Title: Exploiting unique features of microneedles to modulate immunity

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Abstract

Microneedle arrays (MNAs) are small patches containing hundreds of short projections that deliver signals directly to dermal layers without causing pain. These technologies are of special interest for immunotherapy and vaccine delivery because they directly target immune cells concentrated in the skin. The targeting abilities of MNAs result in efficient immune responses—often more protective or therapeutic—compared to conventional needle delivery. MNAs also offer logistical benefits, such as self-administration and transportation without refrigeration. Thus, numerous preclinical and clinical studies are exploring these technologies. Here we discuss the unique advantages of MNA, as well as critical challenges - such as manufacturing and sterility issues - the field faces to enable widespread deployment. We explain how MNA design parameters can be exploited for controlled release of vaccines and immunotherapies, and the application to preclinical models of infection, cancer, autoimmunity, and allergies. We also discuss specific strategies to reduce off-target effects compared to conventional vaccine delivery routes, and novel chemical and manufacturing controls that enable cargo stability in MNAs across flexible intervals and temperatures. We then examine clinical research using MNAs. We conclude with drawbacks of MNAs and the implications, and emerging opportunities to exploit MNAs for immune engineering and clinical use.

1. Introduction

1.1 Vaccines have stopped many diseases but require new advances to address difficult-to-target pathogens.

Vaccines have prevented or limited the spread of many pathogens, leading to global eradication of once devastating diseases such as small pox and polio[1,2]. These technologies harness the body's natural defense mechanisms by causing effector cells to recognize identifying patterns on pathogens, known as antigens, as pathogenic without experiencing the disease. Importantly, vaccines require integration of multiple classes of immune signals to drive efficient, protective responses while avoiding attack of self-tissues[3-5]. Despite the efficacy of vaccines in preventing many infectious

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diseases, there are numerous pathogens for which there are no vaccines, such as human immunodeficiency virus (HIV). Other pathogens rapidly mutate, such as influenza and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), requiring regular vaccine boosters. Additionally, many vaccines do not fully prevent against disease, such as the vaccine for tuberculosis (TB), which can only be administered to a limited population[6].

Immunotherapies, in contrast, are methods to reprogram the body's immune system to effectively mount a response against a disease it is already experiencing. These types of therapies are used to combat diseases such as cancer, autoimmunity, and allergies. These classes of disease are similar in that the immune system incorrectly responds to antigen with respect to the goal needed to combat disease. For example, self-tissue is regarded as harmful in autoimmunity and mistakenly attacked; dysregulated self-tissue is ignored in cancer, and non-harmful foreign material – allergens – are detected as harmful in allergic reactions. While current immunotherapies for these three classes of diseases have improved treatment options[7-9], signficant unmet need remain for each. In cancer, there is need for immunotherapies that are effective against unresponsive tumors and metastases, as well as the relatively narrow groups of patients that meet treatment criteria to receive existing immunotherapies.

Conversely, for autoimmune diseases - during which dysfunctional immune cells attack host tissue - existing therapies are non-curative and focus on managing symptoms. These treatments also have serious side effects due to their lack of selectivity[10]. Allergic reactions, where the immune system detects a non-harmful substance as pathogenic and mounts an excess response, are frequently treated through symptom management or small doses of allergen delivered to tolerize the immune system (i.e., allergy shots). However, in the case of severe, life-threatening allergies – such as those to bee stings and peanuts, these methods are ineffective[8].

From a public health perspective, some of the leading causes of death in developing regions are diseases for which vaccines currently exist, highlighting the need for more robust vaccines that are stable and can be easily transported and administered. Two strategies used to overcome this problem are improved formulations and delivery strategies[11]. Delivery of vaccines via microneedle arrays (MNAs), which are small patches containing short projections to target immune cells, is an emerging strategy to overcome these hurdles.

1.2 Normal immune function allows the body to mount responses against pathogens without attacking healthy host tissue.

During healthy immune function, the body mounts successful defenses against foreign pathogens and eliminate dysfunctional self-reactive (i.e., pre-cancerous or auto-reactive cells) without generating harmful inflammation or side effects. In inflammation, these processes begin with encounter of a foreign pathogen by antigen-presenting cells (APCs) in peripheral tissues APCs recognize antigens, as well as common pathogen-associated molecular patterns that trigger initial inflammatory events specific to the class of pathogen encountered. These signaling events promote APC migration to secondary lymphoid organs (SLOs) including the lymph nodes (LNs) and spleen where APCs present antigens and additional stimulatory inflammatory cues to lymphocytes. These lymphocytes, including T and B cells, differentiate into effector phenotypes or other roles depending on the combination of integrated signals. In autoimmunity and allergic reactions, similar processes take place except that antigens originate from self-tissues or non-harmful species rather than pathogens.

In cell-mediated immunity, APCs such as dendritic cells (DCs) or skin-resident Langerhans cells (LCs) present antigen and costimulatory markers to naïve effector T cells. These T cells can differentiate into different subsets, two of which are cytotoxic ($CD8^+$) T cells which can return to the periphery and destroy cells expressing that antigen, or helper ($CD4^+$) T cells, which assist other types of immune cells in potentiating a response primarily via cytokine production. A subset of these cells remain after vaccine or immunotherapy signals have subsided as memory cells, which can rapidly expand or produce antigen-specific antibodies upon encounter with pathogen expressing that same

antigen. A third subtype, regulatory T cells (T_{REG}s), differ in response to antigen delivered in a dose-responsive manner or with regulatory costimulatory cues. These cells exert tolerizing or anti-inflammatory function and are thus desirable cell subsets to expand to combat autoimmune disease and excess inflammation.

One type of immune cell that $CD4^+$ T cells influence are B cells. B cells in the periphery recognize antigens on pathogens and secrete antigen-specific immunoglobulins, known as antibodies, to potentiate the immune response. These antibodies can recruit other soluble components (complement) to neutralize extracellular pathogens or interact with additional immune cells to eradicate the pathogen or dysregulated cells. Serum levels of antibodies against a delivered antigen are a common way to measure the success of a vaccine or immunotherapy[12].

While the immune system protects us from infections and diseases, these functions can fail in many settings. For example, one way that an infection can become lethal is that the body is not successfully able to recognize and lyse infected cells before the pathogen irreversibly damages critical cells. For example, in certain types of melanomas, the body does not recognize tumor-associated antigens (TAAs) as pathogenic or cannot mount a useful response against cells expressing these molecules. In autoimmune diseases, the body mistakenly targets host tissues, such as myelin in the case of multiple sclerosis (MS) or pancreatic islet cells in the case of type 1 diabetes (T1D). Finally, in allergic reactions, the body mounts a defense against harmless species, increasing the number of histamine-producing cells such as eosinophils and mast cells.

To utilize the immune system to effectively treat and prevent these diseases, vaccines and immunotherapies must be carefully delivered to reach the proper cells in the correct location at the correct time. Geographically, APCs must take up cues at the site of administration and migrate to SLOs, where they can expand effector cells. Immune cues (e.g. antigen and adjuvant) must be taken up simultaneously so that the body mounts the desired response specifically against the antigen. If the immune system encounters either the antigen or the co-stimulatory signals by themselves, undesired tolerogenic effects or broad inflammation can be experienced, respectively[13]. Additionally, the timescale of vaccine delivery is critical as sustained exposure can improve immune responses and outcomes to vaccines[14]. Thus, challenges to achieving the desired immune responses include co-delivering antigen and adjuvant to immune-cell rich populations across a broad time domain.

1.3 Skin provides an ideal location to deliver vaccines owing to a niche of concentrated, specialized immune cells

Because the skin is uniquely dense with multiple specialized immune cells compared to blood, muscle, or fat, the dermal layers are an emerging target for vaccine and immunotherapy delivery. First, the skin contains multiple types of APCs, enabling faster, more potent activation of immune responses compared to the tissues vaccines are conventionally delivered to, such as muscle and the subcutaneous fat. LCs are especially dense in the thin (0.05-0.2mm) epidermal layer of the skin, as well as the mucosal layers of the body[15]. Because they are often the first class of APCs to encounter pathogen in the skin, LCs specialize in trafficking quickly and efficiently to skin-draining LNs[16]. LCs are also involved in promoting tolerance by inducing regulatory effector T cells (T_{REGs}) and activating T follicular helper cells (T_{fh}) to create germinal centers (GCs) within the LNs for antigen-specific antibody production. GCs are specialized microstructures that produce long-lasting plasma cells, which secrete antibodies, and memory B cells. LCs can also activate memory T cells in the skin, without trafficking to immune organs, upon antigen re-encounter[17]. DCs in contrast are located below the epidermis in the thicker (~1.5mm) dermis. They are highly specialized at sampling different species (both self and non-self) throughout the periphery, then presenting this antigen along with costimulatory cues to potently activate effector cells in the LNs and spleen. Compared to the total blood volume within the body, the skin contains approximately ten times more DCs in the upper dermal layers[18]. Thus, the density and prevalence of APCs in the dermal layers make delivering directly to the dermal layers an attractive vaccine administration route.

Poor vascularization of the dermal layers increases its attractiveness as a tissue for vaccine delivery. Because both the dermis and epidermis have few or no blood vessels as well as densely packed cells, vaccines and immunotherapies delivered to these layers are retained for longer periods. This increases the likelihood that a DC or LC can detect the signal and begin mounting an immune response[19].

A variety of clinical and preclinical studies underscore the utility of the skin niche in priming efficient vaccine responses. For example, the first vaccine was against vaccinia virus to protect against smallpox. This type of virus is most effective when delivered through a scratch on the skin (or skin scarification)[20,21]. The monkeypox vaccine represents a recent, relevant example - on August 9th, 2022, the FDA allowed a change in administration route from subcutaneous to intradermal, where data indicates that the desired immune response is achieved with 1/5 the subcutaneous dose[22,23]. This recent example highlights how delivery to the skin can potently activate immune cells in a targeted, specific way to achieve the desired responses. The ideal delivery system for a vaccine would target a high number of APCs in a poorly vascularized space such as the skin but would also address many of the challenges discussed above: specifically, a way to simultaneously deliver antigen and adjuvant to targeted immune cells in a controlled fashion. Microneedle arrays (MNAs), discussed below, fulfill these requirements.

1.4 Microneedle arrays (MNAs) can improve vaccine and immunotherapy outcomes.

Microneedle arrays (MNAs) are small patches with projections sufficiently long to target skin-resident APCs, but too short to reach pain receptors (typically between 100 and 1,100μm long)[24]. These arrays are commonly made of metal or plastic, but can be made of materials as varied as ice[25] or ceramics[26]. Regardless of what material is used to fabricate the array, it must be able to form into sharp points, maintaining mechanical integrity to penetrate dermal layers. Their advantages can be grouped into two broad categories: improved immunologic targeting and public health benefits, as described in **Figure 1**. Both benefits are achieved preclinically through careful engineering and design criteria.

Immunologically, MNAs deliver antigen along with costimulatory cues to skin-resident DCs, LCs, and B cells (**Figure 1A**), which can then migrate to SLOs to drive an immune response by presenting antigen along with costimulatory markers to effector T cells. B cells undergo antibody affinity maturation through licensing by a specific subset of T cells (T follicular helper cells, T_{fh}) in follicles within the LN (**Figure 1B**). Together, these processes improve the number and specificity of cells and antibodies that can neutralize the pathogen. T cells can directly attack infected cells and pathogens, while B cells can shed high-affinity antibodies to neutralize the pathogen **(Figure 1C)**[27]. Antigen and costimulatory cues are delivered into a dense layer of cells in the skin, prolonging exposure to APCs. Together, these features help ensure that a large percentage of cargo is utilized to induce the desired immune response, reducing off-target effects associated with systemic delivery [5,28-30] **(Figure 1D).**

MNAs have inherent and carefully engineered features to achieve these benefits. From a materials engineering perspective, the individual needles projecting from the patch surface enable co-delivery of multiple cues in a solid or small volume format—important characteristics for enabling skin-resident immune cells to take up vaccine components. The material options for creating the matrix, or backing, of the MNA range from polymers to metals to ceramics, and must be strong enough to penetrate the stratum corneum outer layer of skin while remaining immunologically inert[31]. MNAs can be coated, solid, dissolvable, implantable, or a combination of these designs to promote the desired cargo release profile, as visualized in **Figure 1E** and discussed in greater detail in **Section 2: Controlled Release.**

From a public health perspective, MNAs also provide a very efficient way to deliver vaccines compared to traditional routes of administration. Thus, MNAs could help address challenges in regions with limited access to medical professionals. Significant effort is dedicated to ensuring both

component formulations and manufacturing conditions are designed to ensure immunogenicity of the delivered components even after transport or storage in unconditioned climates (e.g., without refrigeration) (**Figure 1F)**. MNAs eliminate medical sharps, have the potential to be applied without a medical professional, and reduce the risk of infection associated with abrading the skin. By reducing the risks and improving the convenience of multiple injections, MNAs can enable unique dosing regimens that traditional routes have difficulty achieving due to logistical complications. For example, MNAs could be applied once daily over 7 days, each containing 1/7th of the desired dose (**Figure 1G**). Prolonged exposure of vaccine components has been shown to improve disease outcomes[32]. Widespread deployment of such regimens with traditional needle injections would require frequent physician visits and likely dramatically reduce patient compliance due to pain and scarring at the site of injection.

In this Review, we examine studies from the past four years to discuss how MNAs can be designed to control the release of vaccine and immunotherapy components. Then, we describe how MNAs are emerging as a potential technology to deliver vaccines and immunotherapies for infection, as well as to treat diseases spanning cancer, allergies, and autoimmunity. We next examine how MNAs can achieve unique dosing regimens and reduce off-target effects owing to their design and logistical benefits. We examine pre-clinical studies showing MNAs maintain or improve disease outcomes compared to traditional injection routes., then discuss how distinct formulation and manufacturing conditions enable stable formulations that maintain immunogenicity. Finally, we assess some of the current shortcomings of MNAs. We conclude this review by examining studies using MNAs in humans, and propose directions that the field may move with this technology.

2. MNAs enable controlled-release mechanisms

It has been well- established that the release kinetics of immune cues and cargo plays a major role in modulating the immune response[13,33-35]. One attractive feature of MNAs is the ability to control the release kinetics of cargo. MNAs typically have two design components: the tines and the backing. The tines are the sharp projections hundreds of microns long that penetrate the skin. In most cases, these are what contain the immunotherapy or vaccine. The backing remains on top of the skin and provides structural support to the tines. Once applied to the skin, depending on the design, the MNA backing can either remain on the skin to enable controlled release of components through the tines. Alternatively, the MNA backing can come off immediately, while the tines can exhibit tunable release kinetics **(Figure 2A)**. For example, tines can be designed to dissolve quickly and release the cargo for a burst release profile. Conversely, a sustained release profile, can be achieved by designing slowly degrading tips to release the cargo over time. Thus, MNAs are tailored for either burst release, tunable release, or sustained release depending on the design. In this section, we will discuss some recent microneedle designs utilized to control the release kinetics of cargo in preclinical models.

2.1 MNAs enable burst release of cargo

In situations where an initial high dose of the drug is required to recruit immune cells, a burst release mechanism is desirable so that the drug/cargo becomes available within minutes to mount an immune response **(Figure 2B)**. Especially for topical applications, burst-release MNAs enable quick release of cargo directly into the dermal layers without pain or the presence of medical professional[36]. One design strategy for burst release kinetics in MNAs is to choose a quickly- dissolving polymer that releases cargo extremely quickly. For example, MNAs fabricated with low M.W. HA led to around 84% of encapsulated cargo cargo (~ 3nm sized Ceria nanozymes) being released rapidly in the first 30s, 99% of the cargo being released in 60s followed the initial rapid release, and complete release being achieved by 300s in PBS[37]. Another group tested the effect of molecular weight of HA for achieving burst-release kinetics from MNAs in *ex vivo* human skin and found that MNA fabricated out of lower M.W. HA (20kDa and 4.8kDa) were completely dissolved in the skin after 10 min application, while the higher M.W. MNA (150 kDa) took 20 min to almost completely dissolve[38] . Another design strategy is to modify the polymer used to fabricate the MNA with a material that dissolves quickly in the skin, such as saccharides, to separate and implant tines. For example, as

shown in **Figure 2B**, HA modified with sucrose enabled rapid separation of diclofenac sodium (a small molecule non-steroidal anti-inflammatory drug)- containing tines from the MNA backing. The HA/Suc modified MNA led to complete separation of the tines from the backing within 30s in a skin mimetic gelatin gel[39]. Choosing the correct polymer allows for burst release of cargo from MNAs and can make the fabrication of MNAs more robust and reproducible. However, this control is limited by the intrinsic properties of the polymers used. Refining the polymer's properties, for example, using a specific molecular weight or using a sugar modified polymer provides greater control over the release rates but adds complexity in manufacturing MNAs.

2.2 MNAs enable stimuli-responsive release

Another way to tune the release kinetics of cargo in MNAs is to use external stimulus or rely on the dermal environment to control cargo release **(Figure 2C)**. This is useful as it adds another degree of control over the release kinetics, enabling improved targeting and localization of cargo. One technique to provide this control is using light as a switch to control the release. For example, several groups have incorporated photo-responsive molecules/particles[40-43], such as black phosphorus[44] along with the immunotherapy into MNAs that either leads degradation of the MNA matrix or release of functional cargo upon activation by specific wavelengths of light. This is different from PTT/PDT as here a light sensitive material is used as a tool to modulate release, not to have a therapeutic effect. In the absence of external stimuli, properties of the skin itself (such as a lower pH) can be used to activate MNAs. For example, magnesium particles incorporated into MNAs led to production of H_2 bubbles upon contact with the lower pH interstitial fluid between dermal layers. This enabled active diffusion of an antibody based checkpoint inhibitor drug (anti-CTLA-4)[45]. Both light- and pH-responsive MNAs can successfully control the release kinetics of therapeutics directly to the dermal layers. These design strategies provide the ability to directly control when and where cargo is released. For this reason, stimuli-responsive MNAs can achieve desired release profiles by changing the relative amount of stimuli responsive particles/cargo.

2.3 MNAs enable sustained release of cargo

Sustained-release MNAs improve the delivery efficiency and bioavailability of many immunotherapies, ultimately improving vaccine efficacy by providing long-lasting signals to immune cells (**Figure 2D**). Furthermore, this type of release profile provides significant public health advantages compared to traditional routes of injection because they enable fewer treatments.

One strategy to enable sustained release in MNAs is to modify the polymer via crosslinking or functionalization[46,47]. For example, a group utilized crosslinked HA MNAs for sustained release of mellitin (a 26 amino-acid polypeptide). They crosslinked HA with methacrylate groups to develop MeHA MNAs that showed a burst release percentage of 56% within 10 min and the rest of cargo exhibited a sustained release profile for 480 min in PBS[48]. Importantly, these studies were performed in PBS; thus, release will likely be slowed in the skin. This time course is important, as in certain diseases sustained release of immunotherapy has been linked to improved disease outcomes[32].

Another strategy that several groups have used to achieve sustained release of cargo using MNAs is to incorporate cargo in nanoparticles[49-51] or microparticles[52,53], then load the particles into MNA tines. Once the polymer matrix degrades, the particles are released. Varying degrees of sustained release can be obtained by changing the size of the particles themselves or the material they are fabricated from. For example, liquid crystalline nanoparticles with rapamycin (an immunosuppressant small molecule) loaded into poly(vinyl alcohol) (PVA): poly(vinylpyrrolidone) (PVP) based MNAs, achieved sustained release of the therapeutic for at least 7 days *in vivo* in mice[54].

While crosslinking, functionalization of the needle matrix itself, or incorporation of nanoparticles allows control over the release kinetics, each have their own advantages. Crosslinking and functionalization, in our opinion, are slightly less complex than incorporating particles, as they does not require one to manufacture two components (the matrix and the particles), which could be a consideration in translation to an FDA-approved manufacturing facility. Additionally, there are many

FDA- approved products on the market that use crosslinked materials to slow degradation within the body[55,56]. Conversely, particles allow for better control over the release characteristics when compared to crosslinking because there are more variables that can be tailored for desired release profiles. For example, pulsatile release, or regularly defined pulses of cargo released over time, can be achieved by loading bilayer engineered particles into MNAs. These particles contain cores and shells or varying diameters, enabling tunable release. Preclinically, such particles are used for the pulsatile release of STING agonists for cancer immunotherapy[57].

The materials themselves, without modifications, can also be used to achieve sustained release of cargo. Polymers that intrinsically dissolve slowly in the skin, or combinations of polymers with varying dissolution rates achieve sustained release of cargo from the microneedles[58-62]. For example, PVA, a swellable polymer, was used to fabricate MNAs. This enabled sustained release of parathyroid hormone for 24h in mice *in vivo*[63]. Using a similar strategy, a group utilized PVA based MNAs containing a long-acting PVP/PEG based NPs loaded with rilpivirine (RPV), to provide a more acceptable choice for treatment of HIV. Based on the results of in vivo studies the MNAs could maintain effective RPV plasma levels over 7 days in humans[64]. Another group utilized chitosan to achieve sustained release of ovalbumin antigen. The group fabricated MNAs with tips made of chitosan loaded with ovalbumin, which in rat skin led to prolonged release of antigen for atleast 4 weeks[65]. This same fabrication strategy could be used to sustain the release of other immunotherapies as well.

Finally, the MNAs themselves can be designed to adhere to the skin for a longer durations, leading to sustained release. One example of this are MNAs designed with suction cups, which increase the contact time with the skin. These MNAs were made with polydopamine (PDA) hydrogels. A peeling test performed after applying the MNAs to porcine skin and found that the suction cup designed MNAs had better adhesion performance than normal conical MNA. In PBS, this design released polymyxin (an antibiotic drug) rapidly in the first 8 hours, then more slowly until a local equilibrium was reached after 2 days. At this point, about 80% of the polymyxin had been released[66]. These recently-employed strategies to achieve sustained release from MNAs enable precise release of components to the skin to achieve the desired response.

2.4 MNAs for Patch-and-Poke delivery

Patch-and-poke delivery involves applying an MNA to the skin, creating channels for topically-administered drug to travel through **(Figure 2E)**. This system is another way to control the kinetics of the cargo, and enable entry of components through the tough outer barrier of skin. Because some therapies are extremely hydrophobic, topical applications make it difficult to cross the stratum corneum and reach immune cells in the dermal layers. For example, an oscillating microneedle pen created channels in porcine skin to deliver immunomodulatory drug[67]. Importantly, this patch- and poke method can be combined with stimuli-responsive MNAs to create burst, targeted entry of components into the dermal layers. For example, anodal iontophoresis (where safe voltage is applied across electrodes in the skin to deliver both charged and uncharged drug) can be performed using the tines on MNAs to penetrate the skin and as electrodes. This can further increase diffusion of drugs into the immune-cell rich layers. For example, a group utilized iontophoresis in combination with microporation to deliver donepezil across the stratum corneum. In this approach, the size and shape of the channels, dictated by MNA tines, controls the diffusion kinetics of cargo. Another way that MNAs improve delivery of topical drugs using the patch-and-poke method is by promoting diffusion of hydrophobic drugs through the stratum corneum[68]. This technique is highly useful for molecules having lipophilicity (log P) higher than three is difficult, as the drug would have a greater affinity for the lipophilic stratum corneum[69]. In this case, the size and shape of MNA tines can control the diffusion kinetics of drugs.

Overall, a variety of release kinetics are achieved using MNAs. MNAs offer strategies to control the release kinetics of cargo. However, it is essential to carefully consider the choice of strategy, taking

into account the specific application requirements and cargo characteristics. Proteins, such as insulin, possess unique properties and sensitivity to external factors, necessitating tailored release strategies. On the other hand, small molecules like rapamycin might have different stability and release requirements. When designing strategies for protein-based cargo, factors such as maintaining structural integrity, preventing denaturation, and preserving biological activity must be considered. Techniques like encapsulation within polymeric matrices or hydrogels, protein engineering for improved stability, and the use of protective coatings can be explored. Each of these strategies comes with its advantages and limitations. For example, encapsulation within polymeric matrices can provide sustained release but may face challenges in maintaining protein conformation and stability. In the case of small molecule cargo, factors such as solubility, release kinetics, and bioavailability play significant roles. Strategies such as the use of microparticles, or nanoparticles, and formulation optimization can be considered. As the MNA field develops, strategies utilizing multiple types of controlled release mechanisms will be employed to precisely control multiple cargos within the patch. For example, one recent study utilized stimuli-responsive materials, delayed release, and prolonged release to precisely tune immunotherapy release. Their patch contained a heat-responsive, dissolvable backing with microparticles to promote hydrogen release when applied directly to a tumor. After dissolution, anti-PD-L1 antibodies were released from a silk-fibrin core to block tumor cell escape[70]. This multi-faceted approach to controlling MNA release will enable greater temporal control over the release of encapsulated cargos.

3. MNAs are being explored to treat infectious, cancerous, allergic, and autoimmune diseases.

Many studies demonstrate that delivery routes (such as intra LN injection or nasal delivery) that specifically target antigens and adjuvants to immune cells can improve antigen-specific disease outcomes compared to non-targeted routes of delivery[71-73]. For this reason, MNAs provide an attractive delivery platform because they promote entry through the tough, stratum corneum outer layer and maximize cargo delivery contact with LC- and DC-rich layers of skin[74]. Immunologically, MNAs target tissue with a high density of specialized APCs and minimize off-target effects. We focus our discussion on recent examples across four disease categories. First, we describe how MNAs are used pre-clinically to enhance vaccine outcomes against infectious diseases, including viruses, bacteria, and parasites. Then, we describe how cancer can be treated using this platform to deliver immunotherapies in preclinical models. Next, we describe how tolerance against autoimmune and allergic diseases can be induced using MNAs in mice. Finally, we explore proof-of-concept studies delivering model antigens with potential to be translated to other disease models.

3.1 Infectious diseases

Many types of pathogens enter the body through the dermal or mucosal layers, such as malaria from a mosquito bite[75] or Clostridium tetani (tetanus- causing bacteria) from a puncture wound[76]. Interestingly, MNAs deliver vaccine cargo in a similar way to penetrate the skin and access immune cells beneath. Vaccine cargo to treat infectious diseases typically contains a specific protein or polysaccharide antigen as well as adjuvants that signal to APCs - such as DCs, LCs, and B cells, that the antigen is pathogenic. Upon delivery, these cells can migrate to SLOs to present antigen to T cells (**Figure 3A**). Overwhelmingly, MNAs used to deliver vaccines against infectious diseases are dissolvable (additional details on specific materials are described below). This is likely in large part due to surface-area limitations of a coated needle, and the smaller volume of a hollow or loaded needle. Dissolvable needles allow a comparatively large volume of cargo to be delivered to the skin, which is important in driving the desired immune response. Additionally, dissolvable needles enable codelivery of antigens and adjuvants to antigen presenting cells, which soluble components flowed through solid or hollow MNAs have a difficult time achieving. A notable exception is vaccines delivering nucleic acid encoding antigen, which are frequently delivered through metal MNAs that conduct electricity. The type of vaccine cargo for infectious diseases is primarily antigen and adjuvants (exceptions being nucleic acid vaccines). Here, we discuss key classes of viral (**Figure 3B**) and bacterial (**Figure 3C**) infections and the mechanisms by which MNAs induce immunity against

them in preclinical settings. For a discussion of clinical infectious diseases, please see **Section 8. Recent MNA clinical trials and studies with humans.**

3.1.1 Viral Infections

Infants are vaccinated shortly after birth because of how lethal infections (especially those caused by viruses) can be in populations with immune systems that are not fully developed[77]. Despite these efforts, influenza, a widespread virus has caused 25-49 million deaths between October 2022 and January 2023 alone[27]. Because of the frequency at which influenza and other viruses mutate, the pathogenic sequence with which an individual is vaccinated each year may differ enough from natural exposure in subsequent years. Thus, some vaccines must be re-engineered and administered annually so that vaccination and circulating strains match as closely as possible. The successful prevention against the flu with a shorter, less painful needle for I.D. delivery (Sanofi's Fluzone microinjection) rather than conventional intramuscular (I.M.) delivery shows that local targeting of skin-resident immune cells can lead to systemic antiviral outcomes compared to I.M. delivery[78]. Features of MNAs that facilitate this less- painful but equivalent response include the delivery of signals to a dense group of cells (**Figure 3D**), activating multiple types of APCs because of the broad surface area and shallow depth reached (**Figure 3E**), and short projections providing a unique opportunity for the needles to be repurposed as electrodes (**Figure 3F**). Recent preclinical studies in mice use MNAs to deliver influenza antigen and a variety of adjuvants to elucidate the mechanism behind this success. One such study in an aged mouse model showed that a dissolvable poly(vinyl acetate) (PVA) and sucrose MNA delivering inactivated influenza virus and adjuvants significantly increases inflammatory cytokines, such as interleukin (IL) 12. Antibody titers were also increased, indicating activation of the humoral immune response. This led to complete protection against disease challenge **(Figure 3G**), indicating systemic effects accompanying local delivery (**Figure 3H**)[79]. Importantly, this study was conducted in an aged mouse model; despite immunological weakening with age (immunological senescence), MNA vaccination still conferred complete protection (**Figure 3I**). Additional studies focus on the role B cells play in conferring this response. Antigen-specific antibodies, especially IgG2, are increased in mice in response to stainless steel MNAs coated with polymers and inactivated influenza vaccine[80]; further, these responses are robust enough to protect mice against disease even 102 days post vaccination[81] (**Figure 3J**). These studies show the outcomes of some of the key benefits of vaccine delivery via MNAs: First, that MNAs provide long-lasting protection against influenza challenge in preclinical models via both T and B effector cell activation, and that they target dense immune cells to improve vaccine outcomes, even in immunosenescence.

MNAs are also investigated for non-respiratory viral infections and provide long-lasting protection. In a Hepatitis C mouse model, stainless steel MNAs delivering quadrivalent Hepatitus C virus vaccine induced significant levels of memory B cells in pigs 42 days after treatment indicating that they arrays conferred protection at least in part due to an antibody-mediated response[82] **(Figure 3K).** While work done with other viruses (e.g. Dengue virus or Vaccinia virus in mice) indicates that MNAs can activate both B- and T cell- mediated immunity[83,84], additional studies on a variety of viral infections will be needed to examine both arms of the immune system to provide a complete picture of the effect of MNAs. Together, these will provide important preclinical data to motivate future clinical trials against a variety of viral diseases.

More recently, SARS-CoV-2 is another example of a respiratory disease that MNAs successfully vaccinate against in mouse studies. MNA delivery of the S1 coat protein as a subunit vaccine elicited potent, antigen-specific antibody responses against the virus's binding protein in mice[85]. Separate preclinical studies examined how deoxyribose nucleic acid (DNA) encoding a SARS-CoV-2 subunit vaccine could be delivered via an electroporation MNA device (a device that delivers a safe burst of electricity, temporarily creating pores in the cell membrane) to rats. This study showed that MNA delivery achieved a similar immune response, compared with a tenth of the dose delivered via I.M. (**Figure 3F**)[86]. In addition to the immunological benefit, this study also highlights how MNAs can be used to stretch supply of difficult- to- isolate antigens. Together, these studies show that MNAs can effectively and potently potentiate a protective immune response via various mechanisms. Particularly in the case of SARS-CoV-2, these immunological benefits with the public health improvements over

traditional routes of administration provide additional support to MNAs as one of the top 10 emerging technologies in the world[87].

3.1.2 Bacterial and Parasitic Infections

Bacterial and parasitic infections, such as malaria, leishmania, plague occur worldwide, impacting millions of people each year. For example, there were approximately 247 million malaria cases worldwide, leading to 619,00 deaths[88]. MNAs similarly can be used to deliver antigen with adjuvant directly to immune cell-rich niches, improving disease outcomes by enabling effector immune cells to recognize, target, and neutralize pathogens (**Figure 3C**). Here, we discuss some of the latest research elucidating the immunologic benefits of using MNAs to improve disease outcomes against bacterial and parasitic infections.

Recent studies show MNAs provide controlled activation over both the cell- and humoral- mediated immune responses; first, we focus on the cell-mediated response. *In vitro* studies investigated the immune response using a dissolvable MNA loaded with a malaria (parasite) transmission-blocking vaccine. These types of vaccines aim to block the maturation and development of reproduction of the parasites within mosquitoes, thereby preventing the spread of the parasite from mosquitos to humans[89]. When murine DCs were treated *in vitro* with cargo from a dissolvable, gelatin MNA, they successfully expressed critical costimulatory markers CD40, CD80, and CD86 upon treatment[90]. In SLOs, these markers bind to receptors on T cells, facilitating their activation against antigens to produce a cell-mediated response.

Interestingly, MNAs provide a platform to elicit different types of immune responses depending on the desired immune response. For example, in a study where an adjuvant and recombinant Leishmania (parasite) antigen were delivered via dissolvable MNA, both B- and T cell mediated immune responses were activated. The primary mediator of this protection was IL-10, an anti-inflammatory cytokine secreted primarily by T_{REGs}, rather than antigen-specific antibody responses or inflammatory interferon (IFN) γ[91]. This shows that the tunable nature of MNAs can activate different pathways depending on the desired immune response by preventing parasite-laden cells from replicating to prevent parasite proliferation. Together, these examples show that MNAs can be engineered to activate T cells, inducing the desired effect against disease.

MNAs also activate humoral responses against bacterial and parasitic infections. For example, when liposome-encapsulated *Yersinia pestis* (Plague bacteria) vaccine was delivered via hollow, metal commercially-available MNA to the dermal layers of mice, antigen-specific IgG titers as well as pro-inflammatory IFN-γ and TNF-α cytokine levels were increased. Increased IgG titers indicate that B cells are activated by MNAs, while higher levels IFN- γ and TNF-α indicate that T cells also played a role in conferring immunity[91]. This indicates that both antibody- and T cell mediated immunity was conferred against the plague bacteria (**Figure 3K**).

Interestingly, recent studies with parasitic vaccines show how the benefits of MNAs could potentially translate into improved public health outcomes. In the Leishmania vaccine study in mice discussed above, MNAs induced a significantly higher ratio of antibodies more protective against polysaccharides (IgG2) than antibodies more protective against proteins and allergens (IgG1) compared to subcutaneous (S.C.) injection, which had an inverse ratio[91,92]. As B cells are the key immune cell type implicated in polysaccharide antigen immunity, MNAs could potentially provide a more effective way to deliver vaccines that have polysaccharide antigens, such as those against pneumococcal disease[93]. Infant and elderly populations, as well as patients on immunosuppressive therapies, have undeveloped or weakened immune systems and tend to respond poorly to these polysaccharide antigens[94] (**Figure 3I**). Incidentally, these demographics would also greatly benefit from these less- painful, self-administered vaccines as all may have difficulty traveling to a healthcare professional. This highlights both the immunologic and logistical benefits of MNAs. This example provides additional motivation to understand the mechanism behind how MNAs elicit the same or improved immune responses compared to conventional routes of bacterial and parasitic vaccine delivery[94].

3.2 Cancer

Cancer is a collection of diseases in which cells grow uncontrollably and spread to other parts of the body (metastasize). Tumors are the tissues that result from the aggregation of these cells[95]. Because cancer is a dysregulation of cell replication, utilizing the body's own immune system to target these cells without impacting healthy cells has generated great interest as a potential strategy to treat the disease. These strategies generally seek to cause healthy immune cells to recognize tumor-associated antigens (TAAs) as pathogenic, creating selective but safe efficacy.

Although transformative for certain patient populations in specific cancers, immunotherapies must overcome hurdles that the tumor microenvironment (TME) presents. First, tumor cells overexpress checkpoint ligands (i.e., programmed death ligand 1 [PD-L1], cytotoxic T-lymphocyte- associated protein 4 [CTLA-4]). In healthy cells, expression of these ligands serves an important regulatory role to return the immune system to homeostasis; for example, by restraining inflammation and proliferation of activated immune cells after an infection has successfully been eradicated[96]. In cancerous cells, however, these ligands are overexpressed, dampening the immune functions of APCs and effector cells to hinder anti-tumor immunity[97-100]. Next, the TME is heterogeneous both between patients and across tumor sites within a patient. For this reason, causing the body to recognize a small part of the tumor as pathogenic may still enable other parts to remain undetected. This can lead to metastasis[101]. Finally, certain types of cancers, such as triple negative breast cancer, express low levels of tumor-specific proteins and/or lack signature overexpression of receptors in cancerous cells. Even overcoming these hurdles, the dysregulated growth of cancer cells makes the tumor poorly vascularized, acidic, and hypoxic[102]. These local changes hinder the ability of immune cells to infiltrate and function in the TME.

Since the discovery of checkpoint blockade therapy in 1996, immunotherapies against cancer have had great success, and many are used clinically[7]. These therapies rely on preventing checkpoint proteins on immune cells to bind to partner proteins on cancer cells that serve as brakes for the immune system. This allows the immune cells to detect the cancer cells as pathogenic and effectively mount an immune response. MNAs provide an exciting platform with numerous benefits compared to conventional delivery. As discussed below, MNAs are being explored to deliver cancer immunotherapies because of their ability to directly target skin-resident immune cells and make immunotherapy cargo available across a broad surface area for a relatively long time (**Figure 4A-D)**. In an interesting proof-of-concept study where MNAs delivered murine melanoma cells, MNAs induced tumors ten times the size of tumors induced by 125 times more cells delivered via hypodermic needle on the flank[103]. This shows that targeting the immunologically rich niche of the skin across a broad surface area can induce more potent, systemic effects than traditional needle delivery of equivalent cargo. In this section, we discuss how the specific benefits of MNAs (**Figure 4E-I)** enable a variety of anti-cancer immunotherapies to be delivered with improved immunological benefits, ultimately enhancing disease outcomes (**Figure 4J-Q).**

3.2.1 Photodynamic and photothermal therapies

Photodynamic therapies (PDT) involve delivery of photosensitizers. These compounds generate toxic reactive oxygen species (ROS) in the tumor microenvironment in response to near-infrared (NIR) light, destroying tumor cells and releasing TAAs and damage associated molecular patterns (DAMPs) that serve as warning signals to APCs. Photothermal therapies (PTT) seek to achieve these same goals with photosensitizers, which generate heat when exposed to near-infrared (NIR) light. In either case, TAAs and DAMPs are made available to prime APCs. APCs can then migrate to immune organs and present these TAAs along with inflammatory costimulatory markers to effector cells (**Figure 4A**). As a result, effector cells can return to the periphery to eliminate the primary tumor and prevent metastasis[104,105]. This class of therapies are approved by the FDA for cancers such as basal cell skin cancer and non-small cell lung cancer[106,107].

MNAs could provide an alternative to this traditional form of delivery because they target the release of TAAs and adjuvants to APCs in the skin. Two currently approved methods of delivery are systemic (I.V. or oral), which can expose surrounding tissues and healthy cells to accumulation of PTT and PDT[108]. Photosensitizers can also be applied topically, but this is limited in its ability to penetrate below the tough stratum corneum[109] (**Figure 4E**). Recent studies in this area are exclusively focused on nanoparticle or nano- micelle loaded MNAs, as these particles can co-encapsulate multiple photodynamic species with drugs[110]. Dissolvable MNAs across all these studies enabled concentration of immunotherapy into the tips of needles and a relatively large volume of cargo to be contained. Combined with the benefits of MNAs, improved disease outcomes can be achieved at least preclinically Below, we discuss the benefits MNAs provide for overcoming these hurdles in mice and discuss how these advantages, as well as certain limitations, could translate clinically.

First, the small volume and broad surface area of MNAs enable unique cargo encapsulation, protection, and targeting (**Figure 4F)**. Because of the number of projections MNAs contain, photosensitizer cargo can be concentrated in needle tips and implanted into tumors (**Figure 4G)**, enhancing local heating and release of tumor compounds [111]. The microscale projections also allow for self-assembly of dissolving microneedle components *in vivo*, such as by electrostatic interactions (**Figure 4H)**. In one recent example, authors used dissolvable MNAs delivering photosensitizing immunogenic cell death-inducer and autophagy inhibitor directly to tumors in mice. MNA cargo self-assembled into micelles upon delivery *in vivo.* This treatment caused APCs to present costimulatory, rather than regulatory, cues to effector cells throughout the body, prolonging survival of mice (**Figure 4J)** [98].

Next, MNAs have been used to target and penetrate topical tumors to release TAAs and DAMPs from within. For example, MNAs delivering nanoparticles containing polydopamine, a PTT agent, successfully generated reactive oxidative species (ROS) within the applied melanoma tumor. The impact of this was two-fold: first, because ROS are toxic to cells, this treatment caused targeted tumor cell death. Second, because tumor cells contribute to the hypoxic tumor microenvironment, their death enabled immune cells to infiltrate. Ultimately, this improved the survival of mice[112]. This study shows that MNAs, with their short projections and wide surface area, can effectively deliver photosensitizers and drive an immune response to the tumor itself.

This penetration also enables MNAs to create the abscopal effect, or local radiation exerting an effect on a remote, non-radiated body part (**Figure 4K)** One recent pre-clinical example examined this by inducing primary tumors on right flank of mice followed by tumoral induction on the left flank five days later. After one day, mice were treated three times with either dissolvable hyaluronic acid MNAs or the same MNAs containing the photosensitizer indocyanine green (ICG) on the primary tumor, causing TAA release. Their results showed that both primary and distant tumor growth was nearly halted. The study indicated that despite local delivery, APCs were effectively primed and effector cells were able to exert a systemic response to prevent distant tumor growth. In the same paper, the anti-metastatic effect of this treatment was also examined. Mice were induced with primary tumors and five days later, inoculated with lung metastases. ICG-loaded MNAs were applied to the primary tumor and not only prevented primary tumor growth, but also significantly decreased the number of metastatic nodules[113]. These studies show that MNAs can be used to effectively deliver PDT to tumors, causing tumor cell death and systemic responses driven by the immune system.

3.2.2 Checkpoint blockade.

Because of their ability to deliver cargo directly to the site of application, MNAs hold promise in recent pre-clinical studies to deliver combination checkpoint blockade therapies (CBTs). Broadly, these therapies are antibodies that inhibit regulatory ligands on tumor cells, such as PD-L1 and CTLA-4, enabling APCs to detect the tumor as pathogenic and effector cells to effectively eradicate the tumor[7]. These therapies are currently administered intravenously, which means that components are delivered to many parts of the body rather than just the target tumor cells alone. However, because PD-L1 and CTLA-4 are still present on healthy cells and an important part of maintaining homeostasis of the immune system post-infection, targeted delivery may improve the success of these

treatments. MNAs are being explored preclinically to achieve this goal, as exemplified by the recent studies discussed below (**Figure 4B)**.

Like many studies involving infectious diseases show, one main advantage of using MNAs to deliver CBTs is their ability to directly target tumor tissue and potently activate both the cell- and humoral-mediated arms of the immune system. Separate studies show that dissolvable MNA delivery of these therapies encapsulated into nanoparticles causes greater numbers of cytotoxic CD8+ T effector cells to infiltrate tumors compared to conventional intravenous (I.V.) delivery of these blockades. These studies were conducted using a B16 melanoma model and 4T1 breast cancer model, indicating that MNAs can be used for a variety of cancers. Furthermore, these immune cells are more effectively polarized towards an inflammatory (rather than regulatory) phenotype within the tumor, indicating that the blockade achieved its goal[100,114]. Similar studies show that the humoral immune response is also activated (**Figure 4L)**: upon delivery, B cells are able to produce antibodies against the tumor antigen and further promote tumor elimination, reducing tumor size and volume[97]. Future work will more explicitly elucidate whether this direct delivery also enables healthy immune function; for example, if mice are successfully able to mount an immune response to another disease, fight off infection, and return to a healthy state post-infection.

Combination therapies of checkpoint blockade therapies with PDT or PTT are used clinically in non-microneedle forms to improve outcomes. Preclinically, MNAs have been studied to deliver these components because their micro-geometry and small volume of projections enables the colocalization of these therapies (**Figure 4F, 4M)**. In fact, recent preclinical studies have shown that MNA delivery of multiple therapies achieves more effective anti-tumor therapies than either photosensitizer or checkpoint blockade therapy alone[111-113,115]. For example, one study showed that a hollow MNA, made of PVP and PVA to support strength and slow dissolution respectively could be fabricated. This MNA delivered cold atmospheric plasma (CAP, a photosensitizer), an anti-PDL-1 antibody, or both CAP and anti-PDL-1 across the full surface area of a melanoma tumor in mice (**Figure 4I**). While each component slowed tumor growth compared to untreated controls, the combination maximally prevented tumor growth and improved survival. This is at least in part due to higher numbers of infiltrating $CD8^+$ and $CD4^+$ T cells in tumors, suggesting that both B- and T cell mediated immune responses can be activated more potently[99]. Another study utilizing cryomicroneedles, or micromolding of cryogenic mediums into MNA molds, encapsulated antigen-pulsed DCs and an anti-PD1 antibody. MNAs delivering both of these components together resulted in stronger anti-tumor efficacy compared to MNAs delivering either alone[116].

3.2.3 Nucleic acid vaccines.

Nucleic acid vaccines are DNA, mRNA, or small interfering (siRNA) encoding for specific antigen and/or immunostimulatory molecules. In the context of cancer, immune cells take up plasmids encoding tumor antigens. Antigen is manufactured by APCs and presented on their surface to immune cells. The primed cells can then return to the periphery, mounting a response against the encoded antigen. Additionally, the nucleic acid itself can act as an adjuvant because extracellular DNA and mRNA act as DAMPs[117]. Currently, DNA or mRNA vaccines are delivered intradermally (I.D.), S.C., intramuscularly (I.M.), often with electroporation[118]. Because of the risk of broad inflammation associated with these systemic delivery routes and the benefit of delivering directly to immune cells, MNAs are being explored preclinically to deliver this type of cancer immunotherapy (**Figure 3C**). These studies suggest that MNAs are an effective platform to deliver these immunotherapies to improve disease outcomes preclinically, with potential to improve outcomes clinically.

First, MNAs provide benefits over current methods of delivery because they directly deliver components to dense immune cells in the skin — a critical step for a vaccine that relies on being taken up by APCs and presented on the surface (**Figure 4E)**. In one recent study that examined the effects of dissolvable MNAs loaded with prostate stem cell antigen DNA, survival in mice was significantly improved during both prophylactic and therapeutic dosing regimens, with less cytotoxicity compared to conventional, I.M. delivery (**Figure 4J,N**) [119]. These results highlight the targeting benefit MNAs, which can reduce non-specific effects. In a different study, MNAs delivering DNA coding for tumor

antigens successfully prevented internal lung cancer metastases. Interestingly, despite local, external delivery to the backs of mice, this treatment prevented internal lung cancer metastases. This result indicates that a systemic immune response is achieved, despite local delivery to the skin[120] (**Figure 4O,P)**. Together, these pre-clinical studies show that delivery of nucleic acid-based cancer immunotherapies can successfully be delivered using MNAs, enabling potent therapeutic efficacy against cancer in mouse models.

Unlike MNA- delivered DNA vaccines encoding tumor antigen, preclinical studies investigating delivery of small interfering RNA (siRNA) vaccines require delivery directly to tumors. Using this strategy, tumor cells take up the siRNA that suppress translation of checkpoint ligands, which helps to overcome the immunosuppressive environment[121]. Similar to studies where PDT/PTT and checkpoint blockade therapies are delivered directly to tumors on the skin, a key benefit of MNAs is that tumor cells can be targeted within the tumor itself, across a broad surface area (unlike topical delivery or soluble I.D. injection) (**Figure 4E, I, O**).

Finally, MNAs themselves can be used to electroporate cells locally and safely, delivering cancer immunotherapies. One recent study showed that electroporating murine colon carcinoma cells to deliver siRNA coding for a silencing PD-L1 drastically improved survival, reduced tumor growth, and blocked PD-L1 expression compared to untreated controls[122]. This type of MNA functionalization could theoretically also be used to deliver DNA vaccines to healthy skin, targeting APCs located in the dermal layers. Because of their ability to target immune cells, potentiate systemic responses, deliver signals deeply within tumors, and easily repurposed geometry for electroporation, MNAs are an excellent platform to deliver nucleic acid vaccines for cancer.

3.2.4 Direct delivery of tumor antigens and adjuvants

MNAs can directly deliver TAAs and adjuvants to immune-cell rich dermal layers. This type of cargo can be delivered distally from the tumor site, eliminating the need for APCs to overcome the immunosuppressive TME to detect tumor antigens and adjuvants (**Figure 4D**). Despite local, distant application however, this type of delivery still causes systemic immune responses to increase survival overall. Below, we discuss preclinical studies investigating the local application of MNAs exerting a systemic effect.

The micro-geometry of the needles also enables colocalization of TAAs and adjuvants (**Figure 4G)** which is critical for proper antigen presentation (**Figure 4Q)**. Recent studies have utilized this unique feature to combine TAA and adjuvant for small-scale delivery: for example, layers of positively-charged antigen and negatively-charged adjuvant can be coated onto needles forming polyelectrolyte multilayers without the use of non-biologic polymer layers[123]. Another study fused a model tumor antigen with a viral core. APCs were able to recognize the antigen as pathogenic because the viral core was detected as foreign; as such, the core itself served as an adjuvant, enabling antigen recognition[124,125]. With this adjuvant/antigen codelivery directly to immune cells, MNAs can not only promote recognition but also cause potent effector cell responses[126]. In one recent study, prophylactic treatment with MNAs promoted a memory T effector cell response. Because memory cells are implicated in preventing metastasis, this study further shows that MNAs are an effective way to directly deliver tumor antigens and adjuvants to immune cell layers[127].

MNAs have also been used preclinically to deliver tumor lysate, enabling immune cells to interact with portions of the tumor itself sans the immunosuppressive environment. These methods include delivery of tumor membranes surrounding nanoparticles containing adjuvants via rapidly dissolving, poly(vinyl propylene) MNAs[128] or whole tumor cell lysate delivered into pores in the skin made by commercially-available stainless steel MNAs[129]. Interestingly, both these methods enable the rapid delivery of cargo into the skin. These methods prolong survival of mice and increase levels of TAA-specific antibodies compared to untreated controls. Future studies will be required to understand whether quick-releasing material and delivery properties are required to achieve the same efficacy. Ultimately, these studies underscore the ability of MNAs to co-deliver immune signals directly to immune cell-rich layers (**Figure 4F)** successfully causing the body to prevent or eliminate tumor

growth[71] (**Figure 4J**). Because both APCs and T cells contain pathogen-associated molecular pattern receptors, investigating the adjuvant effect on each cell type could promote rational immunotherapy design and delivery to each of these cell types. One important consideration for changing I.V. to MNA delivery of cancer immunotherapies, though, is that some therapeutics necessitate a large volume to be delivered in humans. These drawbacks are discussed in greater detail in **Section 5. MNAs provide immunologic and logistical benefits to conventional vaccine delivery routes.**

Delivering a therapeutic dose of immunotherapy may not be volumetrically or practically possible via MNAs. Nontheless, MNAs at least hold promise in cancers of the skin- and mucosal layers. With a clinical study currently recruiting for the delivery of chemotherapeutic Doxorubicin, the future of MNAs in cancer immunotherapies is promising[130].

3.3 Immune tolerance

In addition to inducing an inflammatory response, MNAs have potential to induce immune tolerance to combat inflammation and autoimmunity. Immune tolerance uses natural mechanisms to prevent responses against innocuous or self-antigens [131,132]. In healthy individuals, the immune system is unresponsive to host tissue. However, during autoimmune disease, autoreactive immune cells attack self-antigen, causing inflammation and pathogenicity **(Figure 5A)**. Currently, there are no cures for autoimmune diseases, and the current treatments help manage the symptoms but do not treat the underlying cause[133]. Treatments for chronic autoimmune disorders such as MS and T1D require lifelong treatment, which leads to a high cost and compliance burden[134].

During allergies, the immune system induces an immune response directed at foreign species known as allergens, which the body perceives as harmful **(Figure 5B)**. This excess and unregulated immune reaction leads to inflammation and other allergic symptoms. Currently, allergies are treated mainly using allergen-specific immunotherapy (AIT), which involves sensitization to allergen by gradually increasing antigenic doses. However, this immunotherapy and most current treatments for allergies carry a risk for side effects[135]. They also involve multiple injections or doses over 3-5 years, leading to a high cost and compliance burden. Thus, for both autoimmune diseases and allergies, promoting immune tolerance that easily cures the condition could lead to better treatments. In this section, we discuss recent experimental strategies using MNAs that are being explored preclinically for autoimmune diseases and allergies to promote tolerance.

Inducing and promoting antigen-specific tolerance could be transformative in developing more effective and selective treatments for autoimmune diseases and allergies. Tolerance is promoted by three key drivers: i) low-dose antigen presentation[136], ii) sustained and persistent antigen presentation[137,138], and iii) antigen presentation without inflammatory cues[136]. MNAs can be designed to achieve the above-mentioned drivers of tolerance, as they target the immunological niche of the skin, can be modified to achieve sustained and persistent low-dose antigen presentation **(Figure 5C),** and offer painless administration of cargo without inflammation **(Figure 5D)**. By exploiting drivers of tolerance, MNAs are an ideal platform to create a tolerogenic environment at the site of application **(Figure 5E)**.

Although explored only recently, a growing collection of skin-focused studies reveal principles through which MNAs can promote tolerance. These typically exploit at least one of the three previously mentioned fundamental drivers of tolerance. For example, a recent study using Proinsulin (PI)-coated stainless steel MNAs to target epidermal LCs utilized the principle of delivering immune cues without inflammation. By exploiting this feature, MNAs were able to induce increased antigen-specific T cell proliferation compared to traditional ID injection of proinsulin in healthy mice, indicating that the immune response was antigen-specific[139]. Another example in a mouse model of Type 1 Diabetes Mellitus showed that by delivering Schistosoma japonicum (a parasitic worm) antigen, the immune response shifted from a more inflammatory (T_H1- polarized) response to a more regulatory (T_H2- type) response. This significantly reduced blood glucose and improved pancreatic injury in mice[140]. In a similar study, a clinically approved hollow MNA delivery system (MicroJet600) delivered self-antigen conjugated to gold nanoparticles to target specific tolerogenic

cell populations in the skin (LCs) and deliver cues without inflammation. This led to reduced activation of naïve T cells against the autoantigen[141].

Beyond changing what is delivered using MNAs, MNAs themselves can also be designed to promote tolerance. One recent study changed the geometry of the microneedle itself as a driver of tolerance to reduce inflammation. They did this by fabricating a polycarbonate slit-designed MNA. When coated with a model antigen, ovalbumin (OVA), these MNAs led to preferential migration of LCs to the draining LNs and lower inflammation compared to a traditional, conical-shaped MNA design[142]. These studies reveal that design criteria for the needles can also promote tolerance; for example, controlling the size of the MNAs to target specific immune cells and or designing a less inflammatory MNA. These design insights can be applied to other autoimmune diseases.

These tolerogenic designs for MNAs have also been applied to allergic settings. For example, in a recent study, peanut- sensitized mice were treated for five weeks with a single dose per week of either peanut protein coated on stainless steel MNAs that target specific layers in the skin or a clinical benchmark (EPIT patch) containing peanut proteins. Treatment with peanut-protein coated MNA was superior to the clinical benchmark upon oral challenge with peanuts[143-145]. A recent clinical trial uses the design principle of targeting specific layers of the skin to promote tolerance. In this clinical trial, an MNA was used to poke holes of a specific size, and then milk protein was applied to the skin to treat milk-protein allergy. This approach led to tolerance specifically towards the milk protein allergen (see also **Section 8: Clinical trials**). A study examining the immune response against house dust mite allergen in mice focused on the ability of MNAs to target skin and reduce inflammation. House dust mite (HDM) allergen was loaded into hyaluronate MNAs and tested in a house dust-mite model of asthma. The MNAs reduced inflammatory cytokines (IL-4, IL-5, and IL-13) and HDM-specific antibodies while increasing regulatory cytokines (IL-10 and TGF-B) compared to the clinical benchmark[146]. The other design advantage of MNAs is their ability to deliver multiple immune cues, which is beneficial in delivering multiple cargos to synergistically modulate the immune response towards tolerance[147]. In one study, stainless steel MNAs were coated with OVA and an agonist (CpG). These MNAs led to desensitization of the mice and the upregulation of regulatory cytokines[148].

These recent studies highlight the ability of MNAs to exploit the drivers of tolerance and treat autoimmune diseases and allergens. Most studies utilize the MNAs' ability to reduce inflammation and target the skin. However, there is still potential to incorporate some other tolerance drivers to create more potent MNA-based immunotherapies.

3.4 Model antigens.

Delivery of model antigens in vaccine formulations enables the use of a broad range of tools, techniques, and disease models unavailable for many specific types of diseases. The most common of these antigens are derived from OVA. Studies with MNAs containing these antigens provide important mechanistic information, specifically regarding the trafficking of immune cells to the site of application and immune organs, as well as how effector cells are polarized to exert systemic responses. Together, these model antigen studies enable rigorous characterization of the body's response to antigen delivery with MNAs which can be applied to disease-relevant antigens. They also enable further rational design of MNA-based vaccine and immunotherapy approaches to infectious, cancerous, autoimmune, and allergic diseases. Specifically regarding materials, the studies examined included dissolvable, solid, and coated MNAs. These varied designs and materials have enabled a broad range of release profiles. With novel applications of MNAs requiring specific materials, release profiles, and volumes, model antigens will provide an excellent test bed to evaluate their efficacy.

Model antigens provide a unique lens to examine the benefits of MNAs, starting with the targeting abilities of the actual needles themselves. A recent study compared how a flat, disc patch loaded with OVA and cholera toxin adjuvant to an identical patch containing microneedle projections. When applied to skin, the projections were critical for increasing the total IgG (antibody) titer compared to the flat patch[149]. This example highlights the penetrative ability of MNAs through the stratum

corneum and to the immune-cell rich layers below. It may motivate future studies investigating how conventionally topically-applied therapeutics can be improved by delivery on, in, or through MNAs.

MNA delivery of model antigens also enables mechanistic study of how local delivery of cues translates to systemic responses. For example, delivery of OVA via a slowly dissolving hyaluronic acid MNA (>10hr for drug to be released) directly to the dorsal skin of mice activates skin resident DCs, causing migration to the LN. There, the DCs can present antigen and cause naïve T cells to become mature $CD4^+$ and $CD8^+$ T cells[150]. This granular study of which types of cells are recruited to the site of application and SLOs throughout the vaccination process is made possible because of model antigen delivery. Studies with these model antigens will provide important information for how changing MNA design improves disease outcomes; for example, how skin- resident DCs are temporally activated with a faster or slower release of vaccine components achieved by crosslinking or otherwise chemically modifying the MNA matrix.

MNA-delivered model antigens also provide a test bed for proof-of-concept ideas with potential applications to other types of antigens and disease models[151-153]. For example, solid plastic MNAs loaded with the model antigen OVA can be used to investigate the efficacy of innate inflammatory cues such as toll-like receptor agonists (TLRas). TLRas act as adjuvants, indicating that co-delivered antigen is pathogenic, and target TLRs found in and on immune cells. Both antigen and TLRa were effectively delivered to the dermal layers after MNA puncture, leading to accumulation in draining LNs in part due to uptake by targeting the skin-resident APCs[154]. Excitingly, differences in APC phenotype were observed by changing the relative ratio of multiple adjuvants[155]. This highlights another advantage of model antigen delivery with MNAs: the *in vivo* effect of adjuvant delivery to APCs can be isolated, contributing to rational design approaches for vaccine adjuvants[156,157]. Knowledge gained from these studies can be applied to other types of disease models.

Model antigen delivery via MNAs can also provide important information about how immune cells migrate in response to prolonged skin delivery of vaccine and immunotherapy components. For example, MNAs loaded with OVA caused infiltration of macrophages and neutrophils to the site of application. Ultimately, this increased B cells and effector $CD4^+$ T cells in LNs, which resulted in higher antibody levels systemically[158].

Underscoring the ability of MNAs to target APCs are recent studies in which MNAs were used to sample the dermal layers for infiltrating antibodies and immune cells against a model antigen[159]. Traditional needle- based sampling techniques use a conventional needle to draw up blood, then identify antigen-specific T cells and antibodies to determine vaccine response. MNAs can be used to draw up interstitial fluid post vaccination and identify T_{RM}s, which are critical for conferring long-lasting immunity. While MNAs for disease detection are outside the scope of this review, their ability to detect immune cells at a comparable level to traditional needle-based sampling techniques underscore the ability of MNAs to target these cells in the dermal layers. This type of mechanistic work will help inform critical future studies with disease-relevant antigens.

4. MNAs reduce off-target effects and enable unique dosing regimens

As previously discussed, one distinct advantage of MNAs is their ability to deliver vaccines and immunotherapies directly to the immune cell-rich niche of dermal layers to achieve improved antigen-specific disease outcomes. Because of this direct targeting and the high percentage of cargo delivered to immune cells rather than other cell types, MNAs enable a reduction in off-target effects and often require less vaccine component mass delivered to achieve similar disease outcomes preclinically. In one study conducted in mice, decreasing doses of a vaccine containing OVA and adjuvant were delivered via vaccine- coated MNA, I.D., S.C., or I.M. Even at the lowest dose (twelve times lower than the highest dose), MNAs elicited the highest OVA-specific IgG titers in the serum of mice[160]. When comparing the amount of cargo in organs, MNA delivery reduced accumulation in non-immune organs compared to other delivery routes of the same dose. This study, as well as others showing similar trends, suggest that MNAs enable unique dosing regimens unattainable by traditional methods.

Conventional delivery has difficulty achieving these same frequent regimens due to off-target effects and logistical difficulties in patient compliance.

Since MNAs are easily transported and relatively painless to administer, there is also potential to improve patient compliance. As **Figure 6A** describes, MNAs could offer the ability for future users to apply MNAs themselves. Without generating medical sharps or leaving their homes. These features are not obtained through conventional needle delivery. Furthermore, a large target area and superficial application ensure that repeated use of MNAs does not result in scarring associated with repeated injections (**Figure 6B).** Generally, MNAs are regarded as less painful than SC, ID, or IM injection. 70% of subjects in a Phase 1 influenza vaccine clinical trial preferred MNA delivery over nasal spray delivery or I.M. injection[161] (see (**Figure 6C** and **Section 8: Recent MNA clinical trials and studies with humans**). Although this idea has not yet been rigorously investigated over an extended time period, future public health studies may test the hypothesis that patients are more compliant and receptive to an MNA- application regimen compared to conventional delivery.

With this biological and public health motivation, many recent studies have investigated how MNAs can be used to reduce vaccine accumulation in non-immune organs. Because cargo is targeted directly to immune cells, MNAs enable frequent, unique dosing regimens to achieve the same immune response as conventional delivery methods with reduced off- target effects. In this section, we first examine recent studies showing how MNA delivery of vaccines and immunotherapies reduces off-target effects, often improving biodistribution to critical immune organs. Next, we link this improved biodistribution with unique dosing strategies, and examine how a lower dose of immunotherapy or vaccine delivered via MNA improves disease outcomes. Together, these studies highlight another critical advantage of MNAs compared to traditional vaccine delivery.

4.1 MNAs enhance cargo uptake by immune cells and drainage to LNs

As discussed in Section 1.2, cargo must typically reach SLOs for immunotherapies and vaccines to be effective. This can occur either by passive drainage or active transport by APCs[71]. Accumulation of vaccine and immunotherapies in non-immune organs and tissues, such as the kidneys or blood, does not aid in producing an immune response and can produce systemic toxicity. Because of their small-volume delivery and direct targeting of densely packed, immunologically rich dermal layers, MNAs are an attractive strategy to reduce off-target effects throughout the body while promoting immune cell uptake of cargo and drainage to LNs[162] (**Figure 6D**). For example, MNA delivery of chemotherapeutics, such as Doxorubicin and paclitaxel, promoted uptake in LNs and decreased uptake in the kidneys compared to a dose-matched I.D. or I.V. injection[163]. Interestingly, both components were delivered via transfersomes, which are ultra-deformable particles that improve encapsulation and delivery of drug across the dermal barrier. Combined with skin-penetrating needles, these structures were able to improve immunotherapy drainage to immune organs. Delivery of a model dye and anti-CTLA-4 via MNA confirmed this result, with significantly higher uptake in draining LNs and significantly lower concentrations in the blood and liver compared to I.V. injection[164,165]. Both studies showed that once in LNs, therapeutics and vaccines delivered via MNAs effectively polarized DCs towards inflammatory phenotypes and B cells to secrete antibodies against the delivered antigen. This improved activation can lead to improved immune outcomes. For example, hollow MNAs delivering a fifth of a full-dose I.M. immunization improved protection against the B. Pertussis bacteria, as measured by the number of colony-forming units in various organs[166].

Despite these lower systemic levels, delivery of vaccine components via MNAs can achieve similar or higher antigen-specific antibody titers compared to dose-matched delivery through conventional routes. In one recent study, mice were immunized against a virus (Polio) and bacteria (Tetanus, Diphtheria, and Pertussis) using a combination vaccine. Vaccine was delivered using either MNAs or a five times higher dose I.M. Results showed that DCs and T cells in both the spleen and lungs secreted significantly more inflammatory cytokines characteristic of an antigen-specific effector cell response when treated with MNAs compared to I.M delivery. MNA treatment also activated B cells to produce significantly more antibodies against all implicated pathogens[166]. This study is particularly

interesting for a few reasons. First, MNAs caused a distant response in organs required to mount a response against the disease. While I.M. injection allows cargo to enter the blood stream more quickly, local delivery still exerted a systemic effect. Second, the response to both bacterial and viral vaccine components delivered in the same device is one example of the versatility of MNAs as a platform to deliver vaccines against a variety of disease antigens.

Collectively, these studies suggest that delivery of immunotherapies and vaccines with MNAs results not only in a directed immune response towards the target antigen or disease, but also eliminates dose toxicity and off-target effects associated with conventional forms of delivery.

4.2 Improved biodistribution enables unique dosing regimens

In addition to enhancing uptake by immune cells and delivery to immune organs, MNAs also enable more frequent and unique dosing regimens because of their ease of application, improving biological outcomes (**Figure 6E**). In one recent study, fractional doses of tetanus toxoid, subunit influenza vaccines, and a measles vaccine were delivered daily via MNAs over the course of seven days. This resulted in higher B- and T cell mediated immune responses compared to a single bolus I.D. injection. Importantly, this study showed that MNAs were able to activate both antibody- and cellular- mediated immune responses against three types (viral, subunit viral, and bacterial) antigens more effectively than bolus injection[167]. Further, a study delivering repeated fractional doses of Diphtheria toxoid to mice via dissolvable, HA MNAs found that unadjuvanted vaccine delivery yielded similar antigen-specific antibody levels to adjuvanted vaccine using traditional S.C. injection[168]. In this way, the mechanical force across a broad surface area highlights the ability of MNAs to act as a "mechanical adjuvant", reducing the need for additional inflammatory cues and potentially reducing off-target vaccine effects[169]. Both studies show that by prolonging the exposure of antigen-presenting cells to cargo, MNAs can enhance the immunogenicity of vaccines.

MNAs are poised to enable these types of frequent dosing regimens because patients can self-apply patches from the comfort of their own homes, in contrast to conventional needle delivery. As such, they provide a promising way to improve immune outcomes via continuous dosing. Future work will be required to examine not only the benefit between MNA delivery and more traditional routes, but also how design criteria can be carefully selected to achieve the desired immune response. For instance, OVA delivered with dissolving MNAs induced significantly higher antibody titers compared to an equal dose of antigen delivered via a hydrogel backing on MNAs [170]. Furthermore, an MNA that targeted LCs residing in the epidermis of the skin specifically induced lower erythema and higher LC migration out of the skin compared to an MNA that targeted the deeper, dermal layers containing more dermal DCs[171]. This study, amongst others, provides an example of how MNA design can be tuned to achieve the desired immune response[172]. Thus, the field is is well-poised to investigate not only how microneedles can improve delivery compared to conventional routes, but also how MNAs can be improved to achieve the desired immune response.

5. MNAs provide immunologic and logistical benefits to conventional vaccine delivery routes

Choosing a delivery route for vaccines and immunotherapies is particularly important for components properly localizing to exert the desired immune response. Recent studies show that the route of administration can dictate which cells are targeted and the type of immune response produced[171,173]. Regardless of route, delivery methods via needle generate sharps, are painful, can cause scarring, deliver to a needle-width surface area, and frequently require a health care professional. All routes result in relatively quick clearance of components from immune-cell niches. MNAs can improve these routes by directly delivering to a large surface area of immune cells, delivering solid- or small-volume cargo to retain components at the site of delivery, are painless, do not cause scarring, and do not require healthcare professionals for administration (**Figure 7A**). Here, we use recent studies to show how these benefits of MNAs lead to improved vaccine and immunotherapy outcomes.

5.1 Intradermal (I.D.) Injection

I.D. injections puncture the epidermis of skin (50-200μm) to deliver drugs to the thicker dermis (1,500-3000μm) (**Figure 7B**). This method of delivery bypasses the LCs in the epidermis, while targeting macrophages and APCs in the dermis. Currently, small pox, tuberculosis (TB), 20acilli Calmette-Guerin (BCG), and rabies vaccines are delivered in this manner[19] while clinical trials for a variety of other diseases are underway. Interestingly, a meta-analysis of 30 influenza studies with 177,780 participants found that influenza virus delivered I.D. caused significantly higher titers of influenza-specific antibodies compared to the same dose delivered I.M.[174], providing motivation for delivering vaccines directly to the dermal layers. As discussed earlier, the switch from S.C. to I.D. delivery of the monkeypox vaccine enabled 1/5 the dose to be delivered to achieve the same immunological outcomes [22]. Despite the promise of this delivery route, MNAs could help overcome some of the shortcomings of this delivery route; specifically, the depth of delivery can vary between physicians, and patients frequently report pain at the site of injection[175]. MNAs hold promise in improving this route of administration while still targeting the same dermal immune niche. Here, we examine recent studies investigating how the physical properties of microneedles (such as increased surface area, solid- or small- volume cargos, broad targeting of immune rich niches, fixed length, and painless application) translate to improved memory immune responses, activation of multiple immune arms, consistent application, and overall improved disease outcomes.

Although I.D. injections may be closest to MNA delivery regarding the depth of tissue targeted, MNAs increase retention of immune components in both skin and LNs compared to an equivalent I.D. injection. This is evidenced in one study by significantly increased antigen signal in draining LNs 14 days after treatment compared to I.D. bolus injection[176]. This increased exposure to antigen also increases interactions between B cells and T cells in GCs[14,177]. In one study comparing an implantable solid silk MNA to I.D. delivery of an HIV vaccine, MNAs generated higher numbers of GC B cells in the lymph node compared to I.D. injection of the same vaccine[176]. The retained needles in skin likely provided a source of vaccine, enabling sustained immune responses. Indeed, presence of these germinal cells are indicative of memory and protection against reinfection. They are also important in coordinating the effector immune response between B and T cells. An increase in HIV-trimer specific $CD8^+$ cells after a prime/boost regimen provides further proof of this memory response and linking of the B and T cell responses. Furthermore, this effect is systemic as evidenced by findings in a similar study where MNA delivery of vaccines components caused B cells to produce higher antigen-specific antibody titers compared to I.D. injection[178].

One possible mechanism for this finding is that MNAs are a fixed length, which is to say that their length can be specifically engineered to reach DCs, LCs, or both[179]. Conventional I.D. injections vary between healthcare professionals and may target different cell populations depending on the depth of application[180]. While the B cell- mediated immune response to MNAs compared to I.D. injection has been well-characterized, examining differences in adaptive effector cells, such as T cells, will provide important information for designing rational vaccine delivery strategies. For example, resident memory T cells (T_{RM}) in the skin have been implicated in preventing cancerous melanoma cells from metastasizing throughout the body. Directly targeting these cells via MNAs may provide a potent way to protect against the formation of new tumors.

5.2 Subcutaneous (S.C.) Injection

In contrast to I.D. injections, S.C. injections are delivered to fat between the skin and muscle tissues using approximately 4-8mm long needles **(Figure 7C).** This route of injection is commonly used for polio, combination measles/mumps/rubella (MMR), and chickenpox vaccines[181]. As the fat tissue between the skin and muscle layers is less dense than either surrounding tissue, vaccine is released relatively quickly compared to I.D. or I.M. injections[182]. S.C. injections can be self-administered, limiting the need for medical professional administration. While immune cell recruitment resulting from S.C. injection has not been widely studied, two clinically successful influenza vaccine adjuvants (MF59 and AddaVax) delivered via this route caused recruitment of neutrophils and DC/ macrophage precursors to the site of injection. These precursors differentiated into DCs in the hypodermal layers,

which trafficked to the LNs to exert a successful immune response[183] . Despite these accomplishments, S.C. injection targets loosely-packed fat cells rather than dense layer of cells which can lead to quick clearance of vaccine components before immune cell activation. While S.C. injection recruits cells, MNA delivery directly targets already-present immune cells in dermal layers[184]. Because of this, recent studies show that MNA compared to S.C. delivery leads to improved humoral- and cellularly- mediated immune outcomes. In this section, we use recent studies to show how MNAs enable these benefits. Then, we discuss how MNA compared to S.C. injection can lead to differential immune outcomes, which can be carefully selected depending on the type of immune response required.

MNA delivery improves immune outcomes at least in part to improved sustained release of vaccine components. In one study, coated MNAs were able to sustain vaccine release at the site of application compared to S.C. injection, as evidenced by increased signal measured by IVIS. This, combined with higher frequencies of DCs in LNs, indicates that prolonged availability of vaccine cargo leads to increased active and/or passive drainage to LNs to promote immune cell uptake[160]. The improved bioavailability of cargo in the immune-cell rich dermal layers enables improved immunological outcomes.

As discussed in previous sections, one critical benefit achieved by prolonged exposure and direct immune cell targeting is activation of both the cellular and humoral arms of the immune system. This targeting leads to higher levels of antibodies against the delivered antigen, which include OVA[185], B. Pertussis toxin[186], and SARS-CoV-2 nucleocapsid protein[187,188]. The adaptive immune response is also improved, as one study delivering model antigen OVA with CpG, a TLR9 agonist and vaccine adjuvant to mice and investigated the resulting immune response. MNA treatment increased not only antigen-specific IgG titers (indicative of B cell activation), but also the frequency of IFN-γ producing $CD8^+$ T cells, IFN-γ serum levels, and IL-2, a critical inflammatory cytokine, compared the same dose S.C. [160]. This study underscores how MNAs activate multiple cell types more effectively than S.C. injection. Together, this dual- activation leads to improved disease outcomes. MNA treatment protected mice from pneumococcus bacteria[189] or tumor challenge[190] equally as well as an S.C. injection of dose-matched vaccine components due to the sustained exposure of vaccine components and activation of multiple arms.

While these studies show that MNAs can improve immune responses to I.M. injection, they also provide insight into the types of immune responses MNAs themselves can elicit. For example, in an airway allergy model using OVA as a model allergen, MNAs significantly increased IgG2a levels (but not IgG1 levels) compared to S.C. injection[148]. This is important because although a peptide protein was delivered, the antibody class (i.e. IgG2a) produced by MNA delivery was one more reactive against polysaccharides, commonly found on bacteria. Carefully selecting the route of delivery to specifically activate the immune system against the target pathogen can improve immune responses, and MNAs provide an additional route with which to improve immune responses. In another example, MNA treatment against B. Pertussis increased IL-17 but decreased IL-10 levels compared to S.C. injection[186]. In disease states, IL-17a acts as an inflammatory mediator between T cell and neutrophil activation[191]. IL-10, or human cytokine synthesis inhibitory factor (CSIF), downregulates markers of inflammation on immune cells and acts as a regulatory cytokine as its name suggests[192]. Again, the route of administration could impact the type of immune response achieved; in extrapolating this to autoimmune applications for instance, a route that inherently increases regulatory cytokine release would be preferred. Future work will need to be conducted to reveal whether this skewing of immune response is applicable only for these pathogens, or whether it holds true across all instances of MNA application compared to S.C. injection.

5.3 Intramuscular (I.M.) Injection

I.M. injection is the most common route of administration for vaccines. This type of injection targets well-vascularized muscle tissue so that vaccine is dispersed relatively quickly into general circulation (**Figure 7D**). Vaccines commonly administered by this route include combination diphtheria, tetanus,

and pertussis (Dtap), Hepatitis A and B, and human papillomavirus (HPV) vaccines[181]. Because this route delivers to well-vascularized tissue, vaccine components are cleared quickly. Additionally, there are relatively few immune cells in muscle compared to skin, so vaccine components rely on general circulation to reach APCs and immune organs. MNAs help overcome these challenges because they directly target immune cells in the dermal layers, prolonging exposure of components to APCs. In this way, they frequently enable a lower dose to be administered to achieve the same immune outcomes including activation of both B and T cells.

Overwhelmingly, studies comparing MNA to I.M. delivery examined the resulting antibody titers after treatment, showing that MNA application increased titers against both bacterial and viral infections; specifically, influenza[26,193-195], OVA[196], tetanus toxoid[197], poliovirus[198], and HPV[199]. MNAs can also skew the type of humoral immune response compared to I.M. injection. For example, influenza vaccine delivered via MNAs skews the subclass of antibody response towards one more reactive against proteins (IgG1) rather than bacterially-associated polysaccharides (IgG2) compared to IM injection[200].

While the antibody-mediated immune response of MNA compared to I.M. delivery has been well-characterized, a few recent studies have begun to examine the cellular-mediated immune response as well. For example, in a study where influenza vaccine was delivered via MNA and I.M., MNAs elicited higher $CD8^+$ T cell-secreted IFN-γ responses in lung and spleen lymphocytes compared to I.M., indicative of an adaptive immune response. In linking B- and T cell mediated immunity, work delivering a COVID vaccine I.M. and via MNA shows that the frequencies of both inflammatory effector $CD8^+$ T cells and helper $CD4^+$ T cells in the spleen are higher when delivered via MNA. This increase in inflammatory helper $CD4^+$ T cells is especially of interest because it provides important mechanistic clues as to how MNAs are able to potently activate the adaptive immune response[201], i.e. by increasing the number of mature, inflammatory helper T cells.

Future work will need to be conducted to elucidate how MNAs change immune cell organization over space and time compared to I.M. injection. For instance, skin-resident LCs and DCs have been implicated in a first line, broadly-acting immune response compared to other cell types. This may result in a stronger transient response by skin resident cells to vaccine delivery, but a slower response time by LN-resident immune cells compared to a systemic injection[14]. Delivery via MNAs may be selected when a rapidly mounted, adaptive immune response is required (such as using MNAs to deliver a vaccine booster after I.M.- delivered prime), and choosing multiple delivery routes within the same vaccine regimen may improve immune responses without changing the components delivered. Characterization of the immune cell structure and function of skin, lymphatics, and lymph nodes will provide important information about rational delivery selection.

5.4 Other delivery routes

Other delivery routes, such as intratumoral (I.T.) injection, intraveneous (I.V.) injection, and oral delivery, have been less studied compared to MNAs in the past three years, likely in large part due to how few vaccines and immunotherapies are delivered via these routes. While I.T. and I.V. routes are common for the delivery of immunotherapies, there is only one disease with an FDA-approved orally delivered vaccine (RotaTeq® and Rotarix®, to prevent rotavirus[202]). Nevertheless, each route possesses unique immunological characteristics, many of which MNAs can improve. In this section, we review the latest research comparing MNA delivery to each of these routes of delivery.

I.T. injection involves direct needle injection of immunotherapies into solid, surface-level tumors (**Figure 7E**). This technique is similar in depth to I.D. injections, but the tumor itself is targeted to promote cargo uptake by cancerous cells. This technique holds promise in delivering inflammatory cues or photosensitizing agents, causing immune cells to recognize the cancerous cells as dangerous and mount a response. However, MNA delivery of vaccine components intratumorally provides numerous advantages. First, *in vivo* imaging system (IVIS) imaging shows that MNAs deliver cargo more uniformly and across a broader surface area of tumor compared to I.T. injection[203]. Because

tumor tissues are heterogeneous and create an immunosuppressive microenvironment, MNAs are potentially more effective in delivery because they can be designed to deliver cargo to both the center and cortex of the tumor rather than across a needle-width area. One study characterizing immune cells infiltrating the tumor suggests that MNAs promote tumor infiltration of higher numbers of DCs and LCs, inducing cytotoxic effector T cells to produce the inflammatory cytokines. This culminated in prolonged survival and reduced tumor burden in mice treated with MNAs compared to I.T. injection[204]. A similar preclinical study investigating how chimeric antigen receptor (CAR) T cells loaded into MNAs infiltrated tumors found that MNA delivery of these cells improved infiltration and immunostimulation compared to intratumoral injection of these cells[205]. Together, these studies suggest that MNA delivery of cancer immunotherapies is a promising way to uniformly deliver immunotherapies to tumors, promoting immune cell infiltration and ultimately improving disease outcomes.

MNA delivery of vaccines and immunotherapies compared to I.V. injection promotes vaccine uptake by immune cells while reducing systemic effects. I.V. delivery involves delivery of a typically large volume of components via an intravenous needle (**Figure 7F**). For immunotherapies, this direct injection into the circulatory system means that drug travels systemically to both immune and non-immune organs. IVIS imaging of fluorescently-labelled cargo delivered either by MNAs or I.V. shows that MNA delivery causes cargo to remain at the place of application for up to 24 hours post-application; I.V. injection caused such dilution of the cargo that no signal was detectable even after 1 hour[206]. This prolonged exposure of components to immune cells means that immune cells can be more strongly activated to improve disease outcomes, specifically in cancer. One study showed this using frozen (cryo) MNAs loaded with DCs primed against a model antigen improved survival and caused mice to more effectively resist tumor growth compared to I.V. injection. This was because activated immune cells could more effectively migrate to immune organs[207]. Together, these studies show that MNAs reduce cargo uptake by non-immune organs and cells to achieve similar outcomes compared to I.V. injection, with significantly lower accumulation in non-immune organs. Despite their promise, one important challenge MNAs must overcome are their ability to contain large volumes of cargo and deliver them slowly, over time. While the therapeutic dose contained in MNAs applied to mice may be enough to achieve favorable disease outcomes, alimetric dosing will be required before these same claims can be made about efficacy in human cancers.

Orally administered vaccines and immunotherapies share the benefit of painless self-administration with MNAs, but must overcome a variety of extreme environmental factors including stomach pH and mechanical force before they can enter either the blood stream or lymphatics. MNAs, in contrast, penetrate through the physical barrier of the stratum corneum layer by their very design and directly deliver cargo to the immune-cell rich layers below, enhancing active transport directly to the lymphatics.

This direct targeting and bypassing of extreme environmental factors improved the concentration of a P. yoelii parasite vaccine in mice over time when delivered with an MNA compared to orally. Therapeutic dosages were maintained throughout the body significantly longer than oral delivery. As a result, mice were completely protected from a challenge with the parasite up to twelve days after infection[208]. While this study shows that MNAs may be an alternative to oral delivery of vaccines and immunotherapies by targeting a different immune-rich barrier (i.e. skin vs. stomach lining), the immunological mechanism behind MNA efficacy compared to oral delivery has not been studied. Additionally, while the above studies show potential for MNA delivery of a parasitic vaccine, work comparing MNA delivery of traditionally orally delivered vaccines will need to occur to draw definitive conclusions about route improvements.

In identifying therapeutics that can best be delivered via MNAs in the future, it is important to consider that MNAs provide excellent tools for locally changing the immune response using a small, dense, amount of cargo. For this reason, the authors hypothesize that the next generation of MNA-delivered therapeutics will be vaccines and immunotherapies able to be delivered in a small volume to locally reprogram APCs and T cells to exert a systemic response. Immunotherapies and vaccines that

rely on antigen and adjuvant delivery are excellent candidates, whereas additional research will be required to determine how biologics that currently require infusions can be administered effectively via MNA.

6. Strategies to improve the stability of immunotherapies and vaccines in MNAs

From a public health perspective, the ability for MNAs to be self-administered could facilitate the administration of vaccines and immunotherapies in areas of the world with limited medical infrastructure or during times of personnel shortages. These areas tend to be less accessible, increasing travel and shipment times, and may lack supporting infrastructure to store vaccines per CDC guidelines[209,210].

Vaccines and immunotherapies must be transported at carefully controlled temperatures to maintain their immunogenicity. This requires expensive equipment and careful planning to ensure the same immune response is elicited upon administration as immediately after manufacture. For example, the CDC advises that "the total time for transport alone or transport plus clinic workday should be a maximum of eight hours", and Pfizer, a major pharmaceutical chain, states that their COVID-19 vaccines can be stored in vaccination centers for up to 20 days after shipment from the plant so long as vaccines remain frozen [211]. The specific temperatures (ranging from -90°C to 8°C) vary depending on the type of vaccine and when it will be used but require constant monitoring to ensure temperatures are within range. Taking these factors into consideration, drug delivery strategies to enable mass global vaccination must retain immunogenicity under potentially sub-optimal shipping and storage conditions.

MNAs provide a platform to overcome the challenge of cold chain storage for certain types of vaccines and immunotherapies, but require careful attention to their formulations and manufacture. First, vaccine- and immunotherapy- containing MNAs deliver solid vaccine components rather than components suspended in an aqueous medium. This is itself an advantage, as degradation is expedited in hydrated form[212]. Second, components must be loaded into or onto needles in an immunologically active form. This contrasts with lyophilized vaccine components, which remain in a vial and can be reconstituted with diluent upon receipt. Here, we examine studies published in the past three years focusing on chemical (**Figure 8A**) and manufacturing (**Figure 8B)** controls that can be used to maintain the immunogenicity of vaccines across a wide temperature range and for extended periods of time within MNAs (**Figure 8C**). Immunogenicity has been determined by characterizing the antigen and adjuvant structures using the methods in **Figure 8D**, and by testing the cellular and whole-body response *in vitro* and *in vivo* using the methods in **Figure 8E**.

6.1 Component formulation modifications to improve stability of vaccine and immunotherapy components in MNAs

Recent studies have examined different component formulations to maintain the immunogenicity of vaccines over time **(Figure 8A)**. Because the leading causes of deaths in many developing countries are infectious diseases, many recent studies seek to improve the stability of MNA vaccines focus on bacterial and viral infections. Lyophilization also promoted the stability of PDT drug 5-ALA over five months, as measured by NMR in one cancer immunotherapy study[213]. Interestingly, advances made in the lyophilization of soluble vaccine comopnents can also be applied to MNA vaccine application. For example, a recent paper developed a novel freeze-drying process for mRNA lipid nanoparticles for COVID-19 vaccination. They found that mRNA nanoparticles were stable at 4 °C, 22 °C, and 37 °C, and that the transfection properties of lyophilized particles were maintained during at least 12 weeks[214]. An exciting possibility is that improved process controls such as this would enable the reconsistiution of the vaccine wherever it is to be delivered within the world, then delivered through a hollow MNA directly to immune cell-rich dermal layers.

Excipients, or vehicle components included in the vaccine, can also improve the immunogenicity. Many recent MNA studies now incorporate rigorous screening processes for various amounts and types of excipients, such as carbohydrates and salts, to improve the immunogenicity of the vaccine[215 216 217 218]. In particular, including trehalose to stabilize Hepatitis B vaccine (HBV) formulations maintained immunogenicity after storage at 40°C for seven days, as measured by antibody titers against the virus in mice[219]. In another example, vaccinia virus, stabilized with PVA and trehalose, could be coated onto MNAs and stored at -20°C for up to 30 days. After this time, it was still able to elicit significant levels of neutralizing antibodies compared to uncoated controls[84]. In fact, MNAs can improve the ability of vaccines over liquid formulations to induce an immune response even at extreme temperatures. For example, HBV vaccines reduced the number of infected tissue culture plates upon re-infection with the virus. A dengue viral envelope protein vaccine similarly induced a stronger immune response, as measured by higher levels of neutralizing antibody titers than liquid formulations[112,220,221]. A flu vaccine, stabilized with sucrose or trehalose, was stable after one year at ambient (25°C) storage conditions, as measured by SDS-PAGE/Western blot and anti-influenza antibody responses in mice after a prime and boost vaccine administration[222]. One recent study systematically analyzed over 52 excipients for a measles/rubella vaccine, primarily focusing on the loss of antigens as measured by RT-qPCR viral infectivity and genome assays[223]. They found that different excipients impacted each type of virus antigen differently, and that not only the immunogenicity but also the ability to recover each type of antigen must be considered when choosing an excipient. To address the inherent immunogenicity of excipients themselves, future work must define the immune response to stabilizing agents, as maintaining stability of vaccine components loaded into MNAs over long periods of time at a wide range of temperatures is particularly important to their usefulness as a global vaccine delivery platform. Importantly, the development of effective excipients may also enable sterilization of MNAs[224]. Solid or hollow metal MNAs can be heat sterilized, while dissolvable MNAs will likely require a gamma ray sterilization technique, depending on the cargo inside[225,226]. Future preclinical and commercial work will be required to evaluate how matrix, excipient, and cargo choice impacts which sterilization techniques can be used.

6.2 Manufacturing condition modifications to maintain immunogenicity of vaccine and immunotherapy components in MNAs

While excipients and salts can be used to improve the stability of vaccines over time, those excipients can have inherent immunogenicity. Thus, adjusting the manufacturing conditions to eliminate the need for excipients is another attractive option to improve the immunogenicity of MNAs. To this end, recent studies investigate how the manufacturing conditions of MNAs are adjusted to maintain the stability of encapsulated vaccine components both pre- and post- fabrication[153] (**Figure 8B**). These strategies vary from supersaturating vaccine components to prevent their crystallization in the needles[227] or loading vaccine components into particles, then into MNAs to ensure their stability[228]. Another strategy is to maintain the entire MNA fabrication process at a lower temperature, retaining the immunogenicity of a flu vaccine or several model antigens without use of a stabilizer in the vaccine formulation. This promotes a more antigen-specific immune response[25,229,230]. Other strategies include using a volatile organic solvent, such as chloroform, during the MNA casting process[231] or altering the pH and buffer composition of the solvent used while drying measles- or rubella-containing films to significantly different viral titers[232]. Interestingly, the pH and buffer that yielded the highest viral titer of Measles was different than the combination that yielded the highest titer of Rubella, indicating that manufacturing parameters must be tuned to the specific vaccine or immunotherapy being delivered. Future work will be required to reveal additional manufacturing parameters critical to the stability of the vaccine within the MNA, and how both manufacturing and formulation strategies can be optimized to best maintain the antigenicity of vaccines and immunotherapies delivered via MNAs.

While recent research is beginning to address challenges that the widespread use of MNA- delivered vaccines and immunotherapies face, there are still many logistical hurdles that have yet to be overcome; in short, manufacture at scale, maintenance of sterility, and a cost competitive with traditional injected vaccines. First, MNAs must be manufactured in quantities and at a cost that is

competitive with traditional injected vaccines. This requires a large capital investment on the part of companies to build and maintain facilities and molds that meet these demands[233]. Next, the sterility of the MNA fabrication, packaging, and shipping must be maintained. Because of the added materials and complexity of adding vaccines and immunotherapies to a device, sterility becomes a large issue and question. Adding to this difficulty is a lack of quality standards in good manufacturing practices (GMP) for MNAs, which do currently exist for traditional injectable vaccines[234,235]. Additional standards and regulation regarding how patients self-administer MNAs will also be required to ensure the full dose of delivered cargo enters the skin[236], which have already been developed for physician- or health care professional- administered vaccines and immunotherapies. It is, however, worth noting that because of the fixed length of the tines on MNAs, all cargo is delivered to the same depth. On the other hand, the depth at which physicians deliver I.D. injections varies[180]. Current pre-clinical studies are invesitaging the best way for patients to apply MNAs (see **Section 8. Recent MNA clinical trials and studies with humans**). Finally, ongoing expenses for packaging and transporting the individual arrays must be considered in addition to the one-time cost of building manufacturing facilities. For dissolvable MNAs or MNAs whose components are sensitive to humidity, dessicants, special packaging, or climate control will be required to maintain the mechanical stability of the arrays through transport[210]. All of these added costs will increase the price of each array. Nevertheless, with increased research, clinical trials successfully validating MNAs for vaccine and immunotherapy delivery, and companies developing MNAs, many of these hurdles will likely be overcome in the coming years.

7. Drawbacks of MNA delivery

Despite the numerous benefits listed above, MNAs have certain disadvantages compared to conventional forms of delivery. First, the inherently small size of needles means that to increase the dose of drug contained within needles, the surface area of MNA must be increased while maintaining integrity of the patch itself. In pre-clinical trials, this means that testing is limited by the available surface area of mice or animals, while in human studies, applying many MNAs to achieve the required dose may not be as convenient as simply applying a single band aid-like device. While MNA patches are flat, the skin that they are applied to is not. Therefore, simply increasing the surface area of a needle may lead to breakage of the patch itself (either during manufacture, transport, or application), resulting in a lower delivered dose than expected. For this reason, additional work on the design and geometry of MNAs is required to increase the volume of dissolvable MNAs or surface area of coated MNAs while still maintaining mechanical stability and skin penetration[237]. Alternatively, a flexible patch with penetrative needles may help increase the potential surface area of an array.

Second, in certain cases, MNA have caused skin irritation at the site of application compared to SC injection in humans immediately after application[238]. This is likely due to the disruption of the epidermis, which contains LCs, important for maintaining homeostasis through tolerizing properties after infection. Additionally, Phase 1 clinical trials show that there are higher levels of circulating cytokines implicated in allergic reactions (IL-5 and IL-13) in MNA-treated patients compared to those treated I.M., which similarly led to short-term irritation at the site of application. Emerging work in the field revealed that by using shorter needles to deliver directly to the LC-rich epidermis (as opposed to using longer needles to disrupt the epidermis, delivering to the DC-rich layers), erythema can be reduced[171]. Additionally, at least one recent study has focused on examining the impact of polymeric MNA matrices on *in vitro* immune response[31], and provides exciting preliminary data to motivate investigating this phenomenon *in vivo*. These findings underscore the importance of MNA design and material selection for achieving the desired immune response. Future studies can enhance this design by perhaps including a flexible, sticky backing to the needle that will enable prolonged delivery while minimizing irritation. Additionally, topical or biological tolerogenic molecules applied behind the cargo delivered can help prevent skin irritation. Despite this drawback, it is equally important to recognize that many forms of needle delivery cause scarring after repeated injections[238]. Longer- term studies will need to be conducted to reveal whether MNAs cause the same problem; however, existing literature does not report this phenomenon.

Finally, MNAs may not be the best route of application for all types of vaccines and immunotherapies for both immunologic and practical considerations. For example, in a daily dosing study, vaccinations with MNAs improved immune responses to tetanus toxoid and subunit influenza vaccines but not a live-attenuated viral measles vaccine in comparison to a bolus I.D. injection[167]. While numerous studies have investigated the mechanism behind this (see **Section 5.1**, "**Intradermal (I.D.) Injection**"), additional work is required to understand the mechanistic interaction between delivery of these vaccines to the dermal layers and the immune system. Practically, many infusion immunotherapies (such as monoclonal antibodies or chimeric-antigen receptor [CAR] T cell therapies for cancer) require large volumes to effectively cause the body to uptake and utilize components. These treatments do not require antigens and adjuvants to drain to SLOs, which is a major advantage of MNAs. Therefore, these therapies may not be best suited for MNA delivery.

Despite these drawbacks, MNAs provide a promising way to deliver many forms of vaccines and immunotherapies. By tuning the MNA design and vaccine or immunotherapy formulations contained therein, many current challenges in the field can be addressed, studied, and overcome.

8. Recent MNA clinical trials and studies with humans

Since microinjections were successfully used to deliver influenza vaccines in Phase I clinical trials in 2015[239], numerous clinical and preclinical studies utilize the same principle of targeting skin-resident immune cells with MNAs to combat viral and allergic diseases. These studies seek to fully investigate the efficacy of protection and the mechanism behind how the body develops an immune response[161,195,240,241] . This section focuses specifically on clinical trials and studies conducted in humans in the past 3 years.

Due to their novelty, MNAs have only begun to emerge in clinical trials within the past two decades. Currently, MNAs delivering influenza vaccine are the only phase 1 clinical trials involving MNAs for immunotherapy and infectious disease in the United States, likely due to their prevalence and ease of correcting a potentially ineffective dose. A recent Phase 3 study recently concluded evaluating the efficacy of an MNA patch delivering peanut protein to the skin of toddlers. This trial was multinational, double blink, randomized and placebo-controlled—a clinical trial benchmark that other immunotherapies and vaccines will need to meet before they are able to be deployed for use[242]. However, multiple clinical trials delivering cancer immunotherapies[130,243], measles, mumps, and rubella vaccines[244], and mRNA COVID vaccines[245] are currently recruiting. Two separate studies delivering influenza antigens through different types of MNAs to humans found that MNAs significantly improved outcomes compared to I.M. injections. In the first, MNAs delivering a third of the I.M. dose conferred higher hemagglutination inhibition (HAI) and memory B cell titers compared to I.M. injection **(Table 1)**[241]. The second had similar findings in dose-matched MNA and I.M. injections, and additionally found that MNAs increased circulating T_{fh} cells and inflammatory cytokines (tumor necrosis factor alpha [TNF-α], IL-10, IL-8, macrophage inflammatory protein 1b [MIP-1b]) significantly compared to I.M. injection. Interestingly, cytokines associated with allergic reactions (IL-5 and IL-13) were also higher in the group receiving MNAs, which explained the transient skin irritation at the site of application[240]. A third study delivered MNA vaccines to patients and investigated whether patients would prefer to have MNA vaccinations in the future. This study found that they did, in fact, prefer these types of vaccinations to I.M. injections.

Beyond influenza, studies are investigating how MNAs can be used in humans to combat allergies and sexually transmitted infections[246]. In addition to the Phase 3 clinical trial delivering peanut protein described above, a study conducted in Japan sought to use MNAs delivering milk protein concentrate (MPC) to promote tolerance, enabling patients to switch to oral immunotherapies. A poke-and-patch method of MNA delivery was used where MNAs were used to puncture the skin, enabling a gel patch containing MPC to target the immune-cell rich populations beneath. This increased the symptom induction threshold in half of the subjects such that they could consume milk and switch to oral immunotherapies[247]. Particularly in younger patients with needle aversion, MNAs could provide a

way to not only less painfully deliver vaccines, but also less invasively and improve allergic reactions as current treatments involve shots.

Additionally, in standardizing self-administered applications and the application of patches to other barrier tissues besides the skin (e.g. mucosal interfaces), it is important to ensure that application is, easy, painless, and effective. For example, a study investigating how MNAs could deliver antiretroviral drugs to the vaginal mucosal layers examined not only the immunological response upon application, but also the mechanical forces experienced upon insertion into the vaginal tissue, which has previously not been studied. This study found that for their unique patch, an application time of 120 minutes was necessary for drug release into the tissue. Furthermore, the researchers performed market research on which of 6 types of applicators potential end-users preferred. These applicators were again unique to the vaginal delivery location and included designs such as tampon-like, finger cap, and a wand applicator[246]. One study investigated user perception of MNAs to deliver flu vaccine, and compared groups that self-administered the vaccine (after a short instructional powerpoint) compared to groups that received either the same MNA or I.M. injection from a healthcare professional. They found that generally, patients felt comfortable self-applying the patch compared to having a healthcare provider apply the patch for them[161]. Future studies will be required to elucidate whether this patient confidence translates to the same clinical outcomes as healthcare provider application. With the end-users of MNAs being patients and not necessarily health care professionals, it is important that MNA design not only be focused on delivering cargo but also on consistency of delivery between patients and accuracy to the layer of skin delivered. While studies investigating ease of use will be important for MNA adoption amongst users, standards must also be developed regarding MNA design criteria for effective self- application, and how individuals are instructured to self-administer. These criteria will likely include ensuring the material is capable of withstanding the force necessary to penetrate the epidermis, that the patient or patient advocate is capable of exerting sufficient force to apply the needle, and that cargo is concentrated in the tips of needles to ensure that it is delivered as intended.

Finally, with concerns across many socioeconomic statuses about the safety and efficacy of vaccines in particular, public health studies will be required to determine how willing people are to receive an MNA vaccination compared to traditional needle injection vaccination. In an initial MNA flu vaccine clinical trial in the United States, researchers found that MNA users would generally prefer MNAs to traditional I.M. injection. However, public health or behavioral psychology studies will need to be conducted on a more representative population of a developed country to determine whether this preference is true of the majority of the population in a given area[248]. Additionally, in underdeveloped countries where the stability of MNAs could make vaccination more accessible, studies regarding public perception of the safety of these patches will be needed to determine whether people will use them once they are successfully delivered[249].

In translating pre-clinical studies to the clinic, a number of important milestones must be achieved. First, future work in humans will be required to understand why MNA treatment elicits a stronger immune response in delivering certain adjuvants and antigens compared to others[250]. Another important consideration in translating MNAs to the clinic is the higher dosage of vaccine required in humans compared to rodents. This requires careful manufacturing to ensure a robust MNA, capable of puncturing the dermis, while still being loaded with enough immune signal to confer an effective response against the antigen. Finally, clinical trials for MNA-delivered vaccines will need to meet safety (Phase 1), side effect and risk (Phase 2), and efficacy (Phase 3) requirements across diverse populations. Double-blind, placebo controlled trials (as opposed to single-blind, drug-plus-treatment-as-usual trials) will be required to accomplish these goals[251,252].

9. Conclusions and future perspectives

MNAs provide a novel, minimally invasive way to deliver vaccines and immunotherapies and improve disease outcomes. Recently, important pre-clinical work investigating the ability of MNAs to induce an immune response and improve disease outcomes has been performed in cancerous,

infectious, autoimmune, and allergic disease models. These studies exemplify the benefits of MNAs: direct targeting of immune cells in the dermal layers decreases off target effects without compromising immune responses, and the simple application allows for unique dosing regimens not achievable by conventional needle delivery. Furthermore, careful component formulations and manufacturing conditions ensure the antigenicity of vaccines delivered through MNAs is preserved under normal transportation conditions. Human studies show that immunotherapeutic MNAs produce desired immune outcomes in both the context of viral infections and allergic reactions. Future work will require a more rigorous investigation of these different benefits, and why they occur. For example, transportation studies to regions of the world where access to healthcare professionals is lacking will reveal whether MNA stability can withstand the mechanical, thermal, and temporal effects of shipment. Additionally, public health studies will reveal whether the ease-of-use of MNA patches results in improved patient outcomes, as we hypothesize they will. With these goals in mind, MNAs are a promising way to revolutionize global availability of vaccines and disease spread.

Keywords

Microneedle, vaccines and immunotherapy, delivery, skin, autoimmunity, cancer, infectious disease, tolerance

Conflict of Interest

C.M.J. is an employee of the VA Maryland Health Care System. The views reported here do not reflect the views of the VA or United States Government. C.M.J. has an equity position with Cartesian Therapeutics. Beyond these declarations, there are no other Conflict of Interest to report. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Microneedle arrays (MNAs) are patches containing short projections long enough to reach dense immune cells below the skin, but short enough to avoid pain receptors. This review focuses on studies in the past 4 years utilizing MNAs to deliver vaccines and immunotherapies to improve disease outcomes.

Figure Captions

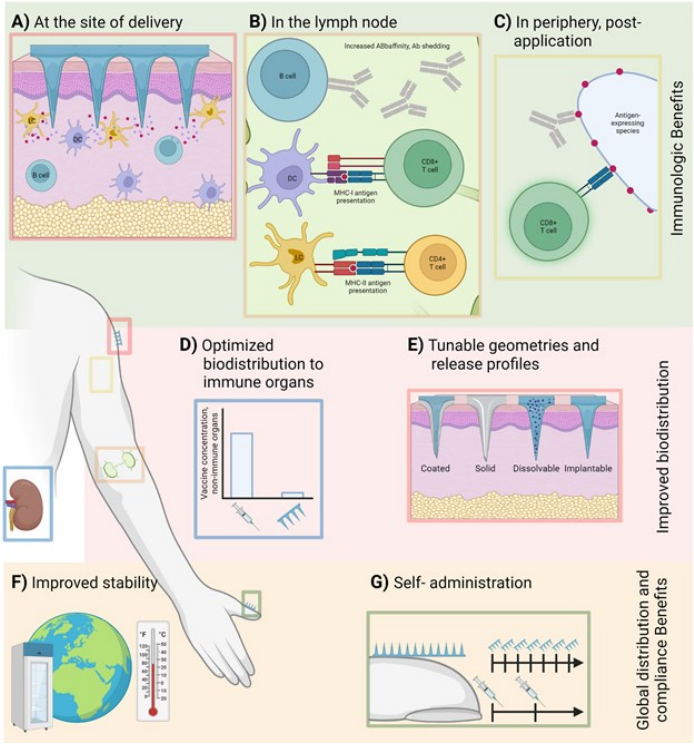

Figure 1:
MNAs provide numerous immunological, systemic, and global distribution benefits. **A)** MNAs activate and recruit immune cells at the site of delivery. **B)** These immune cells can traffic to secondary immune organs, such as lymph nodes (LNs), where antigen-presenting cells interact with T cells. B cells undergo antibody affinity maturation. **C)** In the periphery, post- application, species presenting the same antigen delivered via MNA are recognized and attacked or protected due to signals effector cells experienced in the LNs. These species include cancerous cells presenting tumor antigen, self-tissues presenting self-antigen, and pathogens expressing pathogenic antigen. **D)** MNAs optimize biodistribution to immune organs compared to conventional delivery routes. **E)** This is achieved, in part, by using tunable geometries and release profiles. **F)** Towards making this technology more accessible worldwide, studies have focused on improving the stability of MNAs themselves under a variety of transport conditions. **G)** MNAs can be self-applied, which can lead to improved compliance and unique dosing regimens.

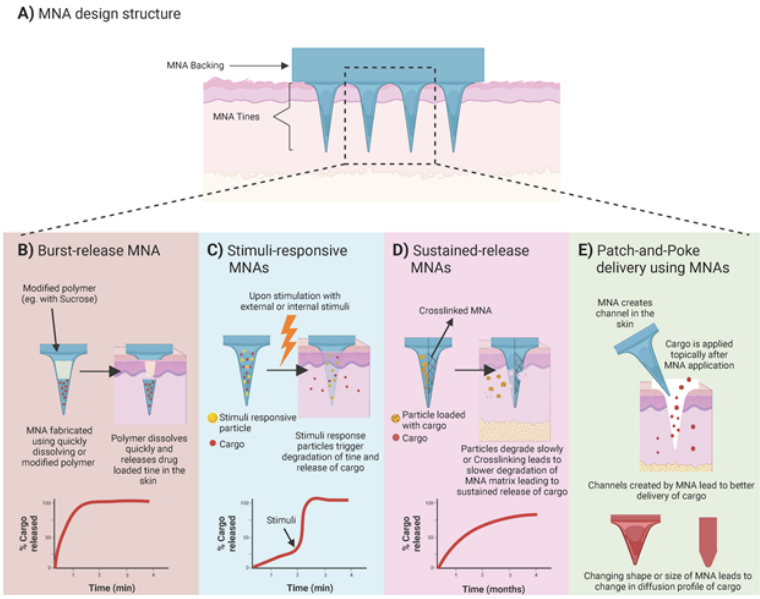

Figure 2:
MNAs can be carefully designed to control release of components, leading to varying immunologic outcomes. **A)** MNAs typically consist of a backing that holds short projections, or tines, together and provides mechanical stability for insertion. **B-E**) A variety of release mechanisms can be incorporated into MNAs to achieve the desired vaccine or immunotherapy release profile.

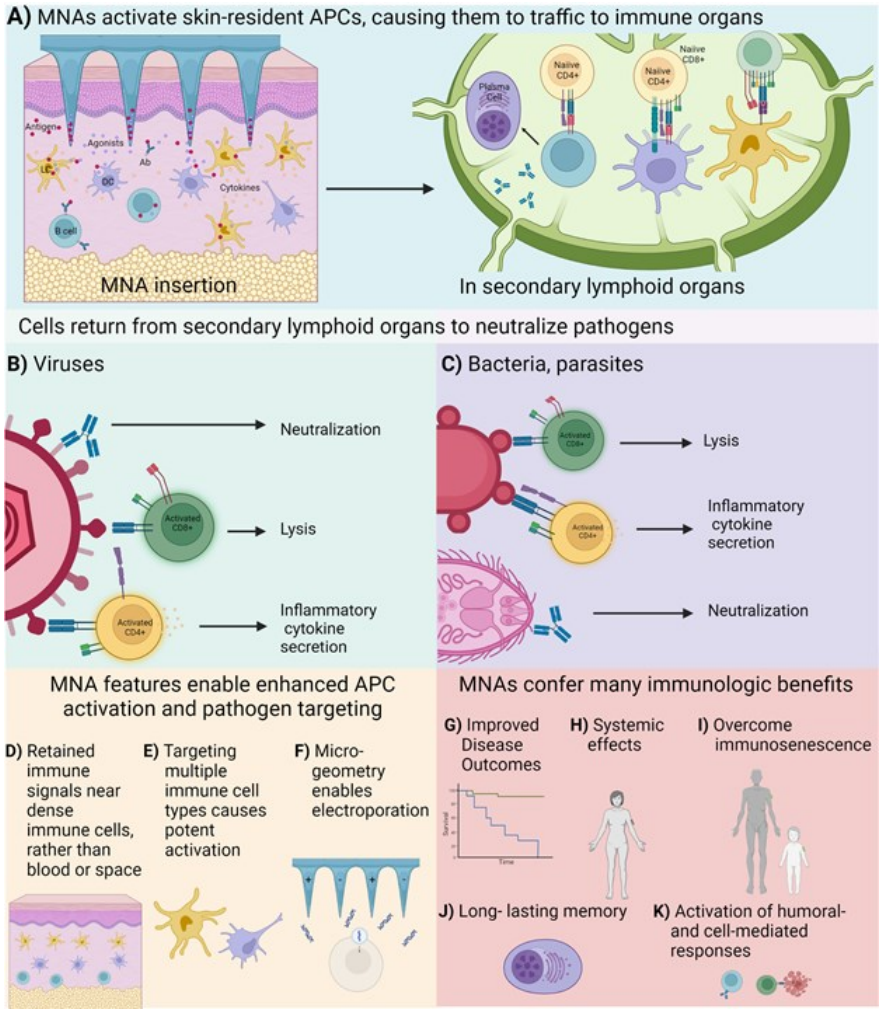

Figure 3:

MNAs can improve outcomes against pathogenic diseases. **A)** Upon MNA insertion, skin-resident antigen-presenting cells (APCs) take up antigen and adjuvant. APCs then traffic to secondary lymphoid organs where antigen and costimulatory markers, generated in response to adjuvant, bind to effector cells. These effector cells can now detect and attack **B)** viruses, **C)** bacteria, and parasites in the periphery, leading to protection. **D-F**) MNAs can be engineered to exhibit a variety of characteristics to improve immune cell activation. **G-K**) This results in numerous immunological benefits.

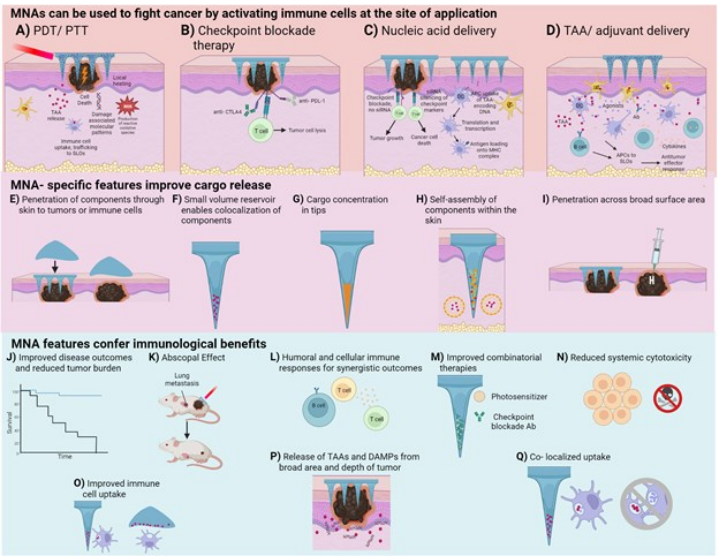

Figure 4:
MNAs can be used to improve cancer disease outcomes preclinically. A variety of immunotherapies, including **A)** photothermal therapy (PTT)/ photodynamic therapy (PDT), **B)** checkpoint blockade therapy, **C)** nucleic acids, and **D)** tumor associated antigen (TAA)/ adjuvants can be delivered either directly to the tumor or distally. This activates skin-resident immune cells, which can then drive systemic responses. **E-I)** Specific features of MNAs enable potent delivery of these therapies and activation of immune cells. **J-Q**) These features confer numerous immunological benefits, which can translate to potent anti-tumor responses.

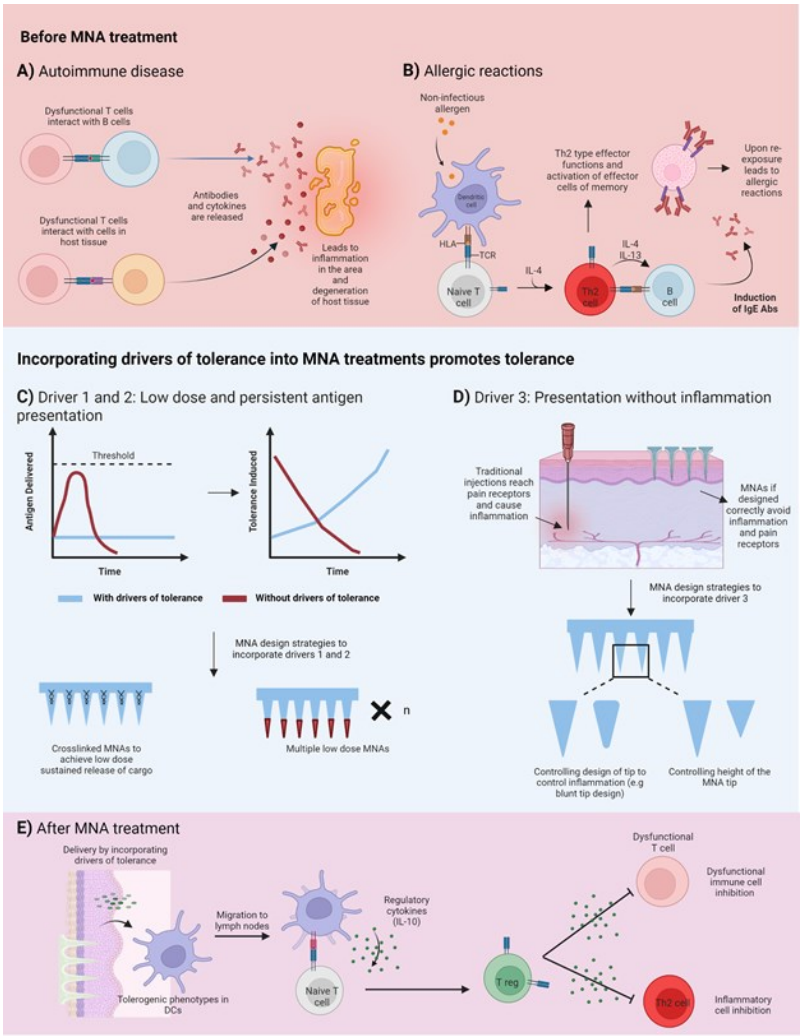

Figure 5:

MNAs show potential to improve autoimmune disease outcomes in preclinical disease models. **A)** In autoimmune diseases, dysfunctional T cells attack host tissues due to mis- regulation of the immune system. **B)** In allergic reactions, antigen-presenting cells (APCs) mistakenly detect foreign non-pathogenic species as harmful. This leads to an allergic reaction. **C-D**) MNAs provide an ideal platform to incorporate drivers of tolerance with immunotherapies to **E)** improve disease outcomes.

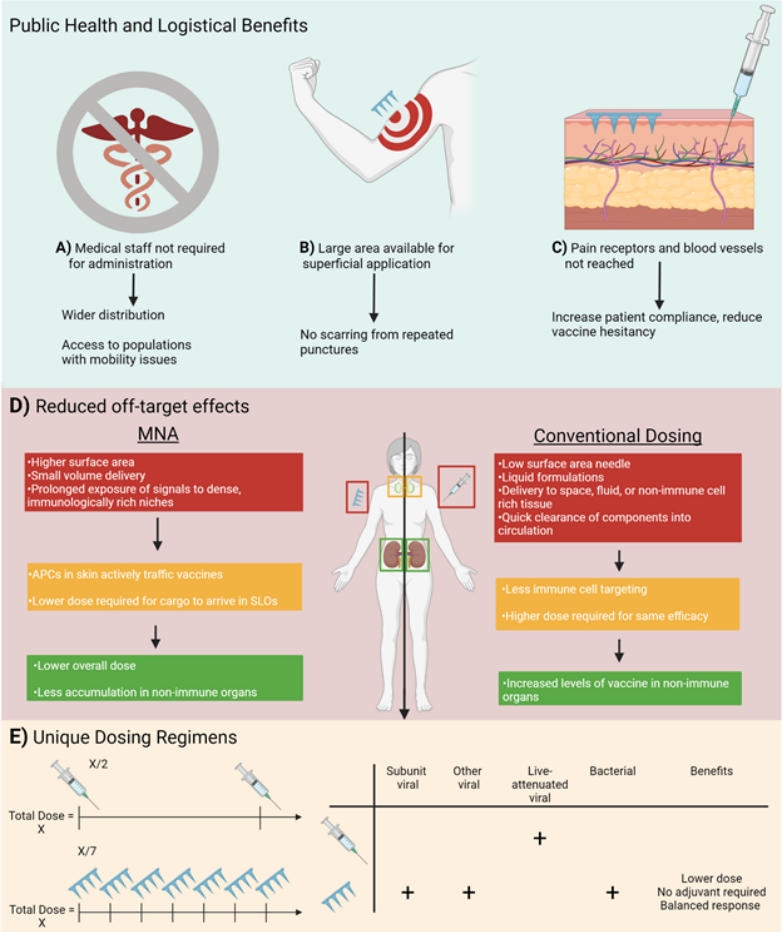

Figure 6:
MNAs provide numerous public health and logistical benefits. Their unique geometry and delivery locations can help reduce off-target effects, enabling unique dosing regimens. **A-C)** Beyond the immunologic benefits conferred by MNAs, these patches also provide numerous benefits to conventional delivery which could ultimately improve patient compliance. With these advantages, **D**) reduced off-target effects and **E**) unique dosing regimens can be achieved that would be difficult or impossible to realize with conventional delivery.

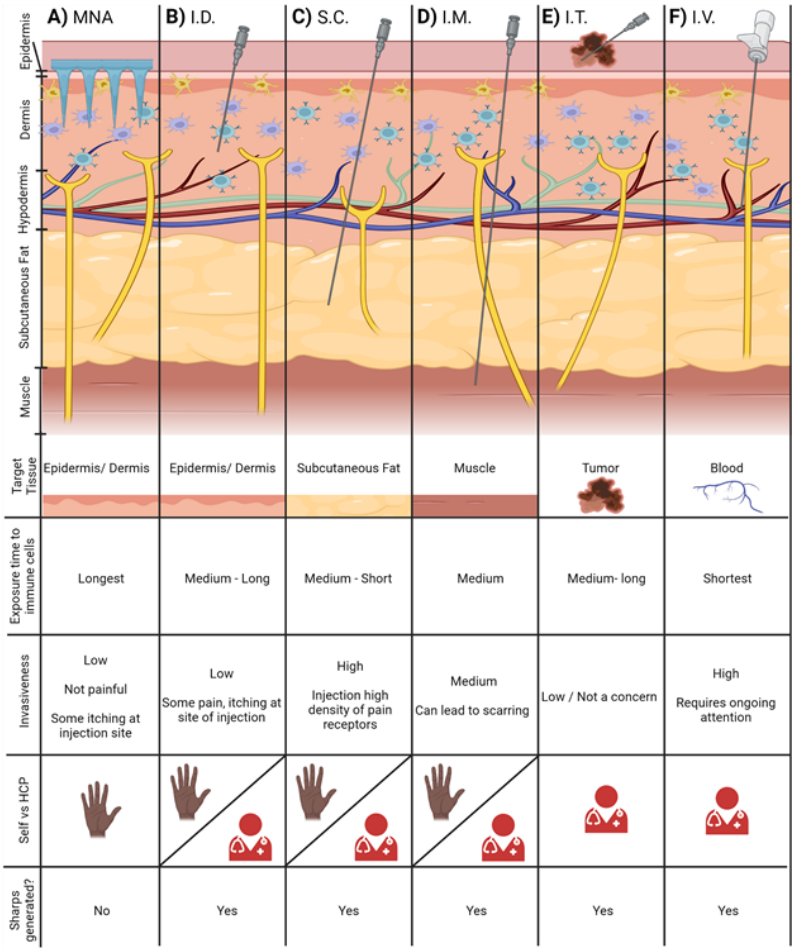

Figure 7:

Recent preclinical studies show that MNAs provide advantages over conventional forms of delivery. **A)** MNAs directly target immune cell- rich dermal layers without the logistical complications of other delivery routes. Depending on the context of delivery, **B)** Intradermal (I.D.), **C)** subcutaneous (S.C.), **D)** intramuscular (I.M), **E)** intratumoral (I.T.), and **F)** intravenous (I.V.) delivery routes all have challenges that MNAs may help to overcome.

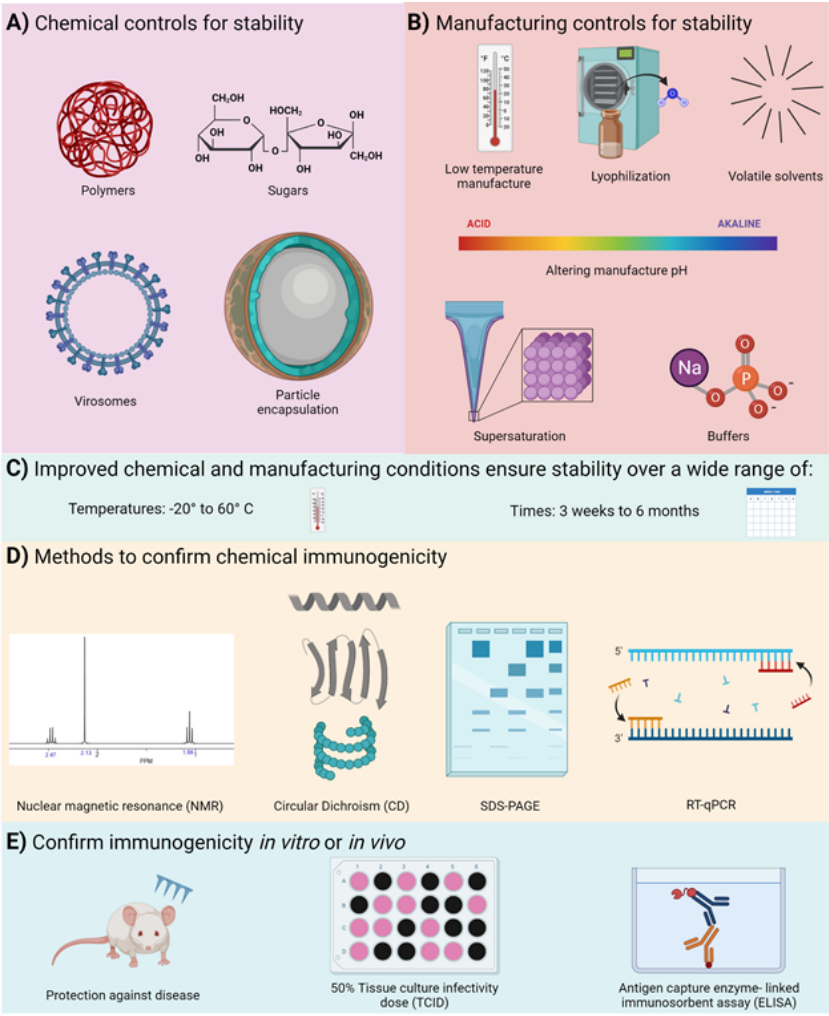

Figure 8:
Due to the potential logistic and public health benefits of MNAs, recent studies have focused on improving and characterizing the stability of MNAs. Both **A)** chemical and **B)** manufacturing controls for stability can be used while fabricating MNAs. **C)** This leads to improved stability over a wide range of temperature and time domains. To characterize this stability, **D)** chemical methods or **E)** in vitro/ in vivo methods are used to ensure that vaccine or immunotherapy components can be detected by the immune system to drive the desired response.

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Table 1. Clinical trials conducted using microneedle arrays to deliver vaccines and immunotherapies between 2019 and 2023.

Pathogen	MNA design	Goal	App. Time	Primary Readouts	Conclusions	Secondary Readouts	Conclusions	Yr	Ref
Influenza H1N1, H3N2, NYMC BX-51 strains Inactivated viruses	**Coated**: Stainless steel MNAs with CMC + vaccine coating	•Determine how cellular, antibody, and cytokine/ chemokine levels in the blood change in MNA vaccination vs IM injection.	20min	•Fold- change of hemagllutinatioation inhibition (HAI) titers •Fold- change of neuraminidase inhibition (NAI) titers •Investigated 3 strains of flu virus: H1N1, H3N2, NYMC BX-51	•MNAs induce significantly higher HAI and NAI titers shortly after vaccination and up to 180 days post vaccination.	•Antibody binding affinity (SPR) •Fold changes in cytokines/ chemokines (TNFα, IP-10, IL-13, MIP-1b, IL-5, IL-8) •Monocytes (CD14+ CD16+) present in blood over time •Inflammatory (IL-2 secreting) helper (CD4+) T cells	•MNAs significantly increase the below parameters compared to conventional (I.M.) injection •Antibody binding affinity •All examined cytokines/ chemokines •Inflammatory helper T cells •Follicular	2021	224

						•Circulating follicular T helper cells (critical for B cell development and antibody secretion) over time •Antigen-specific antibody (IgG)-secreting memory B cells	helper T cells •Memory B cells •In particular, MNAs increase cytokines associated with allergic reactions (IL-5 and IL-13).		
Influenza H1N1 Mono-valent, split inactiv-ated influenza virus vaccine	**Coated**: Polymeric injection-molded MNAs coated with pure vaccine	•Determine whether variable doses of vaccine delivered via MNA induce similar responses to full-dose IM injection •Determine whether where MNA is placed (forearm, FA vs underarm, UA) impacts immune responses	2min	•HAI inhibition titers •Treatment emergent adverse events (TEAEs)	•MNAs delivering 1/6 of the IM dose induce similar HAI titers to IM injection	•Self-reported pain scores •Skin reactions over time •Seroprotection •HA specific FcR binding antibodies (ABs) •HAI AB-secreting memory B cell frequency and type •Cytokine secretion of helper (CD4+) T cells upon stimulation with flu peptides	•Patients tended to report more pain within 1 hr of vaccination when treated with MNAs vs IM injection, but more pain up to 3 days later when treated with IM injection vs MNAs. •After 61 days post vaccination, 90% of patients treated with an MNA containing 1/6D IM injection were seroprotected, vs. 85% of patients treated via IM injection containing 1D. •An equivalent dose of vaccine delivered via MNA induced similar (FA) or higher (UA) levels of HA-specific ABs compared to I.M. injection. •MNAs induced similar levels of memory-specific B cells when applied to either the FA or UA vs. IM	2020	225

							injection. •Generally, MNAs caused increased secretion of TNFα compared to IM injection of the same dose.		
Influenza Inactiv-ated virus	**Dissolvable**: Vaccine + water-soluble materials cast into MNA molds, then water-soluble materials cast to provide a backing.	Determine how well-accepted MNA vaccination vs. IM vaccintaion was to patients.	20min	•MNA use Perceptions •MNA perceived Convenience	•Majority of participants receiving the MNA preferred the MNA to the flu vaccine (55.7% to 64.3% between days 0 and Day 28 post vaccination) •Public perception: MNAs are an effective way to deliver vaccines •Participants agreed that MNA vaccination was easy	N/A	N/A	2020	152
Milk Protein (Allergen)	**Solid**: MNAs used to puncture skin, then crosslinked hydrogel containing milk protein concentrate (MPC) was applied to punctures.	•Determine safety and efficacy of a hydrophilic gel patch for treating severe milk allergies when applied after MNA puncture.	12hr patch applic-ation	•Safety •Efficacy	•Patients with lower levels of allergen-specific Abs were able to safely switch to oral immunotherapies and consume milk after between 16 and 64 weeks.	•MPC-specific antibody levels (IgE)	•All patients had mild erythema at patch site; none had long-lasting adverse events •Poke-and-patch application of the gel reduced the number of allergen-specific ABs in 4 of 8 patients	2019	231