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Review

Challenges and opportunities in the development of mucosal mRNA vaccines

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mRNA vaccines have played a critical role in controlling the SARS-CoV-2 pandemic, and are being actively studied for use in other diseases. There is a growing interest in applying mRNA vaccines at mucosal surfaces as it enables access to a unique immune reservoir in a less-invasive manner. However, mucosal surfaces present several barriers to mRNA uptake, including degrading enzymes, mucus, and clearance mechanisms. In this mini-review, we discuss our understanding of the immune response to mucosal mRNA vaccines as it compares to systemic mRNA vaccines. We also highlight physical and chemical methods for enhancing mRNA uptake across mucosal tissues. Mucosal mRNA vaccination is a nascent field of research, which will greatly benefit from fundamental investigations into the mechanisms of immune activation and the development of technologies for improved delivery.

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Introduction

mRNA vaccines offer a host of complementary advantages to more traditional vaccine modalities such as DNA, protein/peptide, inactivated, and/or attenuated vaccines. These advantages include the speed of production, the ability to express complex antigens in their physiologically relevant state(s), high potency, minimal risk of genomic integration, and the absence of vector-based immunity [1]. Given these characteristics, mRNA vaccines have played a central role in controlling the SARS-CoV-2 pandemic [2,3]; their expanded use throughout diverse populations has helped establish clinical safety and efficacy at an unprecedented rate yet has concurrently highlighted some critical challenges. These clinical observations have spurred a massive expansion of research efforts, which stand to impact a myriad of diseases ranging from infection to cancer and autoimmunity [4–6].

Accordingly, herein we discuss the growing field of mucosal mRNA vaccines. Currently approved mRNA vaccines are administered via systemic injection(s). However, there is emerging interest in the designing of mRNA vaccines that are administered at mucosal sites [7]. Given this, we have attempted to summarize recent work on mucosal mRNA vaccination and in so doing share our perspectives on both opportunities and challenges within this critical area.

Motivation for mucosal mRNA vaccination

Vaccination at mucosal surfaces stands to provide a host of benefits. These include targeting a larger number of diseases, generating a different quality of immune response, perhaps enhancing the immune response, improving patient acceptance, and reducing environmental wastes.

Mucosal surfaces are exposed to a copious number of environmental pathogens and as such host a rich reservoir of immune cells that drain into specialized lymph nodes [referred to as mucosa-associated lymphoid tissues] [8]. Therefore, administering vaccines at mucosal surfaces enables access to these unique immune cells, and the opportunity to induce a potent immune response.

As the vast majority of human pathogens gain entry to the body through mucosal surfaces, the generation of an immune response at this critical interface has been a longstanding goal of vaccination [9]. Canonically, the mucosal immune system was thought to be compartmentalized [10] — a concept supported by the observation that mucosal vaccination at one site can lead to an immune response at the same or distinct mucosal surfaces. Pertinent examples include oral and buccal vaccines that can elicit gastrointestinal and vaginal immunity [11,12] and intranasal vaccines that can yield pulmonary and vaginal immunity [13]. Unfortunately, the induction of mucosal immunity with mRNA vaccines has thus far been suboptimal [14,15]. Hence, methods to boost mucosal immunity of mRNA vaccines are highly desirable.

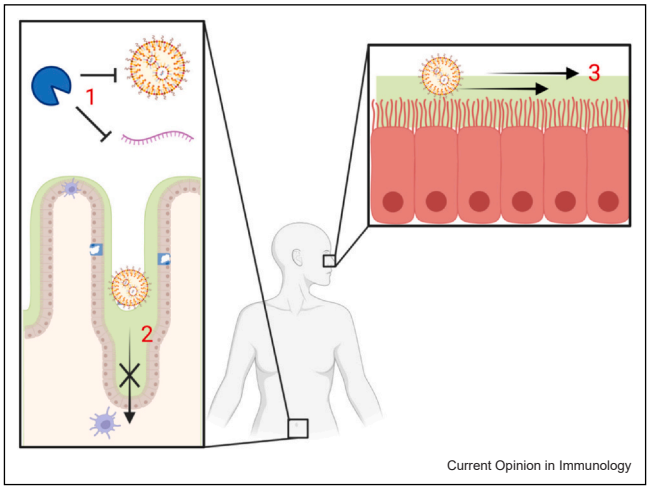
Apart from its potential immunological advantages, mucosal vaccination is anticipated to bring about considerable positive effects on both the patient's experience and the environmental load associated with vaccines. Mucosal vaccines, especially those administered via an oral route, avoid the use of needles. Such an approach may ultimately help improve patient adherence [16,17], reduce needle-stick injuries [18], and potentially reduce bio-sharp waste.

Challenges to mucosal mRNA vaccination

Challenges associated with mucosal mRNA vaccination include those unique to mucosal biology (Figure 1) in addition to those that are innate to systemic mRNA vaccination. Of note, nucleic acids are rapidly degraded by enzymes in mucosal fluids [19], thereby necessitating the protection of mRNA before tissue entry. Orally administered products face additional challenges given that the gastrointestinal environment has diurnal variations, differences based on fasted/fed states, and significant interindividual distinctions [20,21]. Further complicating the situation, gastrointestinal fluid can degrade mRNA carriers such as lipid nanoparticles [22]. Hence, robust product design is imperative in such mRNA vaccines. Next, mucosal surfaces are lined with mucus and this viscoelastic fluid comprises high molecular glycoproteins and enzymes that act as both physical and chemical barriers to the delivery of such vaccines [23]. Furthermore, different mechanisms act to clear materials trapped in the mucus layer (e.g. ciliary clearance in the nasal cavity [24] and salivary dilution in the oral cavity [25]). Hence, mucosal mRNA vaccines have short residence times in which to overcome such transport barriers. Finally, after entry into the tissue, the mRNA vaccine must access the cytoplasm of target cells to ensure transcription [18]. Hence, mucosal mRNA vaccines face a host of obstacles, and a concerted effort is needed to ensure optimal delivery.

Critically, vaccination at mucosal surfaces may also be tolerizing in nature. Immune cells at mucosal surfaces are constantly exposed to microbial antigens, which is thought to have contributed to their tolerizing nature. These cells secrete signaling molecules such as retinoic acid and interleukin-10, which promote immune regulation and/or hyporesponsiveness [26,27]. Hence, mucosal mRNA vaccines will ultimately need to be designed to ensure immune activation and prevent tolerizing effects (i.e. unless one is looking to specifically promote/engage tolerance). In line with such thinking, the most approved mucosal vaccines are live-attenuated or whole-cell-inactivated vaccines, which are highly immunogenic [9].

Figure 1



Challenges to mucosal mRNA delivery. mRNA administered at mucosal surfaces faces several barriers to tissue uptake. This includes (1) degradation by physiological enzymes, (2) entrapment in mucus, and (3) rapid clearance. Overcoming these barriers may improve mucosal mRNA uptake and the efficacy of mucosal mRNA vaccination.

Current experience with mucosal mRNA vaccines

While mucosal mRNA vaccines have been the subject of investigation for over two decades, these systems have garnered less attention than systemic mRNA approaches. Most work has focused on intranasal mRNA vaccines, however, preliminary reports on oral and intravaginal mRNA delivery have also recently emerged.

Intranasal mRNA vaccines

Remarkably, the first generation of intranasal mRNA vaccines comprised naked mRNA. Dimier-Poisson et al. isolated mRNA from *Toxoplasma gondii* and treated mice with a high dose (120 µg of mRNA/mouse) [28]. Mice were subsequently challenged with lethal and sublethal doses of *T. gondii* cysts. Vaccinated mice displayed higher survival rates and fewer brain cysts than unvaccinated mice following both lethal and sublethal challenges, respectively. Protection was attributed to

vaccine-induced production of systemic and mucosal antibodies. Lorenzi and colleagues, using a significantly smaller dose (5–10 μg/mouse) of an mRNA-encoding Hsp65 protein, showed that mice could be protected against an intranasal challenge with *Mycobacterium tuberculosis* [29]. However, the protection afforded by intranasal mRNA vaccines was inferior to that of the standard Bacillus Calmette–Guérin vaccine administered via the subcutaneous route. The authors showed uptake of fluorescently labeled mRNA into pulmonary CD11c+ dendritic cells and local expression of two inflammatory cytokines (viz., TNFα and IFNγ). Taken together, these studies provided indirect evidence of mRNA-mediated protein expression upon intranasal administration and established that the intranasal route may hold promise for mucosal vaccination.

Two key questions in this field are — (1) do intranasal vaccines produce a stronger immune response than systemic vaccines, and (2) is the quality of the immune response to intranasal vaccines inherently different from systemic vaccines? These questions are somewhat complex as it may be challenging to tease out the effects of different delivery efficiencies and different immune cell activation patterns at these sites. Fortunately, a few pivotal studies have recently emerged to shed light on these critical questions. Specifically, we identified studies that showed that intranasal administration of self-amplifying RNA (saRNA) vaccines produced a weaker systemic immune response compared with subcutaneous or intramuscular vaccines, and that this arose in part due to poor uptake across the nasal mucosa. However, delivery across the nasal mucosa could be enhanced using specialized formulations. Finally, in some cases, intranasal mRNA vaccines yielded more potent pulmonary mucosal immunity compared with systemic vaccines. We discuss these studies in greater detail below.

Independent studies from Blakney et al. [30] and Anderluzzi et al. [31] compared immune responses to intranasal and systemic saRNA vaccines formulated as polymeric, solid lipid, and lipid nanoparticles. In both studies, intranasal vaccines yielded weaker systemic and mucosal IgG responses and showed lower systemic T-cell activation than systemic vaccines (i.e. intradermal or intramuscular). Anderluzzi and colleagues also compared the biodistribution of their formulations using a lipophilic fluorescent dye. Following subcutaneous and intramuscular injection, fluorescence was detectable at the site of administration for up to 10 days. In contrast, when administered intranasally, the fluorescence signal migrated to the throat and stomach in just 4 hours. This indicated poor local residence and low tissue uptake. These data provide some explanation for the inferior immune responses following intranasal vaccination and underscore the need for specialized techniques to improve nanoparticle uptake across mucosal surfaces. Some efforts to improve mRNA uptake across the pulmonary epithelium are described below.

Nanocarriers have been specifically designed to overcome barriers associated with mucosal mRNA delivery. Cyclodextrin, a mucoadhesive molecule, was conjugated to polyethyleneimine, which served to complex an mRNA encoding the HIV gp120 protein [32]. In mice, cyclodextrin enabled longer retention of the polyethyleneimine nanoparticles (half-life of ~75 min versus 30 min) at the site of administration in the nasal cavity. The molecular weight of the polymer played a deterministic role in the delivery of mRNA [33]. Intranasal vaccination led to antigen-specific antibody responses in the serum and vaginal lavage. However, no comparison was made between intranasal and systemic routes. Intriguingly, intranasal vaccination did not produce a secretory antibody response in the nasal wash. Concurrently, immune cells isolated from the nasal-associated lymphoid tissues did not secrete IFNγ in response to antigen exposure *ex vivo*. In contrast, splenic cells in vaccinated mice showed robust secretion of the Th1 cytokine. The mechanism for the lack of local response remains unclear.

Another study compared the efficacy of two intranasal lipid nanoparticle vaccines in hamsters, with one formulation containing a specialized lipid for enhanced tissue uptake (details regarding the lipid were not disclosed) [34]. Indeed, the formulation containing the specialized lipid produced stronger systemic antigen-specific IgG and IgA responses, underscoring the importance of tissue uptake. Curiously, both intranasal formulations needed significantly higher doses than that required for intramuscular immunization. Taken together, mucosal immunization at the nasal epithelium is capable of driving immune responses. However, a larger dose of the mRNA vaccine is necessary to obtain immune responses comparable to the systemic vaccines.

The Masopust group compared the quality of immune responses following intranasal and systemic vaccines, by characterizing resident memory T (Trm) cells in the respiratory tract [35]. In mice, intranasal vaccination yielded fewer splenic $CD69^+$ antigen-specific memory $CD8^+$ T cells and fewer pulmonary antigen-specific $CD8^+$ T cells than intramuscular and intravenous vaccinations. However, intranasal vaccination outperformed intramuscular vaccines in generating pulmonary antigen-specific $CD69^+$ Trm cells. Interestingly, in the intranasal vaccination group, a majority of the $CD69^+$ Trm cells stained positive for the integrin, CD103. Intramuscular vaccination also produced lung-resident Trm cells, however at a lower frequency than intranasal vaccination. These studies indicated that intramuscular vaccination produced a stronger systemic immune response, while intranasal vaccination produced a stronger lung tissue-specific memory immune response. This led the

authors to combine an intramuscular prime vaccination with an intranasal boost; critically, this combination yielded high levels of circulating memory and Trm cells.

Although these studies have made significant contributions to this field, there remain many questions regarding methods to improve delivery, safety, and immunogenicity of mRNA vaccines in the nasal mucosa. Additionally, it may be beneficial to understand the mechanisms underlying the immune responses to nasal mRNA vaccines and compare these mechanisms to those involved in systemic vaccines.

Gastrointestinal and intravaginal mRNA delivery systems

While the fields of gastrointestinal and intravaginal mRNA vaccination are in their infancy, we have nonetheless taken the opportunity to review and highlight critical aspects of such platforms for the delivery of mRNA. It should be noted that the immune response to these mRNA delivery systems has not been evaluated in detail, yet we have included these studies as we believe they are promising systems for mucosal vaccination. We also note that there has been considerable work done in the area of oral DNA/plasmid vaccination, yet these articles were not included in this mini-review as the delivery and stability considerations for mRNA are distinct.

Our group has recently described a robotic pill for the oral delivery of mRNA [36]. This system, which we call the self-orienting millimeter-scale applicator (SOMA), is a blueberry-sized device filled with a concentrated dispersion of polymer-based mRNA nanoparticles. The SOMA contains a hollow needle whose base is linked to a compressed spring. Upon ingestion, the SOMA aligns itself on the stomach wall, the spring is decompressed pushing the needle tip into the tissue where the mRNA nanoparticles are deposited. In pigs, SOMA-based oral delivery led to the expression of a reporter protein within the stomach wall.

Systems for intravaginal mRNA vaccination have yet to be described. However, there are systems for intravaginal mRNA delivery, which may ultimately be leveraged for vaccination. Lindsay and colleagues used aerosolized naked mRNA for vaginal administration [37]. The authors used water (not saline) to deliver the mRNA as its hypotonic nature was expected to enhance mRNA transport across the mucus layer [38]. In sheep, aerosolization of an mRNA-encoding luciferase enabled delivery to the female reproductive tract [37]. Interestingly, aerosolization of the mRNA dose seemed critical, as the high-pressure injection of mRNA was found to be ineffective. Remaut et al. developed aerosolized lipid nanoparticles for the intravaginal delivery of mRNA [39]. In pigs, the aerosolized lipid nanoparticles successfully delivered an mRNA encoding the luciferase protein. Further work is needed to understand if these systems can be used for vaccination, what immune cell types are engaged via vaginal vaccination, and the nature and magnitude of immune response engendered by these modalities.

Perspective/outlook

Mucosal mRNA vaccination is an area of active scientific investigation. For clinical translation, several questions regarding efficacy, optimization strategies, and safety must be addressed.

It may not be adequate to repurpose systemic formulations for mucosal vaccination. Formulations used for systemic vaccination may not be able to overcome the transport barriers associated with the mucosa. Further, the local inflammatory response to mucosal vaccines, although comparable to systemic vaccines, may compromise their safety due to the site(s) of resultant inflammation [40]. For example, Acuitas lipids were shown to be inducers of IL6, which drove the formation of germinal centers in the draining lymph node and a potent antibody response [41]. The same cytokine was held culpable for acute mortality following intranasal vaccination in mice [40]. SM102-based lipid nanoparticles (such as those used in mRNA-1273) are potent inducers of the inflammasome pathway and IL1β [42]. This mechanism is critical for its function following intramuscular vaccination. However, IL1β is a key mediator of lung damage, which makes SM102 suboptimal for nasal vaccination [43]. Significant efforts may be needed to overcome this challenge. A potential strategy could be to move away from stimulatory ionizable lipids. Instead, nonstimulatory ionizable lipids (e.g. DLin-MC3-DMA) could be combined with adjuvants that have been safely used for mucosal vaccination (e.g. TLR7/8 agonist [44,45]).

Methods that enable mucus and tissue penetration, while maintaining compatibility with sensitive mRNA formulations, are lacking. Research evaluating chemical penetrants such as tight junction modifiers, mucolytic agents, and/or surface modifications may be valuable. Alternatively, physical methods for enhancing delivery (e.g. microneedles, ultrasound, and/or jet spraying) may be beneficial as well.

Fundamental/mechanistic investigations that help distinguish the type of immune response generated by mucosal mRNA vaccines as compared with systemic mRNA vaccines will be of utmost importance. These studies will help inform the field about the safety of mucosal mRNA vaccines and in so doing help identify the most appropriate applications for these systems.

In sum, the field of mucosal mRNA vaccines is a highly exciting area of research. There exist several mechanistic questions that remain to be fully elucidated. Novel

technologies centered on optimal delivery of mRNA vaccines at mucosal surfaces may yield unparalleled preclinical results and pave the way for translation of this class of vaccine.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Exact Sciences, Horizon, Pavoda, Entreza Inc. CBSET, Avaxia, Lyndra, Novo Nordisk, SNS Nano, Hoffman La Roche, Janssen, Egalet, BMS, Synlogic, Freenome, Suono Bio, Merck, Verily, Eagle Pharmaceuticals Inc., Vivtex, Celero Systems, Bilayer Therapeutics Inc., Teal Bio Inc., Oracle, Wired Consulting, Avadel Pharmaceuticals, Moderna, Syntis Bio, Vitakey, CSL Vifor.

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