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Mono-*N*-carboxymethyl chitosan (MCC) and *N*-trimethyl chitosan (TMC) nanoparticles for non-invasive vaccine delivery

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ABSTRACT

Mucosal application of a vaccine can effectively induce both systemic and mucosal immune responses. In general, mucosal applications of antigens result in poor immune responses. Therefore, adjuvant/delivery systems are required to enhance the immune response. Chitosan is a cationic biopolymer which exerts advantages as a vaccine carrier due to its immune stimulating activity and bioadhesive properties that enhance cellular uptake and permeation as well as antigen protection. Similar effects are also shown by chitosan derivatives. In this study, the nanoparticulate systems were prepared by using differently charged chitosan derivatives, *N*-trimethyl chitosan (TMC, polycationic), and mono-*N*-carboxymethyl chitosan (MCC, polyampholytic) for mucosal immunisation. The derivatives were synthesised and characterised in-house. The aqueous dispersions of the derivatives were also prepared for comparison. The cytotoxicity studies (MTT assay) on Chinese hamster ovary (CHO-K1) cell lines showed that cell viability was in the order of MCC, chitosan and TMC. Nanoparticles were prepared using ionic gelation method and loaded with tetanus toxoid (TT). Nanoparticles with high loading efficacy (>90% m/m), particle size within the range of 40–400 nm, with a negative surface charge for MCC and positive surface charge for TMC and chitosan were obtained. The structural integrity of the TT in the formulations was confirmed by SDS-PAGE electrophoresis analysis. The effective uptake of the FITC-BSA loaded nanoparticles into the cells was demonstrated by cellular uptake studies using J774A.1 cells. Immune responses induced by the formulations loaded with tetanus toxoid were studied *in vivo* in Balb/c mice. Enhanced immune responses were obtained with intranasal (i.n.) application of nanoparticle formulations. Chitosan and TMC nanoparticles which have positively charged surfaces induced higher serum IgG titres when compared to those prepared with MCC which are negatively charged and smaller in size. Nanoparticle formulations developed in this study can be used as promising adjuvant/delivery systems for mucosal immunisation.

1. Introduction

Vaccination is the most cost-effective approach to prevent economic losses and morbidity caused by infectious diseases. The World Health Organization estimates that over 14 million people die each year from infectious disease, about a quarter of all deaths worldwide (Bulletin of WHO, 2000). Mucosal immunisation which is a non-invasive route is an attractive alternative to parenteral immunisation and using the appropriate delivery system it is possible to stimulate both mucosal and systemic immune responses (Gebert et al., 1996). Mucosal vaccination offers also several benefits over parenteral immunisation, including ease of administration, reduced side effects, the possibility of self-administration and especially in developing countries, reduced risk of the unwanted spread of infectious agents via contaminated syringes (Baudner et al., 2003; Ryan et al., 2001). All these benefits result in lower costs and better patient compliance. Yet, the inactivated or subunit antigens applied to the mucosa generally generate poor immune responses. In order to obtain high immune response via mucosal route, it is necessary to utilise an adjuvant/delivery system. Particulate delivery systems have been shown to enhance the immune response following mucosal application (Alpar, 2003; Husband et al., 1996; Vajdy and O'Hagan, 2001). Microencapsulation can also protect antigens and facilitate their uptake into lymphoid tissue (O'Hagan,

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1998). The potential of nanoparticles as a vaccine delivery has also been shown in numerous studies (Prokop et al., 2002; Amidi et al., 2006, 2007).

The mucosal epithelium as well as distal mucosal surfaces of both the nasal-associated lymphoid tissue (NALT) and the gut associated lymphoid tissue (GALT) contains antigen specialised antigen-sampling cells known as the M cells. These cells can transport antigens from the mucosal surfaces into the underlying lymphoid tissues and may represent an efficient potential portal for oral and nasal vaccine delivery (Gebert et al., 1996; Featherstone, 1997). After entering into the MALT (Mucosal Associated Lymphoid Tissue), the antigens are rapidly internalised and processed by antigen presenting cells such as subepithelial dendritic cells, macrophages, and presented to B cells and T cells located in the MALT (Frey and Neutra, 1997). Nasal mucosa is an attractive site for the non-parenteral delivery of antigens for induction of both mucosal and systemic immunity (Jabbal-Gill, 2001).

In general, for mucosal immunisation, chitosan, alginates and PLGA are used as delivery systems (Suckow et al., 2002; Kim et al., 2002; Sayın et al., 2005).

Chitosan is an attractive cationic natural polymer derived from chitin which has a great potential to be used as a delivery system (Illum, 1998; Şenel and McClure, 2004; Sayın et al., 2006). It is non-toxic, biocompatible and biodegradable, and reported to exhibit potential immuno-adjuvant properties (van der Lubben et al., 2001). The degree of deacetylation and derivatisation with various side chains can be a source of manipulation for specific drug-delivery systems. Effective endocytotic uptake and low cytotoxicity were shown for chitosan nanoparticles using different cell culture models (Behrens et al., 2002; Huang et al., 2004). Chitosan derivatives have also been reported as promising for antigen delivery due to their similar properties.

Chitosan is soluble in weak acidic environment, but insoluble at physiological (7.4) or higher pH values being a weak base (pK_a 6.2–7, dependent on the M_w) (Illum, 1998) whereas chitosan derivatives offer a better solubility at neutral pH values (van der Lubben et al., 2001). Modification of chitosan using the amine group substitution by small alkyl groups increases its solubility in aqueous media.

The aim of this study was to develop an antigen delivery system for mucosal (nasal) immunisation using the chitosan derivatives, the negatively charged mono-*N*-carboxymethyl chitosan (MCC) and positively charged *N*-trimethyl chitosan (TMC) and to compare their efficacies *in vivo*. Tetanus toxoid was chosen as the model antigen.

2. Materials and methods

2.1. Materials

Purified tetanus toxoid (5000 Lf/ml) was kindly provided by Division of Bacteriology, NIBSC, UK. Low MW (150 kDa) chitosan was supplied by Fluka, and chitosan glutamate (CS) (Protosan™ UP G 113, Deacaetylation degree >75–90%, Mol. Wt.: 150 kDa) was supplied by Novamatrix, Norway. All other chemicals were reagent grade chemicals.

2.2. Synthesis of TMC

TMC was synthesised by reductive methylation of chitosan using methyl iodide (CH_3I) in the presence of a strong base (NaOH) at 60 °C. The method of synthesis used in the present study was adapted from that previously reported by Thanou et al. (2001) (Sieval et al., 1998). Chitosan and sodium iodide were dissolved in 1-methyl-2-pyrrolidinone on a water bath at 60 °C with stirring. Following dissolution of chitosan, aqueous sodium hydroxide solution and methyl iodide were added while stirring. The product was precipitated using ethanol and subsequently isolated by centrifugation. After washing with ethanol and ether, the product was filtered and vacuum dried. In a second step *N*-trimethyl chitosan iodide initial product was dissolved in 1-methyl-2-pyrollidinone on a water bath at 60 °C with stirring. Sodium iodide, aqueous sodium hydroxide solution and methyl iodide were added with rapid stirring and the mixture was stirring on a water bath at 60 °C. Afterwards, methyl iodide and sodium hydroxide pellets were added and stirring was continued for 1 h. Ethanol was added slowly and the mixture was stirred for 5 min and allowed to precipitate, then filtered and vacuum dried. The product was dissolved in 10% (m/v) sodium chloride solution. The product was again precipitated with ethanol, isolated by centrifugation, filtered and dried. Then, the product was dissolved in water. Afterwards, ethanol was added, product was precipitated and following centrifugation, it was filtered and washed with ether, filtered and dried.

2.3. Synthesis of MCC

MCC was synthesised with the carboxymethylation of chitosan by reacting the free amine groups of chitosan with glyoxylic acid to produce a soluble, gel-forming imine which is then reduced (Colo et al., 2004). Chitosan (MW 100 kDa) was first dissolved in an aqueous acetic acid solution. Then, three equivalents of glyoxylic acid (for every monomer) were added and the mixture was stirred at room temperature for 1 h. Following formation of imine, the pH was raised to 4.5 by adding 1 M sodium hydroxide. Subsequently, an aqueous solution of 5% (m/v) sodium borohydride was added dropwise to reduce the formed imine. The mixture was stirred for 1 h and the product was isolated by precipitation with addition of ethanol. The product was washed on a glass filter under vacuum by ethanol/water aliquots. This product underwent a second step of reaction of carboxymethylation with the same amount of glyoxylic acid, same reaction time and isolation steps as described above.

2.4. Characterisation of the chitosan derivatives

TMC and MCC were characterised by nuclear magnetic resonance (NMR) spectroscopy, and the degree of substitution was determined by ^{1}H NMR on a 600 MHz spectrometer (Bruker, Karlsruhe, Germany).

2.5. In vitro cellular toxicity studies

In order to evaluate the safety of the chitosan derivatives, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cytotoxicity experiment on CHO-K1 (ATCC, CCL61) cell lines was carried out. Sterile, flat bottomed 96-well plates were seeded with CHO-K1 cells to a density of 10^4 cells/well and incubated at 37 °C and 5% CO_2 for 24 h. Chitosan solutions were diluted with the cell culture media F-12 (Hams) from a 20 mg/ml stock to a series of solutions with concentrations of 0, 0.01, 0.02, 0.05, 0.1, 0.5, 1, 2, 5 mg/ml in sterile water. Each concentration was prepared in triplicates. Following incubation for 4 h, the media was removed. The cells were washed with 50 μl of PBS. Fresh culture media of 200 μl was added into each well. The cells were then incubated for another 24 h. MTT (20 μl of 5 mg/ml solution in PBS) was added to each well and the plates were subjected to a further incubation at 37 °C in 5% CO_2 for 4 h. The media was aspirated and the plates were gently tipped. 100 μl DMSO was then added to each well and the plates were incubated at 37 °C for 30 min to dissolve the crystals. The plates were read at 570 nm using a Wallac Victor II microplate reader (1420 Multilabel Counter, Wallac Oy, Turku, Finland). Cells incubated in cell culture medium alone were used as a control to

assay 100% viability. Viability of cells exposed to test materials was expressed as a percentage of the viability seen in control untreated cells.

2.6. Preparation of chitosan (CS), TMC and MCC nanoparticles

Nanoparticles were prepared using ionic gelation method in the presence of Tween 80 (0.5% m/v) as a resuspending agent to prevent particle aggregation (Vila et al., 2004; Jiang et al., 2004). TT was added in the polymer solution during preparation of the nanoparticles (120 limit of flocculation (Lf) of antigen to 10 mg polymer).

CS (Protasan™ UPG 113) and TMC nanoparticles were prepared with the same procedure using polyanionic tripolyphosphate (TPP) as a crosslinker at different concentrations (TMC:TPP ratios between 3 and 6 and CS:TPP ratios between 8 and 14). Two mg/ml CS or TMC was dissolved in distilled water. Then TPP solution was added to give a CS:TPP ratio of 12 and also as a TMC:TPP ratio of 5.5 dropwise to this solution under constant stirring.

MCC nanoparticles were prepared using different concentrations (2 and 4 mg/ml) of MCC. Calcium chloride ($CaCl_2$) was used as the crosslinker. MCC was dissolved in distilled water and $CaCl_2$ solutions at different concentrations (MCC:$CaCl_2$ ratios of 3, 5, 6, 7.5 or 10) were added dropwise to this solution under constant stirring.

2.7. Characterisation of nanoparticles

The morphological and surface characteristics of the nanoparticles were examined by means of transmission electron microscopy (TEM, FEI CM 120 BioTwin, Philips, Netherlands). The particle size and polydispersity were determined using photon correlation spectroscopy (PCS, Malvern Instruments, UK), and zeta potential of nanoparticles was measured by using Zetasizer (Malvern Instruments, UK). The micro-bichinchoninic acid protein assay (microBCA, Pierce, Rockford, USA) and QuantiPro™ BCA assay kit (Sigma, USA) were used for the determination of TT. The amount of tetanus toxoid (TT) loaded to the particles, loading efficiencies (LE) was calculated from the difference between the initial amount and the amount present in the supernatant after centrifugation.

2.8. Cellular uptake studies

Mouse Balb/c monocyte macrophage J774A.1 (ECACC, 91051511) was cultured with Dulbecco's Modified Eagle's Medium (DMEM) supplied with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin in an incubator at 37 °C and 5% CO_2 atmosphere with 95% humidity. Poly-L-Lysine (PLL, 1 mg/ml) solution was sterilised by passing through a 0.22 μm filter and 20 times diluted as a working solution (50 μg/ml). The coverslips were sterilised in 95% ethanol and dried before coating and then were placed in a single layer in a sterile 6-well plate containing the working solution and incubated for 1 h at 37 °C. PLL solution was removed and the surfaces of coverslips were allowed to dry in air. Before placing the particles, macrophages were washed with PBS and then resuspended by scrapping. After collection, 4×10^5 cells in 2 ml of DMEM were added in each well of the 6-well plate with coverslips. Cells were incubated for 18 h and then washed for two times with DMEM-L-glutamine to remove non-adherent cells and FCS. Subsequently, 2 ml of DMEM L-glutamine medium containing 200 μg of nanoparticles loaded with FITC-labelled BSA were added to each well and incubated for 1 h to allow phagocytosis of the particles. After incubation, cells were fixed with cold methanol for 5 min. Then the fixed cells were washed with PBS. The coverslips with fixed cells were mounted on microscope slides and sealed using nail polish. Cell uptake (phagocytosis) was observed using a Zeiss LSM 510 Meta laser scanning confocal microscope. The channels used for the observation were set at a multiple channel function using T1: 488 nm, T2: 633 nm. Pixel time set at 32 μs. The objective was Plan-apochromat 63x/1.4 oil (DIC: differential interference contrast).

2.9. Determination of the protein integrity

The integrity of TT loaded into nanoparticles was analysed by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Nanoparticles were destabilised by adding 1 ml of 10% (m/v) NaCl to 4.5 ml of nanoparticle suspension. Samples were loaded onto a vertical slab gel and subjected to electrophoresis at 200 mV, using Biorad Power Pac 1000 (Biorad, Hemel Hempstead, UK). Gels were subsequently fixed and stained with Coomassie brilliant blue staining solution (50 ml, 0.1%, m/v) (Sigma) for 1 h. Gels were then destained until protein bands were distinct. After destaining, gels were analysed using Gene Genius Bio Imaging System (Syngene, USA).

2.10. In vivo studies

For *in vivo* studies, TT loaded TMC, MCC and CS nanoparticles (Table 1) were administered intranasally (i.n.) or subcutaneously (s.c.) to Balb/c mice ($n = 5$) aged up to 6–8 weeks and weighing about 25 g. Animals were maintained on a normal mouse diet throughout the study. All procedures were performed in accordance with Animal Scientific Procedures Act 1986. For comparison, immune response of the aqueous dispersions of TMC, MCC or chitosan in PBS at 0.2% (m/v) concentration and incorporated with TT (5 Lf TT/mice dose) were investigated following intranasal or subcutaneous administration.

TT was administered either intranasally or by subcutaneous injection (priming dose on day 0 and boosting on day 22). Both the priming and boosting doses were 5 Lf TT/mice dose for each formulation. Mice were lightly anesthetised with fluorethane before intranasal application and 25 μl formulation was applied dropwise into the nares (12.5 μl/nostril). 200 μl of subcutaneous injections were applied to unanesthetised animals. To assess immune responses, serum tail vein blood samples were collected on day 20 and, postboosting titres were obtained on days 42 and 56. The blood samples were centrifuged for 10 min at 10,000 rpm in an eppendorf centrifuge, the serum was collected and stored at −70 °C until analysis for IgG, IgG1 and IgG2a titres. Vaginal washes were also obtained by introducing 100 μl PBS (pH 7.4) into vagina with 200 μl pipettor and flushing it four times. The washes were vor-

Table 1
Nanoparticle formulations

Formulations	ζ-Potential (mV) ± S.D.	*z* Average (nm) ± S.D.	Polydispersity index ± S.D.	Loading efficiency ± S.D. (m/m) %
CS-np (CS:TPP ratio = 12)	+53.8 ± 0.8	397.8 ± 7.8	0.302 ± 0.028	78 ± 4
TMC-np (TMC:TPP ratio = 5.5)	+18.52 ± 1.2	306.0 ± 2.4	0.182 ± 0.039	93 ± 3
MCC-np 1 (MCC:$CaCl_2$ ratio = 7.5)	−29.0 ± 2.5	43.7 ± 1.3	0.438 ± 0.021	95 ± 2
MCC-np 2 (MCC:$CaCl_2$ ratio = 6)	−28.8 ± 2.2	90.9 ± 0.8	0.280 ± 0.013	97 ± 2

np, nanoparticles; MCC, mono-*N*-carboxymethyl chitosan; TMC, *N*-trimethyl chitosan; CS, chitosan; TPP, tripolyphosphate; S.D., standard deviation.

texed and centrifuged for 10 min at 10,000 rpm at 4 °C. The fluid samples were stored after adding sodium azide at −70 °C after centrifugation until being assayed. At day 56, mice were sacrificed and serum and mucosal washes (nasal and vaginal) were collected.

2.11. Enzyme-linked immunoabsorbent assay (ELISA)

Titration of TT-specific IgG, its subunits (IgG1 and IgG2a), and IgA antibody in serum samples and mucosal washes collected from animals was achieved with ELISA (Tierney et al., 2003). Briefly, 100 μl of 0.5 limit of flocculation (Lf) TT in PBS was added to flat-bottom micro-titration plates and allowed to incubate overnight at 4 °C. The plates were washed with PBS containing 0.05% Tween 20 (PBS-T) and were blocked with 2% (m/v) BSA solution for 1 h at 37 °C. After washing with PBS-T, serial 2-fold dilutions of an in-house reference mouse serum (initially diluted to 0.02 IU/ml) and test mouse serum in PBS were titrated across the plate, to a final volume of 100 μl. Plates were incubated for 1 h at 37 °C. At the end of the incubation period, plates were washed with PBS-T and were incubated with 100 μl of goat anti-mouse IgG (Sigma, USA) or 100 μl of goat anti-mouse Ig A (AbD Serotec, UK) diluted 1:2000 with PBS/well, for 1 h at 37 °C. Unbound conjugate was removed by washing with PBS-T and enzyme activity was determined by adding a tablet of 2,2 Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABST) (Sigma, USA) and 5 μl of hydrogen peroxide solution (30%, m/v) in 0.05 mol citric acid buffer/l. All ELISA procedures were performed at room temperature. The absorbance was read at 410 nm on an ELISA reader (Dynex Technologies, MRXII Microplate Absorbance Reader, USA).

For IgG1 and IgG2a antibody subclasses, a similar protocol which is described above was followed. Reference goat anti-mouse IgG1 or goat anti-mouse IgG2a subclasses (AbD Serotec, UK) were diluted 1:4000 with PBS (pH 7.4).

2.12. Statistical analysis

Kruscal–Wallis variance analysis was used to analyse the differences between and within-groups for all data. $p < 0.01$ was taken as significant.

3. Results and discussion

The aim of this study was to develop nanoparticles using chitosan derivatives and compare their potential use in nasal immunisation to that of chitosan nanoparticles. For this purpose, firstly chitosan derivatives were synthesised. The ^{1}H NMR spectra revealed quaternisation degree or methylation of TMC as 57% and carboxymethylation of MCC as 70%. The charge density of chitosan derivatives was defined by the degree of quaternisation for TMC, and by carboxymethylation of MCC. Increasing the number of positive or negative charges on the polymer chain causes the polymeric molecule to expand in solution due to repelling force between the functional groups, this resulting in higher solubility.

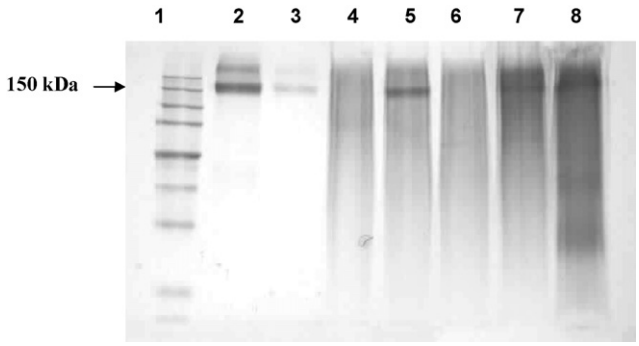

Fig. 1. SDS-PAGE gel displaying the TT determined in nanoparticles after loading process. Loading order: lane 1: molecular weight marker; lane 2: TT standard (high concentration); lane 3: TT standart (low concentration); lane 4: empty TMC nanoparticles; lane 5: TT loaded TMC nanoparticles; lane 6: empty MCC nanoparticles; lanes 7 and 8: TT loaded MCC nanoparticles. (Mol. Wt. of TT: 150 kDa).

3.1. In vitro cellular toxicity studies

Our experimental results showed that the cellular viability with chitosan was higher than that with TMC whereas, with MCC the cell viability was the highest. Higher cellular viability with MCC can be explained with its negatively charged property due to the induction of negatively charged carboxymethyl groups and reduced amino groups. Many cationic polymers have been found to be toxic which has been attributed the interactions with the plasma membrane (Choksakulnimitr et al., 1995).

3.2. Preparation and characterisation of the nanoparticles

The formation of nanoparticles with chitosan and its derivatives by ionic gelation represent an easy method to prepare nasal delivery systems for antigens in mild conditions avoiding heat and use of any organic solvents. Moreover, with water-soluble chitosan derivatives, there is no need for an acidic media to dissolve the polymer. The preparation method involves first mixing the antigen into polymer solution. Crosslinking agent is then added to the suspension under constant stirring. Hence, this preparation method is simple. Due to the complexation between oppositely charged polymer and crosslinker, polymer undergoes ionic gelation and precipitates to form nanoparticles. Preparation conditions play an important role on the antigen, which can be denatured even by limited exposure to heat or organic solvents (Raghuvanshi et al., 2001). Stability problems have been reported for tetanus toxoid in acidic conditions during microparticle preparation (Xing et al., 1996). SDS-PAGE gel analysis results confirmed the structural integrity of the TT in the nanoparticles indicating that the integrity of TT has been maintained during preparation (Fig. 1).

Different initial concentrations of chitosan derivatives (TMC or MCC) and also different crosslinkers (TPP or $CaCl_2$) at various concentrations were used to establish preparation conditions at

Table 2
Properties of the empty MCC nanoparticles

	MCC concentration (mg/ml)	MCC:$CaCl_2$ ratio	z Average (nm) ± S.D.	Polydispersity	ζ-potential (mV)
Trial 1	4	5	Aggregates		
Trial 2	4	3	Aggregates		
Trial 3	2	10	39.6 ± 2.2	0.360	−14.45
Trial 4 (MCC np 1)	2	7.5	37.2 ± 2.9	0.334	−26.05
Trial 5	2	5	150.6 ± 4.6	0.246	−26.57
Trial 6 (MCC np 2)	2	6	116.0 ± 3.4	0.294	−22.20

which nanoparticles were formed. After the preparation of the nanoparticles, three different systems were identified: clear solution, opalescent dispersion and aggregates. The zone of opalescent suspension, which corresponded to a suspension of very small particles, was further investigated.

In this study, it was the first time to develop a nanoparticulate delivery system using MCC. $CaCl_2$ was used as a crosslinker for preparation of MCC nanoparticles. Due to the negative charge of MCC, it was possible to form a nanoparticulate system with $CaCl_2$. Formulations were prepared using 2 mg/ml MCC and at MCC:$CaCl_2$ ratios of 6 and 7.5 for MCC nanoparticles (Table 2) and, 2 mg/ml TMC and at a TMC:TPP ratio of 5.5 for TMC nanoparticles. Aggregates were observed both at higher TMC or MCC concentrations and low MCC:$CaCl_2$ or TMC:TPP ratios.

The particle size, zeta potential and loading efficiency of the nanoparticles are shown in Table 1. For MCC nanoparticles, the particle size was found to be between 40 and 90 nm with a negative surface charge −29 mV (Tables 1 and 2). TEM images confirmed the particle size results and showed non-aggregated nanoparticles (Fig. 2). High loading efficiency results (95–97 ± 2%) were obtained for MCC which can be attributed to the polyampholytic structure of the MCC, having both positively and negatively charged groups on the polymer chain.

For CS and TMC, the nanoparticle sizes were found to be between 300 and 400 nm, with a positive surface charge of +58 and +18 mV, respectively. It was possible to incorporate large amounts of TT into the nanoparticles prepared with chitosan and its derivatives with the loading efficiency of 78 ± 4% for CS, and 93 ± 3% for TMC (Table 1). Due to the positive charge of chitosan and TMC, and negative charge of TT, the antigen loading is expected to be provided by electrostatic interactions.

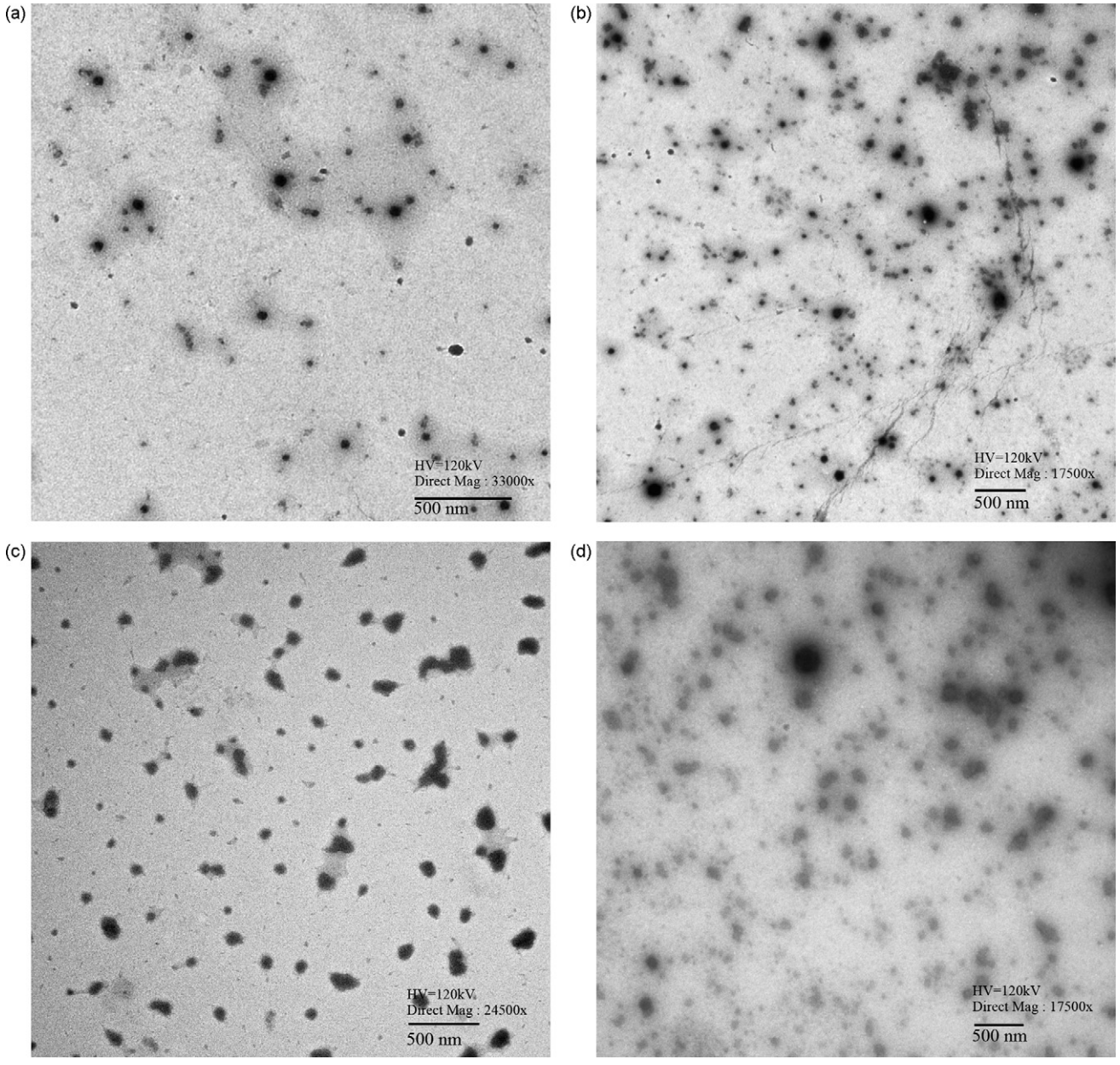

Fig. 2. TEM micrographs of MCC nanoparticles: (a) Empty, and (b) TT loaded (MCC concentration: 2 mg/ml; MCC:$CaCl_2$ ratio: 7.5), (c) TT loaded CS nanoparticles and (d) TT loaded TMC nanoparticles.

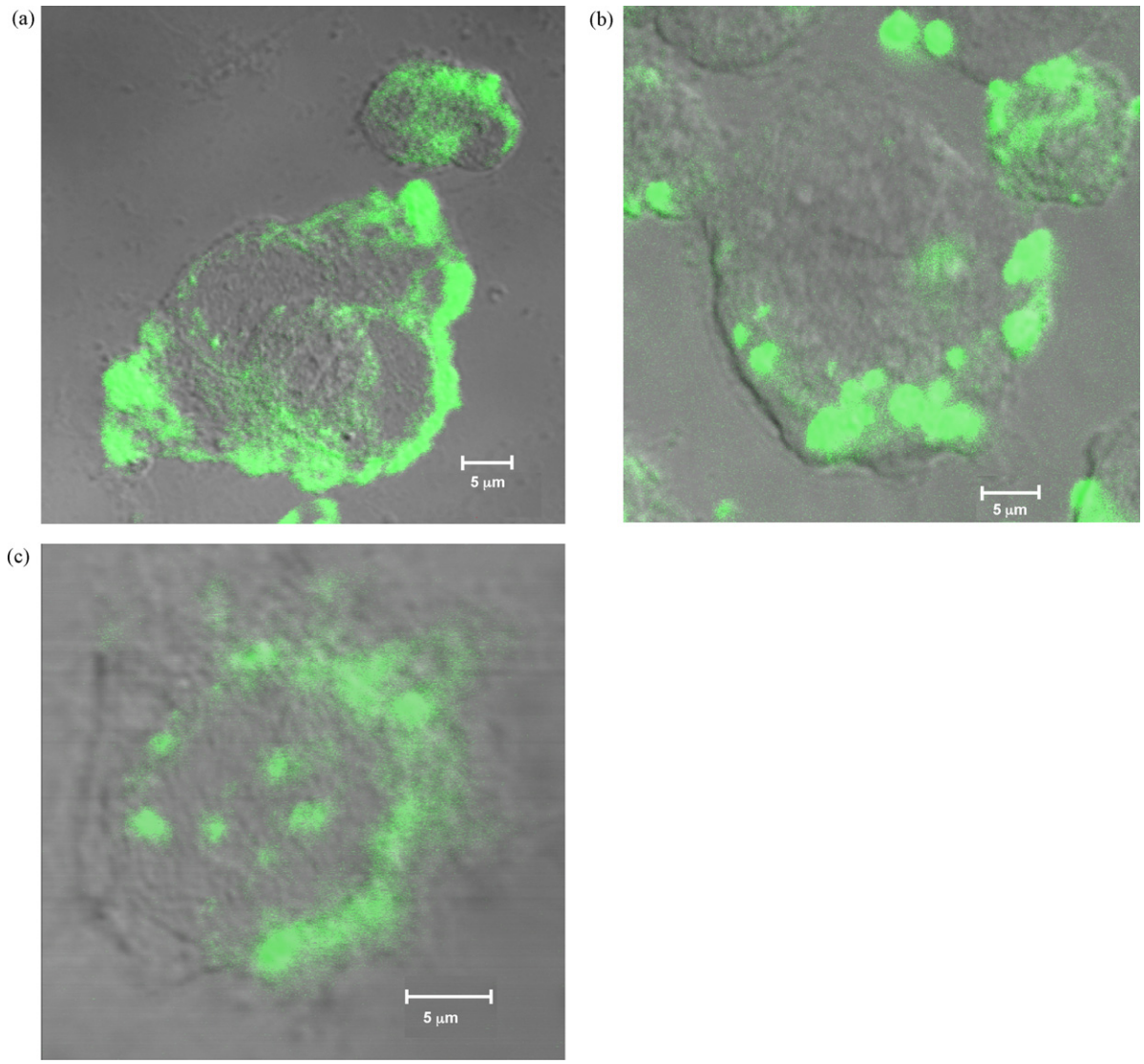

Fig. 3. Uptake of FITC-BSA loaded nanoparticles by J774A.1: (a) CS (b) TMC and (c) MCC.

3.3. Cellular uptake studies

FITC-BSA loaded CS, TMC and MCC nanoparticles were generally all taken up by the murine macrophage J774A.1 cells (Fig. 3). Since cell membranes are negatively charged, TMC and chitosan nanoparticles are expected to be more easily associated and subsequently taken up by the membranes than negatively charged MCC nanoparticles. All nanoparticles were located within the whole cell, taken up by the macrophages. There are several studies in the literature confirming cellular uptake of CS particles due to its mucoadhesive structure (Behrens et al., 2002; Huang et al., 2004; Chowdary and Rao, 2004). Behrens et al. showed that CS nanoparticles had a higher association and internalisation with gastrointestinal tissue cells due to electrostatic interactions compare to the polystyrene nanoparticles.

In addition to the surface charge, there are several other factors affecting the cellular uptake of the nanoparticles, such as particle size (Desai et al., 1997), particle hydrophobicity, and also adhesion molecules, e.g. lectin and B-12, associated onto the particles (Russell-Jones et al., 1999). Particle size is reported to be very important for uptake by the mucosal associated lymphoid tissues (MALTs) (Fell, 1996; Jepson et al., 1996), with more enhanced uptake by nanosize particles compared to the micronsize particles. In general, nanoparticles are expected to cross the mucosal epithelium better than microparticles, since not only M cells overlaying the mucosal associated lymphoid tissue (MALT) but also the epithelial cells are to a lesser extend involved in the transport of nanoparticles (Jabbal-Gill, 2001). The influence of particle size on the cellular uptake is significant. As shown in Fig. 3, the uptake of the MCC particles by J774A.1 macrophages was apparent, even though weaker than TMC and CS, and MCC particles are negatively charged.

3.4. In vivo studies

Increased specific IgG anti-TT titers were obtained with nanoparticles administered intranasally to mice compared to nonencapsulated TT control group (Fig. 4). The high humoral systemic immune responses elicited by the antigen-loaded nanoparticles is most likely based on cellular uptake of the nanoparticles in the nasal epithelium and NALT and subsequent access of the antigen to submucosal lymphoid tissues. CS and TMC nanoparticles resulted in significantly higher levels ($p < 0.01$) of serum IgG than that obtained with MCC nanoparticles. No significant difference was observed between CS and TMC ($p > 0.01$). For MCC nanopar-

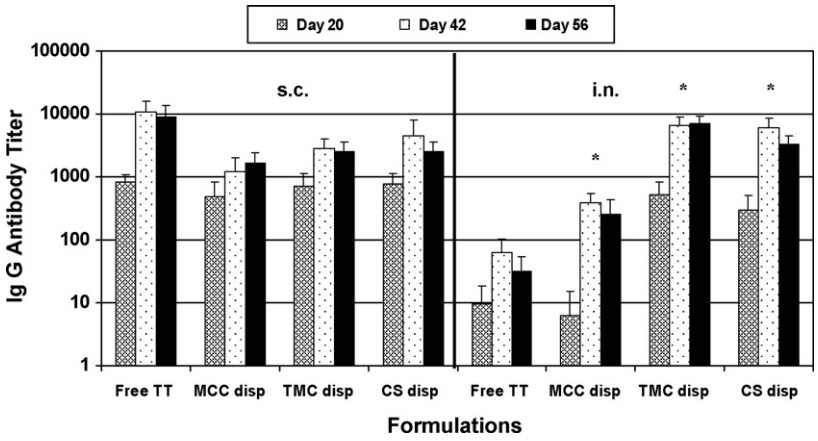

Fig. 4. TT specific serum IgG antibody titers following s.c. and i.n. delivery of TT in dispersions, Booster dose administration: day 22, i.n. application: 5 Lf TT/mice dose was administered in 25 μl; s.c. applications: 5 Lf TT/mice dose was administered in 200 μl ($n = 5$), * significantly higher than free TT ($p < 0.01$).

ticles, the antibody response was found to be significantly higher ($p < 0.01$) with larger particle size (90 nm) than that of the smaller size (45 nm) but still lower when compared to that obtained with CS and TMC. This may be due to both the difference in particle size and also surface charge of the nanoparticles which were significantly smaller than TMC and CS nanoparticles (Table 1).

MCC dispersions and nanoparticles administered nasally were found to induce significantly ($p < 0.01$) lower systemic immune responses than the groups subcutaneously vaccinated with MCC dispersion (Fig. 4). In a previous study by Gutierro et al. (2002) significantly higher immune responses were reported following nasal administration of BSA loaded PLGA nanospheres in relatively larger nanometer size particles (500 nm) than that of free BSA administration. It can be suggested that although a smaller particle size may be necessary for enhanced immune response, it is likely to be a threshold size. Especially at much smaller particles, such as the ones that we have produced (~40 nm), particles generally are prone to aggregation, which may turn nanoparticles into apparent larger size version. Furthermore, while the negatively charged glycocalyx on the apical membrane of mucosal cells can strongly interact with positively charged chitosan and TMC nanoparticles, this cannot take place with the negatively charged MCC nanoparticles. Therefore, this may also contribute to immunological outcome.

The difference we observed between the immune responses of positively charged polymers (CS and TMC), and negatively charged derivative MCC also can be explained by a bioadhesion mechanism. The differences in surface characteristics will affect the molecular interactions; such as the electrostatic, hydrophobic interactions, van der Waals and hydrogen bonding forces, between the interpenetrating bioadhesive polymer chains and the mucin network. Both negatively charged MCC and positively charged CS and TMC possess the electronegative groups associated with hydrogen bonding. However, the anionic MCC nanoparticles will also induce repulsive electrostatic forces with the negatively charged mucin network. These repulsive electrostatic forces would reduce the overall mucoadhesive strength. Charge differences, in conjunction with the differences in molecular contact and flexibility and particle size may explain the differences observed between immune responses of the two differently charged nanoparticle systems. In general, the more toxic potent the adjuvant, this may mean less suited for human use. As seen in MTT assay, the polyampholytic structure of MCC although provided increased cellular viability compared to TMC, it has also showed less potent adjuvant effect.

Especially for the intranasal applications, following boosting, the antibody levels were found to be increased (Figs. 4 and 5). Similarly, enhanced immune responses for various other antigens were reported with TMC and CS nanoparticle formulations (Baudner et al., 2003, 2005).

The aqueous dispersions of CS, TMC and MCC incorporating TT were also investigated *in vivo*. Significantly higher ($p < 0.01$) immune responses were obtained with aqueous dispersions following intranasal application compared to that of control (free TT in PBS solution) (Fig. 4). In our studies, nasal immunisation enhanced the antibody responses with the aqueous dispersions of TT-chitosan and TT-chitosan derivatives compared to that of the control TT-free solution group administered nasally, whereas, with subcutaneous administration, enhanced immune response was not obtained with the dispersions compared to that of the control antigen solution group following subcutaneous administration (Fig. 4). This maybe due to the nasal–mucosal bioadhesive property inherent to chitosan which decrease the clearance rate of formulations from the nasal cavity, nasally administered TT loaded formulations thereby leading to higher antibody titres. On the other hand subcutaneous route, chitosan's bioadhesive property may have not affected the immune outcome. For aqueous dispersions, no significant difference was observed between the immune responses with TMC, MCC or CS following subcutaneous administration. When we compared different routes of administration for the same dispersions, statistical results showed that, for TMC dispersion, enhanced antibody titres, whereas for MCC dispersions, nasal administra-

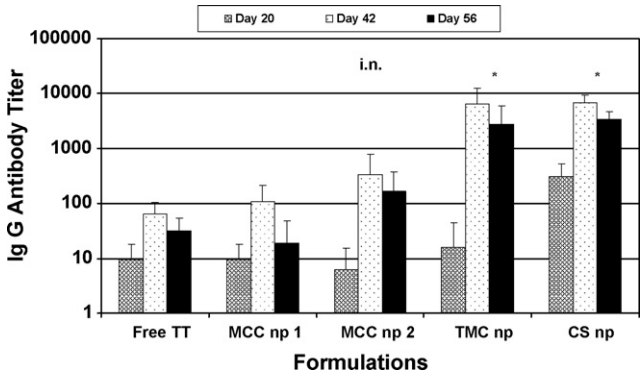

Fig. 5. TT specific serum IgG antibody titers following i.n. delivery of TT loaded nanoparticles ($n = 5$. mean ± S.D.) (particle size of nanoparticles: MCCnp1: 43 nm, MCCnp2: 90 nm, TMC: 306 nm, CS: 397 nm) Booster dose administration: day 22, i.n. application: 5 Lf TT/mice dose was administered in 25 μl np solution, * significantly higher than free TT ($p < 0.01$).

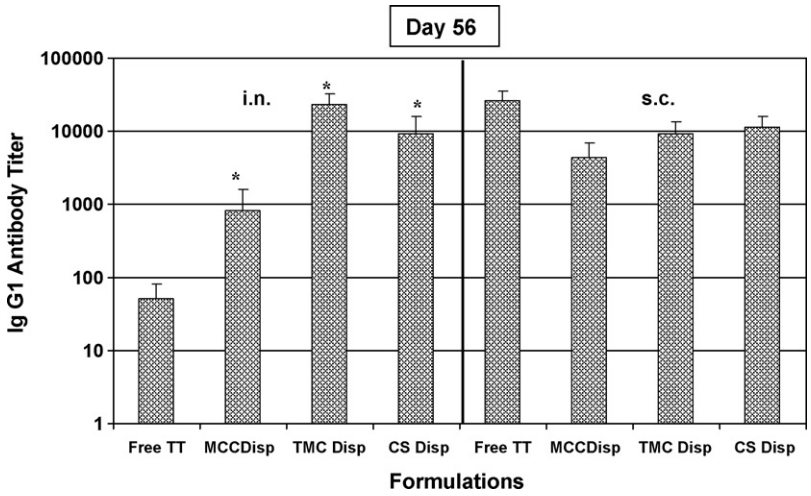

Fig. 6. TT specific serum IgG1 antibody titers on day 56 following i.n. and s.c. delivery of TT in dispersions, ($n = 5$. mean ± S.D.) Booster dose administration: day 22, i.n. application: 5 Lf TT/mice dose was administered in 25 μl; s.c. applications: 5 Lf TT/mice dose was administered in 200 μl ($n = 5$), * significantly higher than free TT ($p < 0.01$).

tion did not enhance the immune response. For CS dispersion, no significant difference ($p < 0.01$) was obtained between subcutaneous and nasal administration route. There are various studies reporting promising results on nasal delivery of several antigens including diphtheria toxoid (DT) (McNeela et al., 2001, 2004; Mills et al., 2003), tetanus toxoid (TT) (Westerink et al., 2002), influenza subunit vaccine (Read et al., 2005), group C meningococcal conjugated vaccine (CRM-MenC) (Baudner et al., 2003), botulinum toxin serotypes A, B and E (Ravichandran et al., 2007) using chitosan in solution form. Zaharoff et al. (2007) have investigated chitosan solution as an adjuvant for subcutaneous vaccination in mice with a model antigen, β-galactosidase and compared to other parenteral vaccine adjuvants, and demonstrated that chitosan was equipotent to incomplete Freund's adjuvant (IFA) and superior to aluminium hydroxide and therefore suggested chitosan as a promising and safe platform for subcutaneous vaccine delivery. Whereas following subcutaneous administration of the aqueous dispersions no significant difference was observed between the immune responses of different formulations. With TMC nanoparticles, it was possible to achieve higher serum immune response compared to that of subcutaneously administered TMC dispersion (Westerink et al., 2002).

The use of chitosan derivatives (especially with TMC) as a strategy to improve the response to nasally administered antigens is likely to be governed mainly by its immunostimulant and absorption enhancer effect jointly aiding the contact of the antigen with intraepithelial and submucosal lymphocytes independent of any pH effects. The potency of chitosan and its derivatives as permeation enhancers were reported as TMC > chitosan > MCC (Thanou et al., 2001). Furthermore, aggregation occurring between the antigen and the polymer in the aqueous dispersion resulting in colloidal structure might also enhance the uptake by M cells.

IgG subclasses, IgG1 and IgG2a have also been determined for all groups. The results obtained following i.n. and s.c. administrations are shown in Figs. 6 and 7. On day 56, higher serum IgG1 titres were obtained with both TMC and CS simple dispersions following intranasal or subcutaneous administration when compared to the control group ($p < 0.01$) (Fig. 6). For MCC and TMC formulations, significantly higher ($p < 0.01$) IgG1 titres were obtained for simple dispersions compared to nanoparticle formulations. Whereas for CS, higher IgG1 titer was obtained for nanoparticles compared to the dispersion of the same polymer. For both routes, IgG1 titres obtained with MCC dispersions and nanoparticles were significantly lower than that of the TMC and CS formulations ($p < 0.01$). Subcutaneous administration of MCC dispersion induced significantly ($p < 0.01$) higher immune response than intranasal administration. With IgG2a titres, no significant differences were observed between the groups ($p > 0.05$) (data not shown).

The serum immunoglobulin G subclass profiles are utilised as an indicative of T helper type 1 (Th1) or 2 (Th2) responses against the antigen (Huang et al., 2001; Zeng et al., 2007). In mice, Th1 cells mediate the prominent production of IgG2a antibody responses, whereas IgG1 antibody production has been shown to be associated with Th2 responses (Abbas et al., 1996). The ratio of IgG2a to IgG1 was calculated and for all formulations IgG2a/IgG1 was found to be smaller than 1 indicated a predominant Th2 response.

In spite of the fact that enhanced systemic antibody responses were obtained with nanoparticles following nasal administration of TT, this was not observed for the local antibody levels. Local Ig A antibody analysed in vaginal and nasal secretions were found to be not significant. Similarly, in a study performed by van der Lubben et al. (2003), following nasal application of DT loaded microparticles, the local antibody levels were not found to be high while enhanced systemic immune response was obtained. For a TT vaccine delivery, the main aim is to enhance the systemic immune response. In our study, higher antibody responses were achieved with the formulations developed using chitosan and its derivatives following nasal

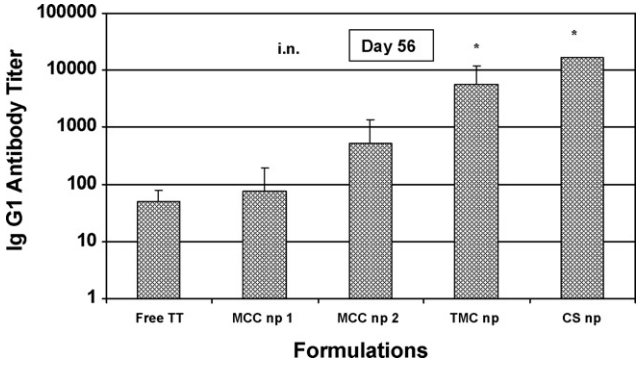

Fig. 7. TT specific serum IgG1 antibody titers on day 56 following i.n. delivery of TT loaded nanoparticles ($n = 5$. mean ± S.D.) (particle size of nanoparticles: MCC1: 43 nm, MCC2: 90 nm, TMC: 306 nm, CS: 397 nm) Booster dose administration: day 22, i.n. application: 5 Lf TT/mice dose was administered in 25 μl, * significantly higher than free TT ($p < 0.01$).

administration. Encapsulation of TT into the nanoparticles would bring advantages over simple dispersion form especially in protecting the antigen from the potential undesired environment at the nasal surface and also to prevent the loss of the antigen before reaching the target M-cells within the delivery system. Moreover, the retention time of the nanoparticles which was longer than that of the simple solutions therefore would allow efficient uptake of antigen through the mucosa thus improving the immune response. Besides, preparation of CS, TMC or MCC nanoparticles do not need any organic solvents which makes it more attractive and safe compared to those prepared with polymers needing an organic solvent for fabrication. Furthermore, there is no need to use any protein stabilisers.

4. Conclusion

In this study, TT-loaded nanoparticles and aqueous dispersions were prepared using chitosan derivatives with different charges (positively charged TMC and negatively charged MCC) and their potentials as adjuvant/delivery system for mucosal immunisation was investigated *in vivo*. This was the first time the negatively charged chitosan derivative, MCC was investigated for mucosal immunisation/delivery.

Both dispersion or nanoparticle systems prepared by chitosan derivatives were found to enhance mucosal immune responses. Our results emphasised that the nature of the surface charge and particle size exert an important role in obtaining an enhanced immune response using carriers prepared with chitosan. MCC induced relatively lower immune responses for TT when compared to TMC and chitosan yet it produced the smallest nanoparticles with much narrower size distribution and high loading capacity. With these desired features, MCC nanoparticles can be suggested as promising delivery systems for diverse range of drugs as well as a gene/protein delivery.

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