Stability of whole inactivated influenza virus vaccine during coating onto metal microneedles

Hyo-jick Choi, Brian J. Bondy, Dae-Goon Yoo, Richard W. Compans, Sang-Moo Kang, Mark R. Prausnitz

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A B S T R A C T

Immunization using a microneedle patch coated with vaccine offers the promise of simplified vaccination logistics and increased vaccine immunogenicity. This study examined the stability of influenza vaccine during the microneedle coating process, with a focus on the role of coating formulation excipients. Thick, uniform coatings were obtained using coating formulations containing a viscosity enhancer and surfactant, but these formulations retained little functional vaccine hemagglutinin (HA) activity after coating. Vaccine coating in a trehalose-only formulation retained about 40–50% of vaccine activity, which is a significant improvement. The partial viral activity loss observed in the trehalose-only formulation was hypothesized to come from osmotic pressure-induced vaccine destabilization. We found that inclusion of a viscosity enhancer, carboxymethyl cellulose, overcame this effect and retained full vaccine activity on both washed and plasma-cleaned titanium surfaces. The addition of polymeric surfactant, Lutrol® micro 68, to the trehalose formulation generated phase transformations of the vaccine coating, such as crystallization and phase separation, which was correlated to additional vaccine activity loss, especially when coating on hydrophilic, plasma-cleaned titanium. Again, the addition of a viscosity enhancer suppressed the surfactant-induced phase transformations during drying, which was confirmed by in vivo assessment of antibody response and survival rate after immunization in mice. We conclude that trehalose and a viscosity enhancer are beneficial coating excipients, but the inclusion of surfactant is detrimental to vaccine stability.

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1. Introduction

Microneedle patches have been studied as a novel means to administer vaccines with increased immunogenicity and simplified logistics [1–3]. The increased immunogenicity is believed to be due to targeting antigen delivery to the skin, which has a rich population of antigen-presenting cells and extensive lymphatic drainage [4–8]. The simplified logistics arise from the easy administration of the vaccine (potentially by minimally trained healthcare personnel or by patients themselves), avoidance of dangerous hypodermic needles and sharps disposal, and small package size to reduce storage, transportation and disposal volumes.

Most research on microneedle patches has emphasized the use of microneedles measuring hundreds of microns in length that are often made of metal and are coated with a vaccine formulation [9–13]. Upon insertion into skin, the vaccine formulation dissolves off the microneedles into the skin within minutes, after which the microneedle patch is removed and discarded. Vaccines are typically coated onto microneedles by applying a liquid formulation that rapidly dries onto the microneedle surface, leaving a thin, solid film of vaccine and coating excipients.

It is well known that biopharmaceuticals can be destabilized during a drying process [14]. Sophisticated chemical formulations and drying protocols have been developed to protect these sensitive compounds during drying by lyophilization and other methods. However, these methods often cannot be directly transferred to use during coating of microneedles because (i) added excipients must be compatible with the coating process, (ii) the total amount of excipients applied to the microneedles must be small (e.g., 1 mg per patch) and (iii) microneedle coatings air-dry quickly (within minutes), leaving little time to modulate drying conditions.

The goal of this study was to investigate the factors and mechanisms involved in influenza vaccine destabilization during the coating of microneedles, especially those associated with the coating drying process. From our study on the stability of influenza vaccine coated...
onto microneedles during long-term storage, we know that thermodynamic processes in the solid coating, such as crystallization and phase separation, play a major role in vaccine activity loss [15]. The tendency of disaccharides to crystallize, as well as the suppression of this process using polymers, has been discussed previously in the context of influenza vaccines [16]. We also found that the surface properties of the coated substrate have an effect on these processes. Finally, we further hypothesize that osmotic stress during the drying process may affect vaccine stability.

These processes are all influenced by the composition of the coating formulation. In our lab, we often coat microneedles using aqueous formulations containing a surfactant (e.g., poloxamer) to facilitate good wetting of the microneedle surface, a viscosity enhancer (e.g., carboxymethyl cellulose) to increase coating thickness and a disaccharide sugar (e.g., trehalose) to stabilize the vaccine during drying [17]. Trehalose has been widely used as a stabilizer of biomolecules under low water conditions [18]. However, trehalose, and any other solute present at high concentration, can also function as an osmolyte to generate osmotic stress on the vaccine during drying of the coating.

This study sought to determine how each of these excipients affects stability of a whole inactivated influenza virus vaccine. We therefore tested a number of different coating formulations with different concentrations of each excipient. Dried coatings were then characterized using a hemagglutination (HA) assay as a measure of antigen preservation in vitro to determine vaccine activity [19,20], light microscopy and X-ray powder diffraction (XRD) to find evidence of crystallization or phase separation, stopped-flow light scattering (SFLS) to observe osmotic shrinking behavior of the virus, and transmission electron microscopy (TEM) to find structural damage to the virus. In vivo immunogenicity and challenge experiments were also carried out in mice to confirm vaccine activity levels.

2. Materials and methods

2.1. Preparation of vaccine

Influenza A virus (A/PR/8/34) vaccine was prepared as described previously [21].

2.2. Coating formulation and in vitro vaccine stability tests

To identify initial vaccine destabilizing factors, influenza vaccine stability tests were performed on titanium (Ti) plates before being tested on Ti microneedles. This model has been used previously to the simplified sample preparation and has been shown to be equivalent to coating on microneedles [15]. The coating formulation used in all experiments was composed of one or more of the following: trehalose-dihydrate (Sigma Aldrich, St. Louis, MO), carboxymethyl cellulose sodium salt (low viscosity, Sigma Aldrich; abbreviated as CMC), surfactant Lutrol® micro 68 (poloxamer, BASF, Mt. Olive, NJ; abbreviated as Lutrol), and inactivated A/PR/8/34 virus in sterile DPBS. All osmolyte solutions were controlled to have 15% trehalose. Only the concentrations of CMC or Lutrol were varied during drying [17].

Test formulations are abbreviated as T15, T15C0.25, T15C0.5, T15C1, T15L0.5, T15L1, and T15L2, where the number following each letter corresponds to the concentration of the excipient in the formulation (after mixing with vaccine) in w/v %. The vaccine-coated Ti plates were then air-dried at ambient conditions. The remaining activity of the virus was then assessed by measuring hemagglutination (HA) activity [15,22].

2.3. X-ray diffractometry

X-ray diffraction (X’pert Pro Multi Purpose Diffractometer, PANalytical, Westborough, MA) (XRD) analysis was used to study structural changes of the vaccine-embedded coating on Ti plates during storage. The measurements were made in the 0–20 mode using a bracket sample holder with a Cu Kα radiation (Cu Kα1 = 1.54059 Å, Cu Kα2 = 1.54442 Å) at room temperature. Data were collected at 20 between 8° and 40° using a step size of 0.008° and an acquisition time of 5 s per step. Samples were measured at 45 KV and 40 mA. X-ray powder diffraction patterns were analyzed using Jade 8 software (MDI Materials Data, Livermore, CA) and shown after background correction. Diffraction peaks were fitted to a Gaussian profile. The degree of crystallinity was calculated by measuring the integrated peak areas [23].

2.4. Viscosity and contact angle measurements

To investigate the effects of CMC and Lutrol on viscosity and hydrophobicity/wettability, 0, 0.25, 0.5 and 1% w/v of CMC and 0, 0.5, 1 and 2% w/v of Lutrol in 15% w/v of trehalose solution were prepared (unless otherwise noted, % w/v has been simplified as % to represent excipient concentration). Viscosity of the coating formulations was measured at room temperature using glass capillary viscometers following the manufacturer’s protocol (Cannon Instrument, State College, PA). Wettability of the above coating solutions was compared by measuring contact angle on both washed and plasma-cleaned Ti plates. One microliter of the solution was dropped on a Ti plate and the contact angle was immediately measured from the analysis of an optical microscope image.

2.5. Osmotic shrinking kinetics of virus

Osmotic leakage properties of the inactivated A/PR/8/34 virus were analyzed by SFLS. The osmotic behavior of the virus was characterized by recording light scattering after a rapid mixing of inactivated A/PR/8/34 virus stock with an equal volume of a separate solution of trehalose-only, trehalose/CMC, trehalose/Lutrol, or trehalose/Lutrol/CMC in sterile DPBS. All osmolyte solutions were controlled to have 15% trehalose. Only the concentrations of CMC or Lutrol were varied in order to compare their effects on the osmotic shrinking behavior of the virus. Analysis was performed by stopped-flow spectrometry (MOS-200/M spectrometer, SFM-20; BioLogic Science Instruments) at a flow rate of 7 ml/s (injection volume 66 μl) at 4°C. The excitation wavelength was set at 546 nm using a 150 W Xe light source and a monochromator (F3.5 grating). The rate constant values (k) were obtained from a single exponential curve fitting of the light scattering spectra (n > 30) using Bio-Kine 32 V4.46 software (Bio-Logic).

2.6. Optical microscopy and electron microscopy

Morphological changes in the dried-vaccine coating on Ti plates and Ti microneedles were monitored using an optical microscope (SZX12, Olympus America, Center Valley, PA) with a CCD camera (RT Slider, Diagnostic Instruments, Sterling Heights, MI). A TEM (Hitachi-2000 FX, Hitachi High Technologies America, Schaumburg, IL) operated at 100 KV, was used to observe the morphology of the influenza vaccine. In preparation, a vaccine suspension was placed on a 3 mm Formvar-amorphous carbon-coated copper grid (Ted Pella, Redding, CA) and
2.9. Immunization and challenge of mice

Laboratories, Hercules, CA). Serum samples were collected on the second week following immunization. Inactivated and live influenza virus (either inactivated or live) with DPBS without excipient caused a dramatic loss of HA activity, down to 12.5% of pre-treatment values (data not shown). As shown in Fig. 1a, addition of 15% trehalose to the coating formulation retained much of the HA activity; 53% and 37% of pretreatment values were observed for inactivated

3. Results

3.1. Functional activity of whole inactivated/live influenza vaccine coated on Ti plates

Our first experiments assessed the effect of coating formulation on the functional activity of influenza vaccine. Coating formulations contained different viscosity enhancer (CMC) and surfactant (Lutrol) concentrations. Functional hemagglutinin activity of the vaccine was assessed via HA assay after drying one day at ambient conditions. Plasma-cleaned Ti plates were used to simulate Ti microneedle surfaces to enable higher-throughput experimentation.

Drying influenza vaccine (either inactivated or live) with DPBS without excipient caused a dramatic loss of HA activity, down to 12.5% of pre-treatment values (data not shown). As shown in Fig. 1a, addition of 15% trehalose to the coating formulation retained much of the HA activity; 53% and 37% of pretreatment values were observed for inactivated

2.7. Water content measurement

The rate of water loss from the coating formulations was measured using an Aquasett coulometric moisture titrator (Photovolt Instruments, Minneapolis, MN) containing Karl Fischer Pyridine-free vessel solution. Data were collected after short-term drying (0–28 min) by preparing samples on a Ti plate and dissolving them in methanol. The dissolved samples were then injected into the titrator using a 1 ml air-tight syringe (Hamilton Reno, NV). In some cases, samples were suspended directly into the titration vessel. Data were collected until the water detection rate fell to within 0.01 μg/min of the background rate.

2.8. Vaccine coating on microneedles

In vivo experiments were performed to evaluate the effects of excipients on vaccine stability using four different kinds of vaccine coatings on plasma-cleaned Ti microneedles: inactivated virus coating with the T15, T15C1, T15L2, and T15L2C1 formulations. To compare the effects of the cleaning method on the vaccine activity, another group of vaccine-coated microneedles was prepared on washed Ti microneedles using the T15L2 formulation.

Ti microneedle arrays of five in-plane needles were fabricated by lithographic masking followed by wet etching [9]. Each needle dimension was 750 μm in length, 50 μm in thickness and 200 μm in width at the base, which tapered to a sharp tip. Microneedles were cleaned using the same cleaning procedures as above for Ti plates. Microneedles were coated using an automated dip-coating apparatus [9], and air-dried at ambient conditions for 1 day before use. The amount of vaccine coated onto microneedles was determined by measuring the absorbance of vaccine samples dissolved off micro needles using a bicinchoninic acid (BCA) assay kit (ThermoFisher Scientific, Waltham, MA) at 560 nm on a microplate reader (iMark, Bio-Rad Laboratories, Hercules, CA).

2.9. Immunization and challenge of mice

Female inbred BALB/c mice (Harlan Laboratories, Indianapolis, IN) 6–8 weeks of age were each immunized once using influenza vaccine-coated microneedles (0.12 μg of total protein contents). Six groups of mice (6 mice per group) were tested: (1) naïve; plasma-cleaned microneedles coated with (2) T15, (3) T15C1, (4) T15L2, and (5) T15L2C1 formulations; and (6) washed microneedles coated with a T15L2 formulation. Mice were challenged 4 weeks after immunization with a lethal dose of mouse-adapted A/PR/8/34 virus (10×LD50) to study survival rates and immune responses. Body weight changes of the mice were then monitored daily. Mice with a body weight loss greater than 25% were euthanized. Animal studies were approved by the Emory University and Georgia Tech Institutional Animal Care and Use Committees (IACUC).

2.10. Antibody responses

Serum samples were collected on the second week following immunization. Influenza virus-specific IgG antibodies were measured using enzyme-linked immunosorbent assay (ELISA) as described [24].

2.11. Statistics

All parameters were recorded for individuals within all groups. Data were analyzed by analysis of variance (ANOVA) and Student’s t test. A P-value of less than 0.05 was considered to be significant. In some cases, median values were compared to validate the results.

Fig. 1. Effect of coating formulations on functional HA activity of influenza vaccine after drying on plasma-cleaned Ti plates. The effects of viscosity enhancer (CMC) (a) and surfactant (Lutrol) (b) on the activity of inactivated influenza virus and live influenza virus were investigated by measuring HA titers at three different CMC concentrations (0.25%, 0.5%, 1%) and three different Lutrol concentrations (0.5%, 1%, 2%) with 15% trehalose. Inactivated and live A/PR/8/34 virus (1 μg of viral protein) coatings were prepared on plasma-cleaned Ti plates and dried at ambient conditions (20–23 °C, 20–45% relative humidity) for one day. HA activity change was calculated with respect to HA titer of the virus in DPBS solution. Unless otherwise indicated, all measurements were performed at 4 °C. Data are represented as the mean± standard deviation (SD) from n = 8–23 replicates.
and live viruses, respectively. The addition of CMC to the formulation significantly increased vaccine stability in a dose-dependant manner (one-way ANOVA, \( P<0.0005 \)) and approached 100% HA activity.

The effect of Lutrol in the formulation was more complex, as shown in Fig. 1b. Addition of a small amount of Lutrol (0.5%) increased vaccine stability relative to a trehalose-only formulation (Student’s t-test, \( P<0.0005 \)). However, further increasing Lutrol concentration decreased HA activity in a dose-dependant manner (one-way ANOVA, \( P<0.0005 \)). This relationship may reflect competing effects of the Lutrol directly on the virus particles and indirectly through effects on the phase behavior or other properties of the trehalose matrix and/or Ti plate interface.

### 3.2. Morphological and microstructural behaviors of coatings on Ti plates

To evaluate the possible role of physicochemical changes to the dried coating on vaccine activity, we examined morphological changes in vaccine coatings after one day of drying. Fig. 2a shows optical micrographs of representative coating samples on plasma-cleaned Ti plates. The coatings prepared from the trehalose-only (Fig. 2a(i)) and trehalose/CMC (Fig. 2a(ii)–(iv)) formulations appear morphologically similar. However, coatings prepared from the trehalose/Lutrol formulations (Fig. 2a(v)–(vii)) contained what appears to be phase separation, as evidenced by the formation of bumps (see insets in Fig. 2a). Moreover, the phase separation becomes clearer and more dominant as the Lutrol concentration increases, which is consistent with a previous report showing that the activity of the amphiphilic poloxamer (Lutrol) induced phase separation during drying processes [25]. The range of bump size was measured to be approximately 6–12 \( \mu \)m, 12–28 \( \mu \)m, and 30–50 \( \mu \)m for the formulations containing 0.5%, 1% and 2% Lutrol, respectively (Fig. 2a(v)–(vii)). Since no bumps were observed in the trehalose-only or trehalose/CMC formulations, it would appear that the Lutrol is responsible for these morphological changes during drying.

To better interpret these macroscale observations, we analyzed vaccine coatings using XRD for the same samples shown in Fig. 2a. Fig. 2b(i) displays XRD spectra measured from the trehalose-only and trehalose/CMC coating formulations. The broad halo peak observed at \( 2\theta = 10–30^\circ \) represents the reflections from the amorphous phase, indicating that the coating matrix made of trehalose-only or trehalose/CMC remained amorphous.

In contrast, Fig. 2b(ii) shows that the trehalose/Lutrol formulations exhibited different phase behaviors depending on Lutrol concentration. That is, XRD spectra revealed similar amorphous halo peaks from 0.5% Lutrol formulation, but crystalline peaks overlapping the amorphous halo peaks were observed with increasing intensity in the 1% and 2% Lutrol formulations. These peaks correspond to the characteristic peaks of crystallized trehalose dihydrate [26,27]. As analyzed in Fig. S1 (in Supplementary data), the addition of 1% and 2% Lutrol to trehalose solution generated 1.6 and 7.3% crystallization of the samples. The driving force for the phase separation and composition of each phase is not known. However, the increased crystallization seen by XRD in Fig. 2b appears to be associated with the phase separation seen by microscopy in Fig. 2a and likewise associated with the decreased HA activity with increasing Lutrol concentrations in Fig. 1b. This observation is
consistent with our previous study showing an association between crystallization and influenza vaccine instability [15].

To better understand the kinetics of crystallization during drying of coatings, time-dependant XRD analysis was performed for representative trehalose-only, trehalose/CMC and trehalose/Lutrol formulations over the first 31 min of drying, as shown in Fig. 3. Consistent with the 24 h XRD data (Fig. 2b), the trehalose-only and trehalose/CMC formulations show an amorphous halo throughout the drying process (Fig. 3a–b). The broad peak at 2θ = 28° present at 3 min is due to the high levels of water still present in the coating. This peak quickly disappears as water evaporates (Fig. S2). In the case of the trehalose/Lutrol formulation, the amorphous phase partially transforms into a crystalline phase within 10 min of drying (Fig. 3c). Moreover, the intensity of the crystal peaks of the coating increased with increased drying time.

3.3. Effects of Ti plate surface cleaning

Because phase changes often nucleate at interfaces and because surfactant is often most active at interfaces, we next examined the effect of changes in the cleaning process of the Ti substrate on which coatings were applied. The previous data came from Ti plates that were washed with organic solvents then plasma-cleaned; the next set of data comes from Ti plates just washed with organic solvents (see Materials and methods) without plasma cleaning (referred to as “washed plates”). We found that the functional HA activity losses of vaccine coated on washed Ti plates (Fig. 4) were, in many ways, similar to those seen on plasma-cleaned Ti plates (Fig. 1). The vaccine activity levels in trehalose-only and trehalose/CMC formulations were independent of Ti plate cleaning method (two-way ANOVA, P = 0.28). The vaccine activity levels for the low-concentration (0.5%) Lutrol formulation were also very similar (Student’s t-test, P = 0.16). However, unlike coatings on plasma-cleaned plates, increasing Lutrol concentration did not affect vaccine activity in coatings applied to washed plates. This suggests that the cleaning method used to remove surface contaminants has a significant effect on the stability of the coated influenza vaccine. The lower HA activity on plasma-cleaned plates compared with that on washed plates at the same Lutrol concentration implies the possibility of the vaccine–metal substrate interaction that enhances destabilization of influenza vaccine, in the case of plasma-cleaned Ti substrate (compare Fig. 4 with Fig. 1b).

To further evaluate the effects of metal substrate cleaning, changes in coating morphology were monitored on washed Ti plates by microscopy, as shown in Fig. 5a. Similar to the plasma-cleaned Ti plates, Fig. 5a(i)–(v) shows coatings with no morphological changes in the trehalose-only and trehalose/CMC formulations. The trehalose/Lutrol formulation did show evidence of phase separation (Fig. 5a(v)–(vii)), but to a lesser extent and with smaller bumps (3–10 μm) compared with the same coatings on plasma-cleaned Ti plates (Fig. 2a(v)–(vii)). The time-dependant XRD spectra in Fig. 5b similarly show the formation of a crystalline phase on washed Ti plates, but to a lesser extent and with slower kinetics compared with the plasma-cleaned Ti plates (Figs. 3c and S3). These results further support the contribution of reduced Lutrol-associated phase transformation, such as crystallization and phase separation, on washed Ti plates to retaining full HA activity of coated vaccines, in contrast to plasma-cleaned Ti plates.

![Fig. 3. Time-dependant X-ray diffraction spectra over time during drying after coating on plasma-cleaned Ti plates at room temperature for (a) T15, (b) T15C1, and (c) T15L2 formulations. Drying times are indicated at the right side of each curve. Peaks on the right part of the spectra (Ti(100), Ti(002)) represent the titanium plate. The vaccine-embedded coating formulation with T15L2 began to crystallize within 10 min upon drying. Spectra are representative of n = 6 replicate samples examined at each condition.](image)

![Fig. 4. Effect of coating formulation on the functional HA activity of inactivated influenza virus after drying on washed Ti plates. The effects of CMC and Lutrol on the activity of the influenza virus (1 μg viral protein) were investigated by measuring HA titers in different vaccine coating formulations after one day of drying at ambient conditions: T15, T15C0.25, T15C0.5, T15C1, T15L0.5, T15L1, and T15L2 (mean ± SD; n = 16).](image)
3.4. Contact angle measurement

To better understand the efficiencies of different cleaning methods and their effects on the properties of the metal plate, we measured aqueous contact angles on washed and plasma-cleaned Ti plates to characterize the hydrophilicity of these surfaces. Representative images of contact angles from three coating formulations on these two surfaces are shown in Fig. 6a, and quantitative results from all seven formulations on both surfaces are shown in Fig. 6b. Regardless of coating formulation, the contact angles on washed Ti plates were much larger than those on plasma-cleaned Ti plates, suggesting that the process of washing produced a relatively hydrophobic surface, whereas plasma cleaning gave rise to a very hydrophilic surface. Surface wettability (i.e., the degree of surface energy and hydrophilicity) is a good indication of the amount of contamination by hydrophobic organic impurities [28]. Therefore, contact angle measurements indicate that plasma cleaning is more effective at removing contaminants from the Ti surface than washing with organic solvents, consistent with previous findings [29].

Closer examination shows that on both surfaces, increasing CMC concentration weakly increased contact angle (two-way ANOVA, $P = 0.002$). Addition of Lutrol significantly lowered contact angle relative to the trehalose-only formulation (Student’s $t$-test, $P < 0.000008$), but there was no additional significant effect on contact angle as a function of increasing Lutrol concentration (one-way ANOVA, $P = 0.35$). Notably, the contact angle was almost zero on plasma-cleaned Ti plate coated with the trehalose/Lutrol formulation, indicating an extremely hydrophilic...
surface. It is known that a 3–7 nm thick oxide layer is spontaneously formed on Ti surfaces due to the highly negative Gibbs free energy of formation \[30,31\]. Thus, the physicochemical properties of Ti relevant in this study depend on this extremely thin titanium oxide film \[32\]. Importantly, titanium oxide has been reported to have a high dielectric constant \(\varepsilon \sim 114\) \[33\] as well as an isoelectric point in the range of 3.5–6.7 \[34\], indicating the presence of a negative surface charge upon exposure to a neutral aqueous solution, such as our coating formulation. As a result, the physicochemically reactive surface can lead to the adsorption of vaccine to the Ti substrate through electrostatic/hydrophobic interactions, similar to the adsorption of other biomolecules (e.g., peptides, proteins, lipids, and cells) to the metal surface \[30,32,35–39\]. Plasma cleaning could cause increased adsorption by removing contaminants and increasing the surface energy. Furthermore, the presence of Lutrol increases the wetting properties of the vaccine coating, which will increase the extent of vaccine–substrate interaction. This accounts for the correlation between the generation of hydrophilic surface and the decrease of vaccine activity in the presence of Lutrol. Therefore, enhanced destabilization of vaccine on the plasma-cleaned Ti surface is believed to be caused by adsorption of vaccine and/or conformational change of the vaccine through its interaction with the Ti substrate.

We have considered the effects of three design features of coatings on vaccine stability and associated phase transformations: viscosity enhancer (CMC), surfactant (Lutrol) and surface cleaning method. Plasma cleaning in combination with surfactants appears to cause an increased vaccine–substrate interaction. However, in the absence of Lutrol, no negative effects were seen with plasma cleaning. The addition of Lutrol on washed Ti surfaces helped stabilize the vaccine, but, in a separate study, we found that Lutrol was associated with phase transformation and vaccine instability during long-term (i.e., weeks to months) storage of influenza vaccine-coated microneedles \[15\]. For this reason, we next focused our analysis on identifying stabilizing effects of the viscosity enhancer CMC in the vaccine coating formulation.

### 3.5. Effects of formulation with both a viscosity enhancer and surfactant on vaccine stability

While CMC alone did not stabilize influenza vaccine during drying (data not shown), use of trehalose in addition to CMC increased vaccine activity with increasing CMC concentration (Figs. 1a and 4). Because surfactant is also needed to effectively coat microneedles \[17\], we next examined formulations composed of all three excipients (trehalose, CMC, and Lutrol) to understand their effects on vaccine.

Fig. 7 shows vaccine stability after drying in coating formulations composed of a constant level of Lutrol and trehalose and varying levels of CMC. Addition of low-concentration (0.5%) Lutrol to the trehalose formulation increased vaccine activity (Student’s t-test, \(P<0.0005\)), consistent with our previous data (Fig. 1b). Addition of CMC at varying levels had no further effect on vaccine activity (one-way ANOVA, \(P<0.0005\)).

We next varied the Lutrol concentration while keeping CMC and trehalose concentration constant (Fig. 7). Increasing Lutrol concentration decreased vaccine activity in the presence of CMC and trehalose (one-way ANOVA, \(P=0.0005\)). This is qualitatively consistent with our previous finding that increasing Lutrol concentration in the absence CMC also decreased vaccine activity (Fig. 1b). However, quantitatively, vaccine activity was higher at elevated Lutrol concentration with CMC than that from formulation without CMC (two-way ANOVA, \(P=0.0005\)). This shows that CMC reduced the damaging effects of high Lutrol concentration in the presence of trehalose.

Because vaccine activity loss in trehalose/Lutrol formulations on plasma-cleaned Ti plates was shown to be associated with phase transformations of the coating and interaction with the metal substrate, we hypothesize that the addition of CMC suppresses these phase transformations and vaccine–substrate interaction. The nucleation and growth of trehalose crystals and the phase separation processes are driven by the diffusion of excipients. Therefore, these results further suggest that CMC suppresses both Lutrol-induced phase transformations and vaccine–Ti plate interactions by increasing coating formulation viscosity and thereby retarding diffusion of excipients (see Fig. S4 for viscosity as a function of CMC and Lutrol concentrations).

### 3.6. Role of osmotic stress in coating formulations

In addition to phase transformations damaging vaccine activity, we also considered the role of osmotic forces on vaccine virus particles. Since the lipid envelope of the inactivated influenza virus functions as a diffusion barrier against solutes, vaccine can be subject to osmotic shocks in natural environment \[40\]. To assess the effect of CMC and Lutrol on the osmotic pressure applied to the vaccine, the osmotic shrinking behavior of the inactivated virus was investigated using SFLS. Osmolyte solutions were controlled to have a final trehalose concentration equal to 15% and concentrations of CMC and Lutrol were varied to compare the effects of CMC and Lutrol on the osmotic shrinking behavior of the virus.

As a control experiment, the SFLS spectrum measured after rapid mixing of virus with the trehalose-only solution is shown in Fig. 8a. The SFLS signal exhibited a step-wise intensity increase over time (Fig. 8a(i)). Since an increase in intensity of the SFLS curve is related to a volumetric decrease, the trehalose-only solution is believed to have created hypertonic osmotic stress on the virus. We believe the first shrinkage at 0 s \(k \sim 1.0\) s\(^{-1}\), Fig. 8a(ii)) corresponds to the shrinkage of the viral envelope by increasing packing density of the membrane and the second shrinkage at 34 s (Fig. 8a(iii)) indicates the resistance of the matrix protein (M1) to further shrinkage, although additional studies are needed to assess this.

Fig. 8b shows SFLS spectra performed on the virus in response to increasing CMC concentration at constant trehalose and Lutrol concentration. Because trehalose is present at a much greater concentration than the other two excipients, the concentration gradient-driven driving force for osmotic pressure is dominated by the trehalose. As shown in the spectra (Fig. 8b(i)), the virus experienced the previously observed step-wise osmotic shrinking behavior. We chose the onset...
3.7. Morphological changes of dried influenza vaccine

To better understand effects of vaccine formulation on the inactivated virus particles after drying on plasma-cleaned Ti plates, morphological changes of the virus were compared before and after drying using TEM analysis (Fig. 9). As a positive control, the virus in DPBS is shown in Fig. 9a. The virus maintained its spherical shape and typical spike-shaped surface morphology due to its antigenic membrane proteins. When using low Lutrol concentration formulations with low- or high-concentration CMC, no morphological differences were seen between samples before (i) and after (ii) drying (Fig. 9b and c), which corroborates to the lack of activity loss under these conditions, as shown in Fig. 7. However, virus particles dried using a high Lutrol concentration formulation also did not show morphological differences before and after drying, even though HA activity loss was seen under those conditions (Fig. 7). This suggests that the observed HA activity decrease during drying may be at the molecular level associated with denaturation of HA antigenic proteins, which cannot be visualized by TEM analysis.

3.8. Effect of vaccine formulation on coating of microneedles with vaccine-coated Ti microneedles applied to the skin

We next performed experiments using vaccine-coated Ti microneedles (700 μm long). Plasma-cleaned microneedles coated with...
trehalose-only (Fig. 10a) and trehalose/CMC (Fig. 10b) formulations had smooth morphology similar to that on plasma-cleaned Ti plates (compare with Fig. 2a(i) and (iv)). Plasma-cleaned microneedles coated with a trehalose/Lutrol formulation (Fig. 10c) exhibited extensive phase separation, similar to that observed on the plasma-cleaned Ti plate (Fig. 2a(viii)). However, bumps were mostly observed near the edge of the coating on Ti plate, but were uniformly distributed throughout the coating film on the microneedles. Coating with a trehalose/Lutrol formulation on washed Ti microneedles further increased phase separation, as evidenced by the formation of larger bumps (sized from 10 to 30 μm, as shown in Fig. 10d) compared with those on the washed Ti plate (Fig. 5a(viii)). Finally, the addition of CMC to the trehalose/Lutrol formulation on plasma-cleaned is shown in Fig. 10e. These needles showed a fairly smooth surface morphology that, despite wrinkled appearance, showed a lower density of phase separation bumps compared with the trehalose/Lutrol formulation in the absence of CMC (Fig. 10c).

3.9. Influenza-vaccine immunization with microneedles and protection against challenge

To confirm our in vitro findings, we performed in vivo experiments using mice vaccinated with Ti microneedles applied to the skin, and a non-vaccinated control. In addition to a naïve, unvaccinated group, five groups of mice were vaccinated with microneedles coated with 0.12 μg of inactivated influenza virus vaccine: T15 (plasma-cleaned), T15C1 (plasma-cleaned), T15L2 (plasma-cleaned), T15L2 (wet-cleaned), and T15L2C1 (plasma-cleaned). A high Lutrol concentration was used to clearly show the effects of surfactants after one day of drying.

3.9.1. Microneedle vaccination with viscosity enhancer is more immunogenic

As Fig. 11 shows, immunization with trehalose-only or trehalose/CMC formulations generated strong antibody responses. In contrast, insignificant antibody production was observed in the groups immunized with trehalose/Lutrol formulations prepared on either washed or plasma-cleaned microneedles. This is consistent with the findings that HA activity was reduced for this formulation on plasma-cleaned Ti surfaces in vitro (Fig. 1b) and that phase transformation was also associated with these formulations on both washed and plasma-cleaned surfaces (Figs. 2, 3c and 5). Immunization with the trehalose/Lutrol/CMC formulation generated high levels of antibody production, which is consistent with our observation that CMC suppresses the destabilizing processes caused by Lutrol and interaction between the vaccine and metal substrate (Fig. 10).

Fig. 10. Five groups of vaccine-coated Ti microneedles with inactivated A/PR/8/34 influenza virus. Plasma-cleaned Ti microneedles were used for coating formulations with (a) T15, (b) T15C1, and (c) T15L2 formulations. For comparison, washed Ti microneedles were separately used for (d) with a T15L2 formulation. To see the effect of CMC addition to T15L2, microneedles were prepared in (e) using a T15L2C1 formulation. Influenza vaccine-coated microneedles with 0.12 μg of protein were air-dried for one day at ambient conditions. Images shown in (c) and (d) indicate the presence of phase separation in both washed and plasma-cleaned Ti microneedles with T15L2. However, as shown in (e), morphological change of the dried vaccine coating was suppressed due to the addition of CMC to the coating formulation. Images are representative of n = 10 replicate samples examined at each condition.

Fig. 11. Comparison of antibody responses after immunization using vaccine-coated microneedles. Total serum anti-A/PR/8/34 IgG levels were determined by reading optical densities using ELISA. IgG was measured from mice bled on the 2nd week following immunization (0.12 μg of viral protein) (mean ± SD, n = 6 mice per group).

3.9.2. Microneedle vaccination with viscosity enhancer confers better protection

The five different groups of vaccinated mice and control mice were challenged after four weeks with a lethal dose of the mouse-adapted A/PR/8/34 influenza virus (10 × LD50) to study post-challenge survival rates. As shown in Fig. 12a and b, the groups of mice vaccinated with microneedles coated with the trehalose/Lutrol formulation (both the washed and plasma-cleaned groups) and the negative control group all exhibited a rapid body weight loss followed by death within 8 days of challenge. This indicates that microneedles coated with the trehalose/Lutrol formulation cannot provide protection against lethal viral challenge. In contrast, 80% of the mice vaccinated with microneedles coated with the trehalose-only formulation survived the challenge with approximately 15% peak body weight loss. However, the groups immunized by microneedles with CMC (both the trehalese/CMC and the trehalose/Lutrol/CMC formulations) were protected 100% against lethal challenge, which is consistent with the antibody response data as well (Fig. 11). This further confirms that inclusion of CMC in the formulations was associated with improved protective immunogenicity, probably due to increased vaccine stability by alleviating osmotic stress, suppression of phase separation, and reducing the affinity between vaccine and Ti plates.

4. Discussion

In this study, the stability of influenza vaccine-coated microneedles was investigated in vitro and in vivo. The purpose of this work was to identify vaccine destabilizing factors during the microneedle coating/drying process, investigate the role of excipients in the current microneedle coating formulation, and design a strategy to maintain protective efficacy of the vaccine during drying.

4.1. Trehalose

Disaccharides have been used often in microneedle coating formulations to protect the functional activity of vaccines from drying-induced damage [12]. Guided by this previous work, this study used 15% trehalose in all coating formulations to take advantage of the sugar-induced stabilizing effect. No significant levels of functional HA activity were detected in coatings made from formulations composed of surfactant and/or viscosity enhancer without trehalose (data not shown). However, about 40–50% HA activity remained for vaccine in the trehalose-only coating formulation (Figs. 1a and 4). Despite this protective effect,
vaccination. As demonstrated in Figs. 1a, 4, and 7, both live and inactivated virus vaccine, both HA activity changes and time-dependent osmotic shrinking behavior were monitored using HA titers and SFLS, respectively, in response to changes in the coating formulation. As demonstrated in Figs. 1a, 4, and 7, both live and inactivated viruses exhibited better stability with increasing CMC content, either with or without the additional presence of Lutrol (Fig. 7). For the same coating formulations, SFLS analysis showed that the presence of CMC in the coating formulation resulted in a longer secondary shrinkage time (Fig. 8b), which indicates decreased osmotic stress. Increasing Lutrol concentration did not generate a meaningful level of osmotic pressure (Fig. 8c). Finally, increasing CMC concentration was also shown to increase coating formulation viscosity, whereas Lutrol had no effect (Fig. S4).

These data show that CMC increased viral stability, decreased osmotic stress and increased coating formulation viscosity. Taken together, these suggest that the increased viscosity imparted by CMC decreased osmotic stress, thereby reducing the destabilization of the virus by osmotic forces. While our data suggest this causative relationship, additional studies are needed to more fully test it.

It should also be mentioned that a higher CMC concentration (≥ 2%) in a trehalose/CMC formulation decreased HA activity (at 2% CMC, ~60% HA activity was observed). It is known that the degree of protection conferred by the sugar depends to a large extent on the amount of hydrogen bonding between the sugars and the lipid head groups and proteins [18]. It is possible that extremely high viscosity at such a high CMC concentration may interfere with the protective effect of trehalose on the vaccine by interfering with or retarding the formation of these bonds.

4.3. Crystallization of coatings

The crystallization of trehalose in the trehalose/Lutrol coating began very early in the drying process (10–14 min after drying, Figs. 3c and 5b). An explanation for this phenomenon can be constructed by looking at the formulation’s glass transition temperature (Tg). It should be noted that obtaining actual measurements of Tg values for the coating formulations at 10 min would be prohibitively difficult, due to the long time needed to perform the measurements and the continuous evaporation of residual water in the sample during that time. Instead, the Gordon–Taylor equation was used to calculate Tg for the trehalose–water system [44],

\[ T_g = \left( \frac{w_{\text{sugar}} T_{g(\text{sugar})} + k w_{\text{water}} T_{g(\text{water})}} {w_{\text{sugar}} + k w_{\text{water}}} \right) \]

where \( w_{\text{sugar}}, w_{\text{water}}, T_{g(\text{sugar})}, \) and \( T_{g(\text{water})} \) represent the weight fractions (w) and the glass transition temperatures of sugar and water, respectively, and \( k = 5.2 \) [45,46].

The water content change is shown as a function of time in Fig. 2b. After 10 min of drying, the water content decreased to 17.3% and 19.8% of the original value on washed and plasma-cleaned Ti plates, respectively. Using reported Tg values of trehalose and water (Tg(trehalose) = 115 °C and Tg(water) = −138 °C [46,47]), the Tg of the trehalose/Lutrol (T15L2) coating was calculated to be approximately ~100 °C on the washed/plasma-cleaned Ti plates after 10 min of drying at ambient conditions. Although this value is lower than room temperature, it is important to note that this value indicates an increased nucleation potential, but not the crystallization rate. This can explain the non-crystallizing behavior of T15 during the early stage of the drying process (Figs. 2b(i) and 3a).

According to previous reports, the addition of poloxamer 188 (Lutrol) did not cause significant changes in the Tg of trehalose [25] or mannitol [48]. This indicates that the contribution of Lutrol to the Tg calculation can be neglected and, thus, the Tg for the trehalose/Lutrol formulations can be approximated as a binary mixture of water and trehalose. In this calculation, phase separation was not considered; i.e., the coating was assumed to have a homogenous composition of trehalose and Lutrol. However, Lutrol-induced phase separation is accompanied by the likely formation of trehalose-rich and trehalose-poor regions. Importantly, since the phase separation process is associated with diffusion of excipients, the enhanced mobility in phase-separated T15L2 is expected to lower the activation energy barrier for crystal growth [49], which would not happen in T15. Therefore, we propose that the greater diffusion of excipients during phase separation is responsible for the higher rate of crystallization in Lutrol-containing samples compared with trehalose-only samples. This is supported by the observed positive correlation between the amount of phase separation and crystallization, as evidenced by XRD analysis (Figs. 2, 3, and 5).

The very low Tg values during drying suggest that plasticizing effects of water provided a thermodynamic driving force for trehalose crystallization in the trehalose/Lutrol coating during the drying
The plasticization effect was probably enhanced by the use of PBS [51]. Additionally, CMC was shown to have a significant effect on the formulation's viscosity (Fig. S4). Since high viscosity is known to kinetically suppress molecular mobility and rearrangement, the addition of CMC would result in a decreased crystallization rate and could explain the lack of crystallization associated with CMC-containing samples during the early stage of the drying process [49,52,53].

4.4. Interfacial effects

The influence of Lutrol on the viral stability depended strongly on the surface properties of the Ti plates. As shown in Fig. 1b, HA activity decreased with increasing Lutrol concentration on plasma-cleaned Ti plates, but not on washed Ti plates. We also found that increasing Lutrol concentration increased phase transformations such as phase separation and crystallization of the vaccine coating on plasma-cleaned Ti plates using optical microscopy and XRD analysis (Fig. 2a(vi)-(vii) and b(ii)).

In a separate study of the long-term stability of influenza vaccine-coated microneedles, the physicochemical properties of the vaccine coating were also found to be critical factors in determining protective immunogenicity of microneedles [15]. Crystallization of trehalose in the vaccine coatings induced activity loss. Importantly, phase-separated samples with large bumps (>15 μm) exhibited a large decrease in functional HA activity, while samples with small bumps (<15 μm) retained their full activity during storage. In our data (Fig. 2a), the low-concentration (0.5%) Lutrol (which did not damage HA activity) had small bumps, whereas the higher concentration Lutrol formulations (which did damage HA activity) had large bumps, which appears consistent with previous findings.

As seen by optical micrographs and XRD analysis (Fig. 2), phase separation observed from the trehalose/Lutrol formulation was usually accompanied by crystallization. Based on this observation, it is not clear whether the phase transformations associated with the phase separation and/or the crystallization might be mechanistically responsible for loss of HA activity. However, such Lutrol-induced HA activity decrease was not seen in vaccine coatings on washed Ti plates (Fig. 4) which had little evidence of phase separation or crystallization (Figs. 5a(vi)-(vii) and S2). In the case of the previous long-term stability test using the trehalose/Lutrol/CMC formulation, a drop in activity was seen in severely phase-separated samples, even though the bumps were not crystallized (as measured by XRD), showing that phase-separation without crystallization could affect vaccine stability [15].

The enhanced stability of the vaccine coating on washed Ti plates can be explained in part by less phase transformation than on plasma-cleaned Ti plates (Figs. 5a(vi)-(vii) and S2) possibly due to decreased nucleation of phase transformations on the washed Ti plate surface. Another possibility is that the electrostatic/hydrophobic interactions of virus with the Ti substrate could be important in influencing stability of the vaccine during the drying process (discussed in Section 3.4). The reactivity of Ti surfaces has been shown to play a critical role in protein adsorption behavior and to cause conformational changes in proteins [35–37]. Thus, based on the observations of 1) the increase in wetting properties of Ti plates by plasma-cleaning, 2) the lower HA activity on plasma-cleaned Ti plates than on washed Ti plates, and 3) the decrease in HA activity under more hydrophilic conditions (i.e., increase in Lutrol concentration), we propose that vaccine–metal substrate interactions occur more extensively on plasma-cleaned Ti plates (especially, in the presence of surfactant) due to the greater contact between the coating solution and Ti substrate, caused by easy spreading. Finally, it is worth noting that although trehalose/Lutrol coatings on washed Ti showed less phase transformation and less HA activity loss than on plasma-cleaned Ti microneedles, trehalose/Lutrol coatings on washed Ti microneedles were not immunogenic and performed just as badly as the coatings on plasma-cleaned Ti microneedles (Figs. 11 and 12). This inconsistency between in vitro HA assay and in vivo immunogenicity assay needs further study.

Questions remain as to why the addition of low concentrations of Lutrol better prevented the HA loss in trehalose-only coatings, possibly induced by osmotic pressure-driven effects (Figs. 1 and 4). A possible explanation is that the surfactant interacted with the viral envelope in a way that stabilized it against osmotic pressure-induced membrane perturbations. This is because Lutrol surfactant has been known to interact with liposomes or cell membranes through its penetration into lipid bilayers [54,55]. However, our previous long-term stability tests showed that even small amounts of surfactant lead to phase separation eventually, even in the presence of CMC [15]. Thus, even though small amounts of surfactant might be a stabilizer in the short term, it is harmful in the long term. In the past, we have included surfactant in formulations to enable uniform coating of microneedle surfaces [9,17]. Here, we have shown that plasma cleaning is at least as effective at reducing contact angles as surfactant (Fig. 6) and thereby is expected to enable uniform coatings too. Additionally, plasma cleaning has no observable negative side effects on vaccine stability in coatings formulated without surfactant. While further research is needed to understand the mechanisms involved, from a practical point of view, formulations containing trehalose and surfactant do not appear suitable for microneedle coating with influenza vaccine.

4.5. Viscosity enhancer (CMC)

An important role of CMC, in addition to reducing osmotic pressure-driven effects, was to suppress phase transformations of the vaccine coating and possibly minimize the interaction of vaccine with the metal substrate, thereby enhancing vaccine stability during drying. The addition of CMC was found to significantly increase viscosity of the coating formulation (Fig. S4). Since crystallization and phase separation of vaccine coatings are driven by diffusion of excipients, a high viscosity can suppress these processes. Moreover, high viscosity can reduce the chance of virus interaction with the metal substrate. Our data consistently show that the addition of CMC to trehalose-only and trehalose/Lutrol formulations increases vaccine stability during drying (Figs. 1a, 4 and 7).

4.6. Coating microneedles

When we compared coatings on Ti plates to coatings on microneedles, all of the microneedle groups showed similar morphology to the Ti plates. Lutrol-induced phase separation was observed with the trehalose/Lutrol coating formulations on both washed and plasma-cleaned Ti microneedles. However, as shown in Fig. 10c and d, extensive phase separation was observed throughout the microneedle coatings, whereas the Ti plates showed phase separation only over a more limited fraction of the coating. This may be because the drops applied to Ti sheets spread over a larger area of approximately 0.15–0.2 cm², whereas the coatings applied to microneedles were limited to the surface areas of the microneedles, which was about 3 × 10⁻³ cm² per microneedle. Because surfactant molecules are known to assemble along interfaces to minimize surface energy of the system [56], less surfactant is expected to assemble along the liquid–air and liquid–metal interfaces on microneedles than on the Ti plates. This can result in an increased surfactant concentration in the rest of coating. This increased surfactant concentration in the bulk coating may cause greater phase separation on microneedles than on Ti plates.

4.7. Immunogenicity

Little or no serum IgG antibody response was detected from the trehalose/Lutrol formulations on both washed and plasma-cleaned microneedles (Fig. 11), which is consistent with the 0% survival rate
after the challenge (Fig. 12). This can be explained by a decrease in vaccine efficacy due to phase transformation on Lutrol-containing microneedles (for both plasma-cleaned and washed) and affinity between vaccine and microneedle substrate (for plasma-cleaned). The high-concentration (2%) Lutrol formulations used in this study had phase separation with large bumps (>15 μm) and crystallization, which damages the vaccine. In addition, the trehalose/Lutrol/CMC group showed a higher level of antibody production and 100% protection against lethal viral challenge compared with the trehalose/Lutrol groups, probably because CMC functions to suppress phase transformations and vaccine–substrate interaction in the coating, which protects the vaccine.

4.8. Coating formulation recommendation

Based on these data generated using whole inactivated influenza virus vaccine, we recommend that a coating formulation should contain 15% trehalose to stabilize the vaccine during drying (based on prior literature [12,19]); and CMC (less than 2%) or some other viscosity enhancer to reduce osmotic stress, suppress phase transformations, and minimize interactions between the vaccine and Ti metal plates. Formulations with Lutrol cause vaccine–destabilizing phase transformations during long-term storage, even in the presence of CMC [15], as well as a decrease in vaccine activity via interaction with plasma-cleaned metal substrates. If the coating is too hydrophobic to coat properly, plasma cleaning can be used to enhance the wettability of the microneedles. However, our data show that in the case of plasma cleaning, the coating formulation should not contain surfactant in order to reduce the vaccine destabilization due to adsorption onto metal substrate. If surfactants are used, it appears to be critical to modify the surface chemistry of the metal substrate after/during plasma treatment to prevent vaccine adsorption [57]. In addition, while our work has been performed using DPBS without calcium and magnesium, the possibilities of enhanced biomolecule–metal substrate interaction via bridging effects of divalent cations [58], pH changes during drying [59,60], and PBS-induced Tg variation/crystallization [51] provide the need for further research on the effects of buffer composition on the stability of vaccine coated onto microneedles.

5. Conclusions

In this work, stability of influenza vaccine during coating onto microneedles was investigated. We examined the role of excipients used in current microneedle coating formulations, focusing on the stabilizing effect of sugar, osmotic pressure, phase transformations of the coating, and suitability of the coating formulation to coat microneedles. This work showed that 1) trehalose significantly protects influenza vaccine during drying, and that 2) osmotic pressure increase caused by high sugar concentration, phase transformation (i.e., phase separation and crystallization) of the vaccine coating, and interactions between vaccine and metal substrates are vaccine-destabilizing factors affecting stability of the vaccine during coating. We further observed that the presence of a moderate amount of viscosity enhancer in the trehalose-only and trehalose/surfactant formulations had a strong stabilizing effect on the vaccine coating during drying by dampening osmotic pressure applied to the vaccine and suppressing phase transformation. We found that the surfactant Lutrol® micro 68 was a cause of phase separation of the coatings, and that this effect was enhanced on the plasma–cleaned metal substrate. Although the stability factors addressed in this study are limited and further research is needed, this study offers useful insight into mechanisms of vaccine damage during coating and presents guidelines for design formulations that preserve vaccine efficacy for influenza vaccine and other vaccines as well.

Competing interests

M.R.P. is an inventor of patents that have been licensed to companies developing microneedle-based products, is a paid advisor to companies developing microneedle-based products, and is a founder/shareholder of companies developing microneedle-based products. This potential conflict of interest has been disclosed and is being managed by the Georgia Institute of Technology and Emory University.

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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.2012.12.002.

References
