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Special Topic Cluster

Intradermal Administration of Influenza Vaccine with Trehalose and Pullulan-Based Dissolving Microneedle Arrays



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ABSTRACT

Most influenza vaccines are administered via intramuscular injection which has several disadvantages that might jeopardize the compliance of vaccinees. Intradermal administration of dissolving-microneedle-arrays (dMNAs) could serve as minimal invasive alternative to needle injections. However, during the production process of dMNAs antigens are subjected to several stresses, which may reduce their potency. Moreover, the needles need to have sufficient mechanical strength to penetrate the skin and subsequently dissolve effectively to release the incorporated antigen. Here, we investigated whether blends of trehalose and pullulan are suitable for the production of stable dMNA fulfilling these criteria. Our results demonstrate that production of trehalose/pullulan-based dMNAs rendered microneedles that were sharp and stiff enough to pierce into *ex vivo* human skin and subsequently dissolve within 15 min. The mechanical properties of the dMNAs were maintained well even after four weeks of storage at temperatures up to 37°C. In addition, immunization of mice with influenza antigens via both freshly prepared dMNAs and dMNAs after storage (four weeks at 4°C or 37°C) resulted in antibody titers of similar magnitude as found in intramuscularly injected mice and partially protected mice from influenza virus infection. Altogether, our results demonstrate the potential of trehalose/pullulan-based dMNAs as alternative dosage form for influenza vaccination.

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Introduction

Influenza is a serious respiratory disease causing seasonal epidemics and occasional pandemics.^{1,2} Vaccination is the most effective measure to prevent or control the spread of influenza.³ Most influenza vaccines are aqueous formulations that are administered intramuscularly or subcutaneously using hypodermic needles. Several

disadvantages have been associated with parenteral vaccine administration such as pain, risks for needle stick injuries, or poor compliance for individuals with needle phobia. In addition to these needle-related issues, currently used liquid influenza vaccine formulations have a limited shelf life and require refrigerated storage and transport, the so-called cold-chain.

The skin could serve as an alternative route for influenza vaccination due to the abundance of Langerhans and dermal dendritic cells in the epidermis and dermis.⁴ In addition, it is easily accessible, albeit that the outer layer of the skin, the stratum corneum, is an efficient barrier for skin penetration of the antigens and adjuvants. To bypass the stratum corneum, microneedle arrays (MNAs) have been developed as a minimally invasive strategy for influenza vaccination.^{5–7} Influenza vaccine administration to the skin with microneedles has been demonstrated to induce superior immune responses compared to traditional intramuscular injection.^{7–9} Besides its immunological advantages, microneedles based formulations may also provide other

Abbreviations: API, active pharmaceutical ingredient; BCIP, 5-bromo-4-chloro-3'-indolylphosphate p-toluidine; DLS, dynamic light scattering; dMNAs, dissolving microneedles arrays; hMNAs, hollow microneedles; IFN, interferon; IL, interleukin; IVIS, in vivo imaging system; MDCK, Madin-Darby Canine Kidney; NTB, nitro-blue tetrazolium chloride; PB, phosphate buffer; RH, relative humidity; TCID, tissue culture infectious dose; TEM, transmission electron microscopy; WIV, whole inactivated virus.

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benefits, including an improved stability of the vaccine. As the antigen is incorporated in the microneedles in the dry state, it is less prone to degradation which may make strict maintenance of the cold-chain superfluous. Furthermore, the use of microneedles averts pain and discomfort during administration and provides the potential for self-administration.¹⁰

Various types of microneedles have been developed for vaccine delivery, including coated MNAs, nanoporous MNAs, hollow microneedles (hMNs) and dissolving microneedles arrays (dMNAs).^{11–17} Among these, dMNAs have gained increased attention, as they are often produced from biocompatible and water-soluble materials. When inserted into the skin, these dMNAs will dissolve into the skin's interstitial fluids, thereby releasing the incorporated active pharmaceutical ingredient (API) or antigen.¹⁸ dMNAs are usually prepared by pouring an aqueous solution containing the dMNA forming material (s) and the API into a mould after which water is evaporated at elevated temperatures. During this process, however, antigens are subjected to several stresses, including heat and dehydration, which may lead to loss of potency. It is well known that several sugars can act as stabilizing excipients during drying of biopharmaceuticals such as vaccines.^{19,20} When dried under the proper conditions, the biopharmaceutical is incorporated in a protecting matrix of the sugar in the glassy state. Among these sugars, the disaccharide trehalose has been most widely used.^{21,22} However, using only trehalose for preparing dMNAs would probably not yield a suitable product. Being a disaccharide, dMNAs prepared from trehalose glass are expected to be too brittle and will not be able to pierce the skin efficiently. In addition, air-drying is a relatively slow process and because the sugar will be in the liquid state, crystallization of trehalose may easily occur resulting in loss of stabilizing properties. We hypothesize that the addition of pullulan to trehalose in preparing dMNAs would solve these issues. Pullulan is a high molecular weight polysaccharide (200–300 kDa) and can gain excellent mechanical properties after making structures from it, which may improve the strength and stiffness of the material.²³ Moreover, pullulan has a high glass-rubber transition temperature, preventing crystallization of the sugar mixture. However, since it is too bulky to form compact molecular coating around antigen, pullulan alone is not an optimal stabilizer for biopharmaceuticals and is too viscous for dMNAs preparation.^{19,20} In previous studies, we found that blends of trehalose/pullulan have favorable properties as they show no crystallization tendency and biopharmaceuticals incorporated in these blends have excellent process and storage stability, especially when exposed to high moisture conditions.¹⁹

Based on these findings, the aim of this study was to investigate whether trehalose/pullulan blends are suitable for the production of stable antigen containing dMNAs capable of penetrating the skin and inducing immune responses. Whole inactivated influenza virus (WIV) was selected as antigen because it contains all the structural viral proteins and is more immunogenic than split or subunit influenza vaccine.²⁴ To assess the stability of the antigen during processing and storage, the hemagglutinating capacity of WIV immediately after dMNA production and after 4-weeks storage at 4°C, 25°C and 37°C was determined. Furthermore, *ex vivo* studies using human skin were performed to evaluate skin penetration, dMN dissolution and intradermal delivery. Finally, WIV encapsulated in dMNAs was administered in the skin of mice and tested for evoked immune responses and protective efficacy against influenza virus infection.

Material & Method

Materials

SYLGARD 184 base [silicone elastomer](#) and curing agent silicone elastomer were purchased from Dow Corning (Midland, MI, USA). Pullulan (average molecular weight 200–300 kDa) and trehalose

were kind gifts from Hayashibara Co., Ltd. (Okayama, Japan). All other excipients and chemicals were of analytical grade. Trypan Blue solution 0.4% (w/v) was purchased from Millipore Sigma (Zwijndrecht, The Netherlands). Vinylpolysiloxanes A-silicone (Elite Double 32) was from the Zhermack Group (Badia Polesine, Italy) and epoxy glue was from Bison International B.V. (Goes, The Netherlands). Tape (packing tape polypropylene, transparent) for stripping skin was purchased from Staples. Silicon microneedle arrays were kindly provided by Tyndall National Institute (Cork, Ireland). IRDye® 800CW NHS Ester was purchased from [LI-COR Biosciences](#).

Production of Placebo dMNAs and WIV Loaded dMNAs

To produce sharp, strong and easily dissolving dMNAs five different trehalose/pullulan weight ratios were investigated to produce dMNAs; i.e. 50:50, 40:60, 30:70, 20:80, and 0:100 w/w. The total concentration of carbohydrates in all five formulations was 15% (w/v) in phosphate buffer (PB, pH 7.4, prepared with 7.7 mM Na₂HPO₄ and 2.3 mM NaH₂PO₄). For preparing dMNAs, 1 µg (from a 2.5% (w/v) suspension) and 12.5 µg (from a 31.25% (w/v) suspension) of WIV were added to 15% (w/v) 50:50 trehalose/pullulan mixture in PB for the storage stability study and for the vaccination study, respectively, which was based on the result of dose selection test (section 3.4).

The production of dMNAs starts from the poly(methyl methacrylate) (PMMA) template which was made by the Fine Mechanical Department (FMD) at Leiden University. It has nine square pedestals. On top of each pedestal, a single silicon microneedle patch (5 × 5 mm²) was placed having nine microneedle tips (3 × 3) with a length of 500 µm and base diameter of 330 µm. In order to prepare polydimethylsiloxane (PDMS) mould, the mixture of silicone elastomer and curing agent (10:1 weight ratio) was poured on the top of the PMMA template and cured overnight at 60°C.²⁵ The minimum volume of dMNA formulation (40 µL) was loaded into the PDMS mould followed by centrifugation for 3 hours at 25°C with 11400 g (Beckman Coulter [Allegra X12R](#) Indianapolis, IN, USA). After overnight drying of the centrifuged mould at 37°C, silicone and epoxy glue were applied onto each array followed by overnight drying at 37°C. Subsequently, the dMNA patch was removed from the mould and the shape of the dMNAs was visualized using a brightfield microscope (Stemi 2000-C, Carl Zeiss Microscopy GmbH, Göttingen, Germany).²⁶

Screening of (Poly)saccharide Formulation Based on Skin Penetration and dMNA Dissolution Tests

To investigate the mechanical strength of the dMNAs skin penetration tests were performed as previously described.²⁶ Briefly, human abdominal skin was collected after reduction surgery from a local hospital, and stored at -80°C after excess fat was removed. Before use, the skin was thawed, wiped with 70% ethanol for cleaning the skin surface, and stretched on parafilm-covered Styrofoam. Next, the dMNA was attached to the impact-insertion applicator (uPRAX Microsolutions B.V., Delft, The Netherlands). An average velocity of 65 ± 1 cm/s was applied on the dMNAs to penetrate the skin (*n* = 3). The dMNA stayed for one second in the skin and then the dMNA was removed. To examine whether the dMNAs penetrated the skin, 75 µL of 0.4% (w/v) trypan blue solution was applied onto the skin. After 45 min, the trypan blue was removed from the skin surface and the stratum corneum was removed by 10 times of tape stripping. The treated site of the skin was analyzed by using a brightfield microscope. As trypan blue makes a blue dot on the penetrated site of the skin, the penetration efficiency was calculated by dividing the number of blue dots by 9 which is the number of microneedle tips in each array. For example, when 8 out of 9 microneedles could penetrate the skin, the penetration efficiency is 8/9 × 100 = 88.9 %.

Next, a dMNA dissolution test was carried out ($n = 3$). To this end, the dMNA was applied onto the skin as described above and retained 15 minutes in the skin. After removal of the dMNA from the skin, the leftover shape of the individual microneedles was analyzed by using a brightfield microscope.

Preparation of Whole Inactivated Virus Vaccine

NIBRG-121, a reassortant of A/California/7/2009 H1N1pdm09 virus, was provided by the National Institute of Biological Standards and Controls, Potters Bar, United Kingdom and propagated in the allantoic cavity of 11-day-old embryonated hens' eggs. The virus was inactivated with β -propiolactone (0.1%) by overnight incubation at 4°C under continuous rotation, followed by dialysis against HEPES buffered saline at 4°C overnight to remove β -propiolactone. Inactivation was verified by inoculating WIV on Madin-Darby Canine Kidney (MDCK) cells and assessing the presence of amplified virus in the supernatant after 72 hours of incubation by hemagglutination assay as described before.²⁷ The total protein concentration of the WIV preparation was determined by micro-Lowry assay.²⁸

Physical Characterization of WIV

The size of WIV particles in plain solution as well as dispersed in a 1% (w/v) 50:50 trehalose/pullulan containing solution was determined by dynamic light scattering (DLS) (Malvern Zetasizer ZS90, Malvern, United Kingdom). The concentration of WIV was 1 mg/mL based on the result of micro-Lowry assay. Transmission electron microscopy (TEM) images of plain WIV solution and WIV formulated in the trehalose/pullulan solution were captured using a transmission electron microscope with Gatan type UltraScan 4000SP CCD Camera (Philips CM120, Philips, the Netherlands). Liquid WIV samples were applied to formvar-coated TEM grids and stained twice with 5 μ L 2% uranyl acetate.

Stability of WIV During dMNAs Production

In order to investigate whether the drying process during dMNAs production affects the WIV activity, WIV formulated in 15% (w/v) 50:50 trehalose/pullulan dried at 37°C for 48 hours without being incorporated into dMNAs was used to mimic the drying process of WIV during dMNAs production. The hemagglutinating capacities of WIV in dispersion and WIV in dMNAs were assessed immediately after the drying process.

The hemagglutination assay of WIV was performed as previously described.^{29,30} Briefly, WIV incorporated in dMNAs was reconstituted in PB (10mM, pH 7.4). WIV dispersed in PB stored at 4°C was used as a control. Two-fold serial dilutions were brought on 96-well V bottom plates followed by addition of 50 μ L of 1.5% guinea pig red blood cells (Envigo, The Netherlands). Hemagglutination was checked after 2 hours incubation at room temperature (RT). Hemagglutination titers were expressed as the $\log_2$ of the highest dilution where red blood cells agglutination occurred. All measurements were performed in triplicate.

Mechanical Stability of dMNAs and Stability of WIV in dMNAs After Four Weeks of Storage

One of main potential advantages of the vaccine containing dMNA is its thermostability as the formulation secures the antigen stability and mechanical strength. The dMNAs maintain their shape and physico-chemical characteristics at ambient temperatures or even at higher temperatures at low humidity. In order to investigate physical stability, dMNAs were prepared from a 15% (w/v) 50:50 trehalose/pullulan solution without WIV as described in section 2.2. and were

stored for four weeks at the following conditions based on our previous study.³⁰ 37°C/0% relative humidity (RH), RT/0% RH, 4°C/0% RH, and RT/56% RH.

To secure 0% RH, dMNAs were placed in the vacuum bag with silica beads. After purging the vacuum bag with argon gas, the bag was sealed with a vacuum sealer. Three sealed bags were placed at three different temperatures: 37°C, RT, and 4°C. In order to maintain 56% RH, 95 g of sodium bromide was added to 100 mL deionized water and poured into the glass container. dMNAs were placed above the saturated NaBr solution.¹⁹ The container was sealed with the lid and parafilm to maintain the humidity and were stored at RT. The humidity inside of the container was monitored every 24 hours by using a humidity sensor (Digitron 2020R). After four weeks of storage, the physical stability was analyzed by determining the sharpness of the dMNAs using a brightfield microscope, and by skin penetration and dMNA dissolution tests as described above (section 2.3). The stability of WIV in dMNAs was tested by a hemagglutination assay as described in section 2.6. A dispersion of WIV in 15% (w/v) 50:50 trehalose/pullulan solution stored for four weeks at 37°C, RT, and 4°C was used as a control.

Dose Determination of WIV Through Ex Vivo Skin Delivery Test

In order to determine the relation between the encapsulated material in the whole array and the amount administered in the skin, we investigated the dose delivered from dMNAs into the skin. For this purpose, dMNAs were loaded with 1.17 μ g, 2.33 μ g, or 4.66 μ g IRDye800CW. Dye-loaded dMNAs were applied onto human skin for 15 min as described in section 2.3 ($n = 3$). After dissolution the remains of the dMNAs were withdrawn from the skin and the fluorescence intensity of the penetrated skin was measured by using an in vivo imaging system (IVIS, Waltham, MA, USA) using a ICG filter set and an exposure time of 1 s. To determine the relationship between the fluorescent intensity and the delivered dose a calibration curve was prepared. To obtain a calibration curve, various amounts (0.058 to 2.33 μ g) of IRDye800CW were injected into the skin at a depth of 400 μ m using a digitally controlled hMN injection system (DC-hMN-iSystem). This system is used to accurately inject volumes of 1–60 μ L (<10% error) into the skin.³¹ Based on the fluorescence counts in the dMNA-treated skin, the delivered amount of dye was calculated using this calibration curve.

Immunization of Mice with dMNAs

Female CB6F1 (C57Bl/6 x BALB/c F1) mice of 6–8 weeks old were obtained from Envigo (The Netherlands). Experimental use of animals was approved by the Central Committee for Animal Experimentation of the Netherlands (CCD application number AVD105002016599). Mice were co-housed with a total number of 3 mice in individually ventilated cages and had ad libitum access to sterilized tap water and standard diet. All mice were acclimatized for 1.5 weeks before the start of the experiment. Mice were vaccinated with WIV-incorporated dMNAs that were either freshly prepared (<1 day) or upon storage at 4°C /0% RH or 37°C /0%RH for four weeks. As a negative control, trehalose/pullulan based dMNAs without WIV were used. For the positive control group, a freshly prepared aqueous WIV dispersion of 1 μ g in 40 μ L PBS was injected via the intramuscular route. A total number of 12 mice per experimental group was used.

Two days before the dMNAs were applied, the flank of the mice was shaved. Dissolvable microneedles were attached to the piston of a digitally-controlled microneedle applicator (U.A.F.M. v1.0, uPRAX Microsolutions B.V., Delft, the Netherlands) by using double sided tape. Next, the mice were anesthetized with isoflurane and subsequently the applicator was placed onto the shaved skin. The microneedles were applied with a velocity of 1.65 cm/s onto the skin and

the applicator controller was programmed to keep the microneedles in place for 15 minutes.

Virus Challenge and Sample Collection

At day 28 post immunization, mice were lightly anesthetized with isoflurane and blood was drawn by cheek puncture for determination of serum IgG levels. Next, mice were anesthetized and intranasally challenged with a sublethal dose (10^4 Tissue Culture Infectious Dose (TCID)₅₀/mouse) of influenza virus strain A(H1N1)pdm09 (NIBCS) in 40 μ l PBS. At three days post-infection, six mice from each experimental group were sacrificed to determine lung virus titers. Blood was collected by heart puncture for determining serum IgG titers and IgG1 and IgG2a levels. Nasal and lung washes were performed with 1 ml PBS containing Complete[®] protease inhibitor cocktail (Roche, Almere, The Netherlands) for determination of IgG and IgA titers. Lungs were collected in 1 ml EPISERF medium (100 U/ml penicillin, 100 mg/ml streptomycin, 12.5 ml of 1 M HEPES, 5 ml of 7.5% sodium bicarbonate for 500 ml medium, Thermo Fisher Scientific, Bleiswijk, Netherlands) to determine viral load (see below). Spleens were collected in 2.5 ml Iscove's Modified Dulbecco's Medium (Thermo Fisher Scientific, Bleiswijk, Netherlands) containing 10% v/v FBS (Lonza, Basel, Switzerland), 100 U/ml penicillin, 100 mg/ml streptomycin and 50 μ M 2-mercaptoethanol (Invitrogen, Breda, The Netherlands) and used for ELISPOT (see below). Remaining mice ($n = 6$) were followed every morning and afternoon for disease activity score which was based on appearance, behavior, weight loss and movement (Supplementary Table 1). Mice reaching a disease activity score of 12 reached the humane endpoint and were therefore terminated.

Virus Titration

Titration of virus present in lungs was performed as previously described.²⁷ Briefly, lungs were homogenized in 1 ml EPISERF medium with a polytrone (Kinematica, Malters, Switzerland) and centrifuged for 10 min at 1400 rpm at 4°C. Supernatants were snap-frozen in liquid nitrogen and stored at -80°C until further use. Supernatants were applied in 2-fold serial dilutions on MDCK cells to determine lung virus titers.²⁷ Virus titers are represented as log₁₀ titer per gram of lung.

Antibody Responses

To determine serum IgG, IgG1, IgG2a levels or IgG and IgA antibodies levels in lung wash or nasal wash, ELISA was performed as previously described³² using A(H1N1)pdm09 WIV as coating. To detect IgG titers, serum samples were diluted 1:100 and applied in 2-fold serial dilutions on the plates. The 2-fold serial dilutions of nasal and lung washes were prepared from undiluted samples. To detect IgG1 and IgG2a levels, serum samples were applied in a dilution of 1:100 and concentrations were determined from a standard curve. Horseradish peroxidase-linked goat anti-mouse IgG (Southern Biotech, Birmingham, USA) antibody was used to detect bound IgG. IgG titers were calculated as log₁₀ of the reciprocal of the serum sample dilution corresponding to an absorbance of 0.2 at a wavelength of 492 nm.

ELISPOT

Spleens were homogenized using a GentleMACS dissociator (Miltenyi Biotec, Leiden, The Netherlands) and washed with Ammonium-Chloride-Potassium lysis buffer (0.83% NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA) to lyse erythrocytes. Next, a mouse ELISPOT kit (MABTEC, The Netherlands) was used according to manufacturer's instructions to enumerate influenza-specific interferon (IFN)- γ and

interleukin (IL)-4 secreting splenocytes. Briefly, splenocytes (5×10^5 /well) were incubated in Iscove's Modified Dulbecco's complete Medium (with 10% FBS, 1% Penicillin-Streptomycin and 0.1% β -mercaptoethanol) with or without 10 μ g/ml of A(H1N1)pdm09 WIV plus 10 μ g/ml of NP366 peptide (ASNENVETM). After 16h of incubation, IFN- γ or IL-4 secreting splenocytes were detected using alkaline phosphatase-conjugated anti-mouse IFN- γ or IL-4 antibodies, respectively. Spots were developed using nitro-blue tetrazolium chloride (NTB)/ 5-bromo-4-chloro-3'-indolylphosphate p-toluidine (BCIP) substrate and counted with an AID ELISPOT reader (Autoimmune Diagnostika GmbH, Strassberg, Germany).

Statistics

Statistical analysis on results was performed using Graphpad Prism version 8.4.1 (La Jolla, CA, USA). Data was tested with Kruskal-Wallis and Dunn's post-hoc test. $p < 0.05$ was considered as significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Results

production of dMNAs and Screening of (poly)saccharide Formulations for dMNAs

The ideal dMNA should be sharp and strong to allow for penetration of the skin. Furthermore, the microneedle should show rapid dissolution in the skin to release the antigen. In order to choose the most suitable (poly)saccharide formulation which provides both mechanical strength and fast dissolution of dMNAs, we produced five placebo dMNAs with different trehalose/pullulan ratios (50:50; 40:60; 30:70; 20:80 and 0:100). Microscopic evaluation revealed that all nine microneedles in each array displayed sharp conical tips around 500 μ m of height and 330 μ m of base diameter as the PMMA template (Fig. 1A-E). In subsequent skin penetration tests, all five dMNA showed a similar average level of penetration efficiency of more than 96% (Fig. 1F-J, Table 1). These results indicate that all dMNA under evaluation had sufficient mechanical strength to penetrate human skin.

Next, a microneedle dissolution test was performed to investigate whether the difference in trehalose/pullulan ratios influences the dissolution rate of microneedles in the skin. While all five types of microneedles showed more than 70% dissolution after 15 min dissolution in the skin there were some differences in the residual left-overs on the backplate for the different trehalose/pullulan ratios. As shown in Fig. 1(K-O), the degree of dissolution decreased with an increasing pullulan proportion in the dMNA formulation. Hence, the microneedles composed of trehalose/pullulan at a weight ratio of 50:50 showed the fastest dissolution rate (Fig. 1K-O), whereas the microneedles composed of pure pullulan displayed the lowest dissolution rate.

As microneedles prepared from trehalose/pullulan at a weight ratio of 50:50 showed the fastest dissolution among the five different trehalose/pullulan ratios, and as 50:50 trehalose/pullulan ratio may also provide better stability of WIV as indicated in our previous paper,¹⁷ this ratio was selected as the dMNA formulation for further studies.

Physical Characterization and Stability of WIV in dMNAs

The size and morphology of WIV particles were analyzed by DLS and TEM, respectively. Plain WIV and WIV dispersed in a 50:50 trehalose/pullulan solution had a similar particle size (166.1 ± 1.4 nm and 168.0 ± 3.7 nm, respectively), which means that formulating WIV in the trehalose/pullulan solution did not have a significant effect on the average diameter of the WIV and did not lead to clumping of the

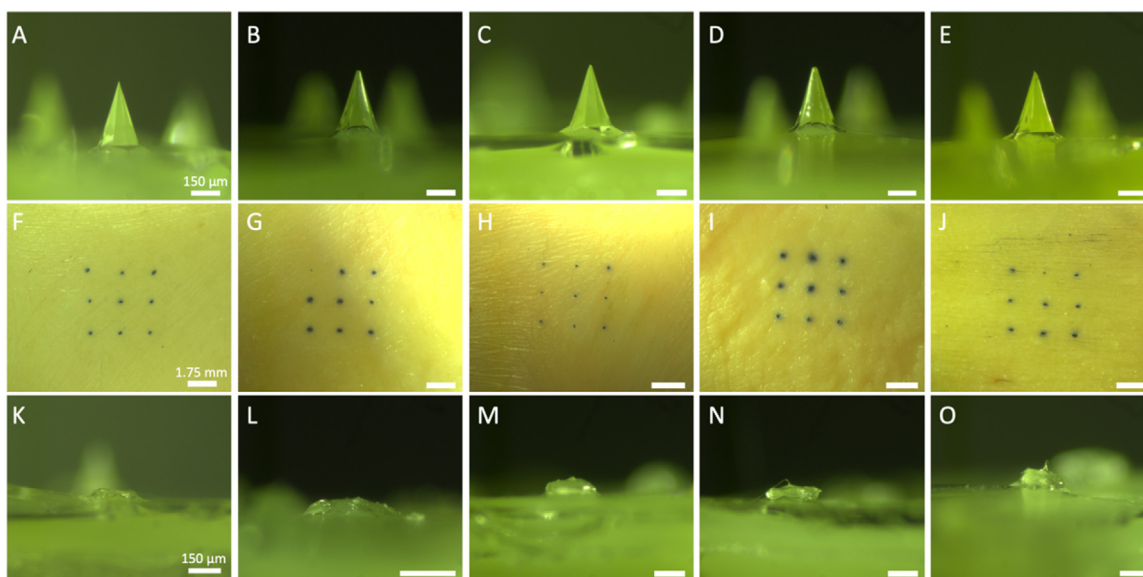


Fig. 1. Freshly produced dissolvable microneedles (A-E), human skin ex vivo penetrated with a dMNA (F-J) and left over microneedle after 15 min of dissolution (K-O) in ex vivo human skin. Microneedles produced with a trehalose:pullulan ratios of 50:50 (A,F,K), 40:60 (B,G,L), 30:70 (C, H, M), 20:80 (D,I,N) and 0:100 (E,J,O) were used. Scale bar of A-E and K-O = 150 μm, and F-J = 1.75 mm.

Table 1

Penetration efficiency rates of dMNAs produced with five different trehalose:pullulan formulations ($n = 3$).

Trehalose:Pullulan ratio	Penetration efficiency rate (%)
50:50	96 ± 6.4
40:60	96 ± 6.4
30:70	100 ± 0
20:80	100 ± 0
0:100	96 ± 6.4

virus particles. TEM analysis indicated that the morphology of WIV particles in buffer and in the trehalose/pullulan solution was similar (Fig. 2A and B). This indicates that the presence of trehalose/pullulan does not affect the morphological appearance of WIV particles.

The preservation of the functional properties of WIV (binding to sialic acid residues) during the production of the dMNAs was assessed by hemagglutination assay. Although WIV in dMNAs showed excellent process stability, there was a slight decrease in hemagglutination titers after production of dMNAs, i.e. from Log_2 8.4 to Log_2 7.3 (Fig. 2). In order to explore the reason for this slight reduction, the hemagglutination titers of WIV dispersed in 15% (w/v) 50:50 trehalose/pullulan solution after the same drying process but without being incorporated into dMNAs were analyzed. WIV from these samples induced the same hemagglutination titers (Log_2 7.3) as WIV incorporated in dMNAs. Hence, the reason for the slight decrease in the activity of WIV during the production of the dMNAs may be due to the drying process.

Storage Stability of dMNAs and WIV

To investigate the impact of temperature and humidity on the mechanical strength of dMNAs, the dMNAs were stored at four different environmental conditions for four weeks: 37°C/0% RH, RT/0% RH, 4°C/0% RH, and RT/56% RH, which explains the penetration efficiency of 96% or more (Table 2). The tips of the microneedle became blunt when the needles were subjected to higher moisture levels (RT/56% RH) reducing penetration efficiency to only 25.9% (Table 2; Fig. 3D).

Therefore, dMNAs stored at RT and 56% RH were discarded for further experiments.

For the next experiments, WIV loaded dMNAs were stored at RT/0% RH, 37°C/0% RH, and 4°C/0% RH for four weeks. Besides, the storage stability of WIV incorporated in dMNAs was investigated by hemagglutination inhibition assay (Fig. 3I). No significant reduction of WIV activity in dMNAs was observed during storage at 4°C, RT and 37°C. After storage at 4°C for four weeks, WIV in dMNAs exhibited a slightly lower hemagglutination titer than WIV dispersion. This minor reduction was probably due to the drying process during dMNAs production as described above. In contrast, after 4-weeks of storage at RT and 37°C, WIV in dispersion completely lost its hemagglutination activity. This substantial reduction of activity when kept in dispersion indicates the minimal intrinsic instability of WIV in an aqueous suspension at RT or higher. Incorporation of WIV in the (poly)saccharide based dMNAs, hence rendering it in the solid-state, strongly improved its storage stability as was also shown in previous studies.^{20,21}

Dose Selection of WIV Through Ex Vivo Skin Delivery Test

Next we investigated which percentage of loaded substance in the dMNAs can be delivered into the skin. To this end three different amounts of IRDye800CW were loaded into dMNAs. After insertion, dissolution, and withdrawal of the dMNAs, the fluorescence in the skin was measured. Subsequently, the delivered amount of dye from the dMNAs was calculated from the calibration curve obtained from the fluorescence of the standards injected by the hMN system. The delivered amount of dye increased with the loaded amount of dye in dMNAs (Fig. 4). An amount of 0.11 ± 0.01 , 0.19 ± 0.02 , or 0.35 ± 0.002 μg of dye was delivered from 1.17 μg, 2.33 μg, and 4.66 μg of dye loaded dMNAs, respectively. It was calculated that 8–10% of the amount of the encapsulated dye was delivered into the skin, which was independent of the amount of dMNA-incorporated dye. From these data, it was concluded that using the current process and setup a surplus of 10–12.5 fold the amount of antigen should be loaded in dMNAs. Therefore, to deliver 1 μg of WIV to mice, we decided to load 12.5 μg of WIV to each dMNA for the immunization study.

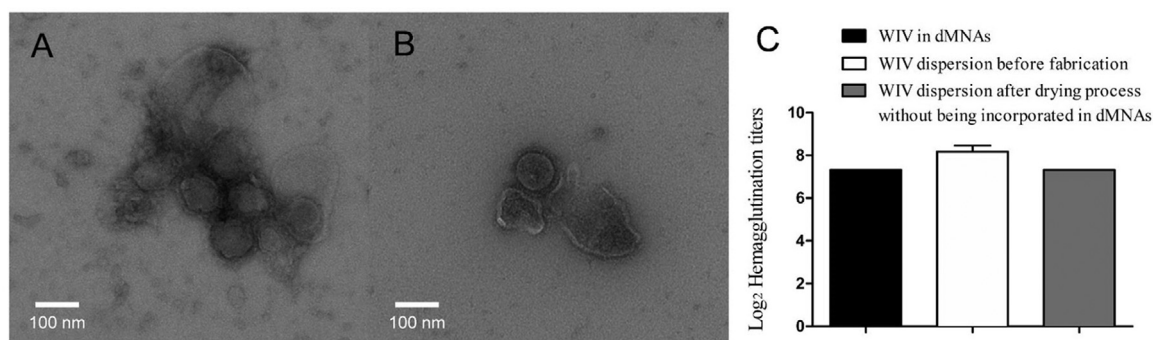


Fig. 2. Physical and functional characterization and process stability of whole inactivated influenza virus in dMNAs. TEM images of WIV in buffer (A) and WIV formulated with trehalose/pullulan blend (B). Hemagglutination titers of WIV dispersion in 15% (w/v) 50:50 trehalose/pullulan solution before production (white) and re-dispersed WIV after 48 hours drying with (black) and without (grey) incorporation into dMNAs (C; $n = 3$). dMNAs = dissolvable microneedle arrays. TEM = transmission electron microscopy. Scale bar = 100 nm.

Table 2

Penetration efficiency rates of dMNAs which were stored at four different conditions for four weeks ($n = 3$): 37°C/0% RH, 25°C/0% RH, 4°C/0% RH, and 25°C/56% RH.

Storage condition	Penetration efficiency rate (%)
37°C/0% RH	96 ± 6.4
25°C/0% RH	100 ± 0
4°C/0% RH	100 ± 0
25°C/56% RH	25.9 ± 6.4

Immune Responses Evoked by Immunization with dMNA

Next, mice were immunized with dMNAs that were either freshly produced, or stored at 4°C /0% RH or 37°C /0%RH for four weeks and the evoked immune responses were investigated. All dMNAs were designed to deliver an amount of 1–1.25 μ g WIV into the skin after an application time of 15 min. As a negative control, placebo dMNAs without WIV were used. For the positive control group, 1 μ g of WIV was injected through the intramuscular route.

First, the A(H1N1)pdm2009-specific IgG titers in the serum were determined at 28 days post the single immunization. As shown in Fig. 5A, all dMNAs appeared effective in inducing influenza-specific IgG, with antibody titers being of the same order of magnitude as induced by intramuscular injection. No significant differences were measured in antibody levels raised by immunization with freshly prepared dMNAs or dMNAs stored under different conditions. The experimental groups immunized with the different WIV-containing dMNAs included, however, several non-responders (Fresh: 3, 4°C: 1, 37°C: 4).

To get more insight into the immune response elicited by dMNAs, we determined IgG subclasses, measured mucosal antibody responses, and evaluated cellular immune responses. These assays were performed in mice three days post A(H1N1)pdm2009 virus challenge. Evaluation of serum IgG subclasses IgG1 and IgG2a (Fig. 5B and C) revealed that all responder mice except two had developed IgG1 titers. Yet, due to variation and presence of non-responders the difference in IgG1 titer with the placebo control group reached statistical significance only for the group immunized with dMNAs stored at 4°C and the group immunized via intramuscular injection with WIV ($p < 0.01$). No significant difference in IgG1 levels was seen between the different dMNA-immunized groups. IgG2a levels were generally low, and differed significantly from the levels in the placebo control group only for the group immunized with dMNAs stored at 4°C ($p < 0.05$). This was unexpected since WIV usually is a strong inducer of IgG2a. The result was, however, not due to the administration form since intramuscularly immunized control mice showed similarly low levels of IgG2a. With respect to mucosal antibodies, influenza-specific IgG was detected in lung washes (Fig. 5D) but not in nasal washes (Supplementary Fig. 1) of dMNA or intramuscularly immunized mice at three days post-infection. Mucosal IgA was neither detected in lung nor in nasal washes from these mice (Supplementary Fig. 1).

Besides antibody responses, cellular immune responses also play an important role in the protection against A(H1N1)pdm2009 infection.³³ To evaluate whether dMNAs can successfully induce influenza-specific cellular immune responses and whether different storage conditions affect the ability of the dMNAs to do so, ELISPOT assays were performed to reveal the number of IFN- γ and IL-4

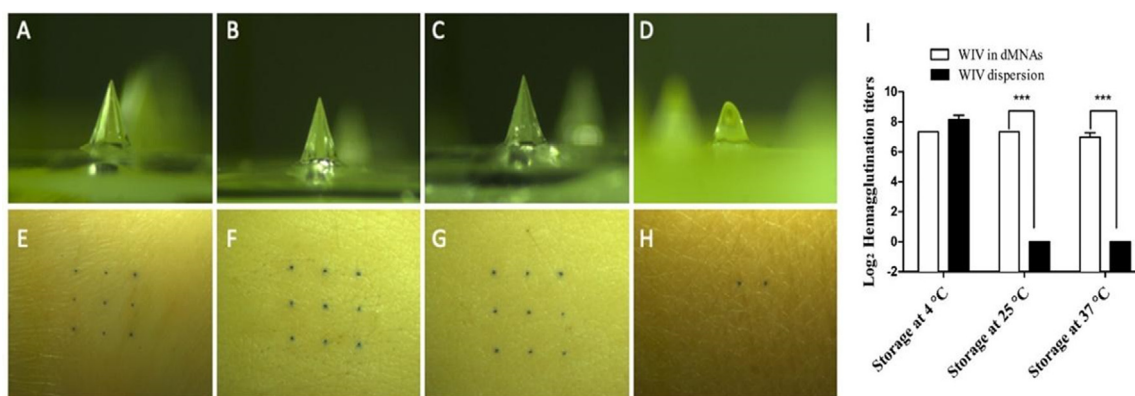


Fig. 3. Impact of storage on mechanical stability of dissolvable microneedles and functional stability of WIV. Microneedles after four weeks of storage at 37°C/0% RH (A), 25°C/0% RH (B), 4°C/0% RH (C), and 25°C/56% RH (D) and skin penetrated with dMNAs which were stored at 37°C/0% RH (E), 25°C/0% RH (F), 4°C/0% RH (G), and 25°C/56% RH (H). Storage stability (as measured by hemagglutination assay) of WIV incorporated in dMNAs or kept in aqueous dispersion after four weeks of storage at 4°C, RT or 37°C, respectively (I; $n = 3$).

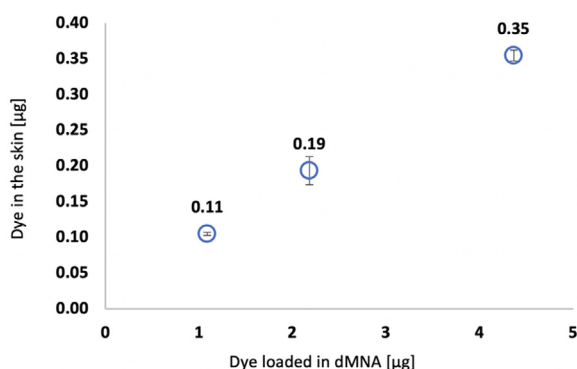


Fig. 4. Delivered amount of cw800 dye from dMNAs to human abdominal skin. 1, 2, and 4 nmol of IFDye800CW in dMNAs ($n = 3$) were delivered to the human abdominal skin through 15 min of dissolution. The absolute amount of delivered dye increased with the loaded amount of dye in dMNAs, and the percentages of delivered dye were calculated at 10–12.5% (w/w).

producing splenocytes. As shown in Fig. 5E, neither intramuscular injection of WIV nor immunization with the different dMNAs resulted in a significant increase of the number of influenza-specific IFN- γ producing splenocytes as compared to mice immunized with placebo dMNAs. However, the numbers of IL-4 producing splenocytes (Fig. 5F) were significantly increased in mice immunized with dMNAs stored at 4°C (1.86 fold; $p < 0.05$) and in mice intramuscularly injected with WIV (2.89 fold; $p < 0.05$). Furthermore, the responder mice that were immunized with either freshly prepared dMNAs or dMNAs stored at 37°C did also show an increase in IL-4 secreting splenocytes that was at a similar level as was shown for mice that were immunized with dMNAs stored at 4°C. Additionally, no significant differences were measured in the number of IL-4 secreting splenocytes induced by the differently stored dMNAs and intramuscularly injected WIV.

Taken together, our data suggest that dMNAs, even when stored for weeks under high temperature conditions, are still able to evoke humoral and cellular immune responses in mice. Yet, the reason for the reduced reproducibility of antibody induction when vaccinating via dMNA needs to be further investigated and optimized.

Protective Efficacy of Immunization with dMNAs

In order to determine whether dMNA-induced immunity was capable of protecting mice from virus replication in the lungs and from disease symptoms, the virus load of A(H1N1)pdm2009 in the lung was measured and mice were followed for disease severity. As shown in Fig. 6A, viral load on day 3 post infection was significantly lower in mice immunized with dMNAs stored at 4°C (40.5 fold; $p < 0.05$) or immunized with freshly prepared dMNAs (40.29 fold; $p < 0.01$) than in mice immunized with placebo dMNAs. A similarly reduced lung viral load was also found in the responder mice immunized with dMNAs stored at 37°C. Moreover, no significant differences in lung virus titer were measured among groups immunized with the different dMNAs. Surprisingly, intramuscular injection of WIV did not reduce viral load in the lungs of mice in this experiment.

The remaining mice were followed for survival and disease activity score for 14 days post-infection with A(H1N1)pdm2009 virus (Fig. 6B–G). Mice immunized with placebo dMNAs showed a high level of disease symptoms as five mice from this group needed premature euthanasia (Fig. 6B) because they reached the humane endpoint (Fig. 6G). Surprisingly, intramuscularly injected mice also showed a high disease activity score (Fig. 6F), nevertheless only one mouse reached the humane endpoint in this group. Immunization with dMNAs stored at 4°C protected fully from severe infection and death from A(H1N1)pdm2009 infection, whereas immunization with

other dMNAs partly protected mice from A(H1N1)pdm2009 infection (Fig. 6B). Three mice immunized with freshly prepared dMNAs, including two non-responder mice, (Fig. 6C) and two mice immunized with dMNAs stored at 37°C, including one non-responder mouse, (Fig. 6E) reached the humane endpoint. However, the responder mice from those dMNA groups showed a similar reduction of disease symptoms as mice that were immunized with dMNAs stored at 4°C. Together, these findings demonstrate that successful application of the different dMNAs on mice reduced viral load in lungs and protected from influenza at a similar level, irrespective of the storage conditions.

Discussion

In this study, we investigated whether trehalose/pullulan blends are suitable for the production of stable dMNAs capable of penetrating the skin and inducing immune responses. Our data demonstrate that dMNAs prepared from a trehalose/pullulan weight ratio of 50:50 were sharp and stiff enough to penetrate the skin and showed a fast dissolution rate in the skin. Furthermore, WIV could successfully be incorporated in these trehalose/pullulan-based dMNAs. The mechanical stability of trehalose/pullulan-based dMNAs and the stability of incorporated WIV could be maintained up to storage temperatures of 37°C. However, the penetration efficiency of those dMNAs dropped when exposed to environmental moisture. Moreover, trehalose/pullulan-based dMNAs, irrespective of storage temperature (4°C or 37°C), induced immune responses in mice that were of similar magnitude as found in intramuscularly injected mice and provided (partial) protection against influenza virus infection.

Based on the dissolution and penetration tests, we selected a trehalose/pullulan blend with a weight ratio of 50:50 for the dMNA formulation as it showed the fastest dissolution rate in the skin among the five different trehalose/pullulan ratios tested. The dissolution test showed a decreasing dissolution rate of microneedles when the pullulan percentage increased and the trehalose percentage decreased, suggesting that pullulan has slow dissolution properties while trehalose due to its known high dissolution rate in water augments microneedle dissolution.³⁴ Unfortunately, dMNAs composed of pure trehalose are too brittle to penetrate the skin and carry drugs.³⁵ For that reason, pullulan was mixed with trehalose to enhance the mechanical strength of microneedles. Our data show that the penetration efficiency of dMNAs in the skin increased by mixing pullulan with trehalose, irrespective of the percentage of pullulan. Various polymers such as polyvinyl alcohol,³⁶ hyaluronic acid,³⁷ or polyvinylpyrrolidone³⁸ can also be mixed with trehalose to increase the mechanical strength of microneedles. However, the advantage of using pullulan over these polymers is that the trehalose/pullulan mixture has high antigen stabilizing properties.¹⁹ Therefore, the use of a trehalose/pullulan blend at a weight ratio of 50:50 can generate dMNAs combining the antigen stabilizing capacities of trehalose and the physical and mechanical properties of pullulan.

In the storage stability test, the penetration efficiency of dMNAs significantly dropped after four weeks of storage at RT/56 % RH while it was maintained at low humidity conditions irrespective of storage temperature (37°C/0% RH, RT/0% RH, 4°C/0% RH). At 56%RH, the shape of microneedle tips changed from sharp to blunt. Apparently, at this RH the microneedles absorbed such an amount of water, which would dramatically decrease the glass transition temperature (T_g) of the trehalose/pullulan mixture.³⁹ Due to this plasticizing effect, the T_g dropped close to RT resulting in viscous flow.

The WIV could be incorporated in dMNAs produced from a trehalose/pullulan blend with good process stability as indicated by a hemagglutination activity of WIV that was only slightly decreased after production. In order to investigate whether other stress factors during the preparation of microneedles (e.g., centrifugation) can

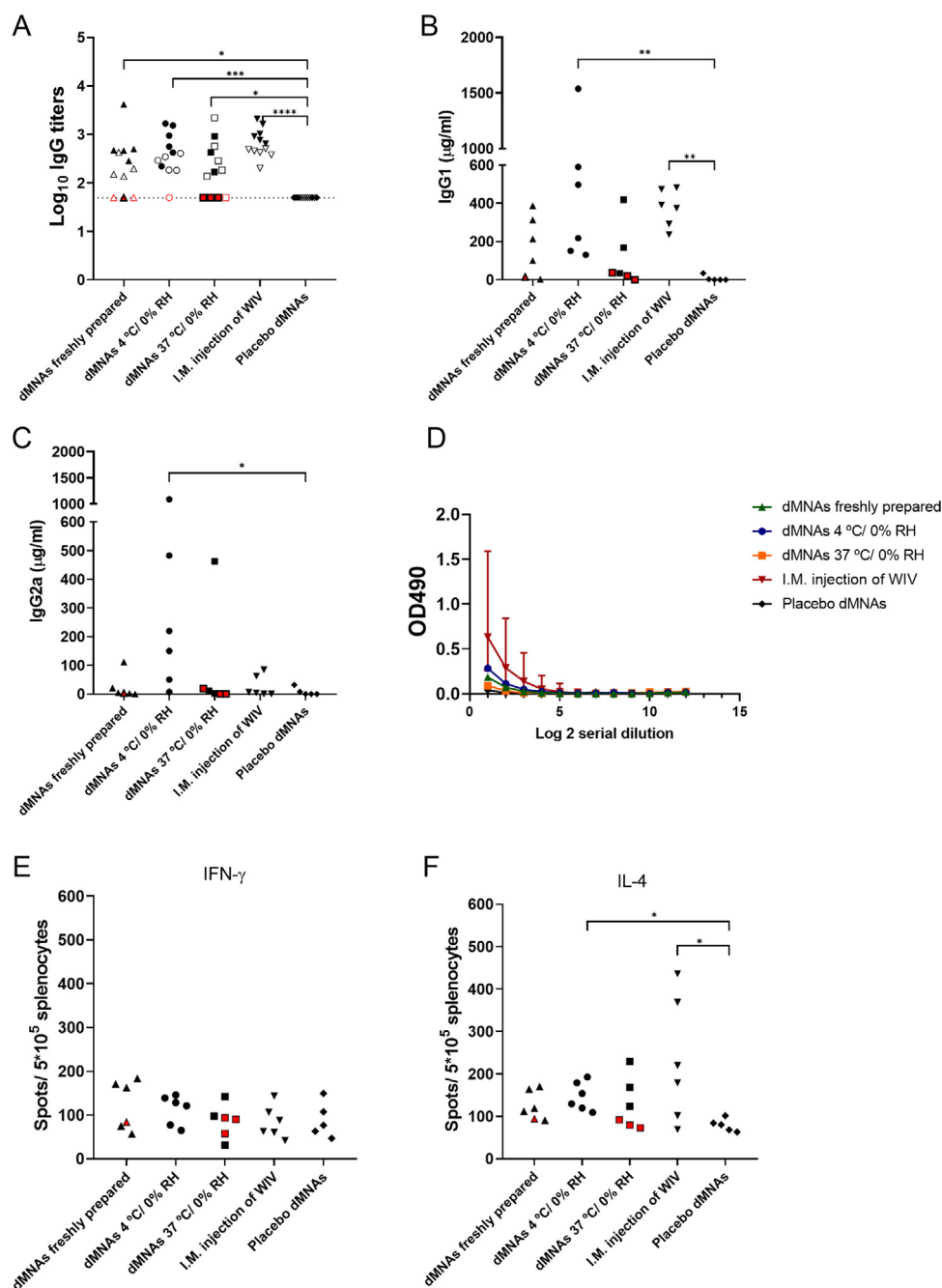


Fig. 5. Immune responses evoked by immunization with dMNA. Mice ($n = 12$ /experimental group) were immunized with whole inactivated virus (WIV) derived from A/California/07/2009 (H1N1) administered via dMNAs (trehalose/pullulan 50:50) or through intramuscular injection ($n = 12$). dMNAs were used freshly prepared or after storage for four weeks at 4 °C/0% RH or 37 °C/0% RH. The control group received placebo dMNAs ($n = 11$). At day 28 post immunization, IgG levels in blood were determined (A). Then, mice were challenged with A(H1N1)pdm09 virus and sacrificed three days post challenge (filled symbols, $n = 6$) or followed for up to 14 days post challenge (open symbols, $n = 6$). Serum of mice sacrificed at three days post challenge was analyzed for IgG1(B) and IgG2a (C), lung washes were analyzed for IgG levels(D). Spleens were collected and analyzed for IFN- γ producing splenocytes (E) and IL-4 producing splenocytes (F). Statistical comparisons between the mice immunized with different dMNAs, between mice immunized with the different dMNAs groups and intramuscularly injected mice, or between mice immunized with the different dMNAs groups and placebo mice were performed using Kruskal-Wallis with Dunn's post-hoc test (* $p < 0.05$, ** $p < 0.1$, *** $p < 0.01$, **** $p < 0.001$). Non-responders identified by absence of influenza-specific IgG in serum at day 28 are presented in red in this and the following figure.

negatively affect WIV activity, WIV dispersed in 15% (w/v) 50:50 trehalose/pullulan solution was dried at 37 °C for 48 hours without being incorporated into microneedles to mimic the drying process of microneedles. WIV in these samples showed the same hemagglutination titers as WIV incorporated in dissolving microneedles. Therefore, the slight decrease in the activity of WIV during the production of the microneedles can be solely ascribed to the drying process. In this respect, it should be emphasized that the drying procedure applied

to produce the microneedles exposes the biopharmaceutical to a combination of a high temperature (37 °C) during a long process time (two days). These are harsh conditions when compared to commonly used drying techniques employed for the preparation of biopharmaceutical products, like freeze-drying (low process temperature) and spray drying (short process time).⁴⁰ Furthermore, WIV as aqueous dispersion fully lost its hemagglutinating capacity after four weeks of storage at 25 °C and 37 °C, which indicates poor stability of WIV in an

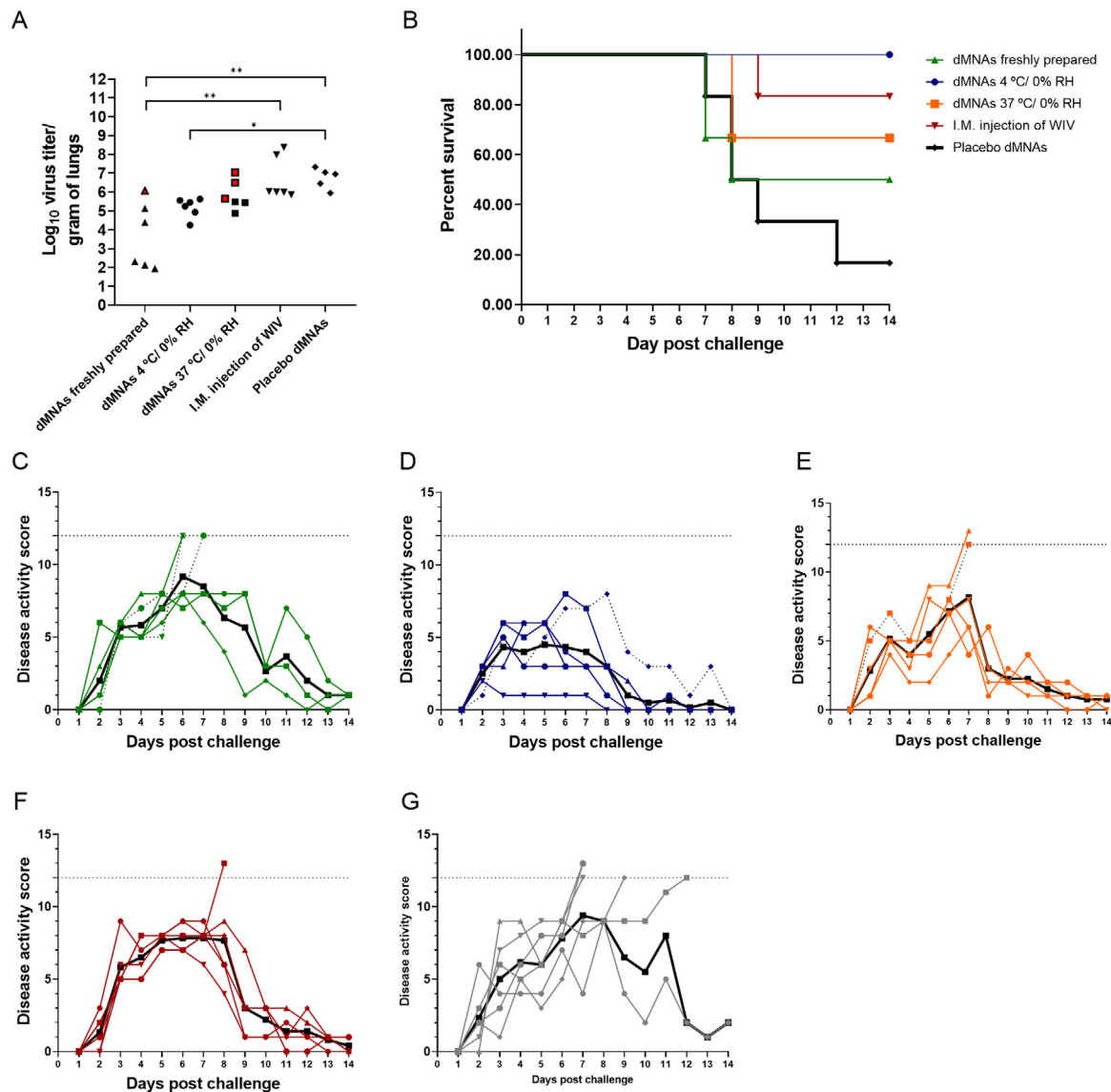


Fig. 6. Protective efficacy of immunization with dMNAs. Mice ($n = 12$), as described in the legend of Fig. 5, were challenged with the A(H1N1)pdm09 virus at 28 days post immunization. Lung virus titers (A) were assessed three days post A(H1N1)pdm09 virus challenge ($n = 6$ per group). Survival rate (B) and disease activity score (C–G) were followed during 14 days post A(H1N1)pdm09 virus challenge in mice that were immunized with dMNAs stored at 4 °C/0% RH (C), application of dMNAs stored at 37 °C/0% RH (D), application of freshly prepared dMNAs (E), intramuscular injection of WIV (F) or administration of placebo dMNAs (G) ($n = 6$ per group). Mice reaching a disease activity score of 12 or higher reached the human endpoint and were terminated. Data includes non-responders (dotted lines) and average disease activity score (thick lines). Statistical comparisons in virus titer between the mice immunized with different dMNAs, between mice immunized with the different dMNAs groups and intramuscularly injected mice, or between mice immunized with the different dMNAs groups and placebo mice were done using Kruskal-Wallis with Dunn's post-hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Non-responders identified by absence of influenza-specific IgG in serum at day 28 are presented in red.

aqueous dispersion at these temperatures. In contrast, WIV incorporated in the trehalose/pullulan matrix of the microneedles fully retained its activity after storage for four weeks at temperatures up to 37 °C. These results indicate that the trehalose/pullulan combination not only provides excellent stabilization during the harsh process conditions of production of the microneedles, but also during subsequent storage.

Our data also demonstrate that a single-dose vaccination of mice with WIV-loaded dMNAs evoked humoral and cellular immune responses and provided protection from influenza infection by reducing viral load in the lungs and disease symptoms. Although prime-boost vaccination regimes are often used to protect from influenza infection,⁴¹ single-dose protection from viral infection in mice after intradermal immunization was also corroborated by other studies using high-density microarray patches containing SARS-CoV-2 spike

protein⁴² or single intradermal injection with live attenuated influenza vaccine.⁴³ However, in contrast to these studies, the current study showed only partial protection of mice from influenza infection after single-dose immunization, for both dMNA and intramuscular immunization. This could be due to the unexpectedly low immunogenicity of the used WIV and the fact that it induced a Th2-type immune response (high IgG1 and IL-4 levels) instead of a Th1-type response (high IgG2a and IFN- γ levels) usually observed for WIV preparations²⁴ and more favorable for protection.^{44,45} A lower immunogenicity of WIV was also observed in trehalose/pullulan-based dMNAs in the hemagglutination assay of this study and was suggested to be related to the drying process of dMNAs. However, the non-dried WIV that was intramuscularly injected to mice also showed a lower immunogenicity, indicating that the drying process did not play a role in the low immunogenicity of WIV in mice. Furthermore,

the hemagglutinin assay solely focuses on the hemagglutinin protein and not on other components of WIV that are also essential for its immunogenicity, such as ssRNA that induce Th1-type responses by activating TLR7.²⁴ Future studies should therefore test for the stability of the different components of WIV to ensure high immunogenicity of WIV which may induce increased levels of immune responses after single administration of the dMNAs. Nonetheless, our results provide evidence that trehalose/pullulan-based dMNAs stabilize the incorporated antigen and can evoke protective immunity against influenza virus infection in mice even after a single-dose immunization. Temperature changes during vaccine storage and transport are a major problem in low- and middle-income countries and lead to substantial loss of vaccine activity.⁴⁶ Due to the excellent antigen-stabilizing properties of the trehalose/pullulan blend the developed dMNAs would not need a cold chain. With further optimization of immunization with the dMNAs, intradermal immunization with trehalose/pullulan-based dMNAs may become a viable alternative to intramuscular immunization with hypodermic needles, with the potential to increase vaccine coverage rates, especially in low- and middle-income countries.

Altogether, our study demonstrates the potential of trehalose/pullulan as the main material to produce antigen-containing dMNAs able to induce an immune response after application. Our research shows the potential of trehalose/pullulan-based dMNAs as an alternative administration form for the classical parenteral influenza vaccination. The knowledge on trehalose/pullulan-based dMNAs can be used to increase the vaccine coverage rate, especially by avoiding the poorly functioning cold-chain systems in developing countries.⁴⁷ In addition to developing countries, the trehalose/pullulan-based dMNAs may also benefit other countries as this technology prevents pain and discomfort during administration and provides the potential for self-administration.¹⁰

Declaration of Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary Materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.xphs.2022.01.033.

References

1. Somes MP, Turner RM, Dwyer LJ, Newall AT. Estimating the annual attack rate of seasonal influenza among unvaccinated individuals: a systematic review and meta-analysis. *Vaccine*. 2018;36(23):3199–3207. <https://doi.org/10.1016/j.vaccine.2018.04.063>.
2. Bhide Y, Tomar J, Dong W, et al. Pulmonary delivery of influenza vaccine formulations in cotton rats: site of deposition plays a minor role in the protective efficacy against clinical isolate of H1N1pdm virus. *Drug Deliv*. 2018;0(0):533–545. <https://doi.org/10.1080/10717544.2018.1435748>.
3. Vemula SV, Sayedahmed EE, Sambhara S, Suresh K. Expert review of vaccine approaches conferring cross-protection against influenza viruses. *Expert Rev Vaccines*. 2017;16(11):1141–1154. <https://doi.org/10.1080/14760584.2017.1379396>.
4. Kang SM, Song JM, Kim YC. Microneedle and mucosal delivery of influenza vaccines. *Expert Rev Vaccines*. 2012;11(5):547–560. <https://doi.org/10.1586/erv.12.25>.
5. Kommedreddy S, Baudner BC, Oh S, et al. Dissolvable microneedle patches for the delivery of cell-culture-derived influenza vaccine antigens. *J Pharm Sci*. 2012;101(3):1021–1027. <https://doi.org/10.1002/jps>.
6. Choi H, Song J, Bondy BJ, et al. Effect of osmotic pressure on the stability of whole inactivated influenza vaccine for coating on microneedles. *Plos One*. 2015;1–22. <https://doi.org/10.1371/journal.pone.0134431>.
7. Sullivan SP, Koutsouanos DG, Del Pilar Martin M, et al. Dissolving polymer microneedle patches for influenza vaccination. *Nat Med*. 2010;16(8):915–920. <https://doi.org/10.1038/nm.2182>.
8. Frew PM, Paine MB, Roupheal N, et al. Acceptability of an inactivated influenza vaccine delivered by microneedle patch: Results from a phase I clinical trial of safety, reactogenicity, and immunogenicity. *Vaccine*. 2020;38(45):7175–7181. <https://doi.org/10.1016/j.vaccine.2020.07.064>.
9. Esser ES, Pulit-Penalosa JA, Kalluri H, et al. Microneedle patch delivery of influenza vaccine during pregnancy enhances maternal immune responses promoting survival and long-lasting passive immunity to offspring. *Sci Rep*. 2017;7(1):1–11. <https://doi.org/10.1038/s41598-017-05940-7>.
10. Rodgers AM, Cordeiro AS, Donnelly RF. Technology update: dissolvable microneedle patches for vaccine delivery. *Med Devices Evid Res*. 2019;12:379–398. <https://doi.org/10.2147/MDER.S198220>.
11. Ita K. Dissolving microneedles for transdermal drug delivery: advances and challenges. *Biomed Pharmacother*. 2017;93:1116–1127. <https://doi.org/10.1016/j.biopha.2017.07.019>.
12. Hoang MT, Ita KB, Bair DA. Solid microneedles for transdermal delivery of amantadine hydrochloride and pramipexole dihydrochloride. *Pharmaceutics*. 2015;7(4):379–396. <https://doi.org/10.3390/pharmaceutics7040379>.
13. Mönkäre J, Reza Nejadnik M, Baccouche K, et al. IgG-loaded hyaluronan-based dissolving microneedles for intradermal protein delivery. *J Control Release*. 2015;218:53–62. <https://doi.org/10.1016/j.jconrel.2015.10.002>.
14. Shin JH, Noh JY, Kim KH, et al. Effect of zymosan and poly (I:C) adjuvants on responses to microneedle immunization coated with whole inactivated influenza vaccine. *J Control Release*. 2017;265(May):83–92. <https://doi.org/10.1016/j.jconrel.2017.09.010>.
15. de Groot AM, Platteel ACM, Kuijt N, et al. Nanoporous microneedle arrays effectively induce antibody responses against diphtheria and tetanus toxoid. *Front Immunol*. 2017;8:1789. <https://doi.org/10.3389/fimmu.2017.01789>.
16. Schepens B, Vos PJ, Saelens X, van der Maaden K. Vaccination with influenza hemagglutinin-loaded ceramic nanoporous microneedle arrays induces protective immune responses. *Eur J Pharm Biopharm*. 2019;136:259–266. <https://doi.org/10.1016/j.ejpb.2019.02.002>.
17. Du G, Hathout RM, Nasr M, et al. Intradermal vaccination with hollow microneedles: A comparative study of various protein antigen and adjuvant encapsulated nanoparticles. *J Control Release*. 2017;266:109–118. <https://doi.org/10.1016/j.jconrel.2017.09.021>.
18. Kim YC, Park JH PM. Microneedles for drug and vaccine delivery. *Adv Drug Deliv Rev*. 2012;64(14):1547–1568. <https://doi.org/10.1016/j.addr.2012.04.005>.
19. Teekamp N, Tian Y, Visser JC, et al. Addition of pullulan to trehalose glasses improves the stability of β -galactosidase at high moisture conditions. *Carbohydr Polym*. 2017;176:374–380. <https://doi.org/10.1016/j.carbpol.2017.08.084>.
20. Tonniss WF, Mensink MA, de Jager A, et al. Size and molecular flexibility of sugars determine the storage stability of freeze-dried proteins. *Mol Pharm*. 2015;12(3):684–694. <https://doi.org/10.1021/mp500423z>.
21. Mensink MA, Frijlink HW, van der Voort Maarschalk K, Hinrichs WJ. How sugars protect proteins in the solid state and during drying (review): Mechanisms of stabilization in relation to stress conditions. *Eur J Pharm Biopharm*. 2017;114:288–295. <https://doi.org/10.1016/j.ejpb.2017.01.024>.
22. Olsson C, Jansson H, Swenson J. The role of trehalose for the stabilization of proteins. *J Phys Chem B*. 2016;120(20):4723–4731. <https://doi.org/10.1021/acs.jpcc.6b02517>.
23. Kawahara M, Mizutani K, Suzuki S, et al. Dependence of the mechanical properties of a pullulan film on the preparation temperature. *Biosci Biotechnol Biochem*. 2003;67(4):893–895. <https://doi.org/10.1271/bbb.67.893>.
24. Geeraedts F, Goutagny N, Hornung V, et al. Superior immunogenicity of inactivated whole virus H5N1 influenza vaccine is primarily controlled by toll-like receptor signalling. Subbarao K, ed. *PLoS Pathog*. 2008;4(8):e1000138. <https://doi.org/10.1371/journal.ppat.1000138>.
25. Leone M, Priester MI, Romeijn S, et al. Hyaluronan-based dissolving microneedles with high antigen content for intradermal vaccination: formulation, physicochemical characterization and immunogenicity assessment. *Eur J Pharm Biopharm*. 2019;134:49–59. <https://doi.org/10.1016/j.ejpb.2018.11.013>.
26. Lee J, van der Maaden K, Gooris G, et al. Engineering of an automated nano-droplet dispensing system for production of antigen-loaded dissolving microneedle arrays. *Int J Pharm*. 2021;120473. <https://doi.org/10.1016/j.ijpharm.2021.120473>.
27. Tomar J, Patil HP, Bracho G, et al. Advax augments B and T cell responses upon influenza vaccination via the respiratory tract and enables complete protection of mice against lethal influenza virus challenge. *J Control Release*. 2018;288:199–211. <https://doi.org/10.1016/j.jconrel.2018.09.006>.
28. Audouy SAL, van der Schaaf G, Hinrichs WJ, et al. Development of a dried influenza whole inactivated virus vaccine for pulmonary immunization. *Vaccine*. 2011;29(26):4345–4352. <https://doi.org/10.1016/j.vaccine.2011.04.029>.

29. Tomar J, Biel C, de Haan CAM, et al. Passive inhalation of dry powder influenza vaccine formulations completely protects chickens against H5N1 lethal viral challenge. *Eur J Pharm Biopharm.* 2018;133(October):85–95. <https://doi.org/10.1016/j.ejpb.2018.10.008>.
30. Tian Y, Bhide YC, Woerdenbag HJ, et al. Development of an orodispersible film containing stabilized influenza vaccine. *Pharmaceutics.* 2020;12(3):245. <https://doi.org/10.3390/pharmaceutics12030245>.
31. van der Maaden K, Heuts J, Camps M, et al. Hollow microneedle-mediated micro-injections of a liposomal HPV E743–63 synthetic long peptide vaccine for efficient induction of cytotoxic and T-helper responses. *J Control Release.* 2018;269:347–354. <https://doi.org/10.1016/j.jconrel.2017.11.035>.
32. Liu H, Patil HP, de Vries-Idema J, et al. Enhancement of the immunogenicity and protective efficacy of a mucosal influenza subunit vaccine by the saponin adjuvant GPI-0100. Ambrose Z, ed. *PLoS One.* 2012;7(12):e52135. <https://doi.org/10.1371/journal.pone.0052135>.
33. Dong W, Bhide Y, Sicca F, et al. Cross-protective immune responses induced by sequential influenza virus infection and by sequential vaccination with inactivated influenza vaccines. *Front Immunol.* 2018;9. <https://doi.org/10.3389/fimmu.2018.02312>.
34. Lee JW, Choi S-O, Felner EI, Prausnitz MR. Dissolving Microneedle Patch for Transdermal Delivery of Human Growth Hormone. *Small.* 2011;7(4):531–539. <https://doi.org/10.1002/sml.201001091>.
35. Tian Y, Visser JC, Klever JS, et al. Orodispersible films based on blends of trehalose and pullulan for protein delivery. *Eur J Pharm Biopharm.* 2018;133:104–111. <https://doi.org/10.1016/j.ejpb.2018.09.016>.
36. McGrath MG, Vucen S, Vrdoljak A, et al. Production of dissolvable microneedles using an atomised spray process: Effect of microneedle composition on skin penetration. *Eur J Pharm Biopharm.* 2014;86(2):200–211. <https://doi.org/10.1016/j.ejpb.2013.04.023>.
37. Kim HK, Lee SH, Lee BY, et al. A comparative study of dissolving hyaluronic acid microneedles with trehalose and poly(vinyl pyrrolidone) for efficient peptide drug delivery. *Biomater Sci.* 2018;6(10):2566–2570. <https://doi.org/10.1039/c8bm00768c>.
38. Dillon C, Hughes H, O'Reilly NJ, McLoughlin P. Formulation and characterisation of dissolving microneedles for the transdermal delivery of therapeutic peptides. *Int J Pharm.* 2017;526(1–2):125–136. <https://doi.org/10.1016/j.ijpharm.2017.04.066>.
39. Drake AC, Lee Y, Burgess EM, et al. Effect of water content on the glass transition temperature of mixtures of sugars, polymers, and penetrating cryoprotectants in physiological buffer. *PLoS One.* 2018;13:(1) e0190713. <https://doi.org/10.1371/JOURNAL.PONE.0190713>.
40. Chu LY, Ye L, Dong K, et al. Enhanced stability of inactivated influenza vaccine encapsulated in dissolving microneedle patches. *Pharm Res.* 2016;33(4):868–878. <https://doi.org/10.1007/s11095-015-1833-9>.
41. Wei C-J, Boyington JC, McTamney PM, et al. Induction of broadly neutralizing H1N1 influenza antibodies by vaccination. *Science (80-).* 2010;329(5995):1060–1064. <https://doi.org/10.1126/science.1192517>.
42. MCMillan CLD, Choo JJY, IDRIS A, et al. Complete protection by a single-dose skin patch—delivered SARS-CoV-2 spike vaccine. *Sci Adv.* 2021;7(44):eabj8065. <https://doi.org/10.1126/sciadv.abj8065>.
43. Lee AC-Y, Zhang AJ, Li C, et al. Intradermal vaccination of live attenuated influenza vaccine protects mice against homologous and heterologous influenza challenges. *npj Vaccines.* 2021;6(1):95. <https://doi.org/10.1038/s41541-021-00359-8>.
44. Cristillo AD, Ferrari MG, Hudacik L, et al. Induction of mucosal and systemic antibody and T-cell responses following prime-boost immunization with novel adjuvanted human immunodeficiency virus-1-vaccine formulations. *J Gen Virol.* 2011;92(1):128–140. <https://doi.org/10.1099/vir.0.023242-0>.
45. Bungener L, Geeraedts F, ter Veer W, et al. Alum boosts TH2-type antibody responses to whole-inactivated virus influenza vaccine in mice but does not confer superior protection. *Vaccine.* 2008;26(19):2350–2359. <https://doi.org/10.1016/j.vaccine.2008.02.063>.
46. Galazka AM, Milstien JB, Zaffran M, Thermostability of vaccines. <https://apps.who.int/iris/handle/10665/64980>.
47. Brooks A, Habimana D, Huckerby G. Making the leap into the next generation: A commentary on how Gavi, the Vaccine Alliance is supporting countries' supply chain transformations in 2016–2020. *Vaccine.* 2017;35(17):2110–2114. <https://doi.org/10.1016/j.vaccine.2016.12.072>.