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Diphtheria toxoid and *N*-trimethyl chitosan layer-by-layer coated pH-sensitive microneedles induce potent immune responses upon dermal vaccination in mice

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ABSTRACT

Dermal immunization using antigen-coated microneedle arrays is a promising vaccination strategy. However, reduction of microneedle sharpness and the available surface area for antigen coating is a limiting factor. To overcome these obstacles, a layer-by-layer coating approach can be applied onto pH-sensitive microneedles. Following this approach, pH-sensitive microneedle arrays (positively charged at coating pH 5.8 and nearly uncharged at pH 7.4) were alternatingly coated with negatively charged diphtheria toxoid (DT) and *N*-trimethyl chitosan (TMC), a cationic adjuvant. First, the optimal DT dose for intradermal immunization was determined in a dose-response study, which revealed that low-dose intradermal immunization was more efficient than subcutaneous immunization and that the EC50 dose of DT upon intradermal immunization is 3-fold lower, as compared to subcutaneous immunization. In a subsequent immunization study, microneedle arrays coated with an increasing number (2, 5, and 10) of DT/TMC bilayers resulted in step-wise increasing DT-specific immune responses. Dermal immunization with microneedle arrays coated with 10 bilayers of DT/TMC (corresponding with ± 0.6 μg DT delivered intradermally) resulted in similar DT-specific immune responses as subcutaneous immunization with 5 μg of DT adjuvanted with aluminum phosphate (8-fold dose reduction). Summarizing, the layer-by-layer coating approach onto pH-sensitive microneedles is a versatile method to precisely control the amount of coated and dermally-delivered antigen that is highly suitable for dermal immunization.

1. Introduction

Intradermal immunization has several benefits over conventional immunization by intramuscular bolus injection, as the skin is easily accessible and intradermal immunization is potentially pain-free. Moreover, superior immunization responses resulting from intradermal immunization as compared to conventional intramuscular immunization were reported for hepatitis B [1,2], influenza [3–8] and polio [9–11]. This may be caused by the specialized dendritic cell subsets that are present in high numbers in the skin [12]. To deliver a sufficient amount of antigen to these cells, the physical barrier of the skin, the stratum corneum, must be overcome [13]. For this purpose microneedles can be used, which are needle-like structures used to pierce the stratum corneum in a minimally-invasive way. Several technologies for microneedle-mediated vaccine delivery are under development [14], such as coated microneedle arrays.

The amount of antigen delivery by coated microneedle arrays may be limited, because of the limited surface area available for coating. Therefore, different approaches have been developed to increase the amount of coated antigen. For example, dipping low-density microneedle arrays in an antigen-containing viscous solution leads to thick coatings containing a high amount of antigen (2 μg) [15,16]. However, applying the dip-coating technique to high-density microneedle arrays results in blunting of the microneedles, which may impair the skin penetration ability of the microneedles [15,17–19].

An alternative coating approach resulting in a thin coating is to

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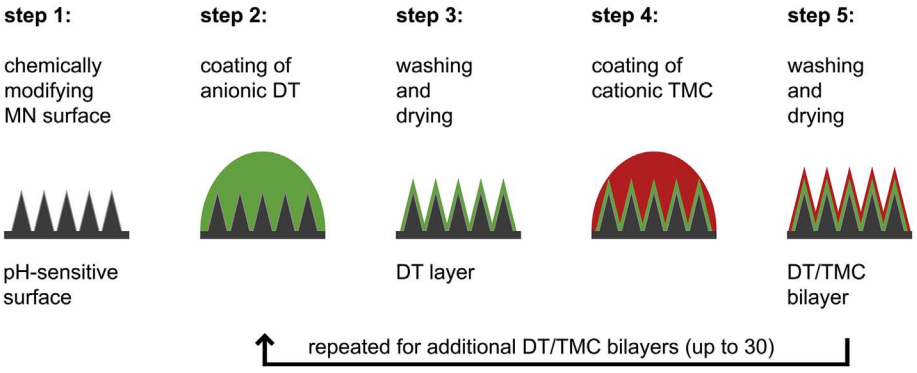

Fig. 1. Schematic illustration of the layer-by-layer coating approach of pH-sensitive microneedle surfaces.
To make use of the electrostatic interactions in the layer-by-layer coating approach, a 1 mM EDTA buffer at pH 5.8 was used as coating buffer, with DT or TMC in a concentration of 40 μg/mL. The first layer of DT was coated by applying a 50 μL droplet of the DT solution onto the pH-sensitive surface of the microneedle array for 30 min at room temperature. Then, the microneedle arrays were washed with 450 μL coating buffer. Subsequently, the microneedle arrays were dried under a flow of nitrogen gas. After drying, a layer of TMC was applied onto the DT-coated surface of the microneedle array, followed by washing and drying, as described above. Then a subsequent DT/TMC bilayer was coated onto the surface of the microneedle arrays, as described above, until the preselected number of DT/TMC bilayers was coated onto the microneedle arrays. The amount of coated DT or TMC, was determined by measuring the fluorescence of DT or fluorescently labeled DT-AF488, DT-IRDYE800 or TMC-RHO in the non-adhered fraction in each of the wash samples.

modify the microneedle surface to control the antigen coating onto microneedle arrays and subsequent release into the skin by pH-dependent electrostatic interactions. In this approach, a negatively charged antigen adheres to a microneedle surface that is positively charged at a coating pH of 5.8. Upon piercing these antigen-coated pH-sensitive microneedles into skin, the electrostatic interactions between the antigen and the microneedles surface cease. This is caused by shifting the pH from a slightly acidic one (of the coating solution) towards a slightly alkaline one (physiological pH (7.4) of the skin). This pH-change induces a change in microneedle surface charge from positive to neutral, respectively [20]. Such pH-sensitive microneedle surface modifications allow to efficiently coat thin layers of antigens onto high-density microneedle arrays and for a controllable and effective antigen delivery.

However, the limitation of this manner of antigen-coating is that only low amounts of antigens can be coated onto microneedle arrays. This limitation can be overcome by using a layer-by-layer coating approach [21–23]. To effectively increase the amount of antigen coated onto microneedle arrays, multiple layers of antigen and polymer are stacked onto one another, utilizing electrostatic interactions between antigen and polymer. Using this approach, high-density microneedle arrays were successfully coated with inactivated polio vaccine (IPV) particles and the adjuvant *N*-trimethyl chitosan (TMC) [24]. However, this approach was not yet examined for subunit vaccines such as diphtheria toxoid (DT), which is used in all pediatric vaccination programs worldwide.

In this study, a layer-by-layer coating approach of DT (a negatively charged antigen above a pH of 5) and the adjuvant TMC (a positively charged polymeric adjuvant) on high-density microneedle arrays modified with a pH-sensitive microneedle-surface was developed. It was demonstrated that the amount of DT coated onto the microneedle array was reproducible and the dermally-delivered amount of antigen was tuned by selecting the number of coated DT/TMC bilayers. Furthermore, the DT/TMC-coated microneedle arrays were capable of inducing potent and functional DT-specific immune responses upon dermal application in mice.

2. Materials

The materials are provided in Supplementary data S1.

3. Methods

3.1. Preparation of fluorescently labeled DT, aluminum phosphate-adsorbed DT and (fluorescently labeled) TMC

To visualize DT and/or TMC adhesion to microneedle arrays and release into skin, DT and TMC were fluorescently labeled. DT was fluorescently labeled with AlexaFluor® 488 (DT-AF488) according to the manufacturer's protocol. TMC with a 15% quaternization degree was synthesized as described elsewhere [25], and was fluorescently labeled with rhodamine B isothiocyanate (TMC-RHO) as previously reported [26]. To quantify the delivery of DT into skin, DT was fluorescently labeled with a near-infrared fluorescent label IRDye® 800CW (DT-IRDYE800) according to the manufacturer's protocol. Aluminum phosphate-adsorbed DT (DT-ALUM) was prepared in a DT:alum ratio of 1:30 (w/w) and the adsorption was between 70 and 80% as determined by a DT-specific ELISA, as described elsewhere [27].

3.2. DT/TMC layer-by-layer coating approach for high-density microneedle arrays

To coat DT and TMC onto high-density microneedle arrays *via* a layer-by-layer coating approach, the following procedures were used. High-density silicon microneedle arrays with 576 microneedles on a 5 × 5 mm backplate (microneedle density of 2304 microneedles/cm^2) and a microneedle length of 220 μm were kindly provided by Robert Bosch GmbH (Stuttgart, Germany). In order to produce pH-sensitive microneedle surfaces with a surface pK_a of 6.9, the surfaces of microneedle arrays were chemically modified with a pyridine functional group, as explained in detail elsewhere [20,24]. Consequently, at a coating pH of 5.8, the microneedle surfaces are positively charged, while (unlabeled and fluorescently labeled) DT is negatively charged and (unlabeled and fluorescently labeled) TMC is positively charged. Subsequently, a layer-by-layer coating approach was used to coat multiple layers of negatively charged DT and positively charged TMC by electrostatic interactions onto the microneedle arrays, as schematically represented in Fig. 1. Optimization of the coating procedure is described in Supplementary data S2. Some studies required the microneedle arrays to be coated with fluorescently labeled DT and fluorescently labeled TMC. For these studies, plain DT was replaced by either fluorescently labeled DT-AF488 or DT-IRDYE800 and plain TMC was replaced by fluorescently labeled TMC-RHO. The fluorescence of (unlabeled and fluorescently labeled) DT and fluorescently labeled TMC was measured on a Tecan Infinite M1000 plate reader (Männedorf, Switzerland) at excitation and emission wavelengths shown in Table 1.

Table 1
Excitation and emission wavelengths of DT and TMC used for fluorimetric assays (for details, see Methods section).

Compound	Fluorescence	Excitation wavelength (nm)	Emission wavelength (nm)
DT	Intrinsic	280	320
DT-AF488	Extrinsic	490	525
DT-IRDYE800	Extrinsic	774	789
TMC-RHO	Extrinsic	550	580

3.3. Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) imaging of DT/TMC coated microneedle arrays

SEM imaging of DT/TMC coated microneedle arrays was performed to inspect the microneedle tip sharpness, geometry and surface morphology of the microneedles on a FEI NOVA nanoSEM 200 in high-voltage mode (10.0 kV). To this end, 4 microneedle arrays coated with 5 bilayers of DT/TMC and as control, 4 microneedle arrays with only a pH-sensitive surface (plain microneedle arrays) were imaged.

To visualize the distribution of DT and TMC on the surface of DT/TMC coated microneedles, CLSM imaging of microneedle arrays coated with 5 bilayers of fluorescently labeled DT-AF488/TMC-RHO was performed. CLSM was performed by utilizing a Nikon TE2000-E inverted microscope and a Nikon C1 confocal unit (Amsterdam, The Netherlands), in conjunction with a Nikon CFI Plan-Apochromat λ DM 20 × (numerical aperture 0.75) objective. An argon laser (488 nm, laser intensity 75 and gain 106) and a 515/30 nm emission filter were used to visualize fluorescently labeled DT-AF488, while a diode-pumped solid-state laser (561 nm, laser intensity 75 and gain 95) and a 590/50 nm emission filter were used to visualize fluorescently labeled TMC-RHO. Nikon NIS Elements software version 4.20.00 was utilized for scan acquisition and analysis. The xy resolution was 1.10 μm/pixel and xyz scans were taken in steps of 2 μm.

3.4. Antigenicity and structural integrity of coated DT

To assess whether coated DT remained antigenic, DT or fluorescently labeled DT-AF488 released *in vitro* from 4 microneedle arrays coated with 5 bilayers of DT/TMC or DT-AF488/TMC-RHO, was examined by DT-specific ELISA or fluorescence measurements, respectively. To assess the structural integrity of coated DT, the integrity of *in vitro* released DT-AF488 was determined by measuring the molecular weight by non-reducing SDS-PAGE. *In vitro* release was triggered by submerging DT/TMC coated microneedles in release buffer (5 mM EDTA with 0.9% (w/v) sodium chloride) at pH 7.4, which induces a change to a (nearly) neutral surface charge on the pH-sensitive microneedle surfaces, loss of electrostatic interactions of the DT/TMC bilayers with the microneedle surface and subsequently release of DT and TMC, as explained in detail elsewhere [20]. More detailed procedures for this experiment are provided in Supplementary data S3.

3.5. Delivery of DT/TMC into the skin by DT/TMC coated microneedle arrays

3.5.1. Visualization of fluorescently labeled DT-AF488/TMC-RHO delivery into human skin

Human skin was obtained from a local hospital and prepared as described in Supplementary data S4. To examine whether fluorescently labeled DT-AF488 and TMC-RHO can be delivered into human skin by coated microneedle arrays, microneedle arrays coated with 5 bilayers of fluorescently labeled DT-AF488/TMC-RHO were applied onto *ex vivo* human skin fixed on Styrofoam, by impact insertion using an in-house designed microneedle applicator. An average insertion speed of 0.5 m/s by the microneedle applicator was set on a uPRAX Microsolutions microneedle applicator controller (The Hague, The Netherlands). After insertion, the microneedle arrays were fixed onto the skin by applying a force of 5 N on top of the microneedle array. After 90 min, the microneedle arrays were withdrawn from the skin and immediately prepared for CLSM imaging by fixing the skin in a sample holder. The skin was imaged by CLSM using the same equipment as described above, except that a Nikon CFI Plan-Apochromat λ 4 × (numerical aperture 0.2) objective was used. A laser intensity of 75 and gain 95 for the 488 nm laser were used to visualize fluorescently labeled DT-AF488, while a laser intensity of 75 and gain 86 for the 561 nm laser were used to visualize fluorescently labeled TMC-RHO. The xy resolution was 5.18 μm/pixel and xyz scans were taken in steps of 5 μm. The focal plane of the scans was parallel to the surface of the skin.

3.5.2. Quantification of dermally-delivered DT

To study the effect of the number of DT/TMC bilayers on the dermally-delivered DT dose, microneedle arrays coated with an increasing number of fluorescently labeled DT-IRDYE800/TMC bilayers were applied onto *ex vivo* human or mouse skin. The delivered DT dose was quantified by using an intradermally injected calibration curve.

For this purpose, an in-house developed system consisting of a single hollow microneedle and a hollow microneedle applicator was used in conjunction with a uPRAX microneedle applicator controller and a syringe pump (NE-300, Prosense, Oosterhout, The Netherlands). This system allowed for intradermal microinjections at a pre-determined skin depth and an accurate volume [10]. The near-infrared fluorescence of the delivered DT-IRDYE800 was measured in a Perkin-Elmer IVIS Lumina Series III *in vivo* imaging system (Waltham, MA, USA), by using a 745 nm excitation wavelength and an ICG emission filter, acquisition time 4 s, F-stop 2, binning 4 and field of view of 12.5 cm. Perkin-Elmer Living Image software version 4.3.1.0 was used for image acquisition and analysis. Background measurements were taken from control regions of the skin.

Quantification of DT delivered into *ex vivo* human skin by DT/TMC coated microneedle arrays was performed as follows. Microneedle arrays coated with 5 bilayers of fluorescently labeled DT-IRDYE800/TMC were applied onto *ex vivo* human skin for a period of either 30 or 90 min. A calibration curve in *ex vivo* human skin was obtained by intradermal microinjections with an injection volume varying between 0.5 and 20 μL at a DT-IRDYE800 concentration of 150 μg/mL and at an injection depth of 150 μm.

As all immunization studies were performed in mice, quantification of DT delivered by DT/TMC coated microneedle arrays into *ex vivo* mouse skin was also examined. Microneedle arrays coated with either 1, 2, 5, 10 or 20 bilayers of fluorescently labeled DT-IRDYE800/TMC were applied onto *ex vivo* mouse skin for a period of 90 min. A calibration curve in *ex vivo* mouse skin was obtained by intradermal microinjections with injection volumes varying between 0.1 and 10 μL at a DT-IRDYE800 concentration of 300 μg/mL and at an injection depth of 150 μm.

3.6. Immunization studies

Animal studies were performed in compliance with the guidelines and regulations enforced by Dutch laws and the Dutch animal ethic committee. These studies were approved by the "Dierexperimentencommissie Universiteit Leiden" under number 14166. Female BALB/c (BALB/cAnNCrl, strain code 028, 6 weeks of age on arrival) were obtained from Charles River, Saint-Germain-sur-l'Arbresle, France. The mice were housed in groups of 4 in a controlled environment subjected to the guidelines of the animal facilities of the Leiden Academic Centre for Drug Research, Leiden University. The mice were randomly assigned to the immunization groups (8 animals per group) and were first acclimatized for 2 weeks after arrival before a study started.

3.6.1. Dose-response study of intradermally- and subcutaneously-delivered DT

To determine the required amount of DT to be delivered by the DT/TMC coated microneedle arrays for sufficient DT-specific immune responses, a DT dose-response study was performed. DT-specific immune responses were determined after intradermal or subcutaneous immunization. DT doses ranging between 0.02 and 5 μg were examined. Detailed experimental descriptions are provided in Supplementary data S5.

3.6.2. Immunization study with DT/TMC coated microneedle arrays

The potential for a dermally applied DT/TMC coated microneedle

Table 2
Immunization study with DT/TMC coated microneedle arrays.

Immunization route	Application method	Group name	DT dose (μg)
D	Coated MN array	2 bilayers DT/TMC	0.05[a]
		5 bilayers DT/TMC	0.3[a]
		10 bilayers DT/TMC	0.6[a]
ID	Single hollow MN	ID DT control	0.31
		ID DT/TMC control	0.31 (+ 0.31 μg TMC)
SC	Conventional 26G hypodermic needle	SC DT control	0.31
		DT-ALUM[b]	5 (+ 150 μg $AlPO_4$)
		PBS pH 7.4[c]	0

The study parameters of the various immunization groups are summarized below. The following abbreviations are used. D: dermal, ID: intradermal, MN: microneedle, SC: subcutaneous.

[a] As determined by the amount of DT delivered from microneedle arrays coated with fluorescently labeled DT-IRDYE800/TMC into *ex vivo* mouse skin.

[b] DT adjuvanted with aluminum phosphate (as positive control).

[c] PBS pH 7.4 mock treated group (as negative control).

array to induce DT-specific immune responses was investigated and compared to immunization by an intradermal injection with a single hollow microneedle or a subcutaneous injection. All immunization groups are provided in Table 2.

Immunization was performed at day 1 (prime immunization), day 22 (boost immunization) and day 43 (2nd boost immunization). One day prior to immunization by a DT/TMC coated microneedle array or a single hollow microneedle, the mice were shaved (an area of 4 cm^2 on the left posterior flank). Serum was collected from each mouse one day prior to immunization. Venous blood (200 μL) was drawn by tail vein incision and was collected in a 0.8 mL MiniCollect® tube, which was stored on ice before centrifugation at 3000*g* at room temperature for 10 min to isolate serum. At day 63, all mice were sacrificed and bled by incision of the hind leg main artery. This blood was collected in 2.5 mL Vacuette® tubes and stored on ice before centrifugation at 2000*g* at room temperature for 10 min to isolate serum. Sera were stored at − 80 °C until analysis. All mice were immobilized for 90 min by anesthesia with 1% isoflurane (with 100% oxygen as carrier gas) prior to and during immunization. During anesthesia, the eyes of the mice were protected by oculentum simplex.

The following immunization procedures were included in the study:

(1) Dermal immunization was performed by applying a microneedle array coated with 2, 5 or 10 bilayers of DT/TMC to the skin. The selected number of coated DT/TMC bilayers was based on two factors: i) the DT-specific immune responses resulting from DT delivered into the skin by a single hollow microneedle as observed in the DT dose-response study and ii) the amount of fluorescently labeled DT-IRDYE800 delivered by microneedle arrays coated with 2, 5 and 10 bilayers into *ex vivo* mouse skin. The DT/TMC coated microneedle arrays were applied onto shaved mouse skin at a speed of 0.5 m/s by the in-house designed impact insertion microneedle applicator in conjunction with a uPRAX microneedle applicator controller and were held in the skin with dermal tape and a specially designed clothespin for 90 min.
(2) As control to the dermal immunization with a DT/TMC coated microneedle array, intradermal immunization by microinjection with a single hollow microneedle was performed with i) 0.31 μg DT and ii) 0.31 μg DT mixed with 0.31 μg TMC, using an injection volume of 10 μL at injection depth of 150 μm. The injected DT dose was based on the amount of fluorescently labeled DT-IRDYE800 delivered by a microneedle array coated with 5 bilayers into *ex vivo* mouse skin. The TMC dose was based on the nearly 1:1 coating ratio of DT and TMC. The intradermal microinjections were performed as described above.
(3) As controls to the (intra-)dermal immunization, a group of mice received subcutaneous injections of 100 μL *via* a conventional 26G hypodermic needle of i) 0.31 μg DT ii) 5 μg DT and 150 μg $AlPO_4$ (DT-ALUM) as positive control or iii) phosphate buffered saline (PBS) pH 7.4 as mock treatment.

3.7. Determination of DT-specific serum IgG titers and diphtheria toxin-neutralizing antibody titers

To measure DT-specific total IgG, IgG1 and IgG2a titers in serum, an ELISA was performed, of which a detailed description of the procedures is provided in Supplementary data S6. Determination of the antibody titer capable of neutralizing active diphtheria toxin (DTx) was performed as described elsewhere [27].

3.8. Statistical analysis

Graphs were plotted as mean (± SEM or ± 95% confidence interval for the immunization studies) and statistics were performed by using GraphPad Prism (v.7.00, GraphPad Software, LaJolla, CA, USA). To statistically compare groups amongst each other, Kruskall-Wallis tests with Dunn's post-hoc tests were performed because DT-specific IgG midpoint titers and DTx neutralizing antibody titers were non-normally distributed. Differences were considered significant at $p < 0.05$.

4. Results

4.1. DT/TMC layer-by-layer coating approach for high-density microneedle arrays

Variations in the coating procedure were examined in order to maximize the amount of coated DT for each individual bilayer (see Supplementary data S2 for details). The optimal DT and TMC coating concentrations were 40 μg/mL, which were used for further experiments.

To determine whether an increased number of coated DT/TMC layers resulted in an increased amount of coated DT and TMC, microneedle arrays were coated with up to 30 bilayers of fluorescently labeled DT-AF488 and TMC-RHO. As shown in Fig. 2, 30 bilayers of DT-AF488/TMC-RHO were successfully coated onto the microneedle arrays and the amount of coated DT-AF488 and TMC-RHO increased linearly for up to 30 bilayers. The slopes in Fig. 2 represent the mean amount of coated DT-AF488 and TMC-RHO per bilayer, being 334.1 ng/bilayer (R^2 = 0.9963) for DT-AF488 and 251.5 ng/bilayer (R^2 = 0.9983) for TMC-RHO. The coating was not linear in the first layers, as demonstrated by the intersect with the y-axis, which is − 543.8 ng and − 89.0 ng for DT-AF488 and TMC-RHO, respectively. After 30 bilayers of DT-AF488/TMC-RHO were coated, 9.67 (± 0.51) μg DT-AF488 and 7.58 (± 0.38) μg TMC-RHO were cumulatively coated onto the microneedle arrays. Therefore, the ratio of coated DT to TMC was 1.3:1. Furthermore, a coating of 5 bilayers of fluorescently labeled DT-AF488/TMC-RHO resulted in a coated amount of 1.20 (± 0.09) μg DT-AF488, while a coating of 5 bilayers of DT/TMC resulted in a coated amount of 2.08 (± 0.14) μg DT. These results indicate that DT is coated more efficiently onto the microneedle arrays than fluorescently labeled DT-AF488.

4.2. SEM and CLSM imaging of DT/TMC coated microneedle arrays

SEM imaging was performed to determine whether the microneedle tip sharpness, geometry, and surface morphology of microneedles coated with 5 bilayers of DT/TMC were maintained. As demonstrated in Fig. 3A and B, a similar geometry with a tip diameter of < 1 μm and

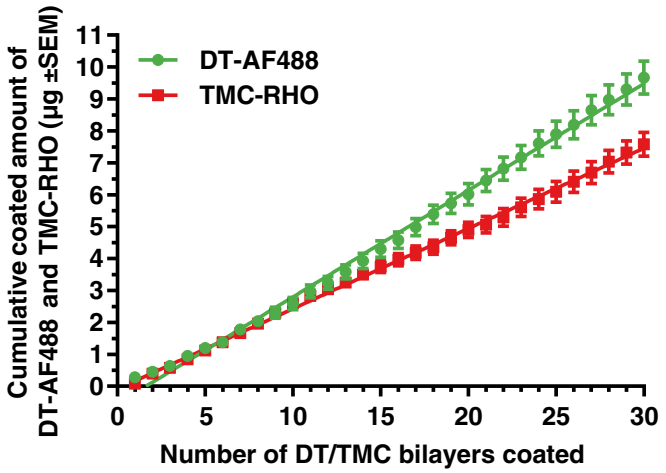

Fig. 2. Coating 30 bilayers of fluorescently labeled DT-AF488 and TMC-RHO.
Cumulative amounts of coated fluorescently labeled DT-AF488 and TMC-RHO by applying 30 DT-AF488/TMC-RHO bilayers onto microneedle arrays *via* a layer-by-layer coating approach. The mean cumulative amounts ± SEM of DT-AF488 (green dots) and TMC-RHO (red squares) coated onto the microneedle array are presented as a function of the number of coated DT-AF488/TMC-RHO bilayers (n = 14). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

surface morphology were obtained for both plain and coated microneedles.

To visualize the distribution of DT and TMC over the surface of DT/TMC coated microneedles, CLSM imaging was performed on microneedles coated with 5 bilayers of fluorescently labeled DT-AF488/TMC-RHO. As demonstrated in Fig. 3C, DT-AF488 and TMC-RHO were indeed both coated onto the microneedles.

4.3. Antigenicity and structural integrity of coated DT

To assess whether coated DT remained antigenic, DT or fluorescently labeled DT-AF488 released *in vitro* from 4 microneedle arrays coated with 5 bilayers of DT/TMC or DT-AF488/TMC-RHO, was examined by DT-specific ELISA or fluorescence measurements, respectively. The release of DT and DT-AF488 (amount released/amount coated * 100%) from the coated microneedle arrays was similar at 28.5% (± 0.5) and 29.0% (± 2.0) (mean ± SEM), respectively, suggesting that the antigenicity of coated and released DT was preserved.

Non-reducing SDS-PAGE was performed to determine the structural integrity of the coated DT. Fluorescently labeled DT-AF488 released *in vitro* from microneedle arrays coated with 30 bilayers of DT-AF488 and TMC-RHO was analyzed together with control samples of DT-AF488 diluted from stock solution and a mixture of DT-AF488 and TMC-RHO taken from stock solutions. The molecular weight of the coated and released DT-AF488 was comparable to that of the DT-AF488 control samples. Moreover, no bands indicative of fragments or covalent aggregates were observed (data not shown).

Altogether, these results indicate that DT retained its structural integrity and antigenicity after it was released from the microneedle surfaces.

4.4. Visualization of fluorescently labeled DT-AF488/TMC-RHO delivery into human skin

To examine whether fluorescently labeled DT-AF488 and TMC-RHO can be delivered into skin from coated microneedle arrays, microneedle arrays coated with 5 bilayers of DT-AF488/TMC-RHO were applied by impact-insertion and held in *ex vivo* human skin for 90 min. Immediately after withdrawal of the coated microneedles, CLSM imaging of the skin at the injection site was performed. From the image in Fig. 3D, showing fluorescence in the conduits in the skin from both DT-AF488 (green) and TMC-RHO (red), it is clear that DT-AF488 and TMC-RHO were successfully delivered into the skin after application and withdrawal of the DT-AF488/TMC-RHO coated microneedle array.

4.5. Quantification of dermally-delivered DT

To quantitatively determine the amount of DT delivered by DT/TMC coated microneedle arrays into the skin, microneedle arrays coated with 5 bilayers of fluorescently labeled DT-IRDYE800/TMC were used. Initially, *ex vivo* human skin studies were conducted to determine the effect of the application period of DT/TMC coated

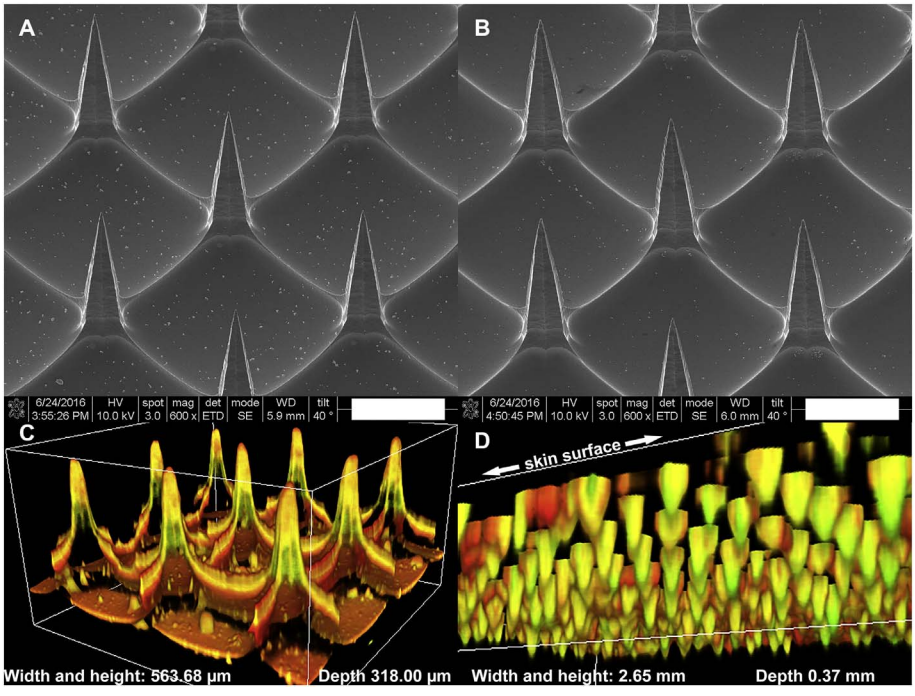

Fig. 3. SEM and CLSM imaging of DT/TMC coated microneedle arrays.
Representative SEM images of plain microneedles (A) and microneedles coated with 5 bilayers of DT/TMC (B) at 600 × magnification, size bars represent 100 µm. Representative CLSM images of (C) microneedles coated with 5 bilayers of fluorescently labeled DT-AF488 and TMC-RHO (10 × objective) and (D) DT-AF488 and TMC-RHO that are released into *ex vivo* human skin, after microneedles coated with 5 bilayers of fluorescently labeled DT-AF488 and TMC-RHO were applied and held in the skin for 90 min and subsequently removed before imaging (4 × objective). The images are overlays of DT-AF488 fluorescence represented in green and TMC-RHO fluorescence represented in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

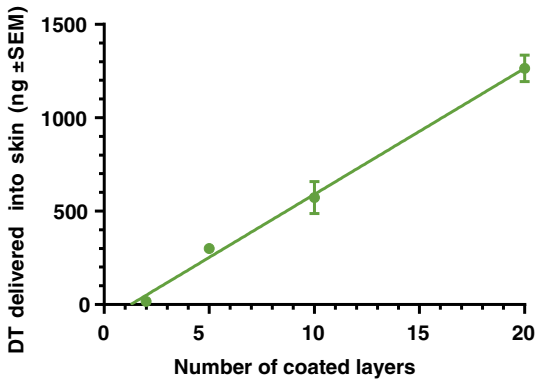

Fig. 4. Quantification of DT delivered into *ex vivo* mouse skin.
Quantification of the amount of fluorescently labeled DT-IRDYE800 (mean ± SEM, $n = 3$) dermally delivered into *ex vivo* mouse skin by microneedle arrays coated with an increasing number of bilayers of fluorescently labeled DT-IRDYE800/TMC.

microneedle arrays on the amount of dermally-delivered DT. After application periods of 30 and 90 min, 184 (± 16) ng and 293 (± 46) ng DT were delivered, respectively. Because a 90 minute application period was superior in comparison to a 30 minute application period, a 90 minute application period was used in subsequent studies.

As the immunization studies with DT/TMC coated microneedle arrays were carried out in mice, subsequent studies were performed on *ex vivo* mouse skin. To determine how the amount of dermally-delivered DT depends on the number of coated DT/TMC bilayers, the amount of dermally-delivered DT by microneedle arrays coated with either 1, 2, 5, 10 or 20 bilayers of fluorescently labeled DT-IRDYE800/TMC was quantified. The amount of dermally-delivered DT-IRDYE800 was linearly dependent on the number of bilayers coated onto the microneedle arrays, as shown in Fig. 4. The slope coefficient for DT-IRDYE800 delivery into *ex vivo* mouse skin was 67.5 ng/layer with a R^2 of 0.9967.

4.6. Dose-response study of intradermally- and subcutaneously-delivered DT

To determine the required amount of dermally-delivered DT for the induction of DT-specific immune responses, a dose-response study was performed. DT was delivered intradermally by using a single hollow microneedle or subcutaneously by a conventional 26G hypodermic needle. The DT-specific IgG responses after the 2nd booster immunization are presented in Fig. 5A (for responses after prime and 1st booster immunization and IgG1:IgG2a ratios see Supplementary data S5). As shown in Fig. 5A, after the 2nd booster immunization, the midpoint (EC50) of the intradermal curve (37 ng) was significantly lower (± 3-fold) than the subcutaneous curve (116 ng). This indicated that higher DT-specific IgG titers using lower DT doses are to be expected from intradermal immunization as compared to subcutaneous immunization. With regard to dose selection, although a DT dose of 0.02 μg already resulted in DT-specific IgG responses, a 0.3 μg dose resulted in similar responses as a 5 μg dose (16.6-fold lower). Moreover, as shown in Fig. 5B, a minimum DT dose of 0.3 μg was required for DTx neutralization titers, whilst a 0.78 μg dose resulted in similar DTx neutralization titers in comparison to a 5 μg dose (6.4-fold lower). Therefore, a minimum dose of 0.3 μg DT is required to be delivered by DT/TMC coated microneedle arrays to induce potent DT-specific IgG titers and DTx neutralization responses.

4.7. Immunization study with DT/TMC coated microneedle arrays

To study the potential to induce immunization of dermally-delivered DT by DT/TMC bilayer coated microneedle arrays, the DT-specific immune responses as a function of the number of DT/TMC bilayers (2, 5 and 10) coated onto the microneedle arrays was investigated in an

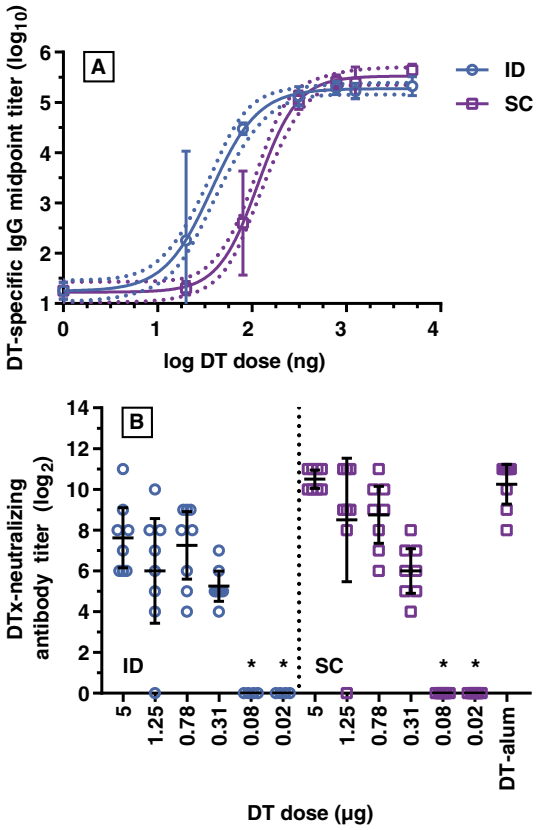

Fig. 5. A dose-response study of DT delivered into the skin.
DT-specific immune responses as a result of intradermal (ID) and subcutaneous (SC) delivery of doses ranging from 0.02 to 5 μg after the 2nd boost immunization. The DT-specific IgG midpoint titers (A) and DTx neutralization titers (B) are represented by blue circles and purple squares, respectively. The DT-specific IgG responses were used to plot a 4-parameter fit (solid line) and a 95% confidence interval of that fit (dotted line) in (A) ($n = 8$). The blue and purple lines represent the fit for intradermal and subcutaneous delivery, respectively. Black lines represent the mean ± 95% confidence interval and stars represent a significant decrease ($p < 0.05$) in (B) ($n = 8$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

immunization study. As 0.3 μg DT is required to induce DT-specific immune responses upon intradermal immunization (based on results of the dose-response study), microneedle arrays should be coated with at least 5 DT/TMC bilayers (see delivery studies in *ex vivo* skin).

The DT-specific IgG titers increased with an increasing number of coated DT/TMC bilayers after every subsequent immunization (prime, boost and 2nd boost), as shown in Fig. 6A–C. The higher response owing to immunization with an increased number of coated DT/TMC bilayers was also reflected in the DTx neutralization titers after the 2nd boost immunization, as shown in Fig. 6D. After every subsequent immunization, DT-specific IgG titers and DTx neutralization titers resulting from dermal immunization with microneedle arrays coated with 10 bilayers of DT/TMC (corresponding with approximately 0.6 μg delivered DT) and subcutaneous immunization with 5 μg DT-ALUM were not significantly different. The addition of TMC to the DT formulation for intradermal immunization with a single hollow microneedle did not result in increased DT-specific responses. As demonstrated in Fig. 6E, a stepwise decrease in IgG1:IgG2a ratio was observed for immunization by microneedle arrays coated with 2, 5 and 10 bilayers of DT/TMC.

DT-specific responses resulting from subcutaneous immunization with or without anesthesia were similar (data not shown), demonstrating that anesthesia had no effect on the DT-specific immune responses. PBS pH 7.4 mock treatment did not result in any DT-specific IgG titers.

Upon comparing immunization routes, dermal immunization with

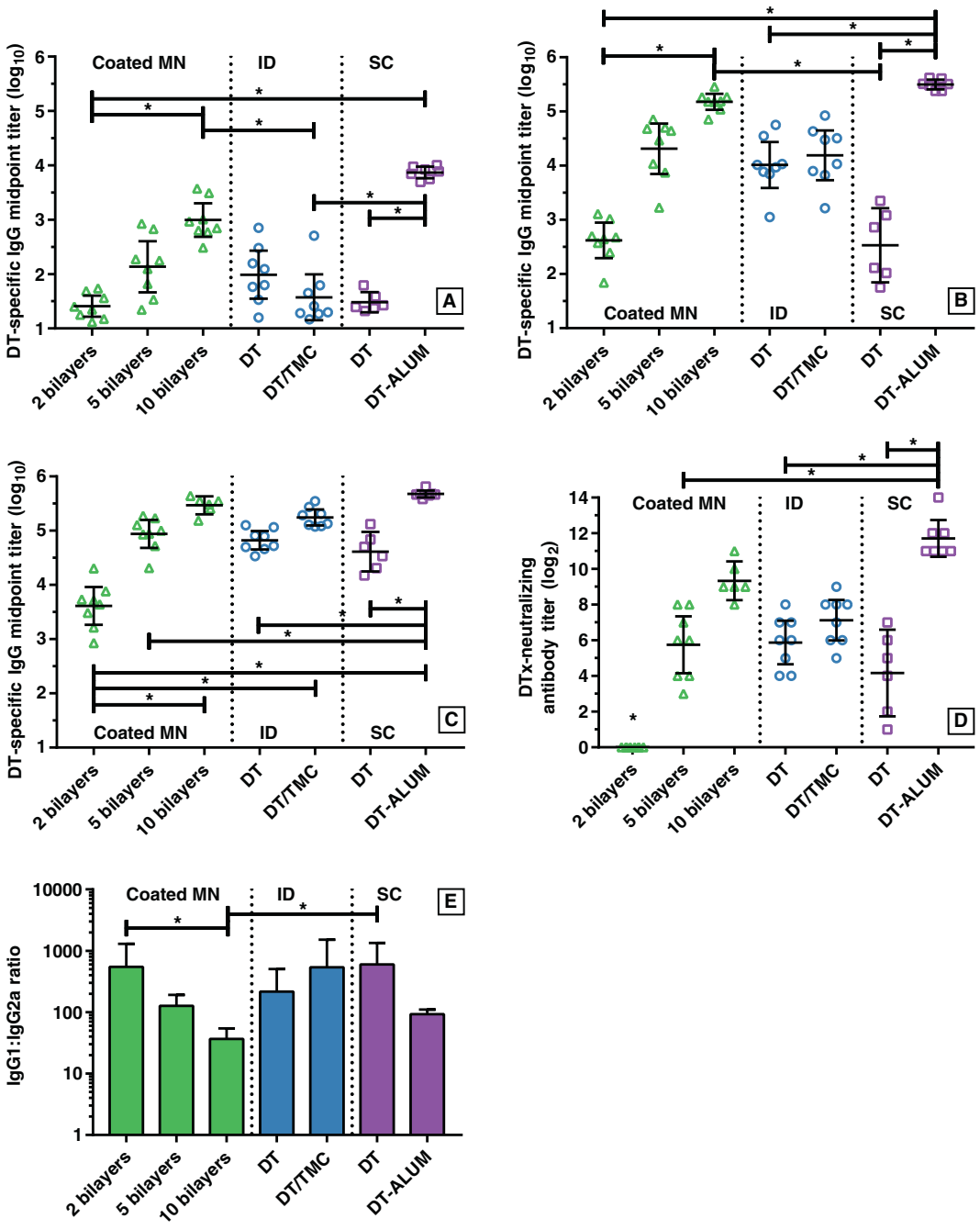

Fig. 6. Immunization study with DT/TMC coated microneedle arrays.
Assessment whether DT/TMC coated microneedle arrays induce DT-specific immune responses, in which a microneedle array coated with an increasing number of DT/TMC bilayers is applied onto the skin and compared to intradermal and subcutaneous control groups (see Table 2). Presented are the DT-specific IgG midpoint titers 21 days past prime immunization (A), boost immunization (B) and 2nd boost immunization (C) resulting from DT immunization *via* the dermal route by a DT/TMC coated microneedle array (coated MN, green triangles or bars), intradermal route by a single hollow microneedle (ID, blue circles or bars) or subcutaneous route by a conventional 26G hypodermic needle (SC, purple squares or bars). DTx neutralization titers (D) and IgG1:IgG2a ratios (E) were determined in serum at 21 days past the 2nd boost immunization as well. Black lines and bars represent mean ± 95% confidence interval ($n = 8$) and stars represent a significant increase ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

microneedle arrays coated with 5 or 10 DT/TMC bilayers or intradermal immunization with a single hollow microneedle resulted in higher DT-specific IgG titers in comparison to subcutaneous immunization after the prime and boost immunization. The DT-specific IgG titers of dermal or intradermal and subcutaneous immunization were only comparable after the 2nd boost immunization. These results indicate that dermal or intradermal immunization resulted in a faster development of IgG responses than subcutaneous immunization. A similar trend was observed for the DTx neutralization titers.

5. Discussion

In this study, it was demonstrated that the DT/TMC layer-by-layer coating approach on pH-sensitive high-density microneedle arrays offers a tunable vaccine delivery system. The amount of DT that was coated and delivered into the skin was reproducible and could be tuned by selecting the number of coated DT/TMC bilayers. Moreover, dermal immunization of mice by using the DT/TMC coated microneedle arrays resulted in strong functional DT-specific immune responses.

5.1. DT/TMC layer-by-layer coating approach for high-density microneedle arrays

The amount of coated DT or TMC incremented linearly with each increasing bilayer of DT/TMC coated onto the microneedle arrays. This was also observed for coating IPV and TMC onto high-density microneedle arrays utilizing a similar layer-by-layer coating approach [24]. This demonstrates that a layer-by-layer coating procedure onto pH-sensitive microneedles is a versatile method to precisely control the amount of coated antigen by selecting the number of coated bilayers. Furthermore, this coating procedure may be applicable, not only to proteins, but also to nanoparticles.

Moreover, the microneedle tips of microneedles coated with 5 bilayers of DT/TMC had a tip diameter < 5 μm, which is required for effortless skin penetration [28]. Coating high-density microneedle arrays while preserving sharp microneedle tips is possible, by using this layer-by-layer coating approach or a nitrogen jet drying coating approach [17,29], while dip-coating approaches resulted in blunt microneedle tips [15,17–19]. Furthermore, viscosity enhancers, lyoprotectants or surfactants are not required for this layer-by-layer coating approach, which simplifies the formulation, while coating a high amount of antigen in a thin layer onto the microneedle arrays.

5.2. Delivery of DT/TMC into the skin by DT/TMC coated microneedle arrays

Using CLSM, we demonstrated that the DT and TMC delivered by DT/TMC coated microneedle arrays were co-localized in the conduits in the skin, but this method is not suitable to quantify the amount of DT within the skin. For this reason, we measured the near-infrared fluorescence intensity in the skin of DT-IRDYE800 delivered by the coated microneedle arrays.

Although precise dosing has been considered critical in dermal immunization [13,14], it only has been convincingly addressed in a limited number of studies [30–32]. In the current study, we demonstrate that a linear increase in coated DT with an increasing numbers of DT/TMC bilayers also resulted in a linearly increased amount of DT delivered into skin, allowing to select a precise dose that is reproducibly delivered into the skin. The delivery efficiency (amount released/amount coated onto the microneedle array * 100%) of 16.7% in *ex vivo* mouse skin was comparable to that reported (between 7.6 and 37.5%) for other dry coating approaches for high-density microneedle arrays [29,32–35].

5.3. Immunization studies

5.3.1. Coated microneedle arrays

Dermal immunization by microneedle arrays coated with 10 DT/TMC bilayers led to similar DT-specific immune responses compared with subcutaneous immunization by 5 μg DT-ALUM. The dose delivered in the skin by these coated microneedle arrays was determined at about 0.6 μg, *i.e.*, about 8-fold lower than the subcutaneous dose. Moreover, coated microneedle arrays are easier to use for (intra-)dermal immunization compared to hollow microneedles, which require a sophisticated applicator [14]. Additionally, dry vaccine formulations generally are more stable than liquid ones [14,15,18,33]. Altogether, this demonstrates the potential of antigen-coated microneedle arrays as an alternative for subcutaneous immunization.

The accuracy at which the DT dose was delivered in the skin by selecting an increasing number of DT/TMC bilayers coated onto the microneedle arrays was reflected in the corresponding increasing levels of DT-specific immune responses. For example, dermal immunization with microneedle arrays coated with 5 bilayers of DT/TMC, corresponding with a DT dose of 0.3 μg delivered into the skin, resulted in similar DT-specific immune responses as intradermal immunization with 0.3 μg DT delivered by a single hollow microneedle.

5.3.2. Application mode of the DT/TMC coated microneedle arrays

A supposed drawback of coated microneedle arrays is the lengthy application period of 90 min in the skin to deliver a sufficient amount of antigen. For this reason, in a pilot study a short-lasting repetitive application mode was investigated to strongly reduce the required application time. By using the impact insertion applicator system, microneedle arrays coated with 5 bilayers of DT/TMC were repeatedly applied onto the skin for 10 applications at a frequency of 1 Hz (1 application per second, 10 s total, with an application time of 0.5 s per application). Intriguingly, 10 repeated applications in 10 s resulted in similar DT-specific immune responses as a single 90 minute application (Supplementary data S7). Therefore, this application mode permits very short application times, which may increase the attractiveness of the use of coated microneedle arrays even more, from a patient compliance perspective.

5.3.3. Influence of TMC

TMC was used as a poly-cation to enable layer-by-layer coating (as DT is negatively charged at coating pH) and because it has been reported to enhance DT-specific immune responses upon intradermal immunization [26]. However, in the current study only a mild increase in DT-specific immune responses (not significant) was observed, likely because much lower DT and TMC doses were used.

5.3.4. Intradermal versus subcutaneous immunization

It became evident in both immunization studies that (intra-)dermal immunization is more efficient at inducing DT-specific immune responses at low doses as compared to subcutaneous immunization. This high immunization efficiency at low antigen doses could lead to dose-sparing and thereby cost reduction of vaccination programs, which is especially of interest to low-income countries [36].

For both intradermal and subcutaneous immunization, humoral immune responses were dominant over cellular immune responses. This is represented by a high IgG1:IgG2a ratio, as humoral immune responses are characterized by the production of IgG1 antibodies, and cellular immune responses are characterized by the production of IgG2a antibodies [37,38]. Surprisingly, in contrast to subcutaneous immunization (less dominant humoral immune responses at low antigen doses), upon intradermal immunization the humoral immune responses became predominant at low antigen doses in a dose-dependent manner.

6. Conclusion

In this study, we examined a layer-by-layer coating approach to coat high-density microneedle arrays with DT as anionic antigen and TMC as counter poly-cation and adjuvant. This coating approach allowed to control the amount of DT coated onto the microneedle arrays and delivered into the skin, whilst maintaining tip sharpness and integrity of the coated DT. After studying dose-response of DT immunization *via* the intradermal and subcutaneous route, it was demonstrated that intradermal immunization at low DT doses resulted in superior immune responses compared to subcutaneous immunization. Furthermore, we have demonstrated that immunization by DT/TMC coated microneedle arrays induced strong DT-specific immune responses. Summarizing, the layer-by-layer coating approach onto pH-sensitive microneedles is a versatile method to precisely control the amount of microneedle-coated and dermally-delivered antigen, which is highly-suitable for dermal immunization.

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writing of the report; and in the decision to submit the article for publication.

Conflict of interest

Koen van der Maaden is co-owner of uPRAX Microsolutions. All other authors declare that they have no conflict of interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2017.07.017.

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