PART II: TMC-BASED FORMULATIONS FOR INTRADERMAL AND TRANSCUTANEOUS VACCINATION

Chapter 5

Efficient induction of immune responses through intradermal vaccination with TMC containing antigen formulations

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Abstract

The function of N-trimethyl chitosan (TMC) in immunisation via the skin is unknown. Therefore we investigated the immunogenicity of both antigen-containing TMC nanoparticles and TMC/antigen solutions after intradermal injection. Nanoparticles were prepared with a size around 200 nm and a positive zetapotential. In vitro, TMC nanoparticles increased the uptake of OVA by dendritic cells (DCs) and both nanoparticles and TMC/OVA mixtures were able to induce upregulation of MHC-II, CD83 and CD86. These activated DCs could induce a Th2 biased T cell proliferation. A solution of plain OVA did not induce DC maturation or T cell proliferation. In vivo, mice were injected thrice with TMC based formulations containing either OVA or diphtheria toxoid (DT), a more relevant antigen. All TMC containing formulations were able to increase the IgG titres compared to unadjuvanted antigen and induced a Th2 biased immune response. When using DT-containing TMC formulations, IgG titres and neutralising antibody titres could match up to those obtained after subcutaneous injection of DT-Alum. In conclusion, both soluble TMC/antigen mixtures and TMC nanoparticles are able to induce DC maturation and enhance immune responses after intradermal injection. This demonstrates that TMC functions as an immune potentiator for antigens delivered via the skin.
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Introduction
Currently, most vaccines in development are subunit vaccines. In comparison to live attenuated vaccines and whole inactivated organisms subunit vaccines are safer, because of their higher purity. However, the high purity reduces the immunogenicity of subunit vaccines, which poses a challenge in designing formulations [1]. Subunit vaccines often require adjuvants to achieve a more effective immune response. Adjuvants are substances or devices that enhance the delivery or immunogenicity of an antigen. Improved delivery can prolong the localisation time of the antigen at the site of action and increased immunogenicity implies better activation of antigen presenting cells (APCs), especially dendritic cells (DCs) [2]. Since it has become clear that APCs have such a central role in the first steps of the immune response, interest into the choice of the administration route has increased. In the muscle, the most common vaccination site, only few APCs are present [3]. Higher numbers are found in the skin and the mucosal membranes, where invading pathogens are usually encountered.

In this study we investigate the skin as immunisation site and the use of N-trimethyl chitosan (TMC) as an adjuvant. In the skin two types of APCs, Langerhans cells (LCs) and DCs are present in the epidermis and the dermis, respectively [4, 5]. The main function of these cells is to take up an antigen, process it and present it to T cells. The presence of LCs and DCs in the skin is one of the great values of vaccination via the skin, which may be a dose-sparing alternative to conventional immunisation routes. For instance, low antigen doses administered intradermally (i.e. injected into the dermis) have been reported to elicit a similar or better immune response compared to higher doses given intramuscularly [6-8]. The only intradermal vaccines currently on the market are against BCG and rabies, and recently Intanza(R) was approved as an intradermal influenza vaccine. An excellent review on recent clinical studies of intradermal immunisation was published by Nicolas et al. [9].

To deliver the antigen to the APCs, it is important to have an interaction of the antigen with a particle [10-13]. Particles do not only increase the exposure time of the antigen to DCs, but can also improve the uptake and maturation of DCs, because their size is more comparable to that of viruses or bacteria. The ideal size of a particle has not yet been determined and may depend on the immunisation route, but some evidence exists that skin APCs preferentially take up small nanoparticles [14]. Nanoparticles can be prepared from natural polymers such as starch [15] and chitosan [16] or synthetic polymers such as D-poly L-lactate (PLA) and poly (DL-lactic-co-glycolic acid) (PLGA) [17]. Chitosan is present in small amounts in some micro-organisms and fungi, and can be derived by deacetylation of the naturally occurring polysaccharide chitin [16]. Its main drawback is the insolubility at a physiological pH. Chitosaccharide chitin [16] can be made more water-soluble by chemical modification.
One possible modification is the introduction of three methyl groups on the NH$_2$ group of chitosan resulting in TMC [18, 19]. This soluble, positively charged derivate has been studied as an adjuvant in vaccine delivery applications for various routes of administration, such as oral [20, 21], nasal [22-25] and pulmonary [26]. However, in all these studies the adjuvant effect of TMC was mainly ascribed to its mucoadhesive properties. In vaccination via the skin this does not play a role and to our knowledge TMC has never been used in transcutaneous (i.e. through application onto the skin) or intradermal vaccination. It has only been shown to enhance the transdermal delivery of low-molecular-weight drugs [27]. Recently, studies performed in our lab showed that TMC nanoparticles can induce maturation of DCs \textit{in vitro} [20]. This indicates that TMC has adjuvant properties besides its function as a mucoadhesive. Our interest is to investigate TMC as an adjuvant in transcutaneous or intradermal vaccination. In transcutaneous vaccination, the efficiency of the delivery system not only depends on the interactions of the delivery system with the DCs, but is also largely dependent on the transport of the vaccine across the skin barrier. The outermost layer of the skin, the stratum corneum, acts as a formidable barrier for the transport of compounds. One of the methods to overcome this barrier is by using microneedles to pierce small conduits in the skin [28]. Although transcutaneous vaccination is our final goal, in this study we will focus on the efficiency of TMC as an adjuvant. Therefore we will avoid the complicating factor of the transport issue across the skin barrier mentioned above. We will explore the immune potentiation of TMC to determine whether the characteristics of the TMC polymer itself or the particulate nature play a prominent role in the adjuvant effect. It is known that TMC nanoparticles can function as an adjuvant using other routes of administration, but whether the TMC polymer itself also functions as an immune potentiator is unclear. For this purpose TMC nanoparticles and mixtures of a TMC and an antigen were compared concerning \textit{in vitro} DC maturation, the \textit{in vitro} T cell proliferation and finally the immune potentiation in intradermal vaccination. Formulations made of TMC with different degrees of quarternisation (DQ) and loaded with ovalbumin (OVA) or diphtheria toxoid (DT) were used.

\textbf{Materials and methods}

\textbf{Materials}

Chitosan (MW 120 kDa) with a degree of deacetylation of 92% was obtained from Primex (Alversham, Norway). Pentasodium tripolyphosphate (TPP), N-(2-hydroxyethyl) piperazine-N’-(2-ethanesulphonic acid) (HEPES) and Tween 20 were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). Ovalbumin grade VII was obtained from Calbiochem (Merck KGaA, Darmstadt, Germany) FITC and Alexa647 labelled ovalbumin (OVA$_{FITC}$ and
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OVA_{AF647} respectively) were purchased from Invitrogen (Breda, The Netherlands). Diphtheria toxin (DTa 79/1) and DT (batch 98/40, protein content 12.6 mg/ml by BCA assay, 1 μg equal to approximately 0.3 Lf) were a kind gift from the Dutch Vaccine Institute (NVI, Bilthoven, The Netherlands). Horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (γ chain specific), IgG1 (γ1 chain specific) and IgG2a (γ2a chain specific) were purchased from Southern Biotech (Birmingham, USA). Chromogen 3, 3', 5, 5'-tetramethylbenzidine (TMB) and the substrate buffer were purchased from Invitrogen. Nimatek® (100 mg/ml Ketamine, Eurovet Animal Health B.V., Bladel, The Netherlands), Oculentum Simplex (Farmachemie, Haarlem, The Netherlands), Rompun® (20 mg/ml Xylazine, Bayer B.V., Mijdrecht, The Netherlands) and the injection fluid (0.9% NaCl) were obtained from a local pharmacy. All other chemicals were of analytical grade.

Animals

Female BALB/c mice (H2d), 8-weeks old at the start of the vaccination study were purchased from Charles River (Maastricht, The Netherlands), and maintained under standardised conditions in the animal facility of the Leiden/Amsterdam Centre for Drug Research, Leiden University. The study was carried out under the guidelines compiled by the Animal Ethic Committee of the Netherlands.

TMC synthesis

TMC with a variable DQ was synthesised by methylation of chitosan by using iodomethane in the presence of a strong base (NaOH) as described previously [29]. In short, 2 g of chitosan and 4.8 g sodium iodide were dissolved in 80 mL 1-methyl-2-pyrrolidinone and after stirring for 20 minutes at 60°C 12 mL 15% NaOH and 12 mL iodomethane were added. The mixture was refluxed for 60 minutes after which the TMC was precipitated with ethanol and diethyl ether. To synthesise TMC with an increasing DQ an additional amount of NaOH (5-14 mL) and 5 mL of iodomethane were added before precipitation. The obtained polymer was purified by dialysis against 1% NaCl for 4 days followed by dialysis against water for 2 days at 4°C. Finally the product was freeze-dried. The purified TMC was analysed by $^1$H-nuclear magnetic resonance (NMR) spectroscopy. For this measurement the TMC was dissolved in D$_2$O and the spectrum was recorded at 80°C with a DMX 400 MHz NMR spectrometer (Brucker, Switzerland). The degree of quarternisation was calculated according to the following equation:

$$DQ = \frac{[(CH_3)_3]}{[H] \times 1/9} \times 100$$

(1)
[(CH₃)₃] is the integral of the trimethyl amino group at 3.3 ppm and [H] is the integral of the hydrogen peaks of the carbon 1 atom of TMC between 4.7 and 5.7 ppm [19].

**Preparation and characterisation of TMC formulations**

Both TMC nanoparticles and mixtures of TMC and antigen were prepared. For nanoparticle preparation TMC of two different DQ was used, namely 15 and 30% (TMC15 and TMC30). TMC nanoparticles were prepared by ionic complexation with TPP as was previously described [20]. Shortly, for a 10 mL batch of nanoparticles an aqueous solution of TPP (1 mg/mL) was added drop wise to 5 mL of TMC solution (10 mg) in 5 mM HEPES pH 7.0 while stirring until the solution became slightly opalescent. OVA and DT loaded nanoparticles were prepared by dissolving the antigen (1 mg) in the TMC solution before adding the TPP solution. The amount of TPP added depended on the antigen and on the DQ of the TMC used (varying between 1.3 and 2.0 mL). After 1 hour of stirring, the nanoparticle suspension was centrifuged for 15 minutes at 10000 g on a glycerol bed and the pellet was resuspended in a 5 mM HEPES buffer adjusted to pH 7.0.

Mixtures of TMC and OVA or DT were prepared by mixing a solution of TMC and the antigen in a 2.5:1 ratio. The size of the nanoparticles and mixtures was determined by dynamic light scattering (DLS) and the zetapotential was determined by laser Doppler velocimetry using Zetasizer® Nano ZS (Malvern Instruments, UK). The amount of TMC and TPP in the nanoparticles was determined with a ninhydrin assay and a phosphate determination, respectively [30, 31].

**Nanoparticle visualisation**

To characterise the morphology of the nanoparticles, they were visualised with scanning electron microscopy. 50 µL of 0.1% w/v nanoparticle suspension was air dried overnight on an adhesive sample holder. Afterwards the samples were gold/palladium sputtered using a sputter coater device K650X (Emitech, Hailsham, UK) and analysed with a JEOL JSM-6700F scanning electron microscope (Jeol, Tokyo, Japan).

**Loading efficiency of TMC nanoparticles**

The amount of encapsulated OVA or DT in the nanoparticles was determined by measuring the amount of protein remaining in the supernatant with a micro-BCA protein assay (Pierce, Rockford, IL, USA) after centrifugation (15 minutes, 10000 g). The same was done with the TMC/antigen mixtures to determine the adsorption of antigen to the TMC polymer. For the OVA_FITC loaded nanoparticles the amount of OVA was determined by measuring the amount of OVA_FITC in the supernatant with a FS920 fluorimeter (ex 488 nm, em 520 nm) (Edinburgh Instruments, Campus Livingston, UK). To avoid differences in
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fluorescence due to pH changes, 25 µl of 5 M NaOH was added to all samples. For both methods a non-loaded nanoparticle suspension was used as a blank to correct for interference by TMC. The loading efficiency was determined with the following equation:

\[
\text{Loading efficiency} = \frac{(\text{Total amount of protein} - \text{Free protein})}{\text{Total amount of protein}} \times 100\% \quad (2)
\]

In vitro stability of TMC nanoparticles

To measure the colloidal stability of the nanoparticles, after centrifugation they were resuspended in 1 ml distilled water and diluted to a final TMC concentration of 1.6 mg/mL in phosphate buffered saline pH 7.4 (PBS: NaCl: 8 g/l, KCl: 0.4 g/l, KH₂PO₄: 0.4 g/l, Na₂HPO₄: 2.86 g/l). The nanoparticles were stored at 37°C and their size was measured after 1, 2, 4, 24 and 48 hours.

In vitro release of ovalbumin from TMC nanoparticles

Nanoparticles containing 250 µg OVA_{FITC} were prepared and after centrifugation (15 minutes 10000 g) resuspended in 1 ml distilled water and diluted to a final TMC concentration of 1 mg/ml in PBS pH 7.4 containing 0.1% Tween 20 (sink conditions). The nanoparticles suspension was stirred continuously in the dark at 37°C for 9 days and every day a 300 µl sample was taken. The samples were centrifuged for 15 minutes at 15000 g and the amount of OVA_{FITC} in the supernatant was determined after the addition of 25 µl of 5 M NaOH.

Generation of human monocyte derived dendritic cells

Monocytes were isolated from whole blood or buffy coat (obtained from blood bank, Sanquin, The Netherlands) by Ficoll and Percoll density centrifugation [32]. These monocytes were purified from platelets by monocyte adherence to 24 well plates (Corning, Schiphol, The Netherlands) followed by washing. To differentiate into immature DCs, the monocytes were cultured for 6 days at a density of 0.5 x 10⁶ cells/well in RPMI 1640, supplemented with 10% FCS, 1% glutamine, 1% v/v Penicillin/Streptomycin, granulocyte macrophage-colony stimulating factor (GM-CSF) 250 U/ml and IL-4 100 U/ml (Invitrogen) at 37°C and 5% CO₂. Medium was refreshed after 3 days.

Dendritic cell association

To assess the effect of nanoparticle encapsulation on antigen uptake, DCs were incubated at 37°C in serum free RPMI 1640 (with 500 U/ml GM-CSF) containing OVA_{FITC} (2 µg/mL) in solution, mixed with TMC15 or TMC30 or encapsulated in TMC nanoparticles. For the in vitro studies formulations containing OVA were used; both because of the availability of
fluorescently labelled antigen and straightforward comparison to previous studies performed in our lab [20]. The DCs were incubated with the formulations for 4 hours. After 1, 2 or 4 hours the cells were washed three times with PBS containing 1% w/v bovine serum albumin and 2% v/v FCS and the association of OVA_{FITC} with DCs was quantified using flow cytometry (FACS Canto II, Becton Dickinson, Breda, The Netherlands). Living cells were gated based on forward and side scatter, OVA_{FITC} association was expressed as the mean fluorescence intensity (MFI) in the FL-1 channel. To verify whether the formulations were not only associated with the DCs, but also actively taken up, the same study was repeated at 4°C. Histogram overlays were created with WinMDI 2.9.

**Upregulation of DC maturation markers**

DCs were incubated for 48 hours in RPMI 1640 containing 500 U/mL GMCSF and 10% FCS with the same formulations as for the DC association study. Since no difference in DC association between TMC15 and TMC30 nanoparticles was observed, only TMC15 was used in this study. LPS (100 ng/mL) was used as a positive control. After 48 hours the supernatant was removed and stored at -20°C until ELISA analysis of IL-6 and IL-12 secretion (PeliKine-compact kit, Sanquin, Amsterdam, The Netherlands). The cells were washed three times with PBS containing 1% w/v bovine serum albumin and 2% v/v FCS and incubated for 30 minutes with 20x diluted anti-HLADR_{FITC}, anti-CD83_{PE} and anti-CD86_{APC} (BD, Breda, The Netherlands) in the dark at 4°C. Cells were washed and the expression of MHC-II, CD83 and CD86 on the cells was quantified using flow cytometry. Living cells were gated based on forward and side scatter, the amount of MHCII, CD83 and CD86 positive cells were expressed as the MFI in the FL-1, FL-2 and FL-4 channel relative to the LPS control.

**Confocal microscopy**

The uptake of antigen by DCs was visualised with confocal laser scanning microscopy (CLSM). DCs were plated at a density of 1 x 10^5 cells to a poly-L-lysine coated petridish with glass bottom and allowed to adhere for 30 minutes. Afterwards the cells were incubated with the formulations containing OVA_{AF647} for 1 hour. After 45 minutes 0.1 mM LysoTracker Green (Invitrogen) was added. Images were processed using a Bio-Rad Radiance 2100 confocal laser scanning system equipped with a Nikon Eclipse TE2000-U inverted microscope and a 40x air objective. The images were captured using an argon laser at 488 nm with a 515/30 nm emission filter and a red diode at 633 nm with a 660 long pass emission filter. Image acquisition was controlled using the Laser Sharp 2000 software (Bio-Rad, Hercules, USA).
T cell activation

To assess the tendency of the formulations to induce a Th1 or Th2 response, CD4+ T cells were purified from PBMC using MACS beads (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer’s protocol. After 48 hrs of incubation of the DCs with the formulations, 5 x 10^3 cells were cocultured with 2 x 10^4 T cells in RPMI 1640 containing 10% FCS and 20 pg/mL Staphylococcus enterotoxin B (SEB; Sigma-Aldrich) in 96-well flat-bottom culture plates. After 5 days, the T cells were transferred to a 24 well plate and IL-2 (10 U/mL) was added. The cells were cultured for 9 more days and on day 14 the T cells were restimulated for 6 hours with 100 µg/mL phorbo1 myristate acetate (PMA) and 0.5 mg/mL ionomycin (Sigma-Aldrich). After 3 hours 5 mg/mL Brefeldin A (Sigma-Aldrich) was added to be able to detect the intracellular production of IL-4 and IFN-γ with flow cytometry. Live cells were gated based on forward and side scatter, the amount of IL-4 and IFN-γ positive cells were expressed relative to the LPS control. As Th1 and Th2 controls a mixture of LPS (100 ng/mL) with Heat-killed Listeria monocytogenes (HKLM; 10^8 U/mL) or with a soluble extract of schistosome eggs (25 µg/mL) were used respectively.

Intradermal immunisation

The immunogenicity of intradermally administered OVA and DT loaded TMC15 nanoparticles and a mixture of antigen and TMC15 was assessed in mice. The in vivo studies were performed with both antigens as DT is a relevant antigen for immunisation and a model to evaluate the protection after vaccination is available [33]. The mice were vaccinated thrice with three weeks intervals. Groups of 8 mice were injected intradermally with a Hamilton syringe equipped with a 30-Gauge needle [34]. A total volume of 30 µL containing 5 µg (1.5 Lf) OVA or DT dissolved in PBS, encapsulated in TMC15 nanoparticles or mixed with an equivalent amount of TMC was injected into the abdominal skin under anaesthesia (by intraperitoneal injection of 150 mg/kg Ketamine and 10 mg/kg Xylazine). As a control 100 µL containing 5 µg of antigen in PBS or in case of DT adsorbed to aluminium phosphate (Adju-Phos®; Brenntag Biosector, Denmark) was injected subcutaneously. The DT adsorbed to aluminium phosphate (DT-Alum) was prepared as previously described and the adsorption was between 70 and 80% [35]. One day before each immunisation blood samples were collected from the tail vein. Three weeks after the last vaccination the mice were sacrificed. Just before euthanasia total blood was collected from the femoral artery. Blood samples were collected in MiniCollect® tubes (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) till clot formation and centrifuged 10 minutes at 10,000 g to obtain cell-free sera. The sera were stored at -80 ºC until further use.
Detection of serum IgG, IgG1 and IgG2a

OVA and DT specific antibodies (IgG, IgG1 & IgG2a) were determined by sandwich ELISA as described previously [36]. Briefly, 96 well plates (Microlon®, Greiner Bio-one, Alphen a/d Rijn, The Netherlands) were coated overnight at 4°C with 100 ng OVA or 140 ng DT in coating buffer (0.05 M sodium carbonate/ bicarbonate, pH 9.6) per well. Afterwards the plates were blocked by incubation with 1% (w/v) BSA in PBS containing 0.05% Tween 20 for 1 hour at 37°C. Two-fold serial dilutions of sera from individual mice were applied to the plates and incubated for 2 hours at 37°C. Plates were incubated with HRP-conjugated goat antibodies against either mouse IgG, IgG1 or IgG2a (Invitrogen) for 1.5 hour at 37°C and antibodies were detected by TMB and measuring optical density at 450 nm. Antibody titres were expressed as the reciprocal of the sample dilution that corresponds to half of the maximum absorbance at 450 nm of a complete s-shaped absorbance-log dilution curve.

Vero cell test

The levels of diphtheria toxin-neutralising antibodies in mouse sera were assessed by a Vero cell test as previously described [33]. First, serum complement was inactivated by heating at 56°C for 45 minutes. Following, twofold serial dilutions of individual sera were prepared in complete medium 199 (CM199, Gibco, Breda, The Netherlands) and applied to 96 well plates (CELLSTAR®, Greiner Bio-One). Then, 2.5 x 10^-5 Lf of diphtheria toxin was added to each well and the plates were incubated for 2 hours at 37°C for neutralisation. Subsequently, medium containing 1.25 x 10^4 Vero cells was added to each well. As a control antitoxin and untreated cells were included. The plates were incubated at 37°C in 5% CO₂ for 6 days and afterwards the presence of living cells was verified by light microscopy. The antibody titres were obtained from the serum dilution factor that still resulted in living cells.

Statistical analysis

Statistical analysis was performed with Prism 5 for Windows (Graphpad, San Diego, USA). Data are presented as mean ± S.D. for all results. Statistical significance was determined either by a one way or a two way analysis of variance (ANOVA) with a Bonferroni post-test, depending on the experiment set-up. The results of the Vero cell test were analysed by a Kruskal-Wallis test with a Dunn's multiple comparison post-test.
Results

Synthesis of TMC with varying degrees of quaternisation

By synthesising TMC according to the one step method described by Hamman and Kotzé [29] we were able to obtain TMC with a reproducible DQ around 15%. By increasing the amount of base (NaOH) in the additional step, the trimethylation could be increased in a controlled manner (figure 1). From this figure it can be observed that initially the chitosan was mostly dimethylated and by adding more NaOH trimethylation was induced. As postulated by Domard et al., the NaOH increases the pH by reacting with the hydroiodic acid formed during the reaction [37]. For that reason, a deficit of sodium hydroxide might limit the trimetylation. By gradually increasing the amount of sodium hydroxide, the DQ could be increased in a controlled manner up to 70%. Recent studies performed by Verheul et al. showed that this method of TMC synthesis induces only a slight decrease in molecular weight due to chain scission [38].

Characterisation of TMC15 and TMC30 formulations

After having optimized the TMC synthesis, nanoparticles were prepared with TMC15 and TMC30 as this is in the DQ range where previously no toxicity was observed [22, 38, 39]. DLS studies revealed that with both TMC15 and TMC30 nanoparticles of a size between 200 and 300 nm could be made, depending on the antigen that was encapsulated. The presence of the nanoparticles was confirmed by SEM images (figure 2C). The nanoparticles were irregularly in shape, which is consistent with previous studies [39]. It was possible to vary the size of the particles to a minor extent by varying the amount of TPP added (data not shown). For TMC nanoparticles containing OVA the ratio (w/w) TMC:TPP to obtain stable nanoparticles was 6.7:1 for TMC15 and 5:1 for TMC30. If more TPP was added, aggregation of the particles could be observed. The same trend was observed if DT was used as an antigen (table 1). Nanoparticles of both types of TMC were positively charged. The zetapotential of the TMC30 nanoparticles was slightly higher than that of the TMC15.
nanoparticles. Since this effect was present regardless of the antigen, it can probably be ascribed to the higher DQ of TMC30.

The colloidal stability of the nanoparticles in PBS was measured during a period of 2 days. We observed a difference between TMC15 and TMC30. In PBS, the size of the TMC15 nanoparticles started to increase and after 24 hours the nanoparticles had already doubled (figure 2A). In contrast, the size of the TMC30 nanoparticles was stable in PBS for at least 2 days (figure 2B).

<table>
<thead>
<tr>
<th>Nanoparticles</th>
<th>Antigen</th>
<th>Size [nm]</th>
<th>PDI</th>
<th>Zetapotential [mV]</th>
<th>Protein loading [%]</th>
<th>TMC [%]</th>
<th>TPP [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMC15</td>
<td>-</td>
<td>219 ± 1</td>
<td>0.13 ± 0.02</td>
<td>16.2 ± 0.8</td>
<td>-</td>
<td>75 ± 1</td>
<td>47 ± 3</td>
</tr>
<tr>
<td>TMC15</td>
<td>OVA</td>
<td>276 ± 6</td>
<td>0.21 ± 0.04</td>
<td>10.6 ± 0.3</td>
<td>69 ± 1</td>
<td>75 ± 3</td>
<td>57 ± 5</td>
</tr>
<tr>
<td>TMC15</td>
<td>DT</td>
<td>211 ± 4</td>
<td>0.15 ± 0.01</td>
<td>12.9 ± 0.8</td>
<td>70 ± 3</td>
<td>56 ± 1</td>
<td>41 ± 2</td>
</tr>
<tr>
<td>TMC30</td>
<td>-</td>
<td>248 ± 9</td>
<td>0.20 ± 0.05</td>
<td>17.4 ± 0.9</td>
<td>-</td>
<td>71 ± 1</td>
<td>43 ± 6</td>
</tr>
<tr>
<td>TMC30</td>
<td>OVA</td>
<td>344 ± 16</td>
<td>0.26 ± 0.03</td>
<td>13.5 ± 1.3</td>
<td>78 ± 5</td>
<td>66 ± 2</td>
<td>52 ± 4</td>
</tr>
<tr>
<td>TMC30</td>
<td>DT</td>
<td>228 ± 2</td>
<td>0.14 ± 0.01</td>
<td>13.6 ± 0.2</td>
<td>78 ± 7</td>
<td>35 ± 2</td>
<td>27 ± 2</td>
</tr>
</tbody>
</table>

The percentage of protein, TMC and TPP shown in the table indicate the amount present in the nanoparticles after purification in comparison to the amount added during preparation.

The release profile of OVAFITC from the TMC30 and TMC15 particles was similar; showing an initial burst release (figure 2D). The burst release of the TMC15 nanoparticles was slightly higher, probably also due to the lower DQ. After the initial release an equilibrium was reached, showing no further release over the next nine days. This release profile is in agreement with results obtained by Amidi et al. who attributed the burst release to
antigen that is loosely bound to the particle surface [39]. This means that most of the antigen is encapsulated in the nanoparticles.

Figure 2. Characteristics of OVA loaded TMC15 and TMC30 nanoparticles. A/B: Size of the TMC15 nanoparticles (A) and TMC30 (B) nanoparticles during 2 days incubation at 37°C. C: SEM images of TMC15 nanoparticles show that the particles have an average size between 200 and 300 nm and are irregularly shaped. D: Release of OVA over time from the nanoparticles in PBS at 37°C. Values are the average ± SD of three independent batches.

DC association and uptake of TMC-ovalbumin formulations

Antigen association to DCs was measured by stimulating immature DCs with OVA_{FITC} formulations. As shown by flow cytometry the association of OVA_{FITC} to DCs increased with time compared to unstimulated DCs. Both types of nanoparticles increased the association tremendously compared to applying a solution of OVA_{FITC} (figure 3A). A 2.5 fold increase could already be observed after one hour and after four hours the association had increased 7.5 fold compared to a solution of OVA_{FITC}. Interestingly, a TMC/OVA_{FITC} mixture did not increase the association of OVA_{FITC} to DCs. No difference was observed between TMC15 and TMC30 regarding their effect on OVA_{FITC} uptake.

Control studies performed at 4°C to inhibit active uptake and CLSM visualisation studies both showed that the positively charged nanoparticles adhered to the DCs. Figure 3B
shows that OVA<sub>FITC</sub> from a solution was only taken up at 37°C, while DCs treated OVA<sub>FITC</sub>-loaded TMC-nanoparticles also had increased MFI values at 4°C. However, association does not completely explain the increased MFI values, because the uptake of OVA<sub>FITC</sub> encapsulated in TMC nanoparticles was slightly higher at 37°C than at 4°C at. Confocal microscopy studies with TMC nanoparticles containing OVA<sub>AF647</sub> confirmed these results (figure 4). Free OVA or in a mixture of OVA and TMC in solution was taken up by DCs and ended up in the lysosomes, as shown by co-localisation of OVA<sub>FITC</sub> and lysosome staining. Overlays made with transmission microscopy showed that if OVA<sub>AF647</sub> was encapsulated in nanoparticles it was mainly present on the surface of DCs. Only a small part of the OVA<sub>AF647</sub> was co-localised with the lysosomes. Similar results were observed for TMC15 and TMC30 (data not shown). The increased association may be favourable for antigen uptake, because the cells are in contact with the antigen for a longer period.

![Figure 3. A: Association of OVA<sub>FITC</sub> with DCs after application of different formulations for 1, 2 and 4 hrs at 37°C. Results expressed as mean MFI ± SEM relative to free OVA<sub>FITC</sub> at t=1 hr (n=6). B: Representatives of the difference between DC association of OVA<sub>FITC</sub> after 4 hrs at 4 and 37°C.](image)

**Effect of TMC15 formulations on DC maturation**

It was already known that nanoparticles can improve the uptake of an antigen [10-13]. However, uptake alone is not enough to induce an immune response. Therefore the ability of the formulations to induce DC maturation was explored. Even though the characterisation and DC uptake studies were performed with both TMC15 and TMC30, for practical reasons in all further studies only TMC15 is used. Since the characterisation and uptake studies showed only minor differences between TMC15 and TMC30, it seems that the difference in DQ in the studied range did not have an influence on the function of the TMC.

Flow cytometry measurements showed that even though a solution of OVA was taken up by DCs, it did not induce upregulation of the expression of the maturation markers CD83,
CD86 and MHCII (figure 5A). LPS, on the other hand showed a distinct increase of the expression of these three markers. If the antigen was either encapsulated in TMC15 nanoparticles or mixed with a TMC15 solution, the levels of CD83 and CD86 increased significantly compared to those after OVA application. A trend of increased MHCII levels could also be observed.

To obtain additional insight into the maturation status of the DCs, the secretion of IL-6 and IL-12 was measured. Both OVA loaded TMC nanoparticles and a mixture of OVA and TMC induced secretion of IL-6 and IL-12 (figure 5B). TMC nanoparticles induced a much higher cytokine secretion than a TMC solution. Even more IL-12 was produced than after LPS stimulation. This confirms that TMC functions as an immune potentiator. To verify that the DC maturation was not caused by LPS contamination in our samples, they were applied to TLR-4 transfected HEK cells. The LPS content was found to be below the detection limit (<0.1 ng/ml, data not shown).

**Figure 4.** CLSM images showing the presence of OVA<sub>AF647</sub> in DCs 1 hr after the application of different formulations. The lysosomes are stained with LysoTracker Green to show possible co-localisation of the OVA and the lysosomes.
Induction of T cell stimulation by TMC formulations

Since the TMC-OVA interaction is based on electrostatic interactions, it is expected that the TMC nanoparticles will be degraded once they reach the lysosomes, releasing the OVA to be processed and presented to T cells. By using the effector DCs obtained after 48 hrs of maturation to stimulate naïve T cells, we studied the T cell response \textit{in vitro}. After 14 days we determined the subsets of effector T cells by analysing the intracellular production of IFN-γ (Th1 biased response) and IL-4 (Th2 biased response). Figure 6 shows that LPS induced a mixture of Th1 and Th2 effector T cells, in agreement with previous studies [40]. The other formulations were compared to LPS. In comparison to LPS, the OVA loaded TMC15 nanoparticles and the mixture of OVA and TMC15 induced more cells to secrete IL-4 than IFN-γ. The TMC-containing formulations were strong inducers of the proliferation of Th2 effector T cells, since the Th1/Th2 ratios were 0.26 for TMC15 nanoparticles and 0.57 for the TMC15/OVA mixture. This was in the same range as an extract of schistosome eggs, a known stimulus of Th2 development, which gave a Th1/Th2 ratio of 0.28 (see figure 6).
Antibody levels after intradermal immunisation

No severe side reactions to the intradermal or subcutaneous injections were observed. For the both the TMC- and alum-containing formulations sometimes a white firmness could be observed at the injection site, most likely due to depot formation. The IgG titres were measured in the sera of the mice before each immunisation and three weeks after the last immunisation. The results obtained with OVA and DT are very comparable. Intradermal or subcutaneous injection with plain antigen gave the lowest titres. TMC15-TPP-OVA nanoparticles were applied subcutaneously and intradermally. Both intradermal and subcutaneous injection of the nanoparticles induced significantly higher antibody titres compared to the plain OVA. No differences between intradermal and subcutaneous could be observed. Besides TMC nanoparticles, also a TMC/OVA mixture was applied. Intradermal application of either TMC nanoparticles or a TMC/OVA mixture induced 5x, 14x and 8x higher anti-OVA IgG titres compared to plain OVA after respectively the prime, first and second boost immunisations (figure 7A).

For DT, after the first immunisation, only 6 out of 8 mice which were injected intradermally with plain DT, showed detectable anti-DT IgG levels (figure 7C). Also here application of the TMC based formulations enhanced the immune response, even more pronounced than for OVA. Intradermal injection of TMC15 nanoparticles containing DT or a mixture of DT and TMC15 induced 200 fold higher titres (p<0.001) after the first immunisation compared to intradermal DT injection. The difference in response was less distinct after the first and second boost, but still respectively 60x and 4x higher IgG levels were obtained (p<0.001). The IgG titres of intradermal TMC15-TPP-DT nanoparticles and a TMC15/DT mixture were comparable to those after subcutaneous DT-alum injection. For both these groups the
titres already reached their maximum values after the first boost. In figure 7B and D the IgG1 and IgG2a titres after the second boost are shown. The ratio of IgG1:IgG2a gives an indication if the immune response is Th1 or Th2 biased. For all groups of both antigens the IgG2a titres were lower than the IgG1 titres and in general developed only after the first boost (data not shown). The IgG1/IgG2a ratio did not differ significantly between the groups and in all cases Th2 biased responses were achieved. The IgG2a titres were slightly higher after DT compared to OVA immunisation and only here significant differences between intradermal application of the TMC formulations and plain OVA were observed (figure 7D). For OVA only an effect of the TMC was present if the nanoparticles were applied subcutaneously (figure 7B).

**Figure 7.** Antibody titres after subcutaneous (SC) and intradermal (ID) vaccination with DT or OVA. Total anti DT (A) and OVA (C) IgG titres after the first and two subsequent boost vaccinations; IgG1 and IgG2a levels against DT (B) and OVA (D) after the second boost. Mean titres ± SD of 8 mice are shown. **/*** p<0.01/0.001 compared to ID OVA or DT. ‡ p<0.001 compared to SC OVA.
The neutralising antibody titres from the Vero cell test confirmed the IgG antibody titre results (figure 8). The TMC based formulations applied intradermally increased the production of neutralising antibodies compared to unadjuvanted DT. This result was most clearly visible after the first boost (figure 8A) when no neutralising antibodies could be detected for intradermal DT, but the TMC based formulations already induced substantial antibody titres. Their neutralising capacity was the same compared to subcutaneous DT-alum, but a trend of faster kinetics could be observed.

**Figure 8.** Diphtheria toxin-neutralising antibody titres after subcutaneous (SC) and intradermal (ID) vaccinations after the first (A) and second boost (B). Serum samples were collected and the titres determined by a Vero cell test. Data are expressed as the highest dilution that was still capable of protecting the Vero cells from the challenge of diphtheria toxin.

**Discussion**

Vaccination via the skin offers great promise as an alternative for intramuscular or subcutaneous vaccination, but to induce an efficient immune response to subunit vaccines, also for this delivery route adjuvants are necessary. Different types of adjuvants have already been used, which evoke or increase the transcutaneous immune response [41-45]. Another adjuvant TMC has already shown great promise in mucosal vaccination, which is thought to be largely due to its mucoadhesive properties. In this study we explored the properties of TMC as an adjuvant in immunisation via the skin. To focus on the adjuvant effect and not on the transport through the skin, which plays a prominent role in transcutaneous vaccination, the formulations were administered by intradermal injection.

The antibody titres after intradermal immunisation show the potential of TMC as an inducer of an immune response after intradermal immunisation. Total IgG, IgG1 and IgG2a titres after immunisation with either OVA or DT loaded TMC15 nanoparticles and...
TMC15/OVA and TMC15/DT mixtures were significantly higher than after immunisation with an antigen solution. From the Vero cell test it can be concluded that TMC15, both in a solution or as a nanoparticle increases the total neutralising antibody titres and their kinetics compared to plain DT. Interestingly, for both antigens a TMC15 solution works as well as TMC15 nanoparticles. This is in contradiction to the results obtained by Amidi et al., who observed a significant increase in IgG titres after intranasal immunisation with influenza-loaded TMC nanoparticles compared to an antigen/TMC solution [25]. Boonyo et al. also showed that a solution of TMC20 and OVA applied intranasally did not induce significantly higher IgG titres compared to OVA alone [23]. In this study we show that by mixing DT and TMC, almost 50% of the DT was associated with the TMC. For OVA this was only 5%. However, antigen adsorption does appear to have no influence on the obtained antibody titres. The promising results obtained with these mixtures indicate that although nanoparticles are very important in for instance intranasal vaccination, this seems not to be the case in intradermal vaccination. Given that by intradermal injection the antigen is immediately at its site of action, nanoparticles might not be necessary. In intranasal delivery the transport across the epithelium also plays a role and nanoparticles are known to enhance the transport across M-cells [46]. Since transport also plays an important role in transcutaneous vaccination, it is interesting to investigate if nanoparticles are an advantage for this delivery route.

To understand the in vivo results, in vitro DC and T cell studies were carried out. TMC nanoparticles, but not a TMC solution, were able to increase the uptake of OVA by immature DCs. This effect could be ascribed to the association of the TMC nanoparticles with the DCs. An increased association was not observed for a TMC solution. The interactions between the TMC and the OVA are stronger in the nanoparticle formulation compared to the mixture. The positive charge of the TMC is responsible for the association to the DCs and therefore the nanoparticles are able to enhance the association of OVA, while a TMC solution is not. The maturation of DCs was shown not to be dependent on the particulate nature of the TMC. Both a TMC15 solution and the TMC15 nanoparticles were able to upregulate the levels of maturation markers and to induce increased IL-12 and IL-6 levels. Furthermore, all TMC based formulations were able to induce Th2 biased T cell proliferation.

If the in vitro and in vivo results are compared, it is clear that both the DC model and the mouse model show that TMC15 is an immune potentiator. It is not possible to draw the same conclusion from the T cell model. In vitro, the TMC15 based formulations are strong inducers of Th2 biased proliferation, but the same is not observed in vivo. This can be due both to the antigens and the mice model that was used. Since immunisation with plain OVA or DT already induces a clear Th2 biased immune response, no further effect of TMC15 on the Th1/Th2 ratio could be observed. Both IgG1 and IgG2a levels were
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increased. The same was observed by Amidi et al after nasal immunisation with influenza antigen loaded TMC nanoparticles [25]. Future immunisation studies with antigens that favour a Th1 immune response or by using for instance C57BL/6 mice can potentially show the effect of TMC on the Th1/Th2 ratio.

Both in vitro and in vivo it was shown that a TMC solution as well as TMC nanoparticles can act as an adjuvant. The mechanism behind this immune modulation remains unclear, but there are different possibilities. It is generally thought that positively charged compounds can act as a ‘danger-signal’ for DCs and function as a signal 0 adjuvant [2]. In vitro DC maturation studies with positively charged poly-L-lysine coated polystyrene microparticles showed enhanced DC uptake and an increased amount of CD83 positive cells [13]. However, negatively charged modified poly(y-glutamic acid) nanoparticles [47] and negatively charged liposomes [48] also induced increased expression of maturation markers. Studies with chitosan, the precursor of TMC, have shown contradictory results as well. It was shown that chitosan increased DC maturation [49], but also studies in which chitosan has no effect on DCs have been published [50, 51].

If TMC as an adjuvant is compared to Alum, the in vivo data show that similar IgG and neutralising antibody titres were obtained after intradermal immunisation with the TMC formulations as after subcutaneous DT-Alum injection. Zaharoff et al. used a solution of chitosan as an adjuvant in subcutaneous immunisation and found it to be superior to aluminium hydroxide [52]. Its adjuvant effect was attributed to the ability of chitosan to retain the antigen at the site of injection. It is possible that TMC has the same function. Further studies should focus on the adjuvant mechanism of TMC and compare it to other known adjuvants.

In conclusion, intradermal immunisation with TMC nanoparticles as well as with TMC solutions can elicit strong IgG and neutralising antibody titres against two different antigens. This demonstrates that the skin is an excellent vaccination site and that TMC based formulations have great potential. In the next step we will focus on the transport of the antigen across the skin barrier. Transcutaneous vaccination studies with the TMC formulations and microneedles, which were already used in previous vaccination studies [36, 53], will be performed.

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References


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