA). Aprotinin and tetramethyl benzidine (TMB) were purchased from Amresco (USA). All other materials used were of analytical or pharmaceutical grade.

2.2. Preparation of TMC nanoparticles

The TMC nanoparticles were prepared by the ionic gelation of TMC with TPP anions. Ten milligrams of TMC and 2 mg of FITC-BSA were dissolved in 5 ml of water. Subsequently, 2 ml of TPP solution (0.6 mg/ml) was added drop-by-drop to the above solution under magnetic stirring at room temperature. Urease loaded TMC nanoparticles were prepared by the same method described above, except that only 0.5 mg of urease and 7.5 mg of TMC was added. Alginate-modified FITC-BSA loaded nanoparticles were prepared by dissolving sodium alginate (0.6 mg) in 2 ml of TPP (0.6 mg/ml) and then adding to the mixture solution of TMC and FITC-BSA. TMC nanoparticle suspensions were centrifuged for 10 min at 10,000 × *g* and 10 °C on a 10 μl glycerol bed, and then resuspended in 5 mM HEPES (pH 7.4) or Hank's balanced salt solution (HBSS) for characterization, permeability investigation or immunization respectively.

2.3. Characterization of TMC nanoparticles

The size and zeta potential of the TMC nanoparticles were measured with a Malvern Zatasize NanoZS90 (Malvern Instruments Ltd., Malvern, UK). The particle-size distribution is reported as a polydispersity index (PDI). The amounts of protein (FITC-BSA or urease) loaded in the nanoparticles were determined as described before (Chen et al., 2007), only the amount of FITC-BSA remaining in the supernatant was assayed by measuring fluorescence using a spectrofluorophotometer (RF-S301PC, SHIMADZU, Japan) at λ_{ex} = 498 nm and λ_{em} = 527 nm. The amount of urease was correlated to the amount of ammonia released from urea in the phenol-hypochlorite urease assay. Therefore, the determination of urease was carried out according to previous literature with slight modification (Mcgee et al., 1999). Supernatants were added to urease buffer (0.05 M PBS [pH 7.5] plus 25 mM urea) in a 25 ml final volume and were incubated at 37 °C for 20 min. The reaction was stopped by removal of an aliquot which was added to a cuvette containing 1.5 ml of solution A (38 g/l of phenol and 400 mg/l of sodium nitroprusside). An equal volume (1.5 ml) of solution B (NaOH

[0.5 M]–NaClO [0.044%, vol/vol]) was added at the same time. Following incubation at 37 °C for 30 min, the absorbance was measured at 625 nm using Cintra 10e UV–vis Spectrometer (GBC Scientific Equipment, Australia). Loading efficiency (LE) and loading capacity (LC) were calculated as follows:

$$\text{LE} = \frac{\text{Total amount of protein} - \text{free protein}}{\text{Total amount of protein}} \times 100\%$$

$$\text{LC} = \frac{\text{Total amount of protein} - \text{free protein}}{\text{nanoparticles dry weight}} \times 100\%$$

2.4. Permeability studies of FITC-BSA loaded TMC nanoparticles using Caco-2 cell monolayer

Caco-2 cells (passages 23) were seeded on tissue culture treated polycarbonate filters (area 4.7 cm^2) in Costar Transwell 6-well plates, respectively (Costar, USA) at a seeding density of 4×10^5 cells/cm^2. Dulbecco's Modified Eagle's Medium (DMEM, pH 7.40; Sigma, USA), supplemented with 1% non-essential amino acids, 10% foetal bovine serum, benzylpenicillin G (160 U/ml) and streptomycin sulphate (100 mg/ml) (all obtained from Sigma), was used as culture medium, and added to both apical and basolateral side of the cell monolayers. The medium was changed every second day and cell cultures were kept at a temperature of 37 °C in an atmosphere of 95% air and 5% CO_2. Filters were used for transepithelial electrical resistance measurements and transport experiments 21–23 days after seeding.

Transport experiments were carried out following procedures described previously (Sandri et al., 2007). One milliliter of FITC-BSA loaded non-modified or alginate modified TMC nanoparticle suspension at either 5 mg/ml or 20 mg/ml in HBSS (buffered with 30 mM HEPES, adjusted with 0.1 M NaOH to pH 7.4) was applied at the apical side of the cell monolayers, and 3 ml of buffered HBSS was applied at the basolateral side of the monolayers. Filters were moved into fresh basolateral medium at 1, 2, 3 h, and all basolateral phases were collected. The permeated nanoparticles were assayed by measuring fluorescence as above mentioned. After 3 h, the cells were washed three times with HBSS. Thereafter, 1 ml of LY HBSS solution (25 μg/ml) was added at apical side, and 3 ml of fresh HBSS at basolateral side. One hour later, the permeated LY was assayed on a spectrofluorophotometer at $\lambda_{ex} = 433$ nm and $\lambda_{em} = 541$ nm. Permeability of nanoparticles and LY was calculated using the following equation:

$$\text{Permeate efficiency(PE)} = \frac{\text{cumulative amount transported}}{\text{initial amount}} \times 100\%$$

During the experiments, the transepithelial electrical resistance (TEER) of the monolayers was measured at 0, 0.5, 1, 2, 3, 4 h, using a Millicell ERS-meter (Millipore Corp., USA).

2.5. Immunization studies of urease loaded TMC nanoparticles in mice

2.5.1. Animal immunization

Female Kunming mice, 6–8 weeks old, were housed under pathogen-free conditions with food and drink provided ad libitum. Mice were immunized as designed (Table 1). All formulations were prepared and/or resuspended in HBSS. Groups of 6 mice were immunized 3 times on days 0, 21 and 42 either by the oral route (i.g.) or by the subcutaneous route (s.c.). For mice treated with formulation 2 and 3, 0.3 ml of 0.1 M carbonate buffer (pH 9.6) was administrated into the stomach 0.5 h before immunization to neutralize the pH.

Three weeks after the final immunization, blood samples of mice were collected from orbit venous plexus and serum samples were obtained by centrifugation at $10{,}000 \times g$ and 4 °C for 10 min. Thereafter, mice were sacrificed and then gastric and intestinal secretions were collected. Gastric secretions were collected in cellulose wicks placed on intact gastric mucosa for 1 min. Then, wicks were moved into lavage medium (PBS pH 7.4 with 0.2 μM aminoethyl-benzene sulfonyl fluoride and 1 μg/ml aprotinin) and secretions were recovered from the wicks by centrifugation (Lee et al., 1999). Intestinal secretions were collected using a modified procedure described by Vila et al (Vila et al., 2004). Lengths (10 cm) of ileum from each mouse were sectioned longitudinally, and the luminal mucosa was carefully scraped into lavage medium, then mixed and centrifuged. Supernatants were used for investigation.

2.5.2. Enzyme-linked immunosorbent assay (ELISA)

The urease-specific IgG in serum and urease-specific IgA in gastric and intestinal secretions were examined by ELISA. For analysis of IgG, 96-well plates were coated overnight at 4 °C with 10 μg urease in 100 μl of 0.05 M carbonate buffer (pH 9.6) and blocked with 200 μl of 1% (m/v) bovine serum albumin for 1 h at 37 °C. After washed three times with PBS-Tween (PBS containing 0.05% Tween 20, pH 7.4), samples (serum diluted serially in twofold steps) were added to the plates at 100 μl/well, and incubated at 37 °C for 2 h. Then the plates were washed and incubated with 100 μl/well of IgG-HRP (diluted 1:500) at 37 °C for 1 h. Thereafter, the plates were washed four times and the specific IgG was detected by adding a mixture of TMB/H_2O_2 (0.05 M citrate buffer containing 0.1 mg/ml TMB, 0.03%, V/V H_2O_2) at 100 μl/well and the plates were subsequently incubated at room temperature for

Table 1
Animal immunization design

Formulation	Administration route	Antigen dose (μg)	Volume (ml)
1. Urease loaded TMC nanoparticles	i.g.	40	0.5
2. Urease + TMC solution	i.g.	40	0.5
3. Urease solution	i.g.	40	0.5
4.Urease loaded TMC nanoparticles	s.c.	40	0.3
5. HBSS (Control)	i.g.	–	0.5

30 min. Then, 2 M sulphuric acid aqueous solution was added at 50 μl/well, and each well was measured for optical density at 450 nm using a BIO-RAD Model 550 microplate reader (BIO-RAD, Japan). For analysis of IgA, a biotin–avidin amplified ELISA (BA-ELISA) method was used (He et al., 2005). After the plates were coated and blocked as previously described, samples (gastric or intestinal secretions diluted serially in twofold steps) were added to the plate at 100 μl/well, and incubated at 37 °C for 2 h. Then plates were washed and incubated with 100 μl/well of IgA-biotin (diluted 1:2000) at 37 °C for 1 h. Subsequently, after the plates were washed three times, avidin-HRP (diluted 1:2000) was added to the plates at 100 μl/well and the plates were incubated at 37 °C for 0.5 h. Then, specific IgA was detected by the same method used in IgG detection. Antibody titers was calculated as the reciprocal $\log_2$ of the last dilution that gave an optical density at 450 nm being 0.2 greater than that of control (mice immunized with HBSS). Comparison between different groups was made by a one-way ANOVA test.

3. Results and discussion

3.1. Characterization of TMC nanoparticles

The physicochemical and protein/vaccine loading properties of different TMC nanoparticles were shown in Table 2. Both FITC-BSA and urease were efficiently associated with TMC nanoparticles. Alginate modification had lead to an increase in LE and decrease in zeta potential and particle size for its negative charge and a strong inter-chain reaction with TMC as previously described (Chen et al., 2007). Recent data showed that nanoparticles smaller than 500 nm may be taken up better by M cells than the generally regarded optimal size range of 1–5 μm (Shakweh et al., 2004). However, due to the presence of glucose in the urease extract, the viscosity of TMC solution was greatly enhanced after urease was added. The increase of viscosity could lead to an increase in particle size (Leonard et al., 2004) and, in our study, PDI values were also largely increased. Therefore, to decrease solution viscosity, the concentration of TMC was reduced from 2 to 1.5 mg/ml to prepare urease loaded TMC nanoparticles with a particle size smaller than 500 nm, and they were not further modified by alginate since alginate is also a viscosity enhancer. Adding of alginate into urease containing TMC solution could lead to the aggregation of the TMC nanoparticles.

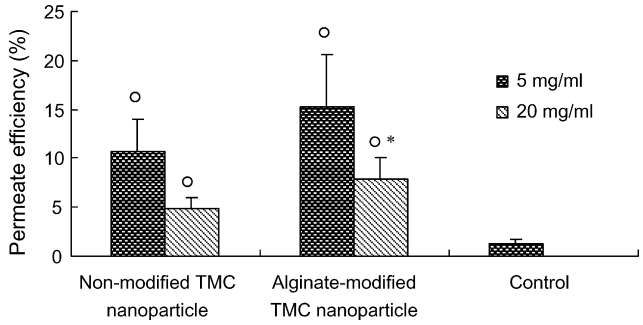

Fig. 1. Observed permeate efficiency of FITC-BSA loaded non-modified TMC nanoparticles, alginate modified TMC nanoparticles at low (5 mg/ml) and high concentration (20 mg/ml) and control (FITC-BSA solution) using Caco-2 cell monolayer. Circles indicated permeate efficiency that were significantly higher ($P < 0.01$) than that of the control. Asterik indicated permeate efficiency that was significantly higher ($P < 0.05$) than that of non-modified TMC nanoparticles. No significant difference was observed between non-modified and alginate modified TMC nanoparticles at 5 mg/ml. All data are mean ± S.D. ($n = 4$).

3.2. Permeability study of FITC-BSA loaded TMC nanoparticles using Caco-2 cell monolayer

To investigate the influence of alginate modification on absorption properties of TMC nanoparticles, Caco-2 cells were utilized as an *in vitro* model of GI epithelium. Fig. 1 showed the permeate efficiency of TMC nanoparticles and control (FITC-BSA solution) calculated as the percentage of FITC-BSA transported through Caco-2 cell monolayer. Both modified and non-modified TMC nanoparticles showed a decrease in permeate efficiency with the increase of nanoparticle concentration, indicating a saturated and limited capability of cellular transport of the nanoparticles. Such phenomenon was also observed by the other groups (Dong and Feng, 2005; Zhang and Feng, 2006). The transport of FITC-BSA was greatly improved ($P < 0.01$) by the presence of TMC nanoparticles no matter they were modified by alginate or not. The permeate efficiency of alginate modified TMC nanoparticles was 1.4- and 1.6-fold higher than that of non-modified TMC nanoparticles at incubated particle concentration of 5 and 20 mg/ml, respectively. It is generally agreed that the nanoparticle transcytosis increases when the particle diameter decreases (Jani et al., 1990; Lamprecht et al., 2001; Florence, 2005) however the mechanism for this phenomenon was not fully revealed yet. It was assumed that particles up to about 100–200 nm can be internalized by receptor-mediate endocytosis, while larger particles have to be taken up by phagocytosis (Couvreur and Puisicux, 1993). Therefore, enhancement

Table 2
The loading efficiency, loading capacity, particle size and zeta potential of different TMC nanoparticle preparations

Preparations	LE	LC (w/w)	Particle size (nm)	PDI	Zeta potential (mV)
FITC-BSA loaded non-modified TMC nanoparticles[a]	94.08 ± 1.88	44.77 ± 0.76	304 ± 6	0.13 ± 0.05	14.9 ± 0.5
FITC-BSA loaded alginate-modified TMC nanoparticles [b]	99.56 ± 0.23	30.71 ± 1.72	181 ± 14	0.23 ± 0.04	6.0 ± 0.3
Urease-loaded TMC nanoparticles [c]	81.57 ± 1.25	10.05 ± 0.32	326 ± 6	0.31 ± 0.04	12.1 ± 0.3

All data are the mean ± S.D. ($n = 3$).
[a] TMC 2 mg/ml, TPP 0.6 mg/ml, FITC-BSA 0.4 mg/ml.
[b] TMC 2 mg/ml, TPP 0.6 mg/ml, sodium alginate 0.3 mg/ml, FITC-BSA 0.4 mg/ml.
[c] TMC 1.5 mg/ml, TPP 0.6 mg/ml, urease 0.1 mg/ml.

of nanoparticle transport was probably because of the reduction of particle size to 100–200 nm after alginate modification.

According to Ma and Lim, soluble chitosan molecules were more effective at disrupting the intercellular tight junction than the chitosan nanoparticles, which might have interacted less effectively with the cellular proteins because of their TPP-crosslinked chitosan chains (Ma and Lim, 2003). The same principle can also be applied in TMC nanoparticles. Alginate could also crosslink with TMC (supported by the reduction of zeta potential) which means alginate might have a negative effect on TMC nanoparticles' ability of opening the TJ. Therefore, we measured TEER value which is believed to be a good indication of the tightness of the junctions between cells (Kotzé et al., 1999b). Fig. 2 showed the effect of TMC nanoparticles on the TEER of Caco-2 cell monolayers. TEER values at all time points (0.5–3 h) were pooled together to evaluate the differences between modified and non-modified TMC nanoparticles, and no significant difference was observed ($P > 0.05$) at the concentration of 5 mg/ml or 20 mg/ml. The TEER values of control were significantly higher than that of non-modified or alginate modified TMC nanoparticles no matter P was calculated when TEER values were pooled together ($P < 0.01$) or at each time point ($P < 0.05$), indicating that alginate modified TMC nanoparticles did not lose the ability of opening TJ. Contrary to previous results observed from TMC solutions that TMC decreased TEER values in a concentration dependant way (Kotzé et al., 1997), in our study, increasing particle concentration from 5 to 20 mg/ml did not lead to further reduction of TEER values. A possible explanation could be that due to the bioadhesive property of TMC nanoparticles (including alginate modified TMC nanoparticles), they were able to adhere to and form a nanoparticle layer on the Caco-2 cell monolayers. Thus, TEER values were decreased regardless of particle concentration. In our study, we find it very difficult to thoroughly remove TMC nanoparticles (both non-modified or alginate modified) of high concentration (20 mg/ml) without damaging the cells, due to their viscosity. Therefore, no increase in resistance, after nanoparticles being removed, was observed in monolayers treated with high concentration of TMC

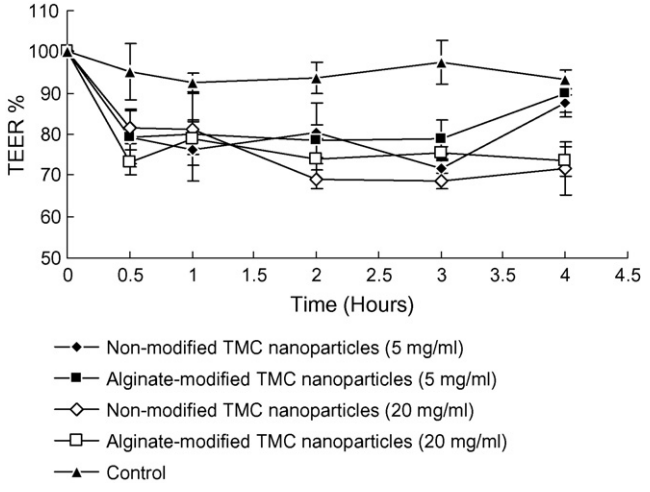

Fig. 2. Effect of TMC nanoparticles on TEER values of Caco-2 cell monolayers. Data presented are mean ± S.D. ($n = 4$).

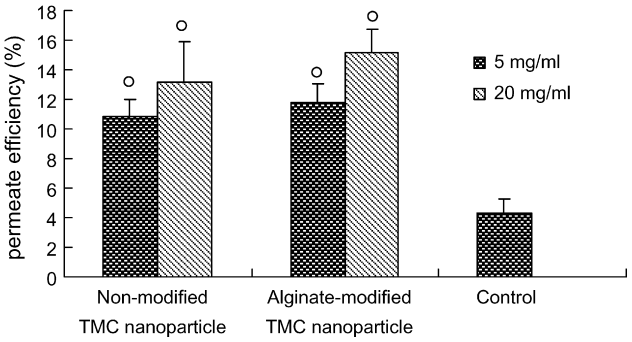

Fig. 3. Observed permeate efficiency of LY after Caco-2 cell monolayers were treated with FITC-BSA loaded non-modified TMC nanoparticles, alginate modified TMC nanoparticles at low (5 mg/ml) and high concentration (20 mg/ml) and control (FITC-BSA solution). Circles indicated permeate efficiency that were significantly higher ($P < 0.01$) than that of the control. No significant difference was observed between non-modified and alginate modified TMC nanoparticles at 5 mg/ml or 20 mg/ml. All data are mean ± S.D. ($n = 4$).

nanoparticles. However, those treated with low concentration (5 mg/ml) of TMC nanoparticles showed an almost complete reversibility towards initial TEER values, indicating the safety of both non-modified and alginate modified TMC nanoparticles.

LY is a fluorescent probe which can only permeate through the Caco-2 cell monolayers when the tight junctions are opened. Therefore, besides TEER values, we also used LY as a marker of the tight junction integrity to investigate the TMC nanoparticles' capacity to enhance drug transport through paracellular pathway. Fig. 3 showed the permeate efficiency of LY after Caco-2 cell monolayers being treated with nanoparticles or control. It confirmed the results in TEER measurement, that alginate modification did not affect TMC nanoparticles' ability of opening TJ. Both non-modified and alginate modified TMC nanoparticles significantly increased the permeability of LY than that of control ($P < 0.01$). In our study, the amount of alginate we used to modify TMC nanoparticles was very small (0.6 mg of sodium alginate compared to 10 mg of TMC), which might explain its weak negative effect on the interaction between TMC nanoparticles and cellular proteins. And also, alginate had greatly reduced particle size, which might redeem this negative effect by enabling more particles to interact with cellular proteins. More importantly, smaller particle size could enhance transcytosis as previously mentioned. Therefore, in our case, alginate modification had strong and positive effect on TMC nanoparticles' ability of enhancing drug transport through transcellular pathway (transcytosis) but very weak effect on TMC nanoparticles' ability of enhancing drug transport through paracellular pathway (through TJ).

3.3. Immunization studies of urease loaded TMC nanoparticles in mice

The anti-urease antibody titers of each formation were shown in Fig. 4. Orally administrated urease loaded TMC nanoparticles were able to induce a significantly higher serum IgG antibody titer than urease solution ($P < 0.01$). It illustrated that i.g. administrated TMC nanoparticles have strong immunostim-

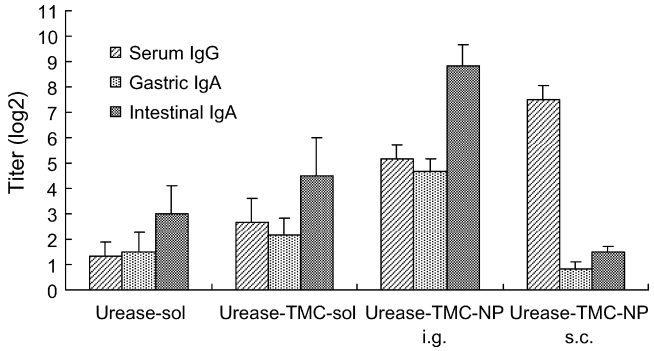

Fig. 4. IgG and S-IgA antibody titers after i.g. administration of urease solution (Urease-sol), urease co-administrated with TMC solution (Urease-TMC-sol), urease loaded TMC nanoparticles (Urease-TMC-NP i.g.) or s.c. administration of urease loaded TMC nanoparticles (Urease-TMC-NP s.c.). Antibody titers presented are geometric mean ± S.E. ($n = 6$).

ulating effect and that they efficiently protected antibodies in gastrointestinal fluids. Although TMC solution was reported to be able to enhance the immunogenicity of antigen (Baudner et al., 2004), in our study, urease co-administrated with TMC solution generated a much lower serum IgG antibody titer than i.g. administrated urease loaded TMC nanoparticles ($P < 0.05$), and only some of the mice (4 out of 6) showed immune responses even after two booster immunizations. This indicated that TMC solution was unable to effectively protect antigen in GI tract, as well as efficiently enhance the immungenicity of the antigen, which probably because of the dilution by the large surface area of GI tract.

Locally produced secretory IgA (S-IgA) antibodies are considered to be among the most important protective humoral immune factors (van der Lubben et al., 2002). They are able to neutralize microbes and their products, thus avoiding mucosa colonization and invasion by pathogens (Phalipon et al., 2002; Magistris, 2006). As it was shown in Fig. 4, although s.c. administrated urease loaded TMC nanoparticles were able to induce the highest IgG titer among all formations, only weak mucosal S-IgA responses were observed in either stomach or small intestine. Orally administrated urease loaded TMC nanoparticles in the contrast were able to elicit the highest S-IgA titers. The results were consistent with those reported by other groups (Bacon et al., 2000; Liao et al., 2003). An explanation might be that most of the IgA antibodies in mucosal secretions were locally produced rather than transudated from serum (Shen et al., 2000; Rydell and Sjöholm, 2005). These observations indicated that mucosal immune response in mice was induced easier through oral administration, thus oral vaccination with TMC nanoparticles can provide better protection to mucosal surface than subcutaneous vaccination.

Urease co-administrated with TMC solution generated similar gastric and intestinal S-IgA titers with urease solution ($P > 0.05$), and significantly lower than those generated by i.g. administrated urease loaded TMC nanoparticles ($P < 0.05$). As mentioned above, TMC solution is able to enhance the paracellular transport of antigens by opening the TJ and its bioadhesive property could prolong the residence time of the antigen at mucosal surface, and these properties might be responsible for its immunostimulating effect (Park et al., 2002; Illum, 2003). However, these properties were both concentration dependent and a low concentration of TMC was not sufficient enough to localize the polymer against the intestinal wall of the small intestine for interaction with the tight junctions and cell surfaces (van der Merwe et al., 2004). Therefore, considering the large surface area of GI tract and the fact that TMC solution was unable to protect antigen from hydrolytic and enzymatic degradation in gastrointestinal fluids, the immunostimulating effect of TMC solution was quiet limited in oral vaccination.

Although TMC nanoparticles might be less effective at opening the TJ than TMC solution due to the TPP-crosslinked polymer chains (Ma and Lim, 2003), in our study they demonstrated much higher immunostimulating effect than TMC solution. Their immunostimulating effect was probably caused by their bioadhesiveness, which was independent of particle concentration, and more importantly the improved uptake of nanoparticles by enterocytes and M cells in gut-associated lymphoid tissue (GALT) due to their particulate nature.

4. Conclusion

Alginate modified TMC nanoparticles were able to further improve the permeability of FITC-BSA than non-modified TMC nanoparticles. It is suggested that, in our case, compared with non-modified TMC nanoparticles, alginate modified TMC nanoparticles had a stronger effect on enhancing drug transport through transcellular pathway but an equal effect on enhancing drug transport through paracellular pathway. The immunization study showed that although s.c. administrated urease loaded TMC nanoparticles were able to elicit highest level of IgG titers, it failed to elicit high level of S-IgA antibodies. In the contrast, i.g. administrated urease loaded TMC nanoparticles generated high titers of both IgG and S-IgA antibodies, indicating TMC nanoparticles are promising carriers for oral vaccination. In light of these results, TMC nanoparticles are potential carriers for oral protein/vaccine delivery.

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