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N,N,N-Trimethyl chitosan nanoparticles for controlled intranasal delivery of HBV surface antigen

Ramesh Subbiah[a,b,1], Prakash Ramalingam[c,1], Subramaniyan Ramasundaram[b], Do Yang Kim[b], Kwideok Park[b], Mohan K. Ramasamy[c,*], Kyoung Jin Choi[d,*]

[a] *University of Science and Technology (UST), 113 Gwahangno, Yuseong-gu, Daejon 305333, Republic of Korea*
[b] *Biomaterials Center, Korea Institute of Science and Technology (KIST), Hawolgok, Seoul 136791, Republic of Korea*
[c] *Department of Pharmaceutics, Nandha College of Pharmacy, Erode, Tamil Nadu 638052, India*
[d] *School of Mechanical & Advanced Materials Engineering, Ulsan National Institute of Science and Technology (UNIST), Ulsan 698805, Republic of Korea*

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ABSTRACT

Hepatitis B virus surface antigen (HBsAg) loaded *N,N,N*-trimethyl chitosan nanoparticles (*N*-TMC NPs) were formulated and studied for controlled intranasal delivery. The size and surface properties of the NPs can be tuned by modifying the concentration of *N*-TMC and found to be 66 ± 13, 76 ± 9 nm for 0.25 and 0.5 wt.% respectively. Loading of 380 and 760 μl of HBsAg yielded 143 ± 33, 259 ± 47 nm sized spherical *N*-TMC NPs with highest loading efficiency and capacity of 90–93%, and 96–97% respectively. *In vitro* drug release analysis ensured 93% cumulative release of HBsAg antigen over prolonged period (43 days). *In vivo* immunological study was performed using 6–8 weeks old female BALB mice and reveals adjuvants efficiency of NPs for antigen is highly stable and better than standard. Obtained results show that *N*-TMC NPs can be extensively used in controlled intra nasal delivery to treat various diseases including hepatitis B and allergic rhinitis.

1. Introduction

Chitosan derived from chitin has emerged as a promising material with unmatchable properties including biocompatible, biodegradable, modifiable, mucoadhesive, antimicrobial, tumor cells recognition, and sustained release of loaded therapeutics (Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004, See Fig. 1). Therapeutic uses of chitosan were primarily limited by its poor solubility and permeability above pH 5.6. *N,N,N*-trimethyl chitosan (*N*-TMC) derived through amine functionalization of chitosan was found to have enhanced solubility, strength, porosity, absorption efficiency, chemical resistant, and non-antigenic properties (Kotze et al., 1998; Mourya & Inamdar, 2009; Muzzarelli & Tanfani, 1985). Consequently, *N*-TMC has been preferred for variety of biomedical applications such as controlled drug delivery system (CDDS) for vaccines, therapeutics and biomolecules (Balmayor, Baran, Azevedo, & Reis, 2012; Koutroumanis, Avgoustakis, & Bikiaris, 2010; Lee & Kim, 2011; Van der Lubben, Verhoef, Borchard, & Junginger, 2001), antimicrobials (Tsai, Chen, Liu, & Lai, 2011; Wu et al., 2011), scaffold matrix for the skin, nerve and bone tissue regeneration (Muzzarelli, 2009, 2011; Radhakumary, Antonty, & Sreenivasan, 2011).

Notably, formulation of *N*-TMC Nanoparticles (NPs) plays a major role in the pharmaceutical field due to excellent physicochemical properties (Hamman, Stander, & Kotze, 2002). For instance, administration of meningococcal vaccine (Baudner et al., 2004), protein (Amidi et al., 2006), influenza subunit antigen (Amidi et al., 2007), tetanus toxoid (Sayin et al., 2008), plasmid DNA for hepatitis B (Khatri, Goyal, Gupta, Mishra, & Vyas, 2008), ovalbumin (Slutter et al., 2010), exotoxin A (Taranejoo, Janmaleki, Rafienia, Kamali, & Mansouri, 2011), antibiotics (Balmayor et al., 2012) using mucoadhesive *N*-TMC NPs were reported. Only few reports have been documented the use of *N*-TMC solution and microparticles (100 to >1000 nm) in nasal vaccine delivery (Kang, Cho, & Yoo, 2009; Van der Lubben et al., 2002). Particularly, the delivery of HBsAg (Hepatitis B Virus surface antigen) vaccine using *N*-TMC NPs is not documented yet. Hepatitis B is a serious infectious disease which in chronic infection causes cirrhosis, hepatocellular carcinoma, and even death if left untreated. Report reveals 4 million clinical cases of acute Hepatitis B virus (HBV) ensues every year (Robinson, 1995; WHO, 2001). Though parenteral vaccines derived from plasma or recombinant DNA of HBsAg are able to induce protection in about 95% of recipients (Centers for Disease Control and Prevention, 1998), specific drug choice has not been evaluated for

* Corresponding authors.
E-mail addresses: thambiavn@gmail.com (M.K. Ramasamy), choi@unist.ac.kr (K.J. Choi).
[1] These authors contributed equally to this work.

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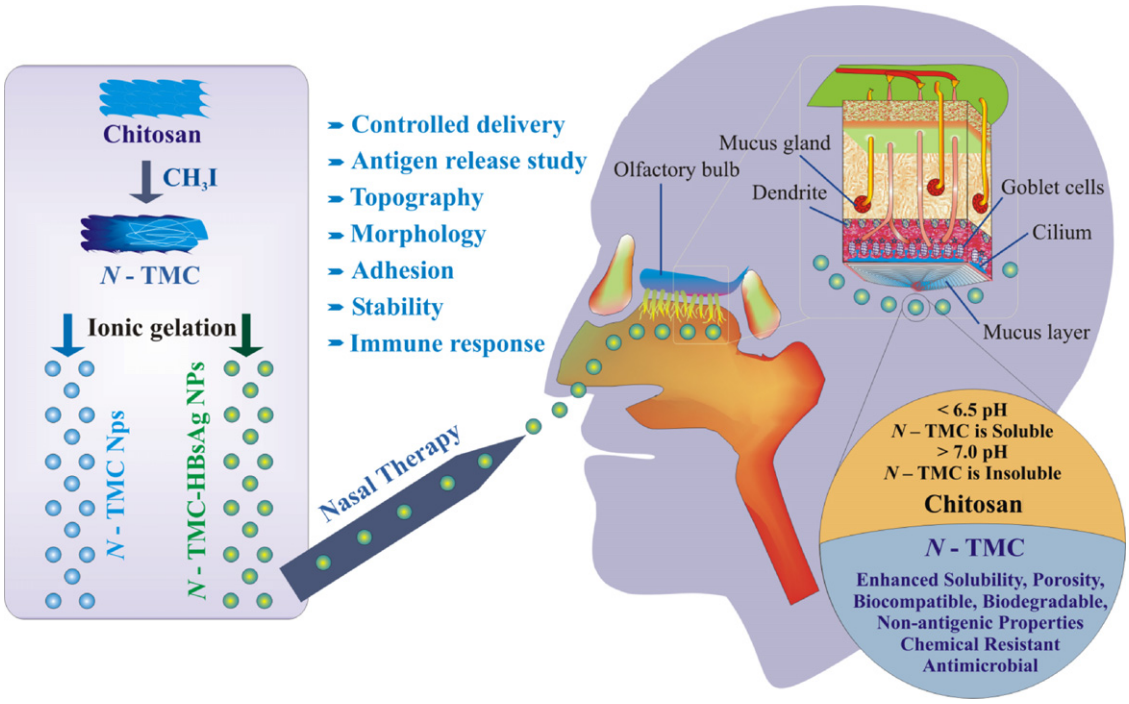

Fig. 1. Schematic flowchart represents the *N*-TMC NPs synthesis, HBsAg loading and intra nasal delivery of HBsAg loaded *N*-TMC NPs. The model nasal delivery and transportation mechanism of NPs in the nasal septum area, criteria for the nasal delivery and the study design have shown. Anatomy of nasal area and the advantage of *N*-TMC NPs over chitosan have illustrated schematically.

Hepatitis B (Mahoney & Kane, 1999). Also the current parenteral vaccination techniques exhibit low immune response, inefficient site for uptake and patient non-compliance. Hence an additional and alternative route of administration such as Intra nasal (*i.n.*) DDS can be employed to eliminate the colligated difficulties (Fig. 1, Illum, 2003).

Nasal mucosa possess abundant nasal associated lymphoid tissue (NALT), dentritic cells, large surface area, and low proteolytic enzymes that serve as a primary defense system against pathogens (Kang et al., 2009; Slutter et al., 2010). It can exhibit high drug concentration, permeation, no first-pass effect and compliance administration without enzymatic destruction (Al-Qadi, Grenha, & Remunan-Lopez, 2011; Taranejoo et al., 2011). Moreover, antigens encapsulated NPs ensure enhanced uptake and controlled release of antigens from the nasal vasculature membrane with strong immunogenicity and improved systemic therapeutic responses (Costantino, Illum, Brandt, Johnson, & Quay, 2007; Kyd, Foxwell, & Cripps, 2001). The possible route of administration for vaccination and therapy through nasal mucosa is schematically illustrated in Fig. 1. Above all *i.n.* DDS should exhibit maximum residing time, <100 nm size, ability of drug release in the nasal cavity with mucoadhesive properties, surface charge, and polymer biodegradation. Though *N*-TMC conjugated therapeutics show enhanced cellular uptake by NALT (Slutter et al., 2010), prolonged nasal residence time together with controlled release of antigens can be achieved exclusively by encapsulation of antigen into *N*-TMC NPs. In this context, the present investigation effort has been made to formulate *N*-TMC NPs with HBsAg for Hepatitis B *i.n.* vaccination. The size of *N*-TMC NPs (<85 nm) has been tuned by adopting suitable formulation strategy utilizing ionic gelation method. The morphology of formulation and its constituents were studied in detail using field emission scanning electron microscope (FESEM) and Bio-Atomic force microscope (Bio-AFM). The HBsAg loading efficiency, *in vitro*, *in vivo* release pattern and immune response produced after *i.n.* HBsAg vaccination has also been evaluated using 6–8 weeks old female BALB mice. Results reveal that *N*-TMC NPs can be used efficiently for controlled *i.n.* delivery of HBsAg vaccine and as CDDS for various diseases.

2. Experimental

2.1. Materials

Chitosan 150 cps (India sea foods), Plain Hepatitis-B antigen (HBsAg; Biological E Pvt. Ltd), Alum adsorbed Hepatitis B vaccine (Shantha Biotech), Glycerol, Bromophenol blue, Methyl iodide, Potassium Di hydrogen phosphate, 1-Methyl 2-pyrrolidinone (Loba Chemicals), Coomassie brilliant blue, Glycine, Polyacrylamide, Tris base, Mercapto ethanol (Himedia (P) Ltd), Disodium hydrogen phosphate (Merk), Ethanol (Changshuyangyan chemical), Diethyl Ether, Methanol, Sodium hydroxide (NaOH), Sodium dodecyl sulfate (SDS; Sd fine chem), Hydrochloric acid (HCl), Potassium chloride, Sodium iodide, Sodium chloride (NaCl; Nice Chemicals (P) Ltd), Tri poly phosphate (TPP; CD fine chemicals), Tween 80, Tween 20 (Ranbaxy Fine Chemicals Ltd) were purchased. The volume-adjustable micropipettes (Eppendorf AG, Germany) and Milli-Q water with resistance 18 MΩ were used in all our experiments.

2.2. Synthesis of N-TMC polymer

The *N*-TMC was synthesized by two step methylation (Amidi et al., 2006). 2 g of chitosan and 4.8 g of sodium iodide were dissolved in 80 ml of 1-methyl-2-pyrrolidinone on a water bath at 60 °C with continuous stirring. After the chitosan was dissolved, 11 ml of 15% aqueous sodium hydroxide solution was added, and then 11.5 ml of methyl iodide, the resultant mixture further stirred for 1 h. The product was precipitated using 70–100% ethanol and subsequently isolated by centrifugation. After washing, the material was dissolved in 40 ml of deionized water, to which 250 ml of 1 M HCl in ethanol (96%) were added carefully to exchange the iodide for chloride. *N*-TMC polymers with higher degree of quaternization (DQ) were prepared with 1 or 2 additional steps (R1 or

R2). A further step involved an addition of 5 ml of methyl iodide and 5 ml of 20% (w/v) aqueous NaOH prior to precipitation of the product from the solution mixture at the end of reaction step (R). The obtained product was dissolved in 40 ml of a 10% NaCl aqueous solution, instead of HCl to exchange the iodide. Centrifugation and washing with 70–100% ethanol and subsequently with ether yielded a white, water-soluble powder, which was dried at 40 °C under vacuum.

2.3. Evaluation of chemical structure and DQ

The purified *N*-TMC was analyzed by Bruker 300 MHz ^{1}H nuclear magnetic resonance spectroscopy in D_2O at 80 °C with a NMR spectrometer. Samples (5 mg) of each synthesized polymer were dissolved in 700 μl of D_2O in a NMR tube and the NMR spectrum of *N*-TMC was analyzed at 80 °C with suppression of the water peak. The DQ of *N*-TMC polymer was calculated from the ^{1}H NMR spectra of the different products using the following equation.

$$DQ = \left[\frac{[CH_3]_3}{H} \times \frac{1}{9}\right] \times 100$$

where DQ (%) is the degree of quaternization as percentage, $[(CH_3)_3]$ is the integral of the signal at 3.3 ppm attributed to the nine hydrogen atoms of the methyl groups pertaining to trimethylated amino groups. [H] is the integral of signals between 4.7 and 5.7 ppm [reference signals] representing the protons attached to the carbon of glucopyranose ring of the glucosamine unit (Sayin et al., 2009).

2.4. Preparation of N-TMC NPs

N-TMC NPs were prepared in the presence of Tween 80 as a re-suspending agent to prevent particle aggregations during purification. The formulation of *N*-TMC NPs and loading of HBsAg was done by Ionic gelation process technique using TPP as a cross linking agent under stirring. Briefly, an aqueous solution of 2 mg/ml (5 ml) of different concentration *N*-TMC polymer containing 1% (w/v) Tween 80 was prepared in water. Subsequently, different concentrations 1 mg/ml (1.8 ml) of TPP solution (pH 8) was slowly added drop-wise to the *N*-TMC solution (pH 6) while stirring (450 rpm) at room temperature for 1 h, yielding a final pH of around 7. The NPs suspensions were then concentrated and purified (from free *N*-TMC, TPP, Tween 80 and unbound protein) by gradient centrifugation. Aliquots of 1.5 ml NPs suspensions were centrifuged at 10,000 × g for 15 min. The supernatants were discarded and the pellets were re-suspended in phosphate buffered saline (PBS). To study the effect of concentration of *N*-TMC polymer and cross linking agent (TPP), three different concentrations such as 0.25, 0.5 and 0.75% of *N*-TMC polymer and several different concentration of TPP from 0.1 to 0.7 mg/ml were used. The different batches of NPs were prepared by ionotropic gelation method.

2.5. Preparation of HBsAg loaded with N-TMC NPs

6 mg of *N*-TMC was dissolved in 3 ml of PBS containing HBsAg (380 μl) and 0.5% (w/w) Tween 80. Thereafter, 1.5 ml of an aqueous TPP solution (1 mg/ml) was added drop wise to the *N*-TMC–HBsAg solution while stirring. Aliquots of 1 ml of the resulting antigen-loaded *N*-TMC NPs suspensions were centrifuged for 15 min at 10,000 × *g*. The supernatants were then discarded and the pellets were re-suspended in 10 μl of PBS. For the *i.n.* immunizations, 10 μl of suspension containing one dose of HBsAg loaded *N*-TMC NPs in PBS (pH 7.4) was administered in mice (Sayin et al., 2008).

2.6. Characterization

2.6.1. Morphology

The morphology and topography of the particles were examined by FE-SEM (JEOL 7500F) and Bio-AFM (Nanowizard II, JPK). The samples were air dried and sputter coated with platinum before examination by FE-SEM.

2.6.2. HBsAg loading efficacy

The amount of protein entrapped in the NPs was calculated by the difference between the total amount added to the loading solution and the amount of non-entrapped protein remaining in the supernatant. HBsAg concentrations in the supernatants were measured by the μBCA protein assay (Smith et al., 1985). Aliquots of the resulting NPs suspension were centrifuged for 20 min at 18,000 × *g* and 10 °C and the supernatants were then separated from the NPs. The amount of non-entrapped protein remaining in the supernatant was measured by the μBCA protein assay (Pierce, USA) using a micro plate reader with a 570 nm filter. A non-loaded NPs suspension without Tween 80 was used as a blank to correct for interference by *N*-TMC.

The loading efficacy (LE) of HBsAg loaded *N*-TMC NPs were calculated from the following equations:

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

The loading capacity (LC) of HBsAg loaded *N*-TMC NPs were calculated from the following equations:

$$LC(\%) = \frac{\text{Total amount of HBsAg} - \text{free HBsAg}}{\text{Nano particles dry weight}} \times 100$$

2.6.3. In vitro release study [stability study]

HBsAg loaded *N*-TMC NPs were separated by centrifugation at 10,000 × *g* and 4 °C for 15 min. The supernatant was decanted and the NPs were re-suspended in 6 ml of 0.1 M PBS (pH 7.4), then kept at 37 °C under magnetic stirring (100 rpm). At different time intervals, 0.5 ml of the suspension was taken and centrifuged (18,000 × *g*, 15 min). The protein concentration in the supernatant was analyzed by μBCA protein assay. The same volume of fresh PBS buffer was added to the release medium to reach the original volume. A sample consisting of only non-loaded *N*-TMC NPs re-suspended in PBS was used as a blank (Chen, Zhang, & Huang, 2007).

2.6.4. Compatibility study FT-IR spectral analysis

Fourier transformed-infrared spectra (FT-IR) of polymer and formulation were recorded after stability study using FT/IR-420 Jasco (Jasco Inc., Tokyo, Japan) FT-IR spectrophotometer instrument in attenuated total reflection (ATR) mode (Borges et al., 2005).

2.6.5. Estimation of protein integrity

SDS-polyacrylamide gel electrophoresis (mini-protean II cell; Bio-Rad Laboratories Hercules, CA, USA) was performed to evaluate the effect of the preparation process on protein integrity. HBsAg loaded *N*-TMC NPs were destabilized by adding 1 ml of 10% (w/v) NaCl to 6.8 ml of the NPs suspension, resulting in a solution with a protein concentration of 0.3 mg/ml. The protein was electrophoresed at 125 V under reducing conditions, in a 7.5% SDS-Polyacrylamide gel. The protein sample was prepared in electrophoresis loading-buffer (60 mM Tris–HCl, pH 6.8, with 25% glycerol and 2% SDS containing 0.1% Bromophenol blue solution, and h-mercaptoethanol) and heated for 5 min at 95 °C. After electrophoresis, the protein bands were visualized by staining with Coomassie Blue R-250 (Chen et al., 2007).

2.6.6. In vivo immunological study

Immunogenicity of *N*-TMC NPs containing HBsAg was evaluated in 6–8 weeks old female BALB mice. Test parameters were listed in Table 1. Animal care and handling were maintained according to the guidelines established by the Institutional Animal Ethical Committee (IAEC) of Nandha College of Pharmacy, Erode-52. The experiment protocols were approved by IAEC (NCP/IAEC/PG-44/2009) and the experiment procedures were in accordance with IAEC (Khatri et al., 2008).

Three successive *i.n.* immunizations with 1, 14, 28 day's intervals were performed in mice (five per group). Nasal dosing was performed by inserting a small piece of polyethylene tubing (straw, sterile), attached to a Hamilton syringe, 0.2 cm into the nostril (10 μl nanoparticle formulation/nostril) of the non-anesthetized animal (Supine position) and ejecting into the nasal cavity. Secondary immunization was done after 2 weeks with the same dose of formulations. Mice were also immunized with intramuscular (IM) injection of as well as alum adsorbed HBsAg (10 μg/animal) on 1st and 14th days with same formulations for comparison purposes. Blood was collected by retro-orbital puncture (under mild ether anesthesia) after 2 weeks interval of primary immunization and sera were stored at −40 °C until tested by ELISA for antibody. The nasal cavity was then flushed thrice with 0.5 ml of 1% PBS (pH 7.4). Salivation was induced by intraperitoneal injection of 0.2 ml sterile pilocarpine solution (10 mg/ml) in mice. The saliva from mice after 20 min was collected using capillary tube. These fluids were stored with 100 mM phenyl methyl sulfonyl fluoride (PMSF) as a protease inhibitor at −40 °C until tested by ELISA for secretory antibody (sIgA) levels. Finally the antigen-specific antibody responses were determined by using ELISA.

2.7. Statistical analysis

Each group was respectively compared, using independent Student's *t*-test. For all analyses, the probability of type I error less than or equal to 0.05 was considered as statistically significant. Each test was repeated for five separate times.

3. Results and discussion

This study demonstrates the synthesis of *N*-TMC polymer to be used as a CDDS in upper respiratory tract as sketched in Fig. 1. Ionotropic gelation method was used to synthesis *N*-TMC NPs which is reported to be one of the best methods for the formulation of NPs containing antigens (Sayin et al., 2008). To achieve the NPs formulation for HBsAg with *N*-TMC, systematic preformulation studies were carried out using formulation variables such as chemical modification of polymer, polymer concentration, cross linking agent, and antigen. HBsAg loaded *N*-TMC NPs were systematically characterized to evolve morphology, drug loading and releasing efficiency. *I.n.* vaccination and release studies were performed using both *in vitro* and *in vivo* models.

3.1. Preparation of N-TMC polymer from chitosan

Methylation of chitosan using CH_3I was achieved to yield *N*-TMC (Sieval et al., 1998). *N*-TMC polymers with different DQ were synthesized by altering the reaction step. Synthesis of *N*-TMC was confirmed by the ^1H NMR signals appeared at $\delta = 3.5$ and 4.1 ppm, which are corresponding to trimethyl groups of *N*-TMC. ^1H NMR spectrum has given in Electronic supplementary materials (Fig. S1). DQ of the *N*-TMC polymers increased with the number of reaction steps used in the synthesis procedure. The DQ of *N*-TMC obtained from single reaction (*N*-TMC-A; R) synthesis is found to be [21.3 ± 0.98%]. Higher DQ were reached in R1 (*N*-TMC-B; R + 1 Addition step synthesis) [40.0 ± 1.56%] and R2 (*N*-TMC-C; R + 2 addition step synthesis) [62.8%] synthesis process were used.

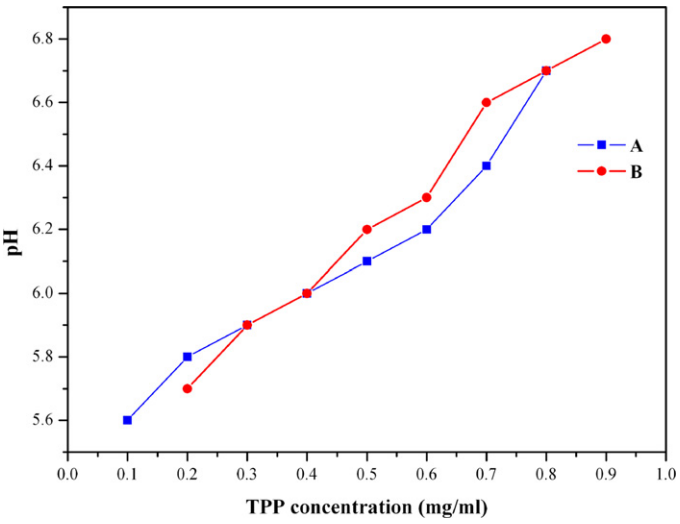

Fig. 2. Effect of concentration of cross-linking agent on the formation of NPs; pH Measurements of *N*-TMC polymer (TMC-A); (*N*-TMC-B).

3.2. Synthesis of N-TMC NPs

Ionic Gelation Method was used for the preparation of NPs and the use of complexation between oppositely charged macromolecules is easy and simple in mild conditions without using heat and organic solvents (Amidi et al., 2006). Moreover the reversible physical cross linking by electrostatic interaction has eliminated the possible toxicity and other undesirable effects of reagents used in chemical cross-linking. Cationic *N*-TMC interacts with polyanionic TPP by electrostatic forces. Adding aqueous acidic solution of cationic *N*-TMC in a drop wise manner to polyanionic TPP solution enhanced the complexation between oppositely charged species and formed spherical NPs. Three different concentration of *N*-TMC such as 0.25 wt.%, 0.5 wt.% and 0.75 wt.% were used to study the effect of concentration of polymer on the formation of NPs which plays a significant role. As shown in Fig. 2, the amount of TPP also had great influence on physicochemical properties such as pH and NPs size. The influence of the DQ of *N*-TMC on the NPs can only be investigated for amounts of TPP in the same range. The polymers with relatively close DQ can be selected to form NPs. DQ of these two polymers were 21.3% (*N*-TMC-A) and 40% (*N*-TMC-B). The pH values of the *N*-TMC solutions (2 mg/ml) and NPs suspensions are modified by adding TPP solutions of different concentrations and NPs could be formed using TPP concentrations of 0.1–0.8 mg/ml for *N*-TMC A and 0.2–0.9 mg/ml for *N*-TMC B. Higher TPP concentrations (>0.8 mg/ml for *N*-TMC A and >0.9 mg/ml for *N*-TMC B) led to aggregation with the increased pH value from 5.6 to 6.8.

3.3. Preparation of HBsAg antigen loaded N-TMC NPs

This mild technique involves the mixing of two aqueous solutions at ambient temperature while stirring without using sonication or organic solvents. The preparation method involves mixing the antigen into polymer solution. TPP is then added to the suspension under constant stirring. Since no acidic media employed for the loading of antigen this preparation method is simple. Due to the complexation between oppositely charged polymer and cross linker, polymer undergoes ionic gelation and precipitates to form NPs. Preparation conditions play an important role on the antigen,

Table 1
Immunological study design.

S. No.	Sample[a]	Volume [μl]	Route of administration	Immunization schedule [days]	Serum sampling [days]	Nasal secretion [days]
1.	Control (normal saline)	10	*i.n.*	1, 14, 28	1, 14, 28, 43	1, 14, 28, 43
2.	Alum adsorbed HBsAg	10	*i.m.*	1, 14, 28	1, 14, 28, 43	1, 14, 28, 43
3.	HBsAg-*N*-TMC NPs	10	*i.n.*	1, 14, 28	1, 14, 28, 43	1, 14, 28, 43
4.	HBsAg-*N*-TMC solution	10	*i.n.*	1, 14, 28	1, 14, 28, 43	1, 14, 28, 43
5.	HBsAg	10	*i.n.*	1, 14, 28	1, 14, 28, 43	1, 14, 28, 43

[a] All formulations were prepared and re-suspended in PBS.

Table 2
Size, loading efficiency, and loading capacity of *N*-TMC NPs loaded with HBsAg.

S. No.	Antigen/polymer	HBsAg concentration [μg/ml]	Particle size	Loading efficacy [%]	Loading capacity [%]
1.	HBsAg/*N*-TMC	380	143 ± 33	93.49 ± 1.19	96.8 ± 1.3
2.	HBsAg/*N*-TMC	760	259 ± 47	90.56 ± 1.66	97.51 ± 3.6

which can be denatured even by limited exposure to heat or organic solvents. Various concentration of HBsAg loaded *N*-TMC-B NPs was investigated and the systems with the highest loading efficacy were used for further studies. It was shown that HBsAg were efficiently associated with *N*-TMC-B NPs. The loading efficacy of HBsAg (380 μl and 760 μl) in the *N*-TMC B was 93.49 ±1.19% and 90.56 ± 1.66 (mean ± SD) and the loading capacity was 96.8 ± 1.3 and 97.51 ± 3.6 (Table 2).

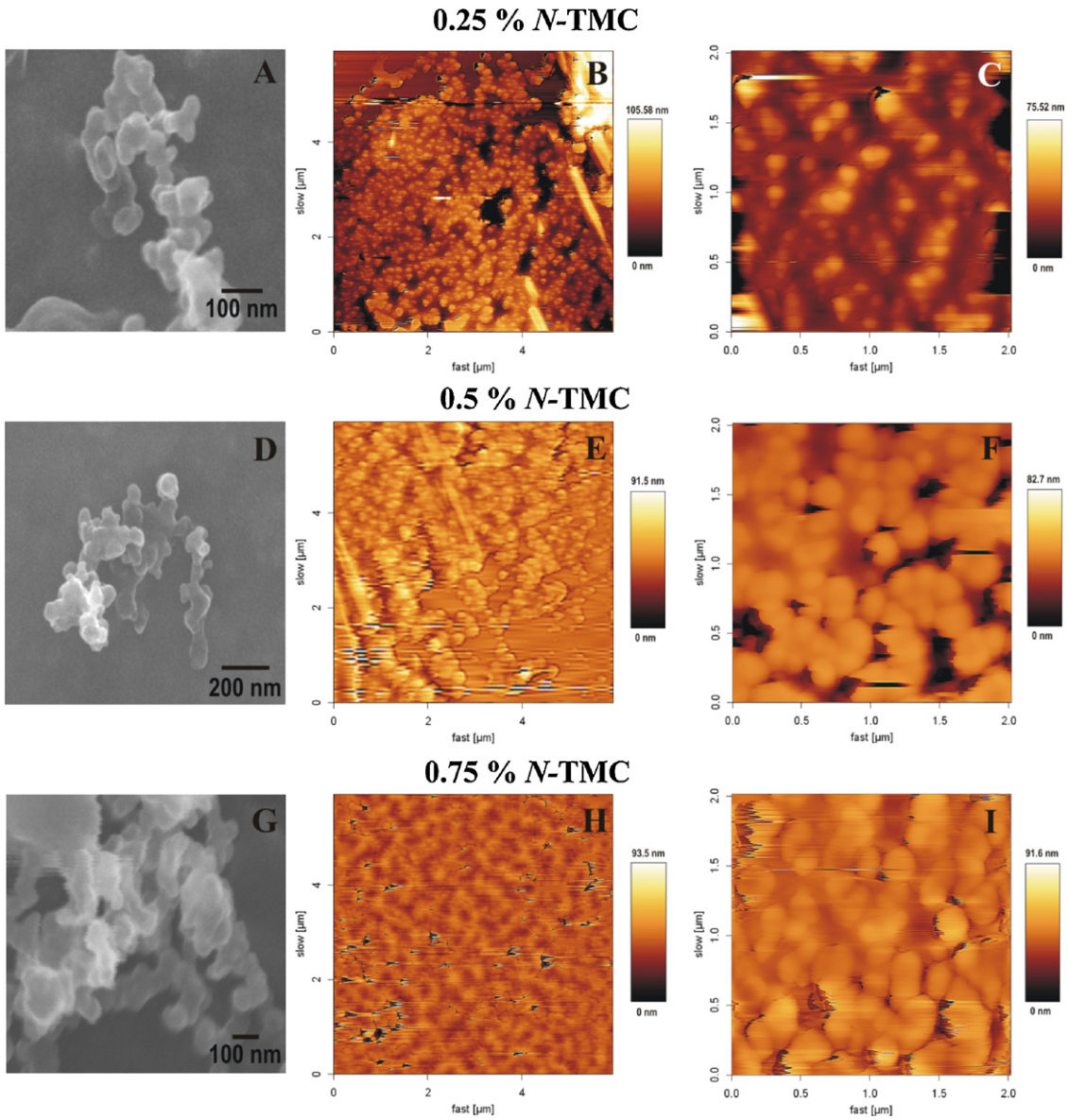

Fig. 3. Morphology and surface topography of *N*-TMC NPs were prepared as a function of *N*-TMC concentration. FESEM (A, D, G), AFM (B, E, H) and magnified AFM images (C, F, I) of 0.25%, 0.5%, and 0.75% of *N*-TMC NPs respectively.

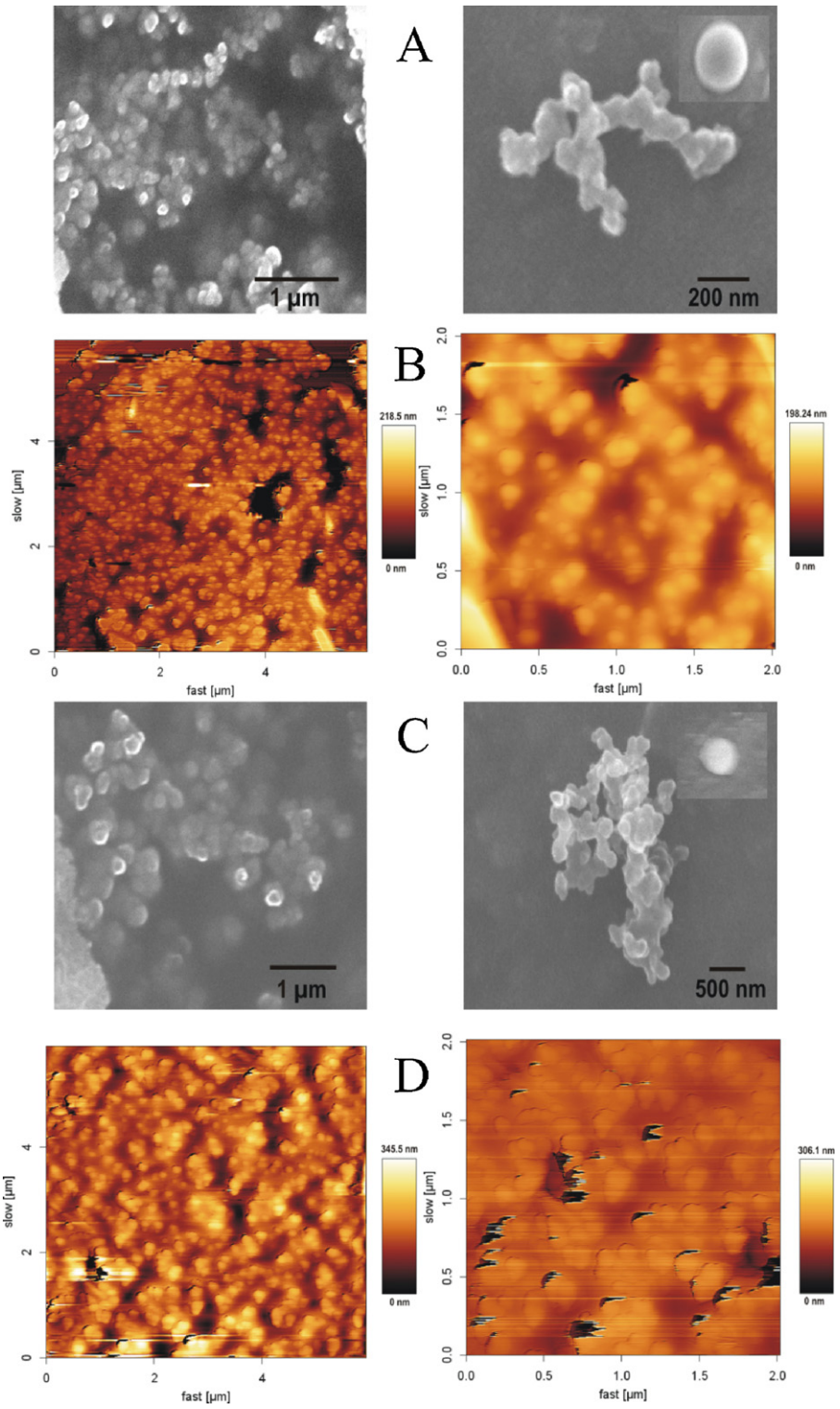

Fig. 4. FE-SEM and Bio-AFM images of (A, B) 380 μl HBsAg loaded *N*-TMC NPs, and (C, D) 760 μl HBsAg loaded *N*-TMC NPs.

3.4. Morphology and topography

The NPs size and enhanced antigen loading efficiency might play a major role in predetermining the nasal residing period and controlled delivery. The size of the NPs increases with increased concentration of polymer. The 0.25 wt.% concentration of *N*-TMC polymer produced 66 ± 13 nm sized spherical NPs with less aggregation with slight rough surface. The 0.5 wt.% *N*-TMC produced 76 ± 9 nm sized spherical shape fine free flowing powder with slightly more aggregation and decreased surface roughness. The NPs synthesized using 0.75 wt.% *N*-TMC shows 85 ± 8 nm sized sticky mass of spherical shaped NPs that shows smoother surface and reduced roughness. The NPs had uniform shape and smooth surface, which became rough after loading HBsAg (Fig. 3). The size of the chitosan derived NPs play a significant role to be used as a drug carrier in to the nasal mucosa. Larger NPs size reduces the surface

Table 3
The results of *in vitro* release μBCA protein assay show that HBsAg were stable in the release medium (0.1 M PBS pH 7.4) for 42 days.

Time in days	Amount of HBsAg released [μg] from *N*-TMC NPs	Percentage release	Cumulative percentage release
0	0	0	0
1	0.11	28.94	28.94
3	0.14	36.84	36.95
7	0.17	44.73	44.87
14	0.20	52.63	52.80
21	0.23	60.52	60.72
28	0.28	73.68	73.91
35	0.33	86.84	87.12
42	0.35	92.10	92.43

area and absorptivity and smaller size reacts with vaccines which lead to easy decomposition and failure of sustained drug release. Optimal conditions were thereby designed to synthesis *N*-TMC NPs of a desired size and stability. The actual size of *N*-TMC NPs was found to be 143 ± 33 nm and 259 ± 47 nm for two different types of antigen loading 380 μg/ml and 760 μg/ml respectively (Fig. 4). The particle size of the loaded NPs depends upon the concentration of antigen. For neat *N*-TMC NPs, the particle size was found to be between 60 nm to 260 nm. FE-SEM and Bio-AFM images confirmed the particle size and results showed non-aggregated NPs. The obtained high loading efficiency (90–93 ± 1.19%) and loading capacity (96–97 ± 1.3%) results evince the possibility to incorporate large amounts of HBsAg into the NPs prepared with *N*-TMC. The morphology and loading results presented here demonstrate that *N*-TMC NPs hold great promise as a delivery system for *i.n.* vaccination.

3.5. In vitro release study

In vitro release of HBsAg from the *N*-TMC NPs was examined using μBCA Protein assay, data listed in Table 3 and plotted in Fig. 5. The results show that HBsAg was stable in the release medium of 0.1 M PBS pH 7.4 for 42 days and indicate that 92.1% of the loaded HBsAg was released on day 42. It is clear that the release of HBsAg from *N*-TMC NPs were found to be sustained for a period of 6 weeks. The *N*-TMC NPs showed 28.9% release of HBsAg within 24 h, followed by 15.89% release in the initial 7 days. However no significant burst release was noticed and the release of antigen from the NPs was constant up to 42 days. The *in vitro* release of antigen is highly

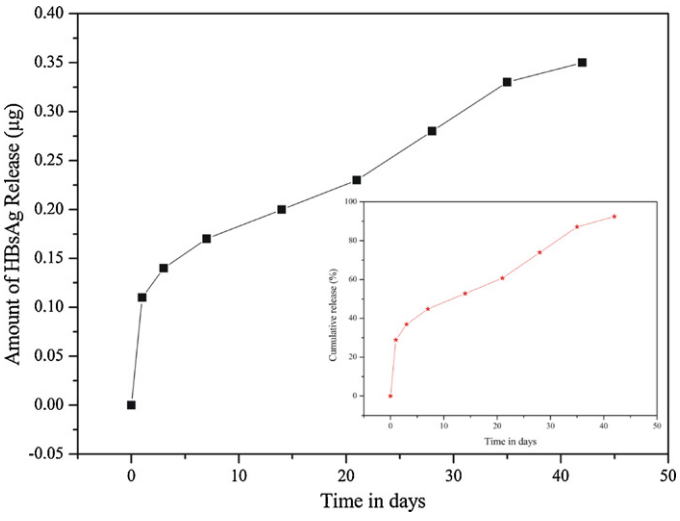

Fig. 5. *In vitro* release of HBsAg from the *N*-TMC NPs was monitored over 43 days. Inset shows the cumulative release of the HBsAg (93%) from NPs.

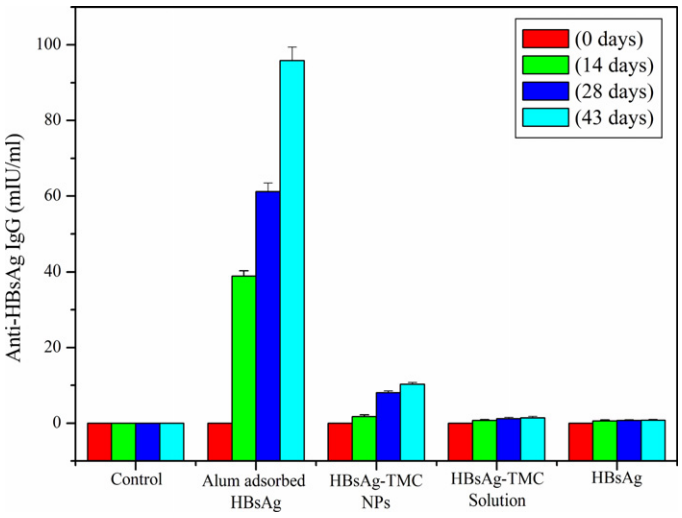

Fig. 6. IgG titers in serum of BALB mice treated with HBsAg *i.m.* or nasally were monitored after 43 days. The comparison between the standard group (II) and the HBsAg-*N*-TMC NPs was made with a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test (*$p < 0.05$).

depending on the concentration of antigen which might result in rapid *in vivo* release (Amidi et al., 2007). The plots of cumulative percentage release Vs time in days shows the release pattern of HBsAg from HBsAg loaded *N*-TMC NPs. The cumulative percentage release at point 92.43% was after 42 days.

3.6. Compatibility study FT-IR structural analysis

The FT-IR spectra of *N*-TMC and HBsAg loaded *N*-TMC NPs were provided in Electronic supplementary materials (Figs. S2 and S3) and interpretation of FT-IR results are shown in Table 4. The presence of polymer in the formulation was well identified by FT-IR spectra. The characteristic peaks due to pure polymer have appeared in NPs formulation, without any remarkable change in their position after successful NPs formulation, indicating no chemical interaction between antigen and polymer. It is also confirmed by polymer during formulation process.

3.7. Antigen integrity

The antigen integrity of HBsAg before and after encapsulation was estimated by SDS-PAGE analysis followed by Coomassie brilliant blue staining. The antigen integrity of the HBsAg within NPs was found to be intact before and after antigen loading. Images are provided in Electronic supplementary materials (Fig. S4). SDS-PAGE gel analysis results confirmed the structural integrity of the HBsAg in the NPs indicating that the integrity of HBsAg has been maintained during preparation.

3.8. In vivo study

HBsAg loaded *N*-TMC NPs were used for immunization studies in the mice. Antibody responses generated by *i.n.* immunization. The method used to measure antibody level was ELISA. From results among the groups immunized with Alum adsorbed HBsAg injection, HBsAg loaded *N*-TMC NPs, HBsAg loaded *N*-TMC solution, HBsAg and control (untreated). The protective Anti-HBsAg IgG titers value and Anti-HBsAg IgA titers value results were found and listed in Tables 5 and 6, and plotted in Figs. 6 and 7. In the present work, the *i.n.* administration of PBS with 10 μg of HBsAg without any adjuvant (group V) was not able to induce mucosal or systemic antibodies. Hence an adjuvant is required for the *i.n.* route to

Table 4
Compatibility study FT-IR structural analysis.

S. No.	Transition	IR range [cm^{-1}]	Wave number [cm^{-1}]	
			N-TMC polymer	Mixture [HBsAg loaded with *N*-TMC NPs]
1.	O—H stretching	3570–3200	3431.48	3448.84
2.	C=O stretching	1725–1705	1705.13	1703.20
3.	NH bending	1542	1546.96	1546.96
4.	C—N stretching	1414	1413.87	1425.44

Table 5
Anti-HBsAg IgG titer values.

S. No.	Formulations	R.O.A	Anti-HBsAg IgG [mIU/ml] Days			
			0	14	28	43
1.	Control (normal saline)	*i.n.*	0	0	0	0
2.	Alum adsorbed HBsAg (standard)	*i.m.*	0	38.92 ± 1.4	61.16 ± 2.3	95.88 ± 3.55
3.	HBsAg-*N*-TMC NPs	*i.n.*	0	1.692 ± 0.5	8.06 ± 0.45	10.26 ± 0.47
4.	HBsAg-*N*-TMC solution	*i.n.*	0	0.742 ± 0.19	1.192 ± 0.36	1.392 ± 0.37
5.	HBsAg	*i.n.*	0	0.608 ± 0.24	0.708 ± 0.20	0.798 ± 0.21

Table 6
Anti-HBsAg IgA titer values.

S. No.	Formulations	R.O.A	Anti-HBsAg IgG [ng/ml]/total IgA [μg/ml] Days			
			0	14	28	43
1.	Control (normal saline)	*i.n.*	0	0	0	0
2.	Alum adsorbed HBsAg (standard)	*i.m.*	0	0.62 ± 0.09	0.736 ± 0.1	0.892 ± 0.09
3.	HBsAg-*N*-TMC NPs	*i.n.*	0	30.98 ± 4.52	48.03 ± 5.24	64.26 ± 5.59
4.	HBsAg-*N*-TMC solution	*i.n.*	0	6.74 ± 1.45	8.02 ± 1.26	10.06 ± 1.84
5.	HBsAg	*i.n.*	0	0.402 ± 0.06	0.512 ± 0.03	0.67 ± 0.08

generate a HBV protective immune response. It has been reported that alum (insoluble aluminium salts), one of the few adjuvants approved by the US Food and Drug Administration, is ineffective for the induction of mucosal immunity. Single *i.n.* vaccination with HBsAg-loaded *N*-TMC NPs resulted in strong IgG immune responses, in contrast to free antigen and polymer solution co-administered with soluble antigen. This indicates that *N*-TMC NPs can act as a strong immunostimulator for locally administered vaccines. Moreover, a single *i.n.* immunization of the HBsAg-loaded *N*-TMC NPs induced significantly higher IgG antibody titers ($^{*}p < 0.05$). This makes the *N*-TMC NPs especially attractive as a nasal vaccine delivery system. After a single *i.n.* immunization antigen co-administered with soluble *N*-TMC was much less immunogenic than HBsAg-loaded *N*-TMC NPs. This shows a moderate adjuvant effect of *N*-TMC NPs. Consequently, antigen can be taken up in nasal tissues in a prolonged time (Amidi et al., 2007). Average mice environment (50% relative humidity and 22 °C) was maintained for *in vivo* study and not much change noticed in the mucosal adhesion of experimental groups. It is believed that *N*-TMC NPs might enhance the adherence and residence of HBsAg on the nasal mucosal membrane and can be administered without any adhesion additives. Also, an *i.n.* vaccination is effectively potentiating the induction of IgA antibodies at the mucosal epithelium. The IgA in the nasal lavages indicates vaccine-induced stimulation of IgA-secreting cells of the NALT. It is clear from the results that the *i.n.* administered HBsAg-loaded *N*-TMC NPs were the only formulation that induced IgA titers ($^{**}p < 0.01$) after three immunizations in all mice. In contrast, *i.m.* administered antigen formulations did not show IgA response, that *i.m.* administrations of antigen formulations were not able to induce any IgA response, not even after several booster immunizations.

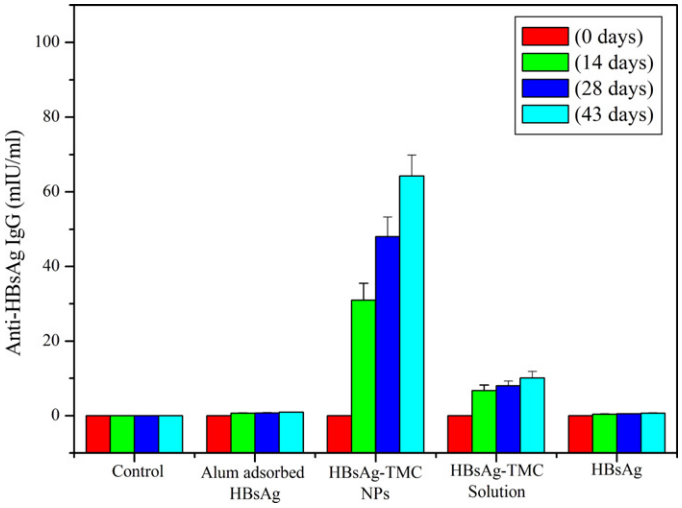

Fig. 7. IgA titers in serum of BALB mice treated with HBsAg *i.m.* or nasally were monitored after 43 days. The comparison between the standard group (Alum adsorbed HBsAg) and the HBsAg-*N*-TMC NPs was made with a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test ($^{**}p < 0.01$).

The prolonged nasal residence time, sustained therapeutic release with significant burst delivery in the initial 24 h with nasal tolerability are the important factors for the *N*-TMC NPs *i.n.* delivery. Moreover the antigen integrity for the successful delivery with enhanced immune response would destine the prompt therapy. The data presented in this study evince that *N*-TMC NPs can be used widely for the *i.n.* delivery of therapeutics for prevention or for treatment such as allergic rhinitis, asthma and chronic obstructive pulmonary disease.

4. Conclusions

Covalent functionalization of methyl group on chitosan with high DQ was achieved and the resultant *N*-TMC polymer was

structurally elucidated using ^{1}H NMR. Ionic gelation method was used to synthesis NPs and subsequently loaded with different concentration of antigen. The effect of polymer, cross linking agent, HBsAg on the formation and physicochemical properties of *N*-TMC NPs were analyzed appropriately. The size and surface properties was tuned by modifying the concentration of *N*-TMC (66 ± 13, 76 ± 9 nm for 0.25 wt.% and 0.5 wt.% respectively) and the results evince that the size increased upon increasing the drug load and hence resulted in controlled drug release. The excellent antigen loading capacity of 380 μl and 760 μl HBsAg was noticed and the cumulative percentage release of antigen was attained around 93% for 43 days. *In vivo* immunological result evinces that the adjuvant efficiency for HBsAg is highly stable and better than standard. Our research continues for the evaluation of bio-nano interface mechanism of NPs adhesion force on nasal cells, cell-NPs interaction and role of surface roughness using *in vitro* models and Bio-AFM force spectroscopy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2012.04.056.

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