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# Microneedle and mucosal delivery of influenza vaccines

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## Abstract

In recent years with the threat of pandemic influenza and other public health needs, alternative vaccination methods other than intramuscular immunization have received great attention. The skin and mucosal surfaces are attractive sites probably because of both non-invasive access to the vaccine delivery and unique immunological responses. Intradermal vaccines using a microinjection system (BD Soluvia) and intranasal vaccines (FluMist) are licensed. As a new vaccination method, solid microneedles have been developed using a simple device that may be suitable for self-administration. Because coated micorneedle influenza vaccines are administered in the solid state, developing formulations maintaining the stability of influenza vaccines is an important issue to be considered. Marketable microneedle devices and clinical trials remain to be developed. Other alternative mucosal routes such as oral and intranasal delivery systems are also attractive for inducing cross protective mucosal immunity but effective non-live mucosal vaccines remain to be developed.

### Keywords

influenza vaccine; microneedles; skin vaccination; mucosal immunization

## Introduction

Both vaccination and antiviral therapy are used to prevent and to treat influenza infection [1]. Nonetheless, vaccination is the most cost-effective public health prevention against infectious diseases [2,3]. The delivery of vaccines and drugs using needles and syringes is a common practice in worldwide. However, there are some concerns about the use of needles and syringes. Each year, the unsafe use of injections is estimated to cause approximately 1.6 million deaths, which include needle-stick injuries, work accidents, and an overwhelming number of infections with blood-borne pathogens such as hepatitis B virus, hepatitis C virus, and HIV due to the improper re-use of needles and syringes [4,5]. Another issue is the needle phobia and the discomfort suffered by children and adults [6,7].

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Skin has unique immunological properties (Fig. 1). Stratum corneum is a physical barrier of the skin (Fig. 1). The epidermis is the skin outer layer with immunological functions and its thickness varies in a range of approximately 50 – 200 μm (Fig.1) [8]. The epidermal cell types include melanocyes, Langerhans cells (LCs; a subtype of antigen presenting dendritic cells) in addition to the keratinocytes (a kind of epithelial cells) the major cell types in the epidermal layer. The outer dermis contains resident hematopoietic-derived cells, including mast cells, dermal dendritic cells (DCs) and macrophages as well as LCs. The dermis also provides the major site for leukocyte extravasation from the blood to the skin [9]. Therefore, skin might provide an attractive site for vaccine delivery [10]. The skin routes for immunization include intradermal, epidermal, and transcutaneous delivery of vaccines. The intradermal route refers to antigen delivery into the dermis via a syringe and needle, or a microinjection system. The term epidermal mostly reflects the delivery of vaccines into the epidermal and outer dermal layers via arrays of microneedles in the form of a patch or using gene-gun technology. Transcutaneous or transdermal vaccination means the topical application of antigen onto the skin [11–13].

As an alternative to intramuscular injection, the intradermal vaccines and the live cold adapted vaccines have been licensed and are widely used in humans [14,15]. The fact that these new routes of licensed influenza vaccines provides further support and rationales for developing new vaccination methods targeting to skin and mucosal sites. With briefly overviewing and comparing these newly licensed vaccines in a liquid form, this review covers recent studies with solid microneedles with a size of approximately 700 μm in length and 150–160 μm in width (Fig. 2) delivering influenza vaccines as an additional alternative to intramuscular delivery and their associated vaccine stability issues due to a phase change from liquid formulation to a dried solid state of vaccines. Also, we discuss mucosal delivery of influenza vaccines and their potential avenues in broadening cross-protective immunity.

## Skin vaccination using microneedles

### Concept of the skin delivery using various forms of microneedles

The initial designs of microneedles are arrays of microneedles that protrude several hundred microns in sizes (Fig. 2). Coated or dissolvable microneedles, or microneedles alone can be used to either to pierce or to make microscopic holes in the skin’s outer layer, straturm corneum [16,17]. Compounds delivered to the skin using microneedles include bovine serum albumin (as a model protein antigen), oligonucleotides or plasmid DNA vaccines, latex particles of viral dimensions, recombinant bacterial proteins and live attenuated virus vaccines [18–22].

Microneedles are fabricated to create micron-scale needles [23,24]. Solid micorneedle vaccines were demonstrated to be used for coating with protein antigens in a dry formulation [25,26]. These coated vaccines can dissolve from microneedles within the skin on a time scale of seconds or minutes (Fig. 3). This concept of solid microneedle vaccines was applied to various model compounds including proteins such as ovalbumin [24–28].

In contrast to solid coated microneedles, dissolvable polymer microneedles with encapsulated drugs or vaccines also have been developed. Dissolvable microneedles

composed of a biocompatible and mechanically robust material have been fabricated from polymers such as polylactic-co-glycolic acid, dissolving sugar, or polysaccharide [29–31]. Dissolving microneedles in the skin leaves behind no sharp and hazardous waste [29]. This concept of dissolvable polymer microneedles has been demonstrated for the delivery of insulin and other model compounds [29,31,32]. In a recent study, a concept of dissolving polymer microneedles was demonstrated to deliver encapsulated inactivated influenza virus to the skin of mice [32]. Immunized mice with dissolving microneedle influenza vaccines showed effective lung viral control and cellular recall responses after challenge, suggesting that dissolving microneedles can provide a simpler and safer vaccination method [32].

## Intradermal influenza vaccination using hollow microneedles

Different from solid microneedles mentioned above, hollow microneedles are composed of a hollow fluid conduit through the body of the individual needle, a vaccine reservoir, and fluid delivery system such as a syringe, pump, or other pressure generating device. One or more hollow needles that are used to flow a liquid formulation into the skin are generally in a range of 1.0–1.5 mm in length (30 – 34 gauge) [33,34]. Intradermal delivery using hollow microneedles has been demonstrated for a variety of compounds and vaccines in animals and in humans [21,22,35]. Intradermal influenza vaccines were first licensed (Table 1) and marketed under various trade names through the commercially available hollow microneedle system, BD Soluvia™ (Becton Dickinson, Franklin Lakes, NJ) [14,36]. This licensed intradermal influenza vaccine is based on a single 1.5 mm stainless steel microneedle in a glass prefilled syringe with a self-deploying safety shield after delivery, which was reported in several studies [8,37,38].

The microinjection system using seasonal trivalent influenza vaccine at a lower dose in adult volunteers induced humoral immune responses (hemagglutination inhibition titers, HAI) comparable to those by the standard intramuscular route. That is, 9 μg or 15 μg of intradermal dose of trivalent seasonal influenza vaccines was comparably immunogenic to the 15 μg standard intramuscular dose for all strains [14,39–41]. In the elderly volunteers, intradermal immunization with 15 μg of trivalent inactivated vaccine using the BD microinjection system is likely to be more immunogenic compared to the intramuscular delivery route [39,42–44] or non-inferior to conventional intramuscular vaccination [45]. In a multicenter, randomized study including 1107 healthy volunteers over 60 years of age, intradermal trivalent inactivated influenza vaccines containing 15 or 21 μg of hemagglutinin per strain were administered using a microinjection system and the strain-specific hemagglutination inhibition geometric mean titers were determined in comparison with the standard intramuscular immunization [42]. Seroprotection and seroconversion rates were significantly higher in each intradermal vaccine [42]. Although the mechanisms in enhancing the immunogenicity of intradermal vaccines are not well understood, dermal dendritic cells might be involved in stimulating cellular immune responses in the elderly [46]. Also, in 112 healthy young children aged 3 to 18 years, intradermal influenza vaccination at one fifth of a standard dose was reported to elicit comparable immunogenicity to full-dose intramuscular vaccination [47].

In a study of skin thickness (epidermis-dermis) to have an insight into the potential site of intradermal vaccine delivery, it was reported that the suprascapular had 2.54 mm, the deltoid 2.02 mm, the waist 1.91 mm of mean skin thickness [48]. Therefore, microneedles with 1.5 mm length would be assumed to deliver the antigen into the dermal layer. The skin sites of deltoid or suprascapular are to be appropriate in the body [48]. The microneedle injection system appears to be easy to perform, reliable and consistent in administering the given dose [37,49]. The systemic reactogenicity (such as muscle pain) of intradermal route is significantly less but minor transient reactions (redness) at the injection sites are more frequently observed [50–53]. The acceptability of intradermal route using a microinjection system is high up to over 95% with satisfaction, most responding that the new intradermal vaccination was less painful and quickly administered [14]. It is thus expected that intradermal vaccines will help increase seasonal influenza vaccination rates in adults. Therefore, clinical trials provide sufficient evidence that delivering vaccines to the dermal layer of skin can be a promising approach for vaccination alternative to intramuscular delivery.

## Influenza vaccination in the skin of mice using solid microneedles

Intradermal injection of influenza vaccines using needles and syringes or microinjection systems has been widely demonstrated in humans [14,51,54–57]. However, its general application may have some limitations due to discomfort associated with needle sticks (in cases of using hypodermic needles and syringes) and/or cost of microinjection devices. As a new influenza vaccination, solid microneedles coated with inactivated influenza vaccines have been demonstrated in mice [58]. In this study, an array of 5 microneedles with 750 μm in length (Fig. 2) was coated with 3.3 μg total protein of inactivated H1N1 influenza virus vaccines at the vaccine concentration of 5 mg vaccine protein per ml. Using 3 sets of 5-needle array, a total of 10 μg of influenza vaccines was successfully delivered to the mouse skin (Fig. 3, [59]). Microneedle vaccinated mice showed comparable vaccine specific antibody responses and protection as intramuscular vaccination [58]. A similarly comparable immunogenicity was observed in another microneedle skin vaccination with 3 to 10 μg inactivated H3N2 influenza virus vaccine as intramuscular immunization [60]. Relatively high doses of influenza vaccines and multiple arrays of microneedles were required probably due to the un-stabilized dry microneedle formulation [58,60].

Stable microneedle formulations were developed to improve the efficacy of microneedle influenza vaccines [59,61]. A trehalose (disaccharide) known to stabilize biomolecules during drying [62] was included in the microneedle coating formulation as a stabilizer (Table 1). As a result, microneedles coated with as low as 0.4 μg of inactivated influenza (A/PR8, H1N1) virus vaccine induced protective immunity, which was superior to the intramuscular immunization with the same vaccine [59,61]. These findings suggest that vaccination in the skin of mice using a microneedle patch improves protective immunity and simplify the delivery of influenza vaccines. This approach has been proved to be applicable to other influenza vaccines such as influenza H1N1 and H5N1 virus-like particle vaccines [63–67] and 2009 H1N1 inactivated influenza virus vaccines [68].

## Immunological responses after influenza vaccination using solid microneedles

A mouse model provides a valuable tool in understanding the detailed host immune responses after delivering vaccines to the skin, which is difficult in humans. With the development of solid microneedle influenza vaccines, we could investigate host immune responses in detail after microneedle vaccination in the skin of mice. Influenza vaccine-coated solid microneedle delivery to the skin of mice could induce virus neutralizing antibody and hemagglutination inhibition (HAI) responses at comparable levels (or higher levels than) to intramuscular route [59,61,65,67]. Enhanced lung virus clearance and less inflammatory cytokine were observed in mice that received microneedle vaccination after challenge infection [59,61] indicating improved protective efficacy of microneedle vaccination. In addition, microneedle vaccination showed significantly higher levels of virus specific recall IgG antibody responses after challenge [59,61], suggesting rapid host anamnestic immune responses in response to the exposure to challenge virus. Whereas, intramuscular immunization showed decreases in levels of virus-specific antibodies at this early time post challenge infection, which might be related with delayed virus clearance.

Regarding cellular immune responses, microneedle vaccination induced higher levels of IFN-γ and IL-4 cytokine-secreting splenocytes compared to intramuscular immunization upon major histocompatibility complex (MHC) II peptide stimulation [59,61], indicating enhanced MHC II-associated $CD4^+$ T helper cell responses, which might be especially important to provide protection in the elderly [69]. Finally, microneedle vaccination in the skin was demonstrated to increase trafficking of dendritic cells to regional lymph nodes, which plays a role in contributing to improved protective immunity [70]. Thus, microneedle vaccination would provide an excellent research tool in studying detailed immune responses after delivery of vaccine antigens to the skin.

## Stability of solid microneedle vaccines and their immunogenicity

We found that the simple process of microneedle coating and drying significantly decreased the stability of the influenza microneedle vaccines, as indicated by the loss of hemagglutination activity, probably due to the phase change from liquid to solid formulation [61,66]. Un-stabilized vaccine yielded much weaker protective immune responses. Immunization using un-stabilized vaccine induced IgG1 antibody as a dominant isotype whereas stabilized vaccines maintaining hemagglutination activity shifted the pattern of antibodies to IgG2a antibodies implicating the T helper type 1 (Th1) immune responses [61,66]. Also, much higher dose (10 μg) of unstabilized microneedle vaccines was required to induce protection [58,60]. Importantly, the addition of trehalose to the microneedle coating formulation was required to retain hemagglutination activity after microneedle coating of influenza vaccines. Microneedle vaccines of a low dose of 0.4 μg inactivated virus with a trehalose stabilizer in the coating formulation conferred significantly enhanced protection [59,61], indicating a positive correlation between the stabilization of coated influenza vaccines and the efficacy of protection. However, it was found that trehalose sugar did not have an adjuvant effect on enhancing immunogenicity [61]. Intact influenza virus vaccines (not exposed to microneedle coating) with and without the addition of trehalose showed similar antibody responses after intramuscular immunization [61], indicating a critical role of trehalose as a stabilizing agent in retaining hemagglutination activity of

influenza vaccines during coating microneedles and drying process but not as an immune adjuvant.

Influenza vaccines provide a useful system utilizing an easy assay of hemagglutination activity to investigate the effects of vaccine integrity and stability on its immune responses after skin vaccination. Solid microneedle vaccine studies of stabilized and un-stabilized influenza vaccines provide evidence that the functional integrity of hemagglutinin in the influenza vaccine may have a significant impact on the types and qualities of host protective immune responses to influenza vaccination [61,66]. Thus, it is speculated that retaining the receptor-binding functional activity of hemaglutinin protein antigens in influenza vaccines is important for the effective induction of protective immune responses. Some lymphoid dendritic cells are more likely to induce Th1 type immune responses whereas non-receptor mediated uptake of vaccines via macrophage cells may induce Th2 type responses affecting the pattern of antibody isotypes [71]. Among the range of immunologic data, the recall immune responses with microneedle vaccination to the skin were significantly stronger than intramuscular immunization [59,61,66]. After taking up transdermally delivered antigens, skin-derived dendritic cells are known to migrate to the systemic and mucosal compartments [72–74], which might be involved in rapid recall immune responses after microneedle vaccination in the skin. Therefore, detailed immunologic study provides deeper explanations for potential improved protective efficacies by vaccine delivery to the skin. This correlation between the functional integrity of hemagglutinin and protective immunity against influenza is further supported by other influenza vaccines such as H1N1 and H5N1 influenza virus-like particle vaccines [63–67,75].

## Coating formulations and stability of solid microneedle vaccines

The stability of microneedle vaccines seems to be related to the composition of coating solution that is composed of 1% carboxymethylcellulose (CMC) sodium salt, detergent Lutrol F-68 NF, and with or without 15% (w/v) D-(+)-trehalose dihydrate stabilizer [59,76,77]. It would be informative to better understand the possible roles of microneedle coating formulation. Sugar combinations of trehalose, mannitol, dextran, and arginine glutamate were developed as a stabilizing formulation for a dry powder form of influenza vaccines [78]. Some carbohydrate compounds such as trehalose, sucrose, glucose, inulin, and dextran were shown to prevent damages caused from drying or freezing of biomolecules [62]. Among the different carbohydrate stabilizers tested, trehalose was found to be the most effective one for stabilizing influenza microneedle vaccines [76]. Addition of trehalose retained 50–80% hemagglutination activity for all three major strains of seasonal influenza A H1N1 and H3N2, and influenza B viruses [76]. In case of a spray freeze drying process, HEPES buffered saline provided a good stability [79].

Drying influenza vaccines in the coating solution caused more damage to hemagglutination activity than drying in phosphate buffered saline indicating the potential effects of components in microneedle coating solution [76]. In the absence of trehalose, hemagglutination activity of vaccines almost disappeared independent of CMC concentrations [64,77]. Although removing CMC from the coating formulation allowed us to retain 100% hemagglutination activity after drying the vaccines in the presence of

trehalose, CMC was nonetheless required to produce thick coatings onto microneedles. Without CMC, the mass of virus coated on microneedles was significantly reduced by more than an order of magnitude [64]. The presence of detergent Lutrol F-68 NF (0.5%) that is also needed for effective coating onto metal microneedles did not show significant effects on stability of microneedle vaccines [64]. Therefore, trehalose and CMC are important excipients in the coating solution for microneedle vaccine formulations.

### Long-term stability of solid microneedle vaccines

Cold-chain delivery and storage is required during influenza vaccination campaign since temperature is an important factor for determining the efficacy of influenza vaccines. In liquid formulation of influenza vaccines, storage at 4°C is needed for maintaining the stability of vaccines as measured by hemagglutination activity and storage at 25°C resulted in a significant loss in vaccine activity [64]. In contrast, microneedle vaccines showed a similar pattern of stability kinetics at 4°C and 25°C [64]. Within a day storage of influenza microneedle vaccines, there were approximately 30% loss in hemagglutination activity at both 4°C and 25°C and 40% loss at 37°C. After 7 days of storage, a low flat point of approximately 30% activity was maintained up to 28 days monitored at 25°C storage. Most importantly, the 100% protective immunity was observed with microneedle vaccines stored at 25°C for 28 days [64]. Therefore, solid microneedle vaccines can be developed as an alternative to cold-chain influenza vaccines (Table 1).

## Epidermal Powder Immunization

As a delivery of vaccines epidermally in the skin, the helium-powered PowderJect device was developed and used to deliver powdered influenza vaccines [80–82]. A powder form of influenza vaccines can be prepared by using different methods such as freeze drying, spray-freeze drying, spray drying, vacuum drying, and supercritical fluid drying [62]. A simple method is air-drying as described for epidermal powder immunization [80,82]. Briefly, vaccine, adjuvant, and trehalose solution are combined, incubated overnight at 4°C with shaking, and plated in a glass petri dish. The vaccine mixture is dried overnight in a desiccator purged with nitrogen gas. The dried solid is collected, ground with a pestle and mortar, and sieved using stainless steel sieves. Dry powders of particulate vaccines are loaded into a trilaminate cassette and used to immunize mice. The helium-powered PowderJect device for delivering powdered vaccines is a reusable model with a 15 cm length composed of an ‘actuation’ button, a helium gas chamber, a vaccine cassette, and a nozzle [80]. The stainless steel gas chamber is filled with helium gas. Once the device is activated, the helium gas is released to rupture the membranes of the trilaminate cassette containing powdered vaccines. The vaccine powders are accelerated to a high speed so that the particles perforate the stratum corneum and land in the epidermis. Epidermal powder immunization was demonstrated to induce cytotoxic T cells, antibody responses in serum and mucosal sites, and protective immunity against influenza viruses in a mouse model [80–82]. The delivery methods of dry influenza vaccine formulations via epidermal powder immunization are successfully tested in phase I clinical trials [83]. However, the complexity of preparation of vaccines and injection device seems to be one of limiting factors for application to humans in general.

## Mucosal Immunization

Many infectious agents enter the body through mucosal surfaces [84]. Mucosal immune responses are being recognized to be important for providing effective protection against pathogens entering mucosal surfaces [85]. Local immune responses at mucosal sites are most efficiently induced by the administration of vaccines onto mucosal surfaces through oral, nasal, sublingual, rectal or vaginal routes. Several mucosal vaccines have been approved for human use. Licensed mucosal vaccines include oral vaccines against poliovirus [86], *Salmonella typhi* [87], *V. cholera* [88], and rotavirus [89], and a nasal spray vaccine against influenza virus [90]. Due to the repeated outbreaks of pandemic influenza viruses and the threat of many mucosal pathogens, the research of testing mucosal vaccines is now increasing with new information on the mucosal immune system.

Epithelial cells are layered on the mucosal surfaces of the respiratory, gastrointestinal, and urogenital tracts exposed to the outside (Fig. 4). Mucosal tissues are sites of intense immunological activity, where dispersed lymphoid and antigen-presenting cells such as dendritic cells (DCs) are present (Fig. 4) [85]. More antibody producing cells are estimated to be present in the intestinal mucosa than in the spleen and lymph nodes [85]. Epithelial cells detect and uptake microbial and/or vaccine components through non-specific endocytosis or pattern recognition receptors such as Toll-like receptors [91,92]. Upon encountering microorganisms or vaccine antigens, together with intraepithelial lymphocytes and underlying dendritic cells and macrophage cells, cytokines and chemokines are produced to trigger innate, nonspecific defenses and to promote adaptive immune responses (Fig. 4) [91,92].

### Oral influenza vaccination

Oral delivery is considered a convenient route for administration of vaccines probably because of its easy acceptability and administration [93]. The most effective oral vaccines are live attenuated poliovirus [86], and live attenuated *Salmonella typhi* [87]. M cells are specialized for endocytosis and rapid transepithelial transport of intact antigens into intraepithelial pockets that contain B and T cells and occasional dendritic cells (Fig. 4). Microparticles that are up to 1 μm in diameter and accessible to M cells are taken up most efficiently (Fig. 4) [94]. Oral immunization of humans with influenza vaccine powder forms was investigated several decades ago. In previous clinical studies, it was demonstrated that ingestion of inactivated influenza virus vaccine powders in enteric-coated capsule formulations stimulated local synthesis of secretory IgA antibody in human nasal and saliva secretions [95–97]. Similarly, the emulsion-inactivated vaccine showed high immunological activity inducing reliable increases in the levels of secretory IgA specific to influenza A and B viruses [98]. However, systemic serum antibody responses were low or not detected in subjects received oral vaccination [95]. Systemic immune responses are needed to meet regulatory criteria for vaccine immunogenicity. In oral vaccine studies using animal models, oral vaccination was shown to induce both systemic and mucosal immune responses as well as protection [99–105]. The efficacy of non-live mucosal vaccines via oral delivery can be significantly increased by incorporating into enteric-coated gelatin capsules and copolymer microparticles, liposomes, or proteosomes [106], which are attractive targets for M cells.

However, there might be a gap in translating results from mouse studies to humans particularly for oral vaccination since serum antibodies were not significantly induced in clinical studies after oral vaccination [107]. Nonetheless, cross-protection against mucosal infection by a variety of respiratory viruses such as influenza might better correlate with the level of mucosal immune responses including secretory IgA antibody in a mouse model [108–111]. Recently, oral vaccination of mice with 25 μg of whole inactivated influenza virus vaccines induced significant levels of serum and mucosal IgG and IgA antibodies cross-reactive to homologous and heterologous virus as well as cross protection [112]. In addition, recall immune responses were observed in orally vaccinated mice upon challenge infection [112]. Therefore, the oral method of influenza vaccination could be superior in providing cross protection if effective serum antibody responses are elicited along with mucosal immune responses in humans.

## Intranasal influenza vaccination

Immunologically, intranasal influenza vaccination is another attractive route as a needle-free vaccine delivery method (Fig. 4). Nasal vaccine delivery offers potential advantages in providing cross protection. The nasal epithelium contains follicular associated lymphoid tissues that are effective in inducing mucosal immune responses. These cells include B cells that produce IgA antibodies at mucosal sites of entry for many respiratory pathogens including influenza. Inactivated influenza vaccines when intranasally delivered to mice have been shown to induce IgG and IgA antibody responses in serum and mucosal sites, which are important for conferring enhanced cross protection [108–111,113,114]. Mucosal IgA antibodies were shown to be effective in neutralizing viruses and in inhibiting hemagglutination of red blood cells by influenza virus [115]. Mice that were intranasally immunized with influenza vaccines were found to protect against influenza viruses that are antigenically different [110,114]. Also, antibodies induced by intranasal immunization were cross-reactive and the ability of the vaccines to produce cross-reactive antibodies showed correlation with the efficacy of cross protection [108,116]. Comparable systemic and mucosal immune responses were demonstrated to be induced after intranasal immunization of mice with suspended whole inactivated virus or virus-like particle vaccines [108,110,111,117].

An early study demonstrated that aerosolized inactivated vaccine provided protection against illness in man after intranasal immunization [118]. However, inactivated and split influenza vaccines are likely to be less immunogenic via the intranasal route compared to the intramuscular delivery, thus requiring the use of mucosal adjuvants. Therefore, various mucosal adjuvants are being developed and tested. Mostly using mouse models, potential mucosal adjuvants for intranasal vaccine delivery that have been tested include detoxified lipopolysaccharide [119], TLR-9 agonist [17], immunostimulatory complexes, MF59 emulsion [120], TLR3 agonists [121], chitosan [9], and bacterial outer membrane complex [122]. Bacterial toxins, such as cholera toxin (CT) and the closely analogous heat-labile enterotoxin (LT) and their derivatives, have been used as potential mucosal adjuvants in experimental models and clinical trials to enhance the immunogenicity of mucosal vaccines [110,123–125]. However, in humans, some side effects including facial paralysis (Bell’s Palsy) and an adverse event of facial nerve disorder when given intranasally were reported to

be associated with intranasal influenza vaccination with an inactivated virosomal vaccine formulated bacterial toxin [126–128].

In contrast to killed and split vaccines, live attenuated influenza vaccines FluMist™ (MedImmune) [15] and Nasovac™ (Serum Institute of India, Ltd., Pune, India) [129] are licensed for intranasal delivery in humans. Humans in clinical trials receiving nasal live attenuated influenza virus vaccines showed cross reactive hemagglutination inhibition and cross protection against variants not present in the vaccine [130,131]. The efficacy data of Nasovac are not sufficiently available yet although there are no serious side effects [129]. FluMist vaccine is generally well tolerated with runny nose/nasal congestion being the most common adverse events mild to moderate in severity. Compared with intramuscular trivalent influenza vaccines, FluMist is likely to increase the incidence of medically defined wheezing in vaccine naïve children aged less than 24 months [132] and is associated with higher frequency of hospitalization in young children aged 6 to 11 months [49]. Thus, FluMist is not approved for young children of less than 2 years old. The meta-analysis of intranasal live attenuated influenza vaccine in children aged 2–17 years demonstrated that live attenuated influenza vaccine showed higher efficacy after 2 doses in year 1 and revaccination in year 2, when compared with inactivated influenza vaccine [133]. Based on results of meta-analysis of clinical studies comparing the efficacies of intranasal live vaccines and inactivated subunit influenza vaccines in all aged groups, both vaccines were similarly efficacious in preventing culture confirmed influenza illness [134]. However, their compartmental responses were different as reporting that the live virus vaccine was lower in inducing serum hemagglutination inhibiting antibodies but higher in levels of local IgA antibodies compared to inactivated virus vaccines [134]. The seroconversion efficacy of FluMist is diminished in adults or in the elderly populations who have pre-existing immunity [135]. Intramuscular trivalent influenza vaccines are recommended in the elderly and the population at high risk of influenza-related complications [136]. Therefore, the choice between the two vaccines needs to be made by weighing the advantage of the attractive intranasal administration of live vaccines against concerns about the risks of using infectious influenza virus at large scale as well as potential gene reassortment with new influenza strains. FluMist seasonal influenza vaccine is one of most successful intranasal influenza vaccine, and effective and well tolerated in children, adolescents, and some adult populations.

In clinical studies with intranasal vaccine delivery, influenza split vaccines adjuvanted with bacterial outer membrane proteins (proteasome) were administered to nostrils of humans using a metered-dose nasal spray pump delivering the vaccine in aerosolized droplets of mean particle size of between 40–50 μM [137]. Individuals who received intranasal proteasome influenza vaccines consistently induced significant increases in serum antibodies specific to influenza as measured by hemagglutination inhibition titers and mucosal IgA antibodies against different strains of influenza virus [138]. The proteasome influenza vaccine delivered intranasally induced significant increases in serum antibody titers as well as mucosal IgA antibodies in healthy adults 18–45 years of age, and proteasome adjuvant formulation was needed to achieve this response [137]. The efficacy of a trivalent intranasal proteosome influenza formulation was determined in an experimental human challenge study using a live influenza virus infection homologous to the vaccine strain. The two dose regimes of intranasal proteosome vaccines were 100% protective against febrile illness while

the single dose showed 65% efficacy as determined with the laboratory confirmation of influenza virus [137]. Development of safe and effective non-live influenza nasal vaccines would be significant since non-live vaccines present no risk of transmission of live virus after vaccination.

### Pulmonary vaccination

Lungs contain local antigen-presenting cells such as macrophage and dendritic cells as well as brochoalveolar lymphoid tissues [139,140]. Pulmonary vaccination was shown to induce both systemic and local IgG/IgA immunity [141]. Aerosol vaccination with inactivated influenza virus droplets ranging in size from 1 μm to 100 μm was assessed in clinical studies [118,142]. After prime-boost immunizations of aerosolized vaccines in humans, substantial levels of cross-reactive mucosal antibodies and satisfactory protection were reported [118,142]. In a comparative study, the efficacy of aerosol administration was relatively lower in protection rates than subcutaneous vaccination in healthy volunteers [143].

In preclinical studies, a small amount of vaccine could induce substantial levels of protective immunity when delivered to the deep lungs in combination with an immune complex adjuvant, and long-term and immune memory responses were demonstrated to be induced by adjuvanted influenza pulmonary vaccination [144–146]. Importantly, pulmonary delivery was shown to be more effective than intranasal immunization, from a study of different respiratory sites of antigen deposition [147]. Consistent with this study, we found that the suspended vaccine volumes used for intranasal immunization significantly influenced the levels of IgG and IgA antibodies detected in sera after intranasal delivery. Use of 50 μl volume of 1 μg of influenza virus-like particle vaccine induced more than 10 fold higher levels of serum IgG antibodies than those from the use of 10 fold less volume (5 μl) of the same amount (1 ug) of influenza virus-like particle vaccine (unpublished data). It is assumed that use of higher volumes of vaccine solution will make it more delivered to the lower respiratory tract and deep into the lungs. It was reported that aerosol infection of mice induced more pathogenicity than intranasal delivery of the same virus [148]. Also, live attenuated virus vaccine that replicates in the lungs was shown to be more immunogenic and protective immunity in mice [149]. Therefore, it might be possible that intranasal immunization with 50 μl of liquid vaccines in mice make substantial amounts of vaccines available targeted to the lower respiratory tracts and lungs as well as to the nasal cavity. This would provide the rationale explanations for the induction of effective systemic and mucosal immunity and cross protection after intranasal immunization with 50 μl of non-replicating influenza vaccines in a mouse model [108–111,114,150].

In summary, despite some promising clinical trials and successful pre-clinical studies, no pulmonary influenza vaccines are available. Effective inhaler devices/systems with reliable reproducibility need to be developed. Also, additional data should be obtained to substantiate the safety of pulmonary vaccine delivery.

## Expert commentary & five-year view

Compared to mucosal routes such as oral or intranasal immunization [112,150], a small dose of microneedle vaccines is expected to be immunogenic and to provide protective immunity

(Table 1). In a mouse model, solid microneedles were used successfully to deliver various forms of influenza vaccines including soluble hemagglutinin protein antigens [151], inactivated whole influenza viruses [58–60], and virus-like particle vaccines [66,67]. Microneedle vaccines could induce comparable or superior protective immune responses in mice, which are similar to the findings reported from clinical studies of intradermal influenza vaccines (Table 1). Intradermal delivery of influenza vaccines reported promising results with older people or infants less than 1 year old [39,42–44,52], suggesting that skin vaccination could be a promising alternative site to intramuscular route. In addition, solid microneedle vaccines may be developed as a patch-based platform enabling self-administration possible by patient themselves. The simple and small size of microneedle systems would facilitate the storage and distribution to the central locations or even to individual households by the postal service. The solid microneedle vaccines stored for a month at 25°C provided good protection [77], indicating a possible alternative to cold-chain current influenza vaccines. However, there is a significant gap in developing marketable application devices for implementing solid microneedle vaccination.

Despite some promising results with solid microneedle vaccination using mouse models, solid coated microneedle influenza vaccines and their efficacies should be tested and assessed in more relevant animals (pig, guinea pig and monkey models) that are considered to have similar skin physiology as humans. Most importantly, efficacy of solid microneedle vaccination should be tested in human clinical studies. We still do not well understand the protective immune correlates and mechanisms of immune responses after delivering vaccines to the skin. Also, the T cell immune responses would be improved by vaccination in the skin as implicated by intradermal delivery of influenza vaccines to the elderly [42]. Similar results of improved cellular immune responses were obtained with microneedle vaccination in the skin of mice [59,61,67]. However, the capability of microneedle vaccination to induce mucosal immune responses still remains to be investigated. As well, cross-reactive immune responses and cross protection after microneedle vaccinations in the skin are to be carried out in future. More importantly, the stability of microneedle vaccines in dry formulation is a difficult problem to be resolved and needs continued studies. Therefore, substantial limitations exist in regarding the development of coating and manufacturing microneedles in an amenable way for commercialization and mass vaccination. Theoretically a patch form of solid microneedle vaccines might be possible but there is a long way for commercialization of solid microneedles and for their practical applications.

Mucosal immunization is another attractive strategy. Its major rationale is that many pathogens enter the body via mucosal surfaces. In terms of convenience of vaccination and acceptability to humans, oral vaccination is an attractive route. The challenge is how we can develop effective oral influenza vaccines. Vaccines that are administered orally are diluted in mucosal secretions, degraded or destabilized in the acidic stomach condition, attacked by enzymes, and excluded by mucosal barriers. So, relatively 10 to 100 fold large doses of vaccines are needed to induce protective immune responses [112]. M cells particularly adhere to microparticles and actively transport them into Peyer’s patches in intestines. Therefore, ligands targeting M cells, incorporated into microparticles would be a promising approach to increase the efficacy of uptake of oral vaccines in intestines [103,152].

Challenges in developing oral vaccination in humans include the requirement for higher doses of vaccines and lower efficacy in inducing systemic antibody responses [95] in contrast to studies in mice [101,112,153]. Another challenge is to better understand how oral vaccination can induce systemic responses and local mucosal immune responses. In addition to inducing mucosal immune responses, much effort needs to be invested to design and develop effective oral vaccines to enhance systemic immune responses as well by clinical trials.

An ideal vaccination at a single site would be to induce protective immune responses in the systemic circulating system as well as at the relevant mucosal sites. In this aspect, nasal/pulmonary delivery of influenza vaccines has particular potential [118,141]. The advantage of nasal route is its superiority in conferring cross protection against antigenically different influenza viruses compared to intramuscular immunization, indicating that nasal immunization might be particularly effective for protection against a breadth of respiratory pathogens [108,110,111,114]. However, most promising results were obtained from mouse studies where intranasal immunization is carried out by liquid drops without using special devices of spray types. Intranasal delivery of inactivated virosome vaccines to humans was shown to be related with some side effects [154,155]. Nonetheless, intranasal route is still a promising one since the successful intranasal vaccines (FluMist, Proteosome influenza vaccine) have been developed and licensed.

Both mucosal and systemic immune effectors are likely to enhance protection against most pathogens. Therefore, taking advantages of intrinsic immunological properties of delivery sites, effective vaccine strategies might be prime-boost combinations that involve mucosal and systemic routes. Mucosal delivery of vaccines might prime the immune system for both systemic and mucosal responses, probably by stimulating the expression of homing receptors by responding lymphocytes [156]. However, it largely remains to be investigated how prime-boost combinations of different vaccine delivery sites may induce protective systemic and mucosal immune responses.

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### Key Issues

- Delivery of vaccines to the skin is possible and would be easy using a patch form of microneedles as a self-administrative device, which might provide improved or comparable protection as intramuscular vaccination.
- The stability issues associated with coating of vaccines into solid microneedles remain to be resolved.
- Due to differences in skin physiology, additional preclinical studies using more relevant animal models (pigs, monkeys) and clinical studies should be performed before developing commercial microneedle vaccines.
- Oral delivery of influenza vaccines is a feasible option but there is a gap in systemic antibody responses reported between mouse animal studies and clinical trials.
- The requirement of high vaccine doses and repeated immunizations is a challenging problem in developing effective oral influenza vaccine formulations.
- There is a limitation in evaluating and interpreting mouse models for intranasal or pulmonary delivery probably due to the lack of information regarding clear description on sites of antigen deposition (upper and lower respiratory tracts, lungs).
- The safety concerns on adjuvants and the availability of reliable inhalation devices for intranasal and pulmonary vaccine delivery should be addressed as future studies.

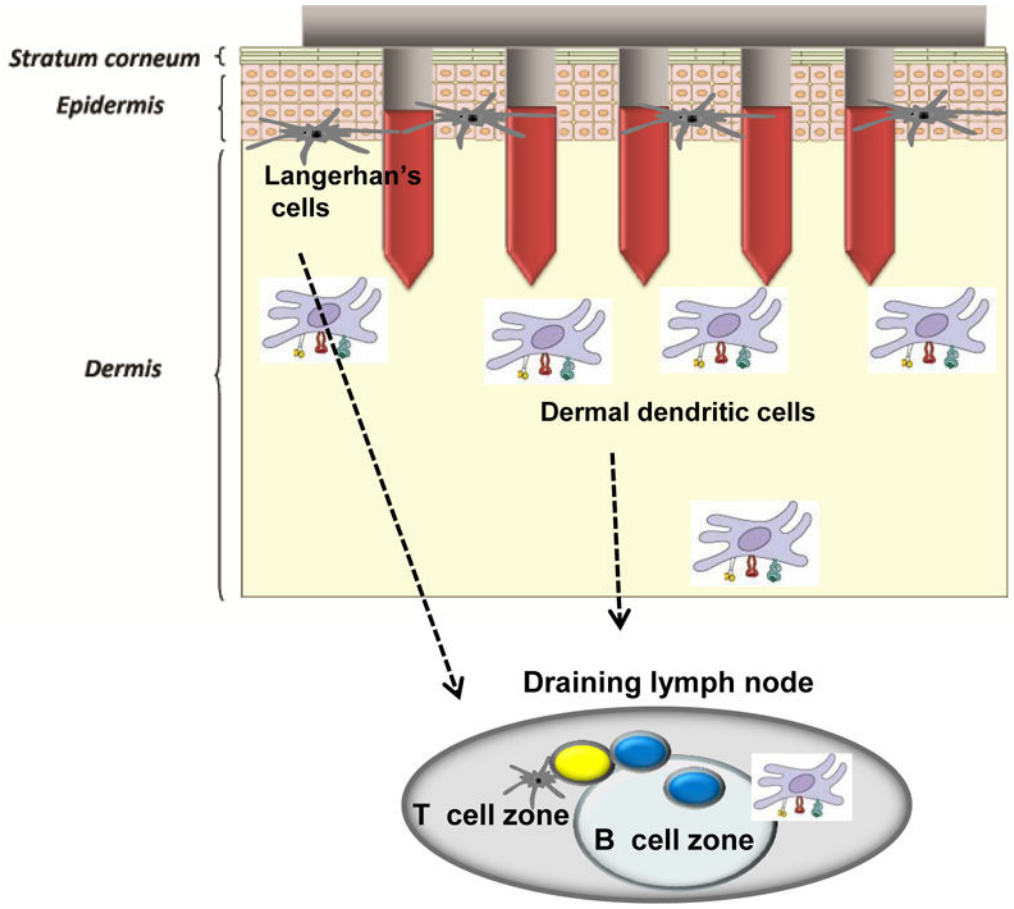


**Fig. 1.**

A diagram of skin layers and microneedle immunization targets. Microneedles with a size of approximately 700 μm in length and 150–160 μm in width are designed to deliver antigens to the epidermal and dermal layers of skin. Microneedle immunization can target epidermal Langerhans cells and dermal dendritic cells (DCs) both resident and recruited from the blood. After capture of antigens, these DCs migrate to the draining lymph nodes and trigger T- and B–cell activation.

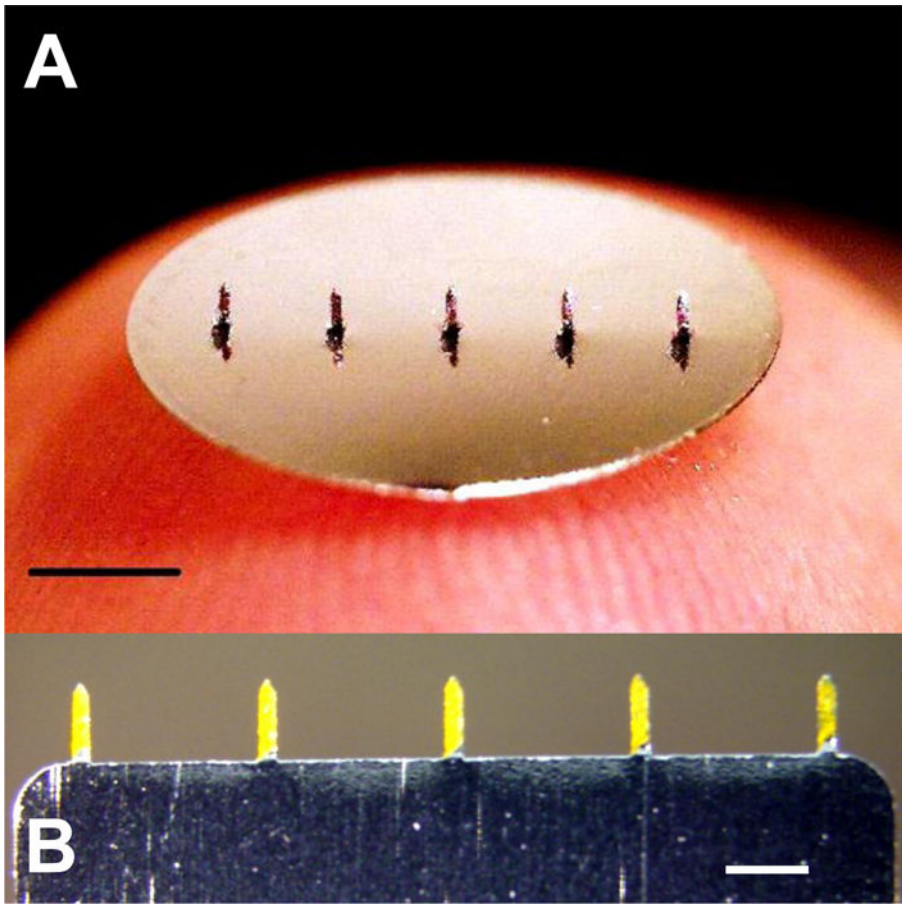


**Fig 2.**

Two different types of microneedles arrays coated with fluorescence. (A) Patch type (scale bar=2mm) coated with sulforhodamine and (B) row-type coated with fluorescein (scale bar=700μm)

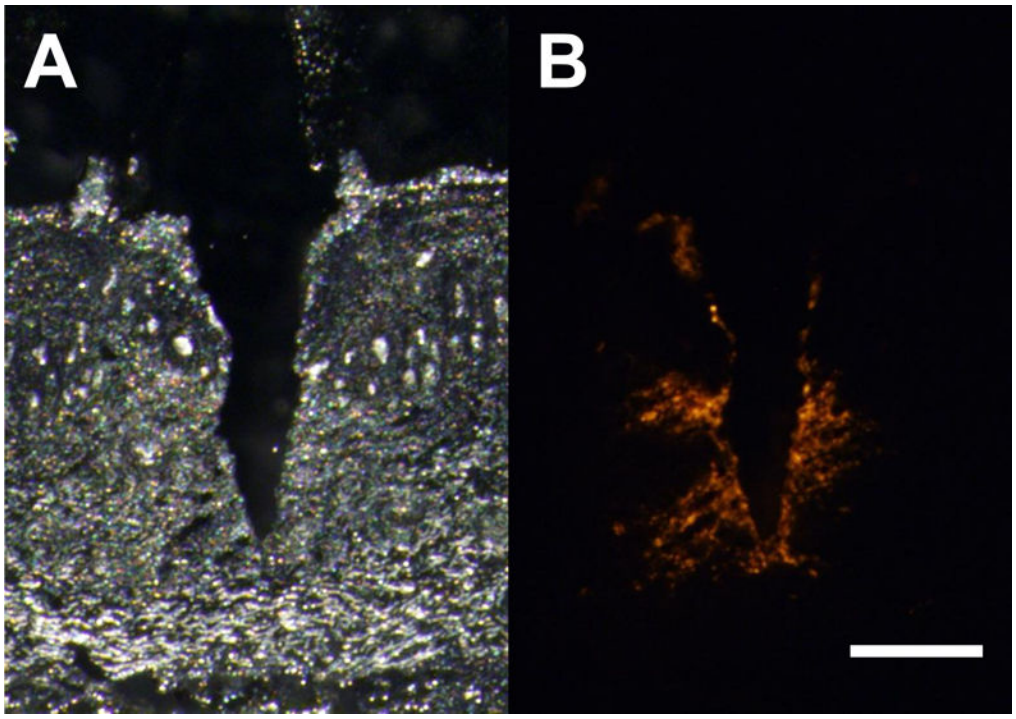


**Fig 3.**

Histology images of insertion and delivery of fluorescence by coated microneedle into guinea pig skin (A) white light and (B) fluorescence images (scale bar=200μm)

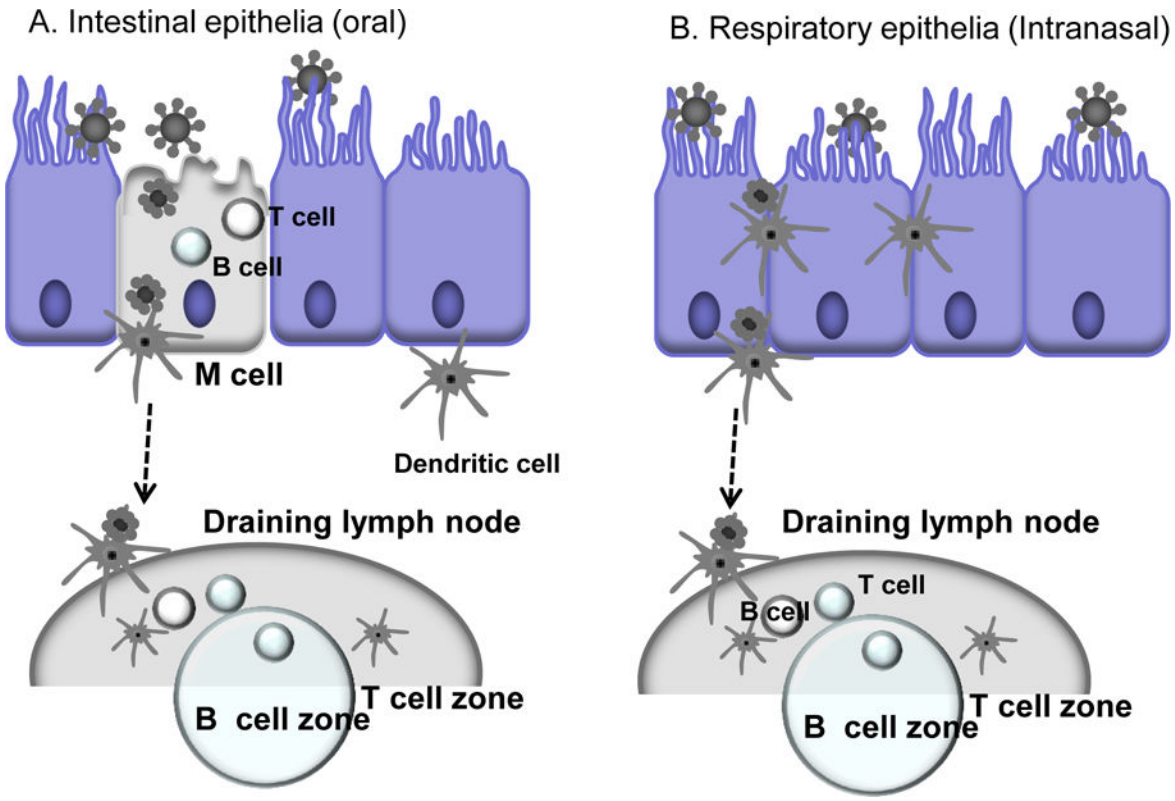


**Fig. 4.**

A hypothetical diagram for antigen sampling at mucosal sites. Mucosal organized lymphoid tissues are important in inducing immune responses after vaccination via mucosal routes [85]. A) Intestinal epithelia: The follicle-associated epithelium contains microfold (M) cells that capture and deliver antigens across the epithelial barrier. The dendritic cells residing at the subepithelium take over antigens from M cells and traffic to the draining lymph node for the induction of mucosal (and systemic) immune responses. B) Respiratory epithelia: Dendritic cells (DC) in the mucosal subepithelial layer are assumed to be mainly involved in sampling most antigens at mucosal sites immediately under epithelia by extending dendrites. DCs from mucosal surfaces travel to the nearest draining lymph node to present antigens to the adaptive immune system.

## Table 1

Intradermal influenza vaccines and microneedle influenza vaccines

| | Intradermal vaccines | Microneedle vaccines |
|---|---|---|
| Target tissues | Dermis | Epidermis and dermis |
| Size | 1.5 mm (BD Soluvia) | approximately 700 μm |
| Formulation | Liquid vaccine | Dried solid vaccine |
| Devices | Microinjection systems (or hollowneedles, needles and syringes) | Microneedles (arrays of steel or dissolving biopolymers) |
| Clinical studies | Yes | No reports |
| License | Yes | No (early preclinical stage) |
| Doses[1] | 9 μg HA in adults (15 μg HA in elderly) | 0.4 – 10 μg total vaccine proteins in mice |
| Stabilizing agent | – | Trehalose sugar |
| Stable storage | Cold-chain | Cold to room temperature |
| Immunogenicity | Comparable or higher | Comparable or higher |
| Protection | – | Comparable or higher |

[1]HA: Hemagglutinin protein; the whole influenza virus was reported to contain 28 – 29% of total viral proteins [110]. Thus, the HA dose in microneedle vaccines in mouse studies is estimated to be 0.12 – 2.9 μg HA.

**Table 2**

Comparative summary of microneedle and mucosal delivery of influenza vaccines

| Delivery | Route | Formulation | Dose | Comments |
|---|---|---|---|---|
| Hollow microneedles | Intradermal | Liquid | Low/comparable | Licensed |
| Solid microneedles | Skin | Solid/stabilizer | Low/comparable | preclinical |
| Dissolving microneedles | Skin | Solid/stabilizer | Low/comparable | preclinical |
| Epidermal powder | Skin | Powder/stabilizer | Low/comparable | Clinic*/preclinic |
| Oral | Oral | Liquid/microparticles | High/adjuvant | Clinic*/preclinic |
| FluMist sprayer | Nasal | Liquid spray | Low (live vaccine) | Licensed |
| Intranasal | Nasal | Liquid spray | High/adjuvant | Clinic*/preclinic |
| Pulmonary | Lung | Liquid/Aerosol | Moderate/adjuvant | Clinic*/preclinic |

* Clinical studies are limited.