OCTOBER 2023 Volume 2, Issue 10

SPOTLIGHT ON

RNA vaccines part 2: addressing ongoing challenges

CONTENTS

SPOTLIGHT: RNA vaccines part 2: addressing ongoing challenges

Spotlight

REGULATORY PERSPECTIVE: Nonclinical development of RNA cancer vaccines: proof-of-concept testing, safety study design, and future prospects
Rondine Allen and Christopher Saeui

INTERVIEW: Developing an effective vaccine formulation using self-amplifying RNA and a nanoparticle emulsion
Amit Khandhar

INNOVATOR INSIGHT: Achieving vaccine equity: challenges and opportunities of multi-modality manufacturing
Katarina Stenklo

INTERVIEW: Optimizing *in vitro* transcription reactions: from fundamental research to mRNA vaccine manufacturing
Craig Martin

Latest articles

EXPERT INSIGHT: Physical methods to overcome tissue barriers in vaccine delivery
Taksim Ahmed, Dylan Freitas, Xisha Huang, Qing Rui Simon Qu, Giovanni Traverso, and Ameya R Kirtane

VACCINEINSIGHTS

RNA VACCINES PART 2:
ADDRESSING ONGOING CHALLENGES

SPOTLIGHT

REGULATORY PERSPECTIVE

Nonclinical development of RNA cancer vaccines: proof-of-concept testing, safety study design, and future prospects

Rondine Allen & Christopher Saeui

RNA-based vaccines are nascent technology currently in development for the treatment of multiple types of cancer. Prior to administration in a clinical trial, nonclinical testing of RNA-based vaccines is required to evaluate activity and characterize safety. This article will discuss:

1. Example types of mRNA cancer vaccines;
2. Commonly used delivery methods for RNAs;
3. Nonclinical approaches for evaluating activity;
4. Considerations for evaluating safety; and
5. Future prospects for RNA cancer therapeutics.

Vaccine Insights 2022; 2(10), 417–427

DOI: 10.18609/vac/2023.55

INTRODUCTION TO mRNA VACCINES

Cancer remains one of the most difficult challenges in modern medicine due to its complex nature and diverse manifestations. Traditional cancer treatment modalities such as surgery, chemotherapy, and radiation therapy have improved clinical outcomes over the past few decades, but they often come with significant

side effects and limited efficacy, especially for recurrent cancers and those that become refractory to these types of treatments. In recent years, immunotherapy has garnered much attention for its potential to harness the power of the immune system to fight difficult-to-treat relapsed and refractory cancers. Within the realm of immunotherapies, mRNA based vaccines have emerged as a new modality to target and eliminate cancer cells. By introducing synthetic mRNA encoding cancer-specific antigens into the body, it is hypothesized that the immune system can be trained to recognize and mount an attack against tumors. For personalized cancer treatments, mRNA technology also has advantages that make it suitable for tailoring treatments to each patient's specific tumor.

A major benefit of using an mRNA vaccine approach is the potential for expeditious product development and manufacturing. Unlike traditional vaccines, which often rely on inactivated or weakened viruses or immunogenic proteins and peptides, mRNA vaccines only require the genetic sequence of a target antigen that can be synthesized into a DNA template for production. This allows developers to rapidly identify tumor targets of interest and manufacture an mRNA vaccine in ways that are both customizable and relatively scalable, potentially making it easier to produce large quantities of product [1]. Moreover, mRNA vaccines have particular safety advantages. The technology does not involve live or attenuated viruses, therefore avoiding virus-related immunotoxicities or the potential for insertional mutagenesis [2]. Further, mRNA vaccines do not alter a person's DNA, and synthetic mRNAs used in these vaccines are labile [3] and typically expected to degrade rapidly within the body, thereby limiting their persistence in cells and tissues.

MECHANISM OF ACTION

The mechanism of action for mRNA cancer vaccines is based on the ability of mRNA to instruct cells to produce specific cancer-related proteins (i.e., antigens). The process for developing an mRNA cancer vaccine begins by identifying tumor-specific antigens (TSA, also known as neoantigens) or tumor-associated antigens (TAAs), which are molecules commonly overexpressed on the surface of cancer cells. Synthetic mRNAs are then designed to carry the instructions for producing these antigens inside of cells. Once an mRNA vaccine is administered, it is endocytosed by immune cells such as antigen-presenting cells (APCs) and dendritic cells (DCs) (Figure 1) [4], where the mRNA can be translated and cancer antigens (i.e., peptides) are produced. These antigens are subsequently presented on cellular surface human leukocyte antigen (HLA) molecules, effectively flagging them as foreign to the immune system. Immune cells such as cytotoxic T cells then recognize cancer antigens presented by DCs and APCs to become activated and initiate an immune response against cancer cells expressing the antigenic peptide.

TYPES OF mRNA CANCER VACCINES

mRNA vaccines for cancer generally fall into two broad classes, based on approach – those intended to target cancer TSAs and those intended to elicit an immune response towards TAAs. As we will discuss further, the type of mRNA cancer vaccine impacts the strategies needed for nonclinical development.

For personalized mRNA cancer vaccines targeting TSAs, the development process typically starts with the identification of the neoantigen using genomic sequencing, proteomics, and other bioinformatics based approaches for each patient [5]. Neoantigens are unique antigens that arise from patient-specific genetic mutations. These mutations can generate novel protein sequences that are absent in normal cells, making them highly specific to a patient's tumor and attractive targets for mRNA based cancer vaccines [6,7]. Once

DOI: 10.18609/vac/2023.55

a neoantigen is identified, the corresponding mRNA sequence can be designed and synthesized to produce the desired personalized vaccine [8].

Likewise, mRNA vaccines intended to target TAAs take a similar approach; however, the antigen being targeted is likely to be commonly overexpressed on tumor cells versus healthy tissues [9]. This type of mRNA vaccine is typically not patient-specific; therefore, these types of vaccines are intended to be administered to more than one subject with the same type of cancer.

mRNA DELIVERY

Although this discussion is unable to cover all possible modalities for mRNA delivery, below are some examples of commonly used methods to deliver RNAs for cancer vaccines.

FIGURE 1

General mechanism of action for RNA-based cancer vaccines.

Lipid nanoparticles

One of the common and popular types of delivery strategy for mRNA based therapeutics, lipid nanoparticles (LNPs) are typically composed of four components [4]:

1. An ionizable lipid;
2. A structural (phospho-) lipid;
3. Cholesterol;
4. A polyethylene glycol (PEG)-lipid.

LNPs serve as a protective envelope around mRNAs during delivery *in vivo.* The positively charged ionizable lipid electrostatically interacts with the negatively charged mRNA, encapsulating it (along with the other lipids) for delivery. Once administered, LNPs deliver mRNAs to target cells through endocytosis. Upon internalization, mRNA–LNPs are shuttled into endosomes where the acidic pH of the endosomal environment causes the ionizable lipid to release the mRNA while concomitantly causing the lipids to disrupt the endosomal membrane, facilitating the release of mRNAs into the cytoplasm of the cell [10].

Polymers

Polymeric delivery of mRNAs involves the use of synthetic polymers to encapsulate and deliver mRNA molecules into cells. Many types of polymers can be used, such as polyethyleneimine (PEI), poly(lactic-co-glycolic acid) (PLGA), or completely novel molecular entities specific to a product's development program [11]. However, the general principles behind polymeric delivery and LNPs are similar to those behind delivery using a lipid-based vehicle, namely electrostatic interaction between the polymer and mRNA for efficient encapsulation, cellular uptake, and release. Polymeric delivery, however, may differ from LNPs by taking advantage of different mechanisms for release ranging from pH-induced release to enzymatic degradation of the polymer [12,13].

Lipopolyplex

mRNA delivery using a lipopolyplex is a technique that utilizes aspects of both LNP and polymer based approaches by using a combination of lipids and polymers to form a complex capable of delivering mRNA molecules into cells [14]. A polymer core that encapsulates mRNA is further encapsulated by a shell consisting of a lipid bilayer. Combining lipids with polymers may impart advantages such as improvements in product stability, longer circulation times, and improved cellular uptake [15].

Electroporation

Transfection of cells with nucleic acids using electroporation is a technique that has been employed for many years. Electric pulses generated by a device are used to facilitate entry of mRNA molecules into cells by creating temporary pores in cellular membranes [16]. In this technique, naked mRNAs are typically administered, and the electroporation device is applied to the site of injection. Electroporation offers advantages of being able to deliver mRNAs into difficult to transfect cells and can be used to deliver mRNA to a broad class of cell types. Parameters such as pulse duration, voltage, number of pulses, and other technical factors with the electroporation device may impact safety, therefore it is important to include use of the intended electroporation device during nonclinical development, as feasible [17].

NONCLINICAL TESTING

Nonclinical studies are important to evaluate the safety and activity of mRNA vaccines prior to initiation of a clinical trial. The nonclinical program typically provides support for the scientific rationale, gives insight into the mechanism of action, evaluates biological

DOI: 10.18609/vac/2023.55

activity, and characterizes the safety profile of the mRNA cancer vaccine product [18].

Proof-of-concept testing

In vitro assessment of mRNA vaccines depends on whether the product is intended to target a TSA or TAA. For product development of TAA-targeting mRNA vaccines, commercially available tumor cell lines can often be purchased that express the TAA, or immortalized cell lines can be engineered to express the antigen of interest. *In vitro* co-culture studies with T cells isolated from peripheral blood mononuclear cells (PBMCs) and whole blood with a tumor cell line expressing the TAA can be designed in ways to demonstrate the potential activity of the mRNA vaccine. HLA matched or partially matched donor DCs can also be used to investigate the mechanism of action of TAA expressing mRNAs. In these types of experiments, DCs are pulsed with the mRNA encoding the TAA, and are then cultured with naïve T cells to induce immune responses towards the cancer associated antigen [19,20]. Primed T cells can then be studied further *in vitro* to characterize immune cell response towards tumor cells expressing the TAA. Assay readouts may include cytokine production, cell proliferation, or cytotoxic activity.

For mRNA vaccines intended to target a TSA or neoantigens, *in vitro* work typically uses a different approach given that each TSA will be patient specific. Consequently, it is not possible to evaluate each neoantigen in nonclinical studies because tumor cell lines that express TSAs are not commercially available and issues with HLA matching may make the practicality of performing such studies very difficult. Alternative approaches are often helpful to demonstrate *in vitro* proof-of-concept (POC) for mRNA products intended to target cancer neoantigens; however, similar to the approach for TAA targeting mRNAs, studies may evaluate cytokine secretion, proliferation, and cytotoxicity following incubation of PBMCs transfected with the TSA-targeting mRNA.

When possible, *in vivo* POC studies are helpful to identify the biologically active dose level range and optimal dosing regimen. For example, studies may evaluate antitumor effects, survival, immune response, or cytokine induction in murine cancer models [21–23]. When selecting an animal species for evaluating a TSA or TAA mRNA vaccine, mRNA-induced biological activity and antigen specific immunological responses are important considerations. Animal models with comparable activation and response to humans may be used to model immunogenicity following administration of the product.

For evaluation of mRNAs targeting TAAs, studies may evaluate anti-tumor efficacy following inoculation in a syngeneic animal model with cancer cells expressing the antigen, or studies can be designed employing humanized mice [24,25]. Following vaccination with the mRNA, tumor growth, immune response, and cytokine production are often assessed to characterize product activity. These types of studies allow for the mechanism of immune stimulation to be investigated in animals that have an intact immune system. For example, mRNA vaccination of HLA matched humanized mice can result in quantifiable antigen specific T cell response and cytotoxic activity[23].

Evaluating TSA-specific anti-tumor activity is often not feasible *in vivo* due to the personalized nature of the product. Instead, anti-tumor studies may utilize murine specific tumor models to investigate antigen specific T cell responses and anti-tumor activity of the mRNA vaccine platform. Studies evaluating *in vivo* T cell response and antigen presentation following vaccination can also be informative for characterizing activity and specificity.

An additional point to consider is that mRNA vaccines are often co-administered with checkpoint inhibitors to enhance immune response against a cancer antigen. To support the rationale for administration of a

TAA- or TSA-targeting mRNA vaccine with a checkpoint inhibitor, co-administration of the mRNA vaccine and the checkpoint inhibitor in an animal model can be used to evaluate the activity and contribution of effect for both products.

Route of administration

The selected route of administration (ROA) will influence the biodistribution, persistence, duration of activity, and dose response of the mRNA vaccine. The ROA is typically based on what will enable delivery of the therapeutic entity to the target cells and generate the desired response, desired safety profile, and ease of administration. In many cases, targeted delivery of the mRNA to immune cells is preferred. Thus, local routes of administration including subcutaneous, intramuscular, intradermal, or intra-tumoral injection are commonly used, along with intravenous administration for systemic delivery.

Studies to determine biodistribution (BD) of the mRNA and the delivery components following administration can be important for understanding the distribution, accumulation, and clearance in both target and non-target tissues, which can also assist in interpreting safety information. Although the target site is primarily at the site of injection, BD in a larger set of tissues can provide additional information on the *in vivo* distribution of the product as a part of a comprehensive development program. This may be of particular importance should safety issues be encountered in nonclinical studies.

mRNA vaccines comprised of novel lipid components or polymers may benefit from additional nonclinical assessments depending on the ROA. For example, intravenous administration of an mRNA vaccine that includes a novel lipid component in the formulation may warrant additional characterization, including assessments for *in vivo* stability, tissue tropism(s), absorption, elimination, and potential for generating specific types of metabolites. Additional safety assessments for mRNA-based vaccines are described below. Considerations for additional nonclinical pharmacology studies will depend on the novelty of the delivery components, expected level of exposure, and the dosing regimen.

SAFETY TESTING

Toxicity studies conducted in healthy animals are often used to assess the safety and tolerability of the mRNA vaccine. The results of the POC studies can be used to help guide the design of safety studies. Typically, definitive safety studies are designed to mimic the proposed clinical trial in terms of using the proposed clinical route of administration, dosing regimen, and identical clinical product, as feasible. The half-life and durability of the encoded antigen are also important considerations when determining the appropriate duration of the safety study.

Data quality, reliability, and integrity should be ensured by conducting definitive safety studies in compliance with good laboratory practice (GLP). Principles of GLP include adherence to a prospectively written protocol, minimization of study bias, and detailed recordkeeping. To adequately characterize and identify safety concerns at the proposed clinical dose levels, dose levels bracketing the clinical dose level range are typically administered when feasible. For safety studies, similar evaluations for both TAA and TSA targeting mRNA vaccines are typically used, such as standard safety endpoints including clinical observations, clinical pathology, histopathology, hematology, and coagulation. Product specific endpoints may include evaluation of antigen-specific or non-specific immune response (i.e., cytokine, chemokine response).

If the mRNA vaccine will be co-administered with a checkpoint inhibitor or another type of immunomodulatory agent, the need for additional toxicity studies investigating the combined products often depends on

 DOI: 10.18609/vac/2023.55

whether adequate safety data are available for the inhibitor as a monotherapy or in combination with relevant products, and the potential for additive toxicity. If safety data are limited for the checkpoint inhibitor or immunomodulatory agent, additional studies may be helpful to support the use of the product combination.

Safety considerations for mRNA vaccines targeting TSA or TAA include the potential for on-target/off-tumor and off-target toxicities. Computational analysis may be performed to determine similarity of the antigen translated from the mRNA sequence to the human proteome in order to predict cross-reactivity to healthy human tissues. *In vitro* assays can also be helpful to confirm targets identified from *in silico* analyses. Additional testing to help characterize these risks may include evaluating for antigen expression in tumor tissue, transcriptome analysis, and epitope modeling that can assist in evaluating potential risks for on-target/off-tumor and off-target risks.

Definitive *in vivo* safety studies are typically conducted in a pharmacologically responsive species. For data interpretability, it is important to include an adequate number of animals per treatment group, while taking into consideration the 'three R's' for reducing, refining, and replacing the use of animals, to the extent feasible. For example, inclusion of equal numbers of each sex per treatment group is often unnecessary unless sex-based differences are anticipated.

Delivery components that comprise the final drug product may pose unique safety concerns. These may include slow clearance, accumulation in tissues following multiple administrations, slow metabolism of novel delivery components that may result in tissue injury, unknown clearance profile of the metabolites, injection site reactions, and potential for acute immune response such as anaphylaxis. Studies to address these types of concerns are typically considered on a case-by-case basis and depend on the attributes of the final product.

Product considerations

The product administered in nonclinical studies is usually identical to the clinical product in terms of the antigen encoded, formulation, and manufacturing process at the current stage of development. Administration of a surrogate product targeting a species-specific antigen may be considered if species specific differences exist that would make administration of the clinical product uninformative. In the case of neoantigen or personalized cancer vaccines developed for a patient's specific mutation, it is often not feasible to evaluate the neoantigen. Therefore, nonclinical studies may evaluate known murine or species-specific TSAs, an analogous product, or a representative human neoantigen that incorporates regulatory, structural, and signaling elements found in the clinical product. If an analogous product is evaluated in the nonclinical studies, it is important that the clinical formulation and manufacturing process is utilized to determine the significance of any abnormal findings.

Clinical dose selection

The clinical starting dose for mRNA vaccines is usually based on the totality of nonclinical data. Nonclinical pharmacology and safety data should be used to inform dose selection for the clinical starting dose. To translate data from animal studies to humans, it is important to provide a scientific rationale for the method of dose level extrapolation and provide data to support the dose level and dosing regimen for the mRNA vaccine. In addition, data can be leveraged from studies where the vaccine was comparatively assessed in *in vitro* assays using human and animal cells. We also defer readers to guidance for therapeutic cancer vaccines [26].

PROSPECTS

While the field of mRNA-based therapeutics has grown exponentially over the last decade,

innovation in the RNA field continues, and development of newer technologies within this space may provide additional benefits. Below, a few emerging RNA technologies that are related to the previously discussed mRNA approaches are highlighted. The nonclinical development of products that employ these types of technologies may benefit from discussions with regulatory authorities throughout the product lifecycle due to the novelty of these products and product-specific issues.

Self-amplifying mRNAs

Self-amplifying mRNAs (saRNAs) are modified mRNAs that have the ability to replicate within host cells, leading to the production of higher quantities of mRNA and subsequently more translated protein (Figure 1). saRNAs are engineered by incorporating genetic elements that encode for non-structural proteins that form a replication complex to produce more copies of mRNA. This idea was derived from the viral genomes of alphaviruses or flaviviruses [27]. Amplification of mRNA allows for enhanced antigen production, thus potentially improving immune responses for vaccines. In addition, due to the self-amplifying nature of the RNAs, lower dose levels may be needed to achieve similar types of activity observed with traditional mRNA vaccines, providing manufacturing and safety advantages [28].

Circular RNAs

Circular RNAs (circRNAs) are a class of RNA molecules that are formed by covalently closing RNA molecules to form a circular structure, as opposed to a linear molecule like mRNA. circRNAs are generated through a process called back-splicing, where a downstream splice site is joined to an upstream splice site, resulting in a circularized molecule [29]. The potential advantage of this change in topology is increased stability compared to linear mRNAs, which is achieved by the resistance of circRNAs to nuclease degradation [28,30]. Although circRNAs are currently being investigated for additional biological functions such as serving as microRNA sponges, RNA-binding proteins, and transcription and splicing regulators, circRNAs might be used in the development of cancer vaccines due to the advantage of a longer RNA molecule half-life that may subsequently lead to enhanced immunogenicity against cancer.

Smart RNAs

Smart RNAs are synthetic RNA molecules with specific chemical or structural alterations that confer desired properties. Such properties can involve chemical modifications such as nucleoside substitutions or backbone modifications to enhance stability, specificity, or resist degradation [30–32]. Functional elements that can be incorporated into smart RNAs include ribozymes with enzymatic activity, aptamers designed to bind to specific targets, or elements encoding for small interfering RNAs [33]. These highly customizable RNA molecules can be used in applications intended to modulate gene expression, such as inhibiting the expression of disease-causing genes, or stimulating the immune system to elicit a desired immunogenic response. Smart RNAs represent a customizable class of RNA molecules, making them a versatile platform for potential cancer vaccine development.

Trans-amplifying RNAs

Trans-amplifying RNAs (taRNAs) are engineered RNAs designed to amplify the expression of a target gene by serving as intermediaries that enhance the production of specific RNA transcripts [34]. taRNAs typically contain sequences that bind to mRNA molecules with high specificity. Once a taRNA binds to its mRNA target, it recruits RNA polymerase to synthesize more copies of the mRNA molecules, leading to amplified levels of the target mRNA molecule. A common challenge with developing vaccines for neoantigens or

 DOI: 10.18609/vac/2023.55

specific tumor markers is low expression on the cell surface that prevents a strong immunogenic reaction following vaccination. Therefore, like saRNAs discussed above, taRNAs may be an attractive strategy to amplify expression of antigens with low abundance on the cancer cell surface.

CONCLUSION

mRNA cancer vaccines represent a new approach that offers several unique advantages in the field of cancer immunotherapy. These vaccines utilize the ability of mRNA to instruct cells to produce tumor antigens that can stimulate a targeted immune response. The development of mRNA cancer vaccines has been facilitated by recent advancements in mRNA synthesis, delivery systems, and our understanding of tumor specific antigens.

A significant advantage of mRNA vaccines is their flexibility and rapid development process. mRNA sequences can be easily modified to encode specific tumor antigens, allowing for personalized and adaptable vaccine design. This adaptability enables the inclusion of multiple antigens or other immune stimulatory factors that can enhance immune response to overcome the challenges associated with tumor heterogeneity.

The nonclinical development of mRNA-based cancer vaccines presents unique challenges that are class-specific and largely a function of whether the vaccine is intended as a personalized vaccine to target a cancer neoantigen (i.e., TSAs), or as a vaccine against a cancer selective target that is broadly overexpressed in a specific tumor type (i.e., TAAs). Nonclinical assessments of these products can be challenging due to issues such as the reliance of these products on HLA presentation and the differences between humans versus non-human species in this aspect of adaptive immune response. For these reasons, nonclinical development programs are often product- and indication-specific. Continued dialogue with regulatory authorities may be helpful during product development to obtain feedback on the nonclinical testing program.

Despite these challenges, the development of mRNA cancer vaccines continues to make significant progress in the fight against cancer. Clinical investigation of mRNA based vaccines has demonstrated their utility during the COVID19 pandemic, and recent publicly reported results show that mRNA cancer vaccines have progressed to late-stage clinical trials [35]. Research into new RNA technologies such as saRNAs, circRNAs, and taRNAs will continue to advance progress of this field.

REFERENCES

1. Rosa SS, Prazeres DMF, Azevedo AM, Marques MPC. mRNA vaccines manufacturing: Challenges and bottlenecks. *Vaccine* 2021; 39, 2190–2200.

2. Pardi N, Hogan MJ, Porter FW, Weissman D. mRNA vaccines—a new era in vaccinology. *Nat. Rev. Drug Discov.* 2018; 17, 261–279.

3. Wang C, Liu H. Factors influencing degradation kinetics of mRNAs and half-lives of microRNAs, circRNAs, lncRNAs in blood *in vitro* using quantitative PCR. *Sci Rep.* 2022; 12, 7259.

4. Deng Z, Tian Y, Song J, An G, Yang P. mRNA vaccines: the dawn of a new era of cancer immunotherapy. *Front. Immunol.* 2022; 13, 887125.

5. Li L, Goedegebuure SP, Gillanders WE. Preclinical and clinical development of neoantigen vaccines. *Ann. Oncol.* 2017; 28, xii11–xii17.

6. Xie N, Shen G, Gao W, Huang Z, Huang C, Fu L. Neoantigens: promising targets for cancer therapy. *Signal Transduct. Target Ther.* 2023; 8, 9.

7. Lang F, Schrörs B, Löwer M, Türeci Ö, Sahin U. Identification of neoantigens for individualized therapeutic cancer vaccines. *Nat. Rev. Drug Discov.* 2022; 21, 261–282.

8. Schumacher TN, Scheper W, Kvistborg P. Cancer neoantigens. *Annu Rev Immunol.* 2019; 37, 173–200.

9. Haen SP, Loffler MW, Rammensee HG, Brossart P. Towards new horizons: characterization, classification and implications of the tumour antigenic repertoire. *Nat. Rev. Clin. Oncol.* 2020; 17, 595–610.

10. Patel S, Ashwanikumar N, Robinson E, *et al.* Boosting intracellular delivery of lipid nanoparticle-encapsulated mRNA. *Nano Lett.* 2017; 17, 5711–5718.

11. Pack DW, Hoffman AS, Pun S, Stayton PS. Design and development of polymers for gene delivery. *Nat. Rev. Drug Discov.* 2005; 4, 581–593.

12. Yang W, Mixich L, Boonstra E, Cabral H. Polymer-based mRNA delivery strategies for advanced therapies. *Adv. Healthc. Mater.* 2023; 12, e2202688.

13. Huang P, Deng H, Zhou Y, Chen X. The roles of polymers in mRNA delivery. *Matter* 2022; 5, 1670–1699.

14. Zhang W, Jiang Y, He Y, *et al.* Lipid carriers for mRNA delivery. *Acta Pharm. Sin. B* 2023; 13, 4105–4126.

15. Persano S, Guevara ML, Li Z, *et al.* Lipopolyplex potentiates anti-tumor immunity of mRNA-based vaccination. *Biomaterials* 2017; 125, 81–89.

16. Silva-Pilipich N, Lasarte-Cía A, Lozano T, Martín-Otal C, Lasarte JJ, Smerdou C. Intratumoral electroporation of a self-amplifying RNA expressing IL-12 induces antitumor effects in mouse models of cancer. *Mol. Ther. Nucleic Acids* 2022; 29, 387–399.

17. Campillo-Davo D, De Laere M, Roex G, *et al.* The ins and outs of messenger RNA electroporation for physical gene delivery in immune cell-based therapy. *Pharmaceutics* 2021; 13(3), 396.

18. FDA. Guidance for Industry. Preclinical Assessment of Investigational Cellular and Gene Therapy Products 2013.

19. Teufel R, Carralot JP, Scheel B, *et al.* Human peripheral blood monuclear cells transfected with messenger RNA stimulate antigen-specific cytotoxic T-lymphocytes *in vitro. Cellular Mol. Life Sci.* 2005; 62(15), 1755–1762.

20. Ponsaerts P, Van Tendeloo VFI, Berneman ZN. Cancer immunotherapy using RNA-loaded dendritic cells. *Clin. Exp. Immunol.* 2003; 134, 378–384.

21. da Silva JR, Rodrigues KB, Pelegrin GF, *et al.* Single immunizations of self-amplifying or non-replicating mRNA-LNP vaccines control HPV-associated tumors in mice. *Sci. Transl. Med.* 2023; 15, eabn3464.

22. Grunwitz C, Salomon N, Vascotto F, *et al.* HPV16 RNA-LPX vaccine mediates complete regression of aggressively growing HPV-positive mouse tumors and establishes protective T cell memory. *Oncoimmunology* 2019; 8, e1629259.

23. Do ASMS, Amano T, Edwards LA, Zhang L, De Peralta-Venturina M, Yu JS. CD133 mRNA-loaded dendritic cell vaccination abrogates glioma stem cell propagation in humanized glioblastoma mouse model. *Mol. Ther. Oncolytics* 2020; 18, 295–303.

24. Wang Y, Zhang L, Xu Z, Miao L, Huang L. mRNA vaccine with antigen-specific checkpoint blockade induces an enhanced immune response against established melanoma. *Mol. Ther.* 2018; 26, 420–434.

25. Choi Y, Lee S, Kim K, Kim SH, Chung YJ, Lee C. Studying cancer immunotherapy using patient-derived xenografts (PDXs) in humanized mice. *Exp. Mol. Med.* 2018; 50, 1–9.

26. FDA. Guidance for Industry: Clinical Considerations for Therapeutic Cancer Vaccines, 2011.

27. Geall AJ, Verma A, Otten GR, *et al.* Nonviral delivery of self-amplifying RNA vaccines. *Proc. Natl. Acad. Sci. USA* 2012; 109, 14604–14609.

28. Dailey GP, Crosby EJ, Hartman ZC. Cancer vaccine strategies using self-replicating RNA viral platforms. *Cancer Gene Ther.* 2023; 30, 794–802.

29. Su M, Xiao Y, Ma J, *et al.* Circular RNAs in cancer: emerging functions in hallmarks, stemness, resistance and roles as potential biomarkers. *Mol. Cancer* 2019; 18, 90.

30. Liu X, Zhang Y, Zhou S, Dain L, Mei L, Zhu G. Circular RNA: An emerging frontier in RNA therapeutic targets, RNA therapeutics, and mRNA vaccines. *J. Control. Release* 2022; 348, 84–94.

31. Liang JC, Bloom RJ, Smolke CD. Engineering biological systems with synthetic RNA molecules. *Mol. Cell* 2011; 43, 915–926.

32. Kushwaha M, Rostain W, Prakash S, Duncan JN, Jaramillo A. Using RNA as molecular code for programming cellular function. *ACS Synth. Biol.* 2016; 5, 795–809.

 DOI: 10.18609/vac/2023.55

33. Dykstra PB, Kaplan M, Smolke CD. Engineering synthetic RNA devices for cell control. *Nat. Rev. Genet.* 2022; 23, 215–228.

34. Beissert T, Perkovic M, Vogel A, *et al.* A trans-amplifying RNA vaccine strategy for induction of potent protective immunity. *Mol. Ther.* 2020; 28, 119–128.

35. Lorentzen CL, Haanen JB, Met Ö, Svane IM. Clinical advances and ongoing trials on mRNA vaccines for cancer treatment. *Lancet Oncol.* 2022; 23, e450–e458.

AFFILIATIONS

Rondine Allen
US Food & Drug Administration (FDA)

Christopher Sauei
Team Leader and Pharmacology/Toxicology Reviewer,
Center for Biologics Evaluation and Research,
US Food & Drug Administration (FDA)

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.
Acknowledgements: None.
Disclosure and potential conflicts of interest: The authors have no conflicts of interest.
Funding declaration: The author received no financial support for the research, authorship and/or publication of this article.

ARTICLE & COPYRIGHT INFORMATION

Copyright: Published by *Vaccine Insights* under Creative Commons License Deed CC BY NC ND 4.0 which allows anyone to copy, distribute, and transmit the article provided it is properly attributed in the manner specified below. No commercial use without permission.
Attribution: Copyright © 2023 Allen R & Saeui C. Published by *Vaccine Insights* under Creative Commons License Deed CC BY NC ND 4.0.
Article source: Invited; externally peer reviewed.
Submitted for peer review: Aug 3, 2023; **Revised manuscript received:** Oct 6, 2023;
Publication date: Nov 9, 2023.

INTERVIEW

Developing an effective vaccine formulation using self-amplifying RNA and a nanoparticle emulsion

Charlotte Barker, Editor, *Vaccine Insights*, speaks to **Amit Khandhar**, Director of Formulations, HDT Bio, about creating a formulation that aims to address key challenges of RNA vaccines, including thermostability and reactogenicity.

Vaccine Insights 2023; 2(10), 395–398

DOI: 10.18609/vac.2023.52

What are the biggest challenges for RNA vaccine formulation and how are you seeking to address those?

AK: There have been some reactogenicity issues with lipid nanoparticle (LNP)-formulated mRNA vaccines, so we are working on making RNA vaccines less reactogenic, while still preserving their effectiveness.

Our formulation uses self-amplifying RNA instead of mRNA. Self-amplifying (sa)RNA adopts a virus' ability to replicate without actually making a virus, swapping out the structural proteins of the virus for the gene of interest—often a vaccine antigen. Our delivery

formulation, LION™ (a cationic oil-in-water nanoparticle emulsion), optimizes surface chemistry, improving effectiveness and reducing reactogenicity.

By combining our saRNA and LION technology, we have created a vaccine platform, AMPLIFY, which we believe has important advantages over mRNA–LNP and saRNA–LNP formulations.

> We can make a vaccine antigen, but we would also like to directly produce antibodies to the antigen, by encoding it in the RNA.

LNPs distribute widely, specifically to the liver, which produces a large amount of systemic interferons and other inflammation markers. High levels of systemic interferons also contribute to the dose ceiling for mRNA. With AMPLIFY, replication occurs in the muscle at the injection site, which is immunologically quiescent, so it can maintain high expression levels. However, the platform still drives an effective immune response by "turning a desert into an oasis" of immune cells as they are recruited to the site of injection.

When characterizing the immune response to AMPLIFY versus saRNA–LNP, an interesting finding was that responses varied between species. We saw similar adaptive immunogenicity with both platforms in mice; however, when we started studying larger animals like monkeys, we saw less reactogenicity and improved immunogenicity, raising the dose ceiling of AMPLIFY compared with saRNA–LNP [1]. Because we can go to higher doses, we are able to incorporate multiple saRNAs to make multivalent vaccines.

Another critical issue is that current mRNA vaccines require cryofreezing, which necessitates an infrastructure not readily available at the global level. We have developed methods for lyophilization—premixing our formulation with RNA and reconstituting it with water for injection—which allows for storage at 2–8° C, or even room temperature.

Q Could you elaborate on the structure of LION?

AK: Emulsion formulations are very stable because of their oil core and we can take advantage of that stability by complexing the RNA to the surface of the particles.

With LNPs, the RNA is inside the nanoparticle and it is manufactured in line. The RNA and the lipids are mixed and the lipids self-assemble around the RNA. With LION, the formulation line is separate from the RNA, and you can mix it at any point before administration. Because the RNA is on the surface of the particle, it can also be swapped out more readily, for example, to adapt to a new virus variant.

Since the RNA is on the outside, it is important to optimize how we complex the LION and RNA. The ratio of the formulation and the mixing kinetics are both important. Depending on the process used to mix it, the particles can be a different size. If we can develop a greater understanding of how those different sizes of particles affect adaptive responses, we can begin to tailor the complex to a desired immune response.

Where is RNA vaccine formulation heading in the next 5–10 years?

AK: The speed at which we are able to produce RNA vaccines will hopefully enable us to respond better to future pandemics. There is a constant battle to identify

> …when we started studying larger animals […] we saw less reactogenicity and improved immunogenicity, raising the dose ceiling of AMPLIFY compared with saRNA–LNP.

what those pandemics will be, but if some pathogens are known and the vaccine formulations are stable we may be able to stockpile vaccines. Therefore, thermostability will become very important.

From a therapeutic angle, another area with a lot of potential is expressing antiviral antibodies. We can make a vaccine antigen, but we would also like to directly produce antibodies to the antigen, by encoding it in the RNA. This would require passive immunoprophylaxis, meaning that it needs to avoid triggering the immune system. If we can get the right amount of antibody in the blood, it could afford immediate protection, working as a prophylactic or even therapeutic. This area of research is very hot right now.

Safety is always an important area of discussion, and we are starting to learn more about how LNPs trigger adaptive responses. A lot is already known, but we are unlocking more information on the mechanisms behind the responses, such as the potential adjuvant effects of the formulation itself. For instance, researchers recently screened a whole library of lipids to see if they could further potentiate the immune response and allow lower doses [2].

Q What is next for HDT Bio?

AK: In addition to our work in further increasing thermostability, one of our next steps is exploring different routes of administration. For example, intranasal administration may give us some desired mucosal responses for certain pathogens.

Another area that we are working on is cancer vaccines. As deep sequencing and RNA production increase in speed, we will be able to tailor vaccines to individuals.

REFERENCES

1. Kimura T, Leal J, Simpson A, *et al.* A localizing nanocarrier formulation enables multi-target immune responses to multivalent replicating RNA with limited systemic inflammation. *Mol. Ther.* 2023; 31(8), 2360–2375.

2. Li B, Manan R, Liang S, *et al.* Combinatorial design of nanoparticles for pulmonary mRNA delivery and genome editing. *Nat. Biotechnol.* 2023; 41(10), 1410–1415.

BIOGRAPHY

AMIT KHANDHAR is the Director of Formulations at HDT Bio. He earned his PhD in Materials Science and Engineering from the University of Washington and, for over a decade now, he has investigated how various nanoparticle technologies interface with biological systems. He is an inventor of HDT Bio's LION™ technology—a clinical stage formulation used for the delivery of self-amplifying RNA vaccines. His current research interests lie in understanding how routes of vaccine administration affect the quality of immune responses, extending HDT Bio's AMPLIFY technology to diverse infectious disease and oncology targets, and designing formulations that enable nucleic acid modalities beyond saRNA.

AFFILIATION

Amit Khandhar PhD
Director of Formulations,
HDT Bio

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.
Acknowledgements: None.
Disclosure and potential conflicts of interest: Khandhar A owns HDT Bio stock options, is an inventor on several filed and issued patents relating to HDT Bio's AMPLIFY technology
Funding declaration: Some of the safety data referenced in the interview was generated by funding support from the US NIH (1R43AI165100-001, 75N93020C00028, 75N93019C00037, R61AI161811, 75N93019C00008).

ARTICLE & COPYRIGHT INFORMATION

Copyright: Published by *Vaccine Insights* under Creative Commons License Deed CC BY NC ND 4.0 which allows anyone to copy, distribute, and transmit the article provided it is properly attributed in the manner specified below. No commercial use without permission.
Attribution: Copyright © 2023 Khandhar A. Published by *Vaccine Insights* under Creative Commons License Deed CC BY NC ND 4.0.
Article source: Invited; externally peer reviewed.
Revised manuscript received: Oct 20, 2023; **Publication date:** Nov 8, 2023.

VACCINEINSIGHTS

RNA VACCINES PART 2:
ADDRESSING ONGOING CHALLENGES

SPOTLIGHT

INNOVATOR INSIGHT

Achieving vaccine equity: challenges & opportunities of multi-modality manufacturing

Katarina Stenklo

Next-generation vaccine platforms comprise different modalities, including viral vectors (e.g., AAV), pDNA), and mRNA. These vaccine platforms have become popular among the biopharmaceutical industry but also entail several manufacturing challenges. This Expert Insight outlines the manufacturing processes of these vaccine platforms, the difficulties faced by manufacturers who choose to implement multiple modalities, factors to consider when working with smaller batches, and the benefits of implementing digitization. Streamlining the manufacturing process with cost in mind may increase the adoption of these new vaccine technologies globally, including in developing countries.

Vaccine Insights 2023; 2(10), 381–393

DOI: 10.18609/vac/2023.051

INCREASING VACCINATION MANUFACTURING WORLDWIDE

With the rising popularity of nucleic acid-based vaccines, manufacturers have more choices than ever before for their vaccine platforms. Adoption of these new vaccine technologies worldwide, particularly in developing countries, could be improved, with several challenges posing barriers to implementation. In 2021, WHO set the target of 70% global COVID-19 vaccination coverage by mid-2022 [1]; however, this was not achieved. The cost of producing mRNA or viral vector-based vaccines is significant, especially for lower-income countries. To meet the WHO target, low-income countries would need to increase their healthcare spend by 56.6% on average, while high-income countries only need to increase their health spend by 0.8% on average [2]. Further challenges applicable to all vaccine manufacturers

include optimization of manufacturing, procurement, distribution, education, and uptake.

Vaccines can be divided into classical vaccine platforms and next-generation vaccine platforms. Next-generation vaccine platforms include viral vectors (e.g., Ebola vaccine), DNA vaccines, RNA vaccines (e.g., mRNA COVID-19 vaccine), and antigen-presenting cells. Classical vaccine platforms include whole inactivated virus (e.g., polio vaccine), live-attenuated virus (e.g., measles, mumps, rubella vaccine), protein subunit (e.g., flu vaccine), and virus-like particle (e.g., human papillomavirus vaccine). Classical platforms are typically based on weakened/inactive viruses or viral proteins, while next-generation platforms are mainly based on gene transfer.

MANUFACTURING mRNA

Promise of mRNA

Compared with traditional vaccine platforms, mRNA vaccines are faster and easier to produce because they do not rely on the production of cell-based components, which typically require several optimization rounds. mRNA vaccines can be produced in as little as five weeks; in comparison, other viral vector platforms can take up to three years. A benefit of the increased speed of mRNA vaccine development is the reduced costs in process development, making the vaccine platform more accessible. To increase the accessibility of mRNA vaccines to low-income countries, WHO created the mRNA vaccine technology transfer hub in Cape Town, South Africa. The hub aims to build capacity in low- and middle-income countries, enabling them to produce mRNA vaccines through a center of excellence and training.

mRNA therapy can be manufactured to cater to different population sizes, spanning from an individual patient (personalized therapy), small to medium populations (e.g., for infectious diseases and oncology), and large populations (e.g., pandemic response). Several considerations around the manufacturing process of mRNA vaccines are required to cater to these different population groups, including the dosage regimen and the flexibility of manufacturing workflow required. A summary of mRNA vaccine manufacturing requirements for different population sizes is shown in **Figure 1**.

The mRNA therapy landscape is evolving rapidly, with increased diversity in indications and therapy types (e.g., vaccine, gene editing, protein, and antibody replacement) for *ex vivo* and *in vivo* cell applications. The majority of mRNA drugs are for vaccines for non-oncological indications (approximately 42%), followed by vaccines for oncological indications (16%) and protein replacement therapies (9%). A flexible manufacturing set-up will enable manufacturers to undertake various mRNA therapies for different indications simultaneously and allow scaling up when product demand increases.

mRNA manufacturing at large & small scales

In addition to manufacturing considerations around population size, mRNA therapy type, and disease indications, the specific manufacturing workflow should be acknowledged and optimized for each therapy. **Figure 2** shows the general mRNA therapy manufacturing workflow, with considerations during development, and strategies for efficient therapy development.

mRNA vaccine manufacturing requirements differ depending on the batch size. Manufacturing large to mid-size batches focuses on increased yield, quality, and capacity, while small-to-mid-scale batches (including personalized therapies) aim to implement focused, integrated, and automated solutions. To scale up production from small to larger batches, it is important to consider minimizing COGs while the optimal batch size is achieved. For personalized therapy, patient end-to-end tracking is required, which can only be achieved through robust system

DOI: 10.18609/vac/2023.051

integrations to patient services, manufacturing, and QC.

The mRNA production process in small-scale manufacturing is diverse and highly dependent on the construct size, capping strategy, and *in vitro* transcription efficiency. Smaller-scale production can be accommodated by a flexible manufacturing platform with separate but connected unit operations, which maximizes flexibility, or as fully

FIGURE 1

mRNA therapy manufacturing considerations based on population sizes.

Category	Considerations
Pandemic response	• Few but large therapies to large populations • Number of doses: one– two, yearly booster shots • 10 L – 100 L IVT scale • Production/year: < 1 kg – 300 kg • Large manufacturing, localized, pandemic preparedness
Other infectious diseases and oncology	• Potentially many different therapies to medium to small populations • Number of doses: one- life long • 0.1 L – 10 L IVT scale • Production/year: < 1 g – 50 kg • Flexible manufacturing, multi product, and/or scaled out
Personalized therapies	• Doses: µg – mg • Number of doses: one– life long • 10 mL - 50 mL IVT scale • Production/year: < 1 g • Collapsed workflow in micro factory

IVT: *In vitro* transcription (an enzymatic process used to synthesize mRNA products).

FIGURE 2

Considerations and strategies for mRNA therapy manufacturing.

	DNA template	mRNA IVT	Purification	LNP formation	Filling
Considerations	• Quality and availability of template DNA • pDNA or synthetic DNA • Template design	• In vitro transcription (IVT) and capping efficiency • Capping strategy • Titer variations • Impurities • Scale	• Nonscalable methods • Capacity • Selectivity • Yield • Product stability • Scale	• Efficiency • Access to lipids • Solvents • Outsource or insource • Limited industry experience and expertise	• High dose volume • Multi-product • Often outsourced • Stability • Quality control
Strategies	– Insourced pDNA manufacturing	– Reagent sourcing and accessibility – Process control to reduce batch-to-batch variability	– Resins, membranes, and filters specifically developed for mRNA combined with traditional technologies – Flexible equipment	– Insourced encapsulation – Scale out to maintain quality with improved timeline – Access to lipid library	– Closed robotic filling equipment – Scale out or scale up filling to match dose output need – Insourced filling capacity

LNP: Lipid nanoparticle; pDNA: Plasmid DNA.

integrated unit operations in a box or RNA printer setting, where everything is connected in a fixed way. This simplifies the process but there will be less flexibility for process changes.

Intensifying manufacturing of mRNA

Intensifying a mRNA manufacturing facility can allow for reductions in batch sizes and process steps, allowing several shorter batches at smaller scale to be carried out. There are a number of opportunities to improve the efficiency of an mRNA manufacturing facility. The manufacturer could consider whether a ballroom manufacturing system (deploying equipment to meet the needs of a specific process by connecting additional operations units or running multiple processes in one closed space) could be used, or whether discrete closed-room segregation is required. Furthermore, single-use products and closed systems reduce contamination.

The FlexFactory™ biomanufacturing train (Cytiva), is an integrated manufacturing platform with flexible single-use equipment that can be automated, including different levels of industrial automation. The optimized manufacturing system provides an end-to-end solution to support smaller-scale mRNA production while complying with GMP policies. For mid- to large-scale vaccine manufacturing, Cytiva also supplies a KUBio™ manufacturing facility, designed to include all the benefits of the FlexFactory. When production plans call for a larger scale of manufacturing, the KUBio BSL-2 modular facility offers a flexible, expandable solution in as little as 18 months. Kubio provides the BSL1 environment for manufacture, as well as accommodating the preparative processes and suitable space for GMP filling.

FIGURE 3

pDNA is used as the starting point for many therapies or directly as a DNA vaccine.

MANUFACTURING pDNA

pDNA is used at the start of the manufacturing process of several vaccines and therapies; for example, it is used as a template for mRNA manufacturing and to produce recombinant protein. pDNA can also be utilized directly as DNA vaccines (Figure 3).

High-quality pDNA manufacturing is needed to produce vaccines. Although GMP certification is not required, there has been a recent drive for manufacturing facilities to comply with GMP principles to ensure that the drug product intended for clinical trials is free from trace amounts of DNA plasmids and fragments. A pDNA GMP manufacturing facility should aim to produce pDNA at the appropriate quality for the specific therapy and allow flexibility for scale and multi-product manufacturing. The challenge in a pDNA GMP manufacturing facility is that different plasmids may require alterations in the developmental process (e.g., the purification strategy) depending on the application. Additional challenges include meeting the required yield and purity goal of the pDNA as well as access to increased manufacturing capacity. Furthermore, manufacturers may opt for integrated solutions to allow more efficient manufacturing, with increased control and oversight on compliance.

Optimizing the pDNA manufacturing process involves several parameters to meet the desired purity and concentration:

- Process time

 DOI: 10.18609/vac/2023.051

- Scalability
- Flexibility
- Increased process control
- Batch cost
- Footprint
- Environmental waste handling

Examining the workflow and optimizing parameters for each pDNA is essential for an efficient manufacturing process. Further considerations to improve pDNA titer and quality require optimization of upstream and mid-stream processes, including:

- Fermentation protocol
- Cell concentration
- Efficient cell lysis to allow pDNA release
- Removal of potentially contaminating material (e.g., host-cell protein, RNA, DNA, and endotoxins)

A two-step pDNA downstream purification can be implemented into the manufacturing workflow to meet the purity and concentration needs of the manufacturer, as outlined in Figure 4. Furthermore, with a flexible manufacturing set up you can support different process with different purification strategies. Cytiva offers biomanufacturing solutions for pDNA, including the FlexFactory and KuBio manufacturing facilities.

MANUFACTURING VIRAL VECTORS

Viral vectors include lentivirus, AAV, and adenovirus. The manufacturing processes of these viruses for clinical use are summarized in Figure 5, where the process for lentivirus and AAV differs from that of adenovirus (and other viruses). Of note, the size of these viruses is also distinct and affects the purification strategy utilized, i.e., AAV is 25 nm, lentivirus is 80–120 nm, and adenovirus is 70–90 nm.

AAV is widely used as a viral vector for gene therapy and, depending on the target organ, several AAV serotypes exist (Table 1). The serotype chosen as a vaccine therapy determines

FIGURE 4

Proposed two- and three-step pDNA purification process.

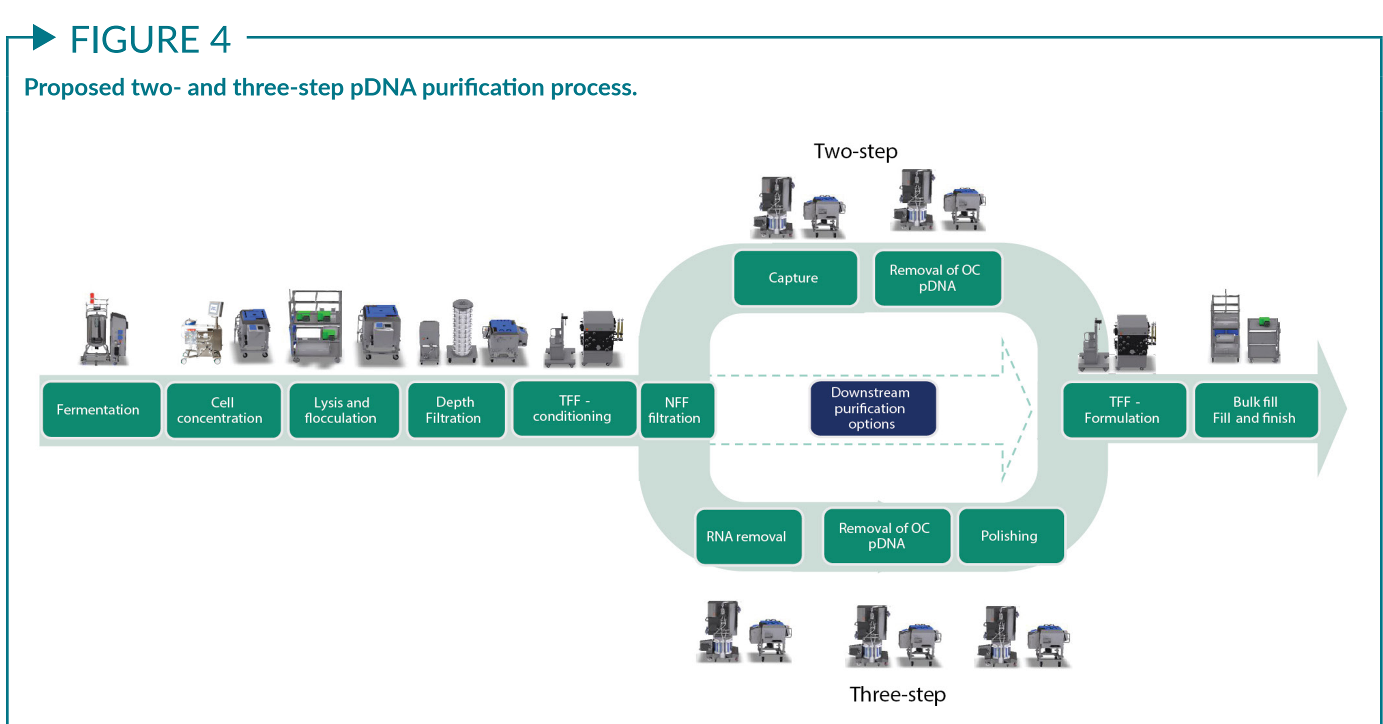


TABLE 1

Target organs determine the selection of serotype.

Tissue	Optimal serotype
CNS	AAV1, AAV2, AAV4, AAV5, AAV8, AAV9
Heart	AAV1, AAV8, AAV9
Kidney	AAV2
Liver	AAV7, AAV8, AAV9
Lung	AAV4, AAV5, AAV6, AAV9
Pancreas	AAV8
Photoreceptor cells	AAV2, AAV5, AAV8
RPE	AAV1, AAV2, AAV4, AAV5, AAV8
Skeletal muscle	AAV1, AAV6, AAV7, AAV8, AAV9

CNS: Central nervous system; RPE: Retinal pigment epithelium.

the developmental process required, including the purification strategy.

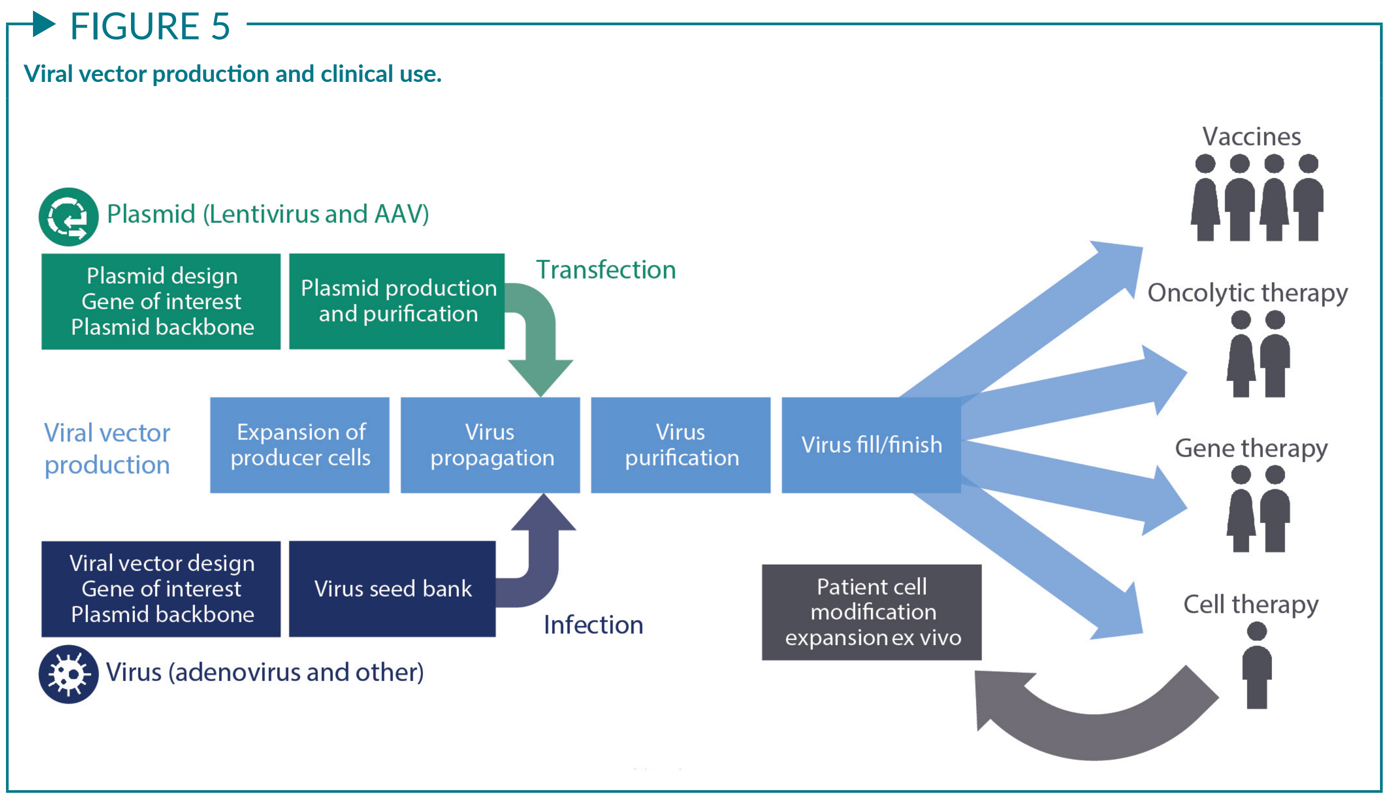
In AAV manufacturing, several challenges must be considered and overcome. A low proportion of full capsids in the harvested material may ultimately reduce the yield/output. At the harvest and filtration step, sufficient lysis of cells is needed to release the virus from the transfected host cells, and reducing the release of host cell proteins and DNA may be a strategy to optimize filtration capacity. At the polishing step, it is necessary for full and empty capsids to be separated, which would need to be optimized for each serotype. Manufacturers may also consider the trade-off between viral genome recovery and the level of full capsid present. Lastly, analytics for each step must be accurate, especially for optimizing the polishing step.

ASEPTIC FILLING

Aseptic filling occurs at the end of the manufacturing workflow. The goal at this stage is to maximize the number of doses/vials from each manufacturing batch and reduce loss

FIGURE 5

Viral vector production and clinical use.



DOI: 10.18609/vac/2023.051

from the final product. Manufacturers may require increased capacity if they implement a multi-product manufacturing process. Cytiva provides the SA25 Aseptic Filling Workcell, a standardized, robotic filling solution integrated into a closed, gloveless isolator for middle-sized vaccine batch productions. The use of robotic work cells in a closed environment reduces the risk of contamination or exposure to hazards and the facility enables storage of products at 80 °C if required.

FUTURE-PROOFING VACCINE INVESTMENTS

Investing in uncertainty can be challenging, especially regarding vaccine manufacturing. Setting up GMP manufacturing for vaccines can be time-consuming, and it may be necessary to start securing manufacturing facilities before it is known what will be produced in the facility. It is recommended to design manufacturing to start with small batches, to help with scaling up, as opposed to setting up a larger but underutilized manufacturing facility.

Manufacturers must communicate with their scientists to ensure they are informed and engaged on how manufacturing will be done. It is also essential to know the quality attributes of the products: the characteristics of a product that are critical to its safety, efficacy, and performance. In some cases, new QC tests may be required to ensure the vaccine meets the necessary quality standards.

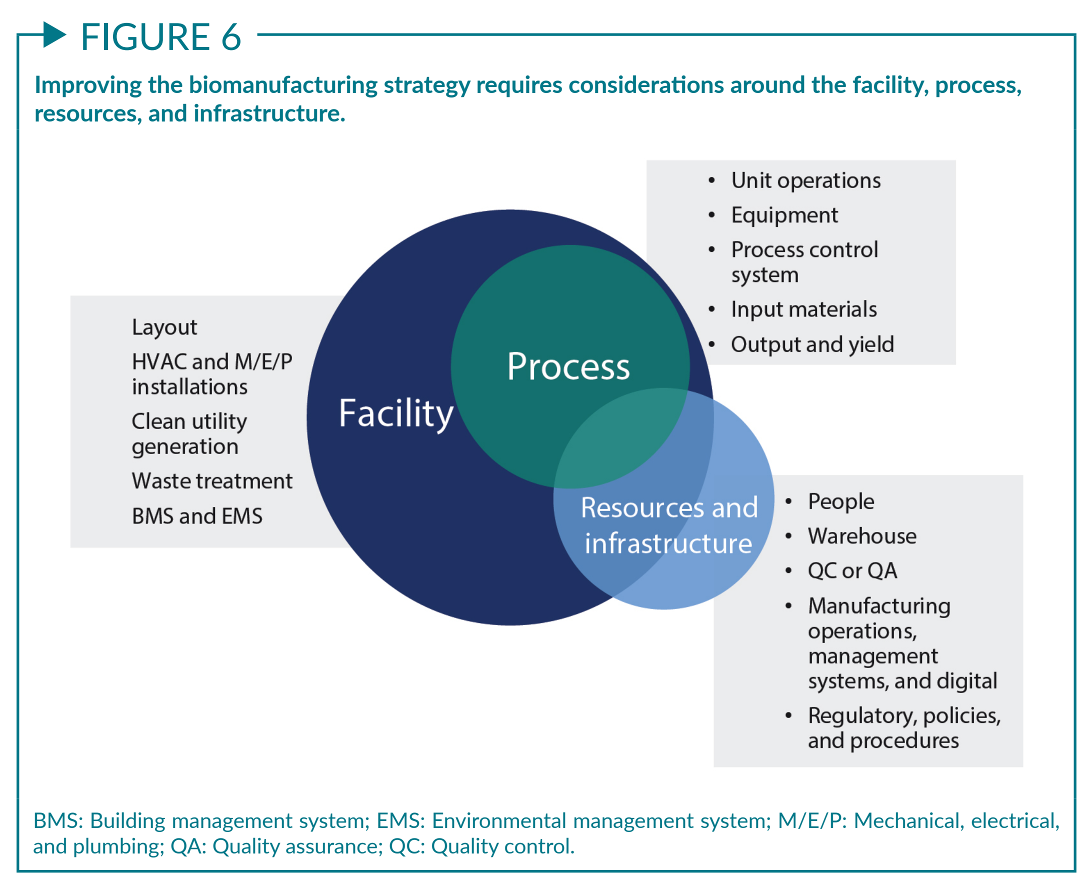
Simplifying the manufacturing strategy can help reduce the time and cost of production. Biomanufacturing incorporates processes, facilities, resources, and infrastructure (Figure 6), all of which are connected.

STRATEGIES FOR MULTI-MODALITY VACCINE MANUFACTURERS

Although challenging, some manufacturers may adopt a multi-modality approach and utilize crossovers in the manufacturing line.

FIGURE 6

Improving the biomanufacturing strategy requires considerations around the facility, process, resources, and infrastructure.



BMS: Building management system; EMS: Environmental management system; M/E/P: Mechanical, electrical, and plumbing; QA: Quality assurance; QC: Quality control.

Process equipment may be similar across the modalities but with different development workflows and processing environments. There are regulatory concerns with certain vaccines being produced alongside their vaccine product, due to concern that multi-modality manufacturing will potentially compromise the end product quality. Consequently, it is necessary to gain regulatory approval before implementing a multi-product approach.

If regulatory bodies approve the multi-modality manufacturing approach, the first step to consider is which processes and materials must be segregated and which can be shared, bearing in mind the potential contamination risks when manufacturing processes are combined. mRNA is produced by an enzymatic reaction that does not involve any cell-based components and therefore may not require as rigorous testing for contaminating host cell proteins as the other cell-based methods. However, mRNA is susceptible to degradation by RNases, which are present in human cells and tissues. A consideration with pDNA is the endotoxin level produced during manufacturing. Moreover, viral vector production requires a BSL2 rated manufacturing space due to their infectious nature.

It may be possible to share buffer preparations that do not come into contact with the product; however, this may depend on the existing capabilities of the manufacturer. The buffers can be either made in-house or purchased externally. If the facility opts for in-house buffer preparation, factors such as labor, storage, buffer volumes, and QC and release need to be considered. Alternatively, purchasing ready-made buffers may enable efficient management.

DIGITIZATION

Digitization of various aspects of the workflow is essential in intensified manufacturing. Manual entries or transcription of paper-based data need four-eye verification when being made digital. Digitized manufacturing enables increased speed and annual throughput, drives down manufacturing costs, particularly labor reduction, and increases the quality of the product by decreasing errors during manufacturing, including fewer deviations in batch releases. Furthermore, the US FDA requires electronic submissions.

Digitization and electronic support of batch records and other documents will become increasingly important contributors to the future of mRNA manufacturing. As the industry moves towards smaller mRNA batches, we may see an increase in workload and paperwork for the QC department, which may delay the delivery of the product. By using digital solutions, the throughput and quality of the manufacturing can be improved, while reducing errors and deviations. The manufacturing cost can also be lowered, mainly by saving labor time.

Another benefit of digital solutions is the ease of process transfer. Using the same digital platform at different stages and sites of manufacturing can facilitate the electronic transfer of thousands of documents involved in a tech transfer. An electronic platform can enable the development of standard operating procedures at the innovation center, which can be further refined at the manufacturing site. Digitization may also enable earlier training and education of the workforce involved in the manufacturing process.

QC PROCEDURES

QC methods should be specific to the product and aligned with the manufacturing goals (Figure 7), to avoid creating a bottleneck. QC capacity should also be scaled according to the manufacturer's needs.

CONCLUSION

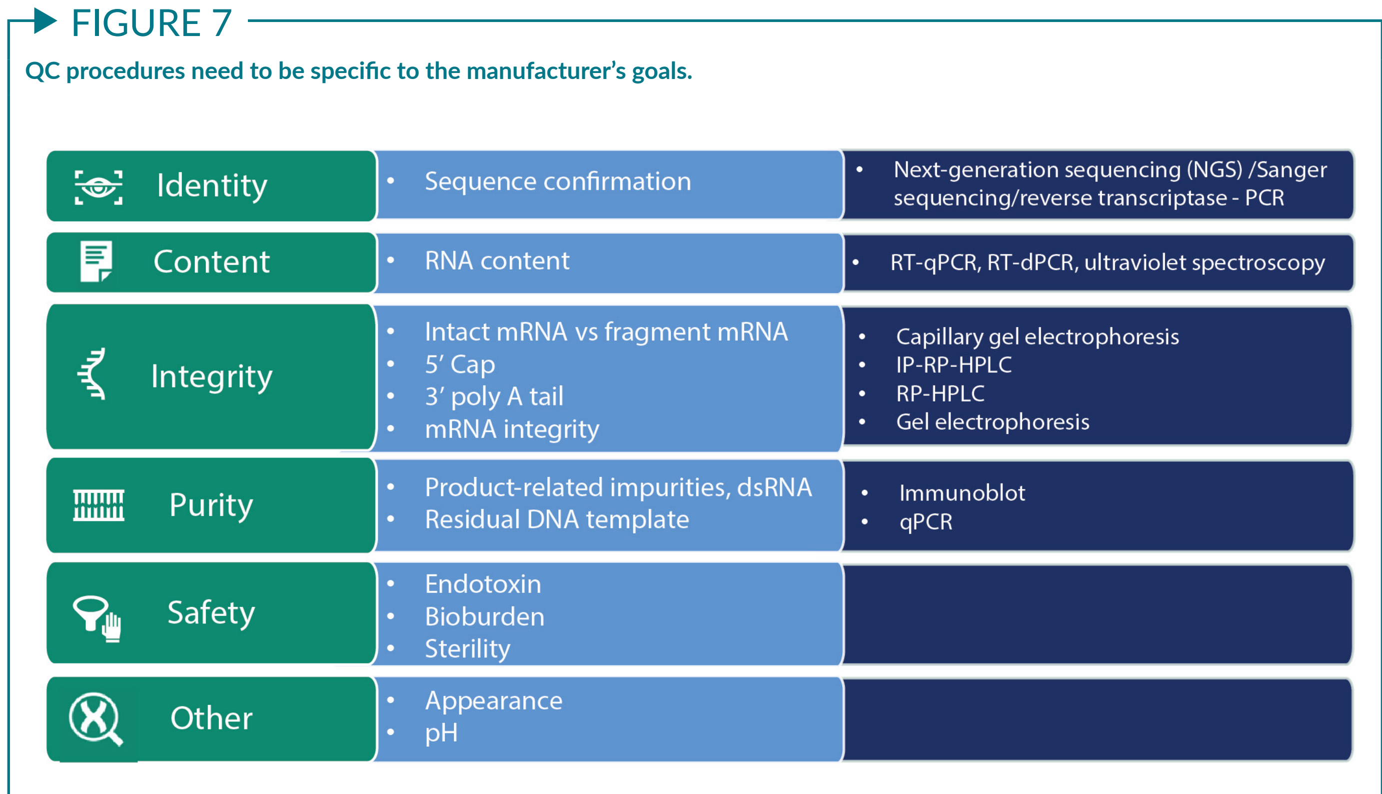
In summary, several challenges must be overcome when implementing new vaccine platforms and multi-modality manufacturing. The manufacturing process can be

DOI: 10.18609/vac/2023.051

future-proofed by considering scalability at all stages. Additionally, standardized and modular equipment platforms can increase flexibility and efficiency, as well as reduce complexity and cost. Digitization and automation can enable reproducibility and high-throughput data recording. Lastly, QC processes and contamination control must be relevant and scaled in proportion to the manufacturing processes.

FIGURE 7

QC procedures need to be specific to the manufacturer's goals.



ASK THE AUTHOR

Katarina Stenklo answers your questions on optimizing the manufacturing of next-generation vaccine modalities.

Q Why are mRNA vaccines faster to produce than traditional vaccine methods?

KS: mRNA is produced through an enzymatic reaction, which eliminates the need for cell lines, stable clones, and other related steps, which are the starting point for other vaccines. Therefore, mRNA can be modified more quickly, reducing the manufacturing time. For viral vectors, monoclonal antibodies, or other products, the cell culture stage may take several months before enough product is obtained, followed by downstream processing. For mRNA vaccines, the equivalent of the upstream process takes only hours rather than weeks.

Q What challenges do you envisage for multi-modality manufacturers in the future?

KS: The possibility of manufacturing multiple modalities in the same manufacturing line may be appealing, but the manufacturer must consider the changeover process. The manufacturer must ensure that the manufacturing line is clean and free of cross-contamination, and maintain an adequate throughput without long interruptions (e.g., for cleaning). An idle manufacturing line during the changeover process can be costly and inefficient.

Q What should manufacturers consider if they want to move to smaller volumes?

KS: Manufacturers who want to move to smaller volumes should take into account several factors. Large-scale equipment is typically qualified for GMP manufacturing whereas some equipment initially designed for lab and development work may not be suitable for GMP purposes. Therefore, manufacturers should ensure that their equipment meets the quality and safety standards for the intended use. Another factor is compliance with GMP requirements regardless of the volume size. Manufacturers should apply the same GMP principles and practices even when working with smaller volumes.

Q How can digitization support manufacturing?

KS: Digitization can support manufacturing in various ways, especially in the context of faster and smaller batch production. Data management involves handling large amounts of data generated by the manufacturing process. Additionally, recording manual steps, such as logging consumables, is required for batch release. If the goal is to accelerate the delivery of products to patients, the batch records should not take weeks to review. Digitization can facilitate this process and will be more cost-effective in the long run.

Q Which parts of the process do manufacturers most frequently outsource and why?

KS: One part of the process that manufacturers often outsource is the fill and finish because it requires specialized expertise and equipment. However, insourcing could be carried out in smaller batches by utilizing specialized equipment, such as those provided by Cytiva. Another part that may be outsourced is the new technology of lipid nanoparticle formation, as the process is not widely known. In this case, manufacturers may start with outsourcing but may later plan to insource it later to gain more flexibility.

Q How long are viral vectors stable with the transgene, and what is the recommended period of storage and usage?

KS: The stability of viral vectors with the transgene may vary depending on several factors, such as the type of vector and the cell line used. Therefore, it is advisable to follow the protocols and recommendations provided by the supplier of the vectors or the backbones. However, it may also be necessary to be validated by the manufacturer to ensure the optimal period of storage and usage for each vector.

Q What are the mRNA purification options? Can mRNA be purified using non-solvent systems to avoid the need for costly facility upgrades?

KS: There are different options for mRNA purification, and some do not require solvents. For instance, some methods use an oligo (dT) ligand paired with affinity purification, which is solvent-free. Other methods use traditional resins or other systems. However, solvents may still be needed for the LNP formation step. The amount of solvents used in manufacturing can be reduced but not eliminated.

BIOGRAPHY

KATARINA STENKLO holds a licentiate degree in biochemistry and has over 20 years of industry experience. She currently works for Cytiva in the manufacturing capacity solutions business unit. She has extensive expertise in developing end-to-end manufacturing solutions that includes process workflows, process equipment, automation, consumables, and facilities. Her focus over the last few years has been developing and designing solutions for the manufacture of new product modalities such as viral vectors, plasmids, and mRNA therapies.

REFERENCES

1. Achieving 70% COVID-19 Immunization Coverage by Mid-2022 - Statement of the Independent Allocation of Vaccines Group (IAVG) of COVAX. WHO December 23, 2021 (accessed Oct 2023).
2. Global Dashboard for Vaccine Equity. UNDP (accessed Oct 2023).

AFFILIATION

Katarina Stenklo
Enterprise Solutions Commercial Activation Leader,
Cytiva

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: The named author takes responsibility for the integrity of the work as a whole, and has given her approval for this version to be published.
Acknowledgements: None.
Disclosure and potential conflicts of interest: Katarina Stenklo is an employee of Cytiva and has no other conflicts of interest to disclose.
Funding declaration: The author received no financial support for the research, authorship and/or publication of this article.

ARTICLE & COPYRIGHT INFORMATION

Copyright: Published by *Vaccine Insights* under Creative Commons License Deed CC BY NC ND 4.0 which allows anyone to copy, distribute, and transmit the article provided it is properly attributed in the manner specified below. No commercial use without permission.
Attribution: Copyright © 2023 Cytiva. Published by *Vaccine Insights* under Creative Commons License Deed CC BY NC ND 4.0.
Article source: This article is a transcript of a webinar, which can be found here.
Webinar recorded: Aug 23, 2023; **Revised manuscript received:** Oct 17, 2023; **Publication date:** Dec 20, 2023.

This is a transcript of a webinar.
You can also watch the recorded webinar:
WATCH NOW

VACCINEINSIGHTS

RNA VACCINES PART 2:
ADDRESSING ONGOING CHALLENGES

SPOTLIGHT

INTERVIEW

Optimizing *in vitro* transcription reactions: from fundamental research to mRNA vaccine manufacturing

Charlotte Barker, Editor, *Vaccine Insights*, speaks to **Craig Martin**, Professor of Chemistry, University of Massachusetts Amherst, about leveraging decades of basic research on RNA polymerases to develop a more cost-effective flow reactor process for RNA manufacture that aims to eliminate double-stranded RNA.

Vaccine Insights 2023; 2(10), 399–404

DOI: 10.18609/vac.2023.53

What have been the overarching themes of your work?

CR: Over the past 35 years, our laboratory has dedicated extensive research to the T7 bacteriophage RNA polymerase as a simplified model system for bacterial and eukaryotic RNA polymerases. Our primary objective has been to gain a comprehensive understanding of its structure, its ability to recognize DNA sequences during promoter binding, the complex processes that occur after promoter binding, promoter escape, and the subsequent production of short RNAs. Our work has also involved the study of elongation

complex stability, where we pause the polymerase midway along the DNA strand to assess its stability.

Additionally, a significant portion of our research involved the examination and understanding of elongation and initiation complexes, and the transition between these two states. We have also delved a bit into the mechanisms of termination, where specific DNA sequences trigger the polymerase to disengage—a topic we are now revisiting due to its practical implications.

My goal has always been to understand the fundamental mechanisms involved, so we focused on a simpler RNA polymerase model with fewer complications, T7. Notably, this aligns neatly with the requirements for manufacturing processes. Highly complex eukaryotic RNA polymerases excel in complex regulatory aspects of biological systems but are ill-suited for industrial-scale operations. The attributes that drew my interest toward T7 as a model system also make it an appealing tool for large-scale manufacturing. With that in mind, we broadened our research focus around 7 years ago to include manufacturing RNA and scaling up reactions.

Q How has your work evolved from fundamental research to more applied work on improving synthesis?

CR: Since T7 RNA polymerase is neither eukaryotic nor bacterial, there was a small niche in funding for many years. Over the past three decades, only a few research groups have been focused on investigating T7 RNA polymerase. 10 years or more ago, the others retired or moved to other systems, leaving only me.

About 8 years ago I was developing a new research direction that used T7 RNA polymerase as a tool for exploring RNA folding, and although this avenue didn't yield significant results, it marked the beginning of my role as an end-user of the enzyme. At that stage, I shifted my focus towards producing higher-quality RNA, not for therapeutic purposes but for fundamental research.

We started to encounter multiple long-standing problems in manufacturing RNA, so we embarked on a mission to comprehend and resolve these challenges. Ultimately, we realized that the solutions had broader implications. In 2019, I secured funding from the NIH with the specific aim of developing more efficient methods for producing RNA using T7 RNA polymerase for potential RNA therapeutics.

Also in 2019, we published a significant paper that elucidated the mechanism behind double-stranded RNA formation [1]. It is important to acknowledge that two research groups in the mid-1990s had previously described this, but the implications of their work were largely overlooked. While we weren't the first to identify the issue, we were able to provide new insights into the process, thanks to the availability of newly accessible and advanced tools. This breakthrough led me to decide to dedicate my research to enhancing RNA manufacturing.

A few months later, the COVID-19 pandemic began, and the two mRNA vaccines that were developed both used T7 RNA polymerase.

What factors lead to unwanted side products like double-stranded RNA in high-yield *in vitro* transcription reactions?

CR: Our aim is for T7 RNA polymerase to bind to a specific promoter sequence on the DNA and synthesize the desired RNA product. This process typically works well, but there is a lower probability event where the polymerase can instead bind to the RNA

product. In this scenario, the enzyme essentially replicates the same process it performs on DNA but with RNA, resulting in the generation of double-stranded RNA.

The issue arises when we consider that this secondary reaction, like any binding reaction, depends on concentration. As we scale up the production of RNA, which is a key goal in manufacturing, we inadvertently drive this secondary reaction. In a typical batch reaction that many researchers use, it is possible to produce around 100 RNAs for each DNA template. Towards the end of the reaction, the concentration of RNA significantly outweighs that of DNA, leading to a proportional increase in double-stranded RNA. It becomes a competition between rebinding the promotor DNA or the RNA, and as the RNA concentration increases, it skews the balance. As enzymologists, we typically focus on examining initial reaction rates rather than pushing reactions to their extremes. Consequently, we didn't frequently observe this secondary reaction at the lab scale.

Q How do you seek to understand and address these issues?

CR: Our approach is to immobilize the enzyme and DNA to create a flow reactor system in which RNA exits the reactor immediately after synthesis, preventing reattachment of the polymerase.

A major challenge was to immobilize the DNA and polymerase molecules in a way that still allowed them to interact effectively. I had confidence in our ability to achieve this based on an experiment we conducted in the early 2000s, which showed that the polymerase can still produce full-length transcripts when tethered to the DNA [2].

Now, we have adopted more modern immobilization techniques that have proven highly effective. In a recent development, we successfully synthesized an 8,000-base RNA using an immobilized enzyme–DNA complex.

In a batch reaction, a fixed amount of enzyme and DNA is used, operating for a specific duration, typically generating approximately 100 copies of RNA per DNA template. After this, the enzyme and DNA are discarded. Our aim is to produce substantial quantities of RNA using the RNA–DNA catalyst within the reactor. Although cost-saving wasn't our primary goal, it has emerged as a valuable benefit of our approach.

The multiple purification steps of the batch process are necessary to remove the polymerase, DNA, and double-stranded RNA but our goal is to eliminate all of these purification steps. If the enzyme and DNA remain in the reactor, there is no need for their removal from the end product. Similarly, if the process doesn't make double-stranded RNA, we don't have to remove it. I believe the cost-saving will come from removing or avoiding these purification steps.

At a recent manufacturers' meeting I attended, methods for double-stranded RNA removal were still a major point of debate; however, my ambition is that this discussion becomes obsolete because there is no detectable double-stranded RNA in the first place.

Given the reduction in capital costs, could this technology be used in lower- and middle-income countries (LMIC)?

CR: I receive funding from both the US NIH and Wellcome Leap, part of the Wellcome Trust. Wellcome Leap's RNA Readiness and Response program aims to develop a streamlined RNA manufacturing process that is not only simple but also has a compact footprint for easy transport.

The vision is that in the event of a disease outbreak in a LMIC, we can load a few of these mobile labs onto a freighter, send them to the affected area, and set up on-site RNA manufacturing. The Gates Foundation shares a similar mission, and there may be other organizations with similar goals. This is a monumental venture and if we can democratize these therapies, the impact would be tremendous.

Cost reduction is undoubtedly an important factor, and we aim to keep the process straightforward. This not only ensures that it doesn't require an army of experts to operate but also that it is entirely safe and compliant with the necessary standards. I am optimistic that my approach will not only find substantial commercial success in the Western world, but also make a meaningful impact in LMIC.

What technologies are making your work possible?

CR: Around 15 years ago, I attempted to develop a flow system and encountered significant challenges that resulted in failure, which was mainly because I lacked expertise in fluidics. Our current success can be greatly attributed to a collaborative partnership with Sarah Perry, Associate Professor of Chemical Engineering at the University of Massachusetts Amherst. Sarah is a fluidics expert and provides invaluable guidance on all aspects of microfluidics in our research. Additionally, some of my students are working within her group, which has proven to be absolutely essential to our progress.

Other researchers have been involved in scaling out microfluidic processes, and we are not pioneers in this regard. Microfluidics has been an established field for some time, and the concept of fluidics manufacturing is not entirely novel either. Chemical engineers often favor flow processes, particularly for applications such as petroleum cracking. Flow reactions are more reliably scaled out, with each reactor behaving similarly.

In the context of lipid nanoparticle formulation, this approach is often implemented on a microfluidic scale. A group at the University of Pennsylvania has made 256 parallel reactors on a small chip that can process 7 L of material per hour. We are performing all of our processes on a similar chip, so as we continue to scale out, our reactor's physical footprint will remain reasonably small.

How are you finding the transition from academia to startup?

CR: I did not set out in academia with the goal of a startup; however, I came to realize that my unique expertise held the potential for a significant impact. So far it has been both an exciting and stressful journey!

The manufacturing world has presented an entirely different set of challenges to the academic world. In academia, there is a risk of being 'scooped' but my previous work primarily focused on an RNA polymerase that few cared about, so competition was not a significant issue.

In manufacturing and industry, you can't disclose anything until it is properly protected as intellectual property. Failing to patent something can hinder your ability to achieve your goals. Living in a world where I can't openly discuss my work is somewhat counterintuitive for someone with an academic background. Sharing our research and findings with others is essentially the product of academia.

As someone who has been involved in both, what are your thoughts on the relationship between fundamental and applied research?

CR: All applied research has its foundations in fundamental research. The funding of fundamental basic research by governments is indispensable for the progress of applied research and this critical relationship can sometimes be overlooked. I want to emphasize that everything I am currently working on is rooted in the fundamental research that my lab has been conducting for over 35 years. Without this foundation, our current work simply wouldn't be possible.

There are many such examples. CRISPR originated from the study of an obscure system that initially had no apparent applications and would never have been discovered by those conducting applied research. It serves as a powerful reminder that governments must continue to invest in research. Industry recognizes this fact and has often advocated for government funding of fundamental research, understanding that their future products and innovations are ultimately reliant on such breakthroughs.

REFERENCES

1. Gholamalipour Y, Mudiyanselage AK, Martin CT. 3′ end additions by T7 RNA polymerase are RNA self-templated, distributive and diverse in character-RNA-Seq analyses. *Nucl. Acids Res.* 2018; 46, 9253–9263.

2. Esposito EA, Martin CT. Cross-linking of promoter DNA to T7 RNA polymerase does not prevent formation of a stable elongation complex. *J. Biol. Chem.* 2004; 279, 44270–44276.

BIOGRAPHY

CRAIG MARTIN completed his Bachelor's degree in Chemistry at the University of California in 1979, before gaining a PhD in Chemistry at the California Institure of Technology in 1984. He went on to complete his Postdoctoral degree in Molecular Biophysics and Biochemsitry at Yale University in 1988, before starting his current role as a Professor of Chemistry at the University of Massachussetts Amherst.

AFFILIATION

Craig Martin
Professor of Chemistry,
University of Massachusetts Amherst

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.

Acknowledgements: None.

Disclosure and potential conflicts of interest: Martin C has two provisional patent applications in preparation and two issued. Martin C is the sole stock owner of RNA4Tx, Inc. Martin C was an invited speaker at three Hanson Wade conferences, where the registration fee was waived.

Funding declaration: Martin C recieved a grant from National Institutes of Health, 1R01GM134042, and funding from Wellcome Leap R3 program.

ARTICLE & COPYRIGHT INFORMATION

Copyright: Published by *Vaccine Insights* under Creative Commons License Deed CC BY NC ND 4.0 which allows anyone to copy, distribute, and transmit the article provided it is properly attributed in the manner specified below. No commercial use without permission.

Attribution: Copyright © 2023 Martin C. Published by *Vaccine Insights* under Creative Commons License Deed CC BY NC ND 4.0.

Article source: Invited; externally peer reviewed.

Revised manuscript received: Oct 24, 2023; **Publication date:** Nov 8, 2023.

EXPERT INSIGHT

Physical methods to overcome tissue barriers in vaccine delivery

Taksim Ahmed, Dylan Freitas, Xisha Huang, Qing Rui Simon Qu, Giovanni Traverso & Ameya R Kirtane

Vaccination represents one of the oldest and most effective public health interventions. Vaccines are routinely administered via systemic injections. Due to the invasive nature of this technique, it introduces several challenges. Vaccine administration without the use of needles would be beneficial. However, vaccine uptake is significantly limited by the presence of tissue barriers. Here, we review physical methods that disrupt these tissue barriers and enable efficient vaccine delivery. Four methods, namely microneedles, needle-free jet injectors, electroporation, and ultrasound. We focus on how these methods compare to needle-based vaccination in preclinical and clinical studies, and discuss their use in mucosal vaccination. In sum, these methods offer an attractive alternative to conventional vaccine delivery strategies; however, much work needs to be done to further improve their efficacy.

Vaccine Insights 2023; 2(10), 363–380

DOI: 10.18609/vac/2023.050

INTRODUCTION

The earliest form of vaccine was administered by a process called variolation [1]. A lance was introduced into a smallpox pustule of an infected individual and then driven into the skin of an uninfected individual. Alternately, smallpox scabs were isolated, dried, and blown into the nose of an uninfected individual [1]. Interestingly, if the dried scab was rubbed onto the skin, it was not as effective [2]. This practice is said to have existed in India and China as long as 1000 BC [3]. Early records from Boston, dating back to the 1700s, indicate that the mortality rate of smallpox was approximately 14% in unvaccinated individuals. In contrast, the mortality rate in vaccinated (variolated) individuals was

only approximately 2% [4]. These anecdotes highlight two points that have been studied extensively in modern vaccinology—first, the viability of the organism dictates immune protection, and second, the mode of administration of the vaccine plays a key role in determining efficacy.

Modern vaccines are commonly administered by injection as this may be convenient for administration across age groups, and because injections allow for maximum bioavailability (defined here as the fraction of the dose of the vaccine entering the body). However, systemic injections have limitations. Individuals suffering from needle phobia may be hesitant to receive injections and may avoid getting vaccinated [5,6]. Needle-stick injuries, especially in young and trainee healthcare providers, are a low-probability but high-risk event [7]. Needle-based vaccination produces significant biological sharp wastes. Some vaccines (e.g., DNA and RNA vaccines) must be introduced intracellularly [8], which is not achieved by injection into the muscle. Finally, systemic vaccination produces only weak mucosal immunity. In contrast, vaccination at mucosal sites can produce an immune response at the site of vaccination and other mucosal sites [9,10]. Hence, there is a critical need to design non-invasive methods to administer vaccines. Adopting these non-invasive methods for mucosal vaccination would be particularly impactful.

Needle-based administration is effective as it overcomes the transport barriers posed by the body's surfaces (Figure 1). Multiple cell layers in the epidermis separate the first line of immune cells from the external environment [11]. Mucosal sites, such as the nasal mucosa and the gastrointestinal tract, are lined by a layer of mucus. Mucus is a hydrogel-like structure containing high molecular weight proteins (e.g., immunoglobulin A [IgA]) and proteoglycans (e.g., mucin) [12]. Together, the epidermis and mucus are formidable barriers to the diffusional entry of vaccines, which often take the form of macromolecules or microscopic organisms.

Biological transport barriers can be overcome by several methods, broadly classified into chemical [11,12] (e.g., cell-penetrating peptides, permeation enhancers) and physical methods. This article will focus on the latter strategy. We identified four physical methods for vaccination: microneedles, jet injectors, electroporation, and ultrasound. For each method, we aimed to understand how their delivery efficiency compares to needle-based administration in the preclinical setting, if mucosal delivery with these modalities is possible, and how these methods have performed in clinical practice (search performed in PubMed database on June 15, 2023; no limits were placed on the dates of the publication). For conciseness, we limited our discussion of preclinical studies to animal species that are developmentally closest to humans and studies that directly compare the physical modality to needle-based administration.

PHYSICAL METHODS FOR DELIVERING VACCINES

Microneedles

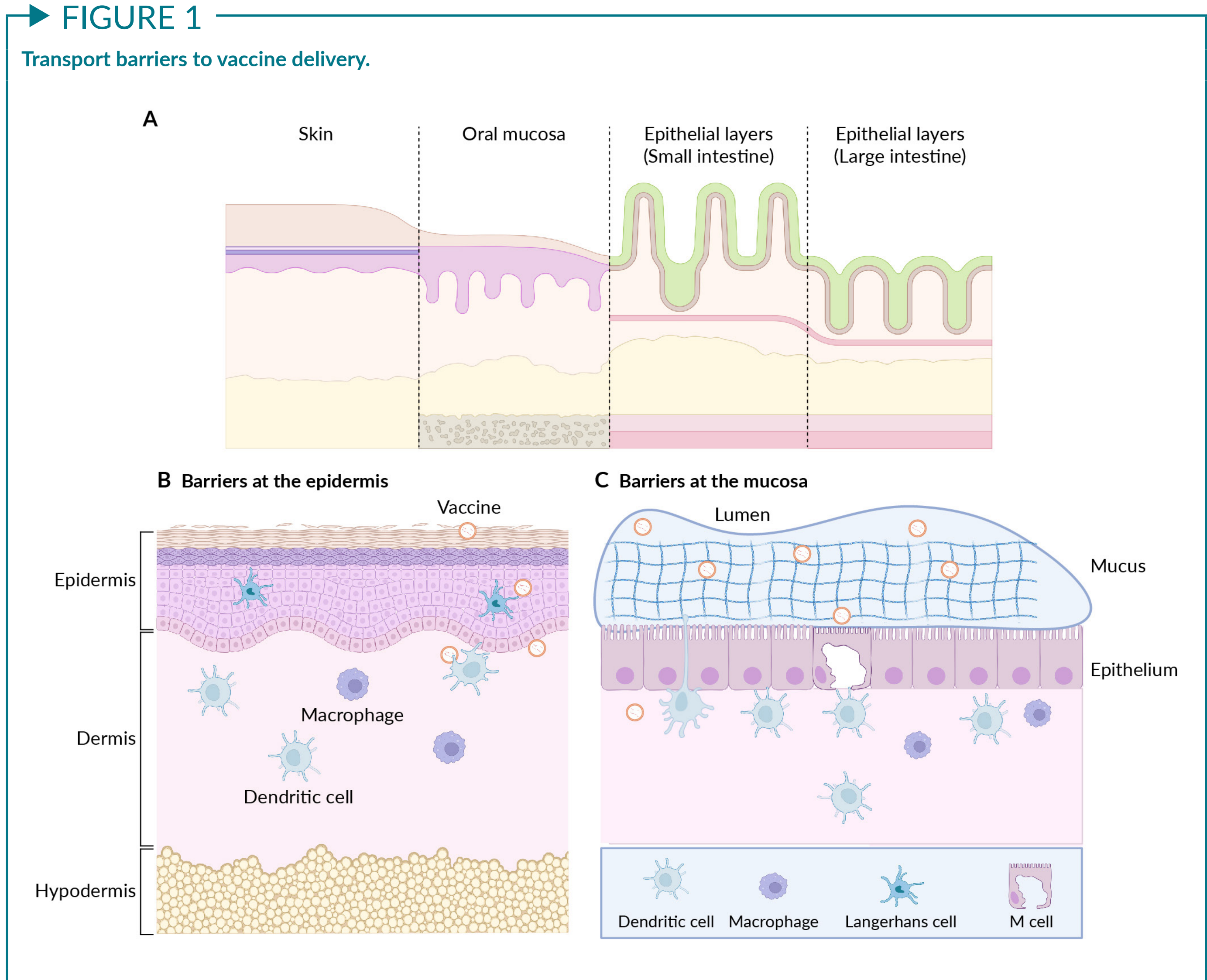
Microneedles are a minimally invasive method used for the delivery of a range of cargoes. Micron-sized needles made from polymers, metals, and/or silicon are used to pierce through the cell layers and introduce the cargo into the tissue [13–15]. The length of the microneedles can be manipulated to prevent contact with the nerve cells and avoid activation of pain receptors [16].

Vaccines can be loaded into the microneedles using different strategies [17]. In matrix-type systems, vaccines are loaded into the body of a dissolving microneedle (Figure 2). Alternatively, the vaccine can be coated on the surface of microneedles. Finally, hollow microneedles attached to an actuation system (e.g., syringe) can also be used. These microneedles are akin to a conventional needle, but introduce the vaccine at a shallower depth into the skin. We note that tattoo needles have also been used for vaccine delivery. Tattoo

 DOI: 10.18609/vac/2023.050

FIGURE 1

Transport barriers to vaccine delivery.



(A) Barriers at the skin and mucosal tissues. **(B)** In the skin, the vaccine must penetrate across the stratum corneum in the epidermis and multiple cell layers before reaching the dendritic cells in the dermis. **(C)** In mucosal tissues, mucus forms a hydrogel-like structure that impedes the transport of the vaccine to the underlying immune cells. Created with [88].

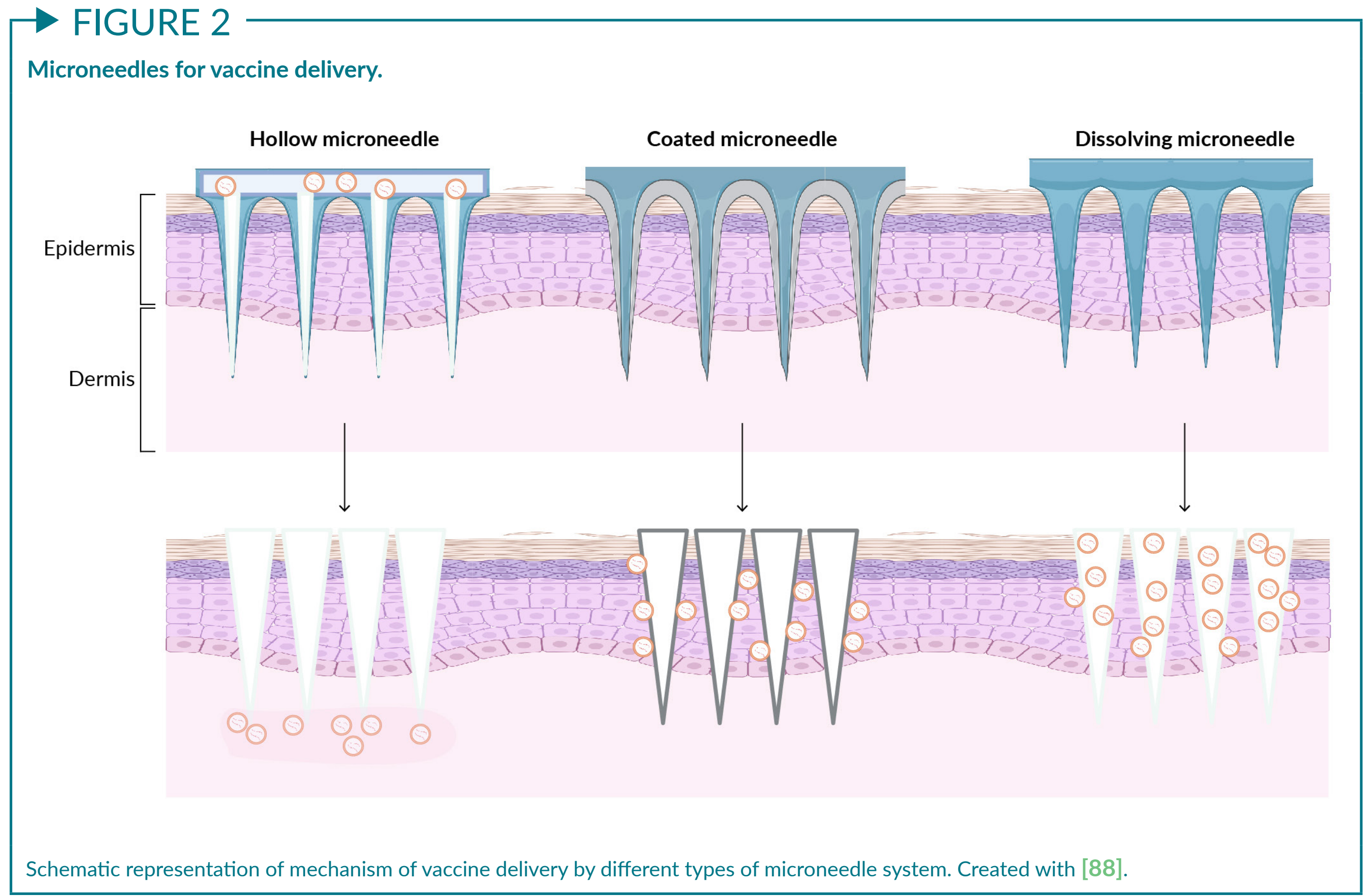
needles employ high-frequency oscillation to penetrate the skin multiple times and deposit vaccine into it [18]. As tattoo needles are somewhat similar to microneedles but less studied, we will not discuss them in much detail in this article. Microneedles have been widely explored in preclinical models for the delivery of nearly all types of vaccines, including subunit [19], protein [20], mRNA [21], DNA [22], and attenuated organism vaccines [23].

Comparison to needle-based administration in preclinical studies

Microneedle array patches were used for the delivery of hepatitis B surface antigen-based vaccine in rhesus macaques [24]. Microneedle-based administration of the adjuvant-free vaccine led to protective antibody responses throughout the study in three of the four animals. In contrast, intramuscular injection produced protective antibody responses in only one of the four animals. The enhanced immunogenicity of microneedles was attributed to the unique depth at which they introduce the antigen. Microneedles deliver the antigen in the epidermis—a rich reservoir of immune cells, especially Langerhans cells [25,26]. In comparison to the epidermis, the muscle has a lower density of immune cells. Indeed, several reports have shown that microneedle-based vaccines produce an immune

FIGURE 2

Microneedles for vaccine delivery.



Schematic representation of mechanism of vaccine delivery by different types of microneedle system. Created with [88].

response equivalent to injections even at significantly lower doses [25,27]. This effect is known as dose-sparing. The low immune response in the injection group was rescued by the inclusion of an alum-based adjuvant. Notably, the antibody titers in the alum-based vaccine group were greater than those seen in the microneedle group. Adjuvants promote immune cell entry at the site of administration, thereby improving antigen uptake into immune cells, even in the muscle.

Microneedles have also been used for the administration of inactivated and attenuated virus-based vaccines targeting polio and measles [28,29]. Microneedles produced comparable antibody titers and protection to subcutaneous/intramuscular vaccination (i.e., no dose-sparing effect was observed).

Taken together, microneedle-based vaccines are efficacious in large mammals. However, we hypothesize that the dose-sparing effect may be contextual, in that dose-sparing may be more evident with weakly immunogenic vaccine platforms such as subunit vaccines. Additionally, microneedles may offer a means to circumvent the use of adjuvants, simplifying the vaccine formulation and perhaps reducing toxicity [30].

Mucosal application

Microneedles have been used for vaccinating at the buccal and sublingual mucosa. The first report describing buccal microneedles used a combination of a protein and DNA vaccine encoding HIV antigens [31]. In rabbits, buccal microneedles produced similar immunoglobulin G (IgG) responses to the intramuscular injection. However, salivary IgA responses were greater with the buccal microneedles. Likewise, recent work in mice showed that administration of spike protein using a sublingual microneedle patch yielded comparable IgG responses but more potent pulmonary IgA responses than intramuscular vaccination [32]. Higher mucosal immune responses with buccal microneedles were likely related to the route of administration and not

DOI: 10.18609/vac/2023.050

the use of microneedles. However, this data suggests that microneedle-based administration of vaccines in the oral cavity is possible. Our work has revealed that microneedle-based buccal administration was preferred to syringe-needle-based intramuscular injections [33]. Key for the successful use of buccal microneedles may be applicators that enable reproducible administration. Furthermore, rapid dissolution of the microneedles in the buccal tissue may be advantageous as this will minimize patient discomfort. In summary, microneedle-based buccal vaccination may prove to be a patient-friendly means of administering a vaccine that elicits a mucosal immune response.

We have recently developed a mm-scale injector that allows for the delivery of mRNA nanoparticles via the oral route and into the gastric mucosa [34]. Using this system, we showed mRNA-mediated production of a model protein in the stomach of pigs. With further optimization and validation, this system could be used for the delivery of mRNA vaccines.

Clinical experience

Microneedles have been the most widely studied physical method for vaccine administration in the clinic. Different types of microneedles, including dissolving microneedles, coated microneedles, and hollow microneedles have been evaluated for their patient acceptability, antigen delivery, and immunogenicity.

The Prausnitz group has employed dissolving microneedles attached to a polymer-based backing membrane [35,36]. A flu vaccine was loaded into the body of microneedles and applied to the skin with a thumb push. After a 20-minute application, only approximately 10–20% of the dose was recovered on the patch, indicating excellent transfer into the skin. Patient acceptability of the microneedle patch was greater than intramuscular injections. In some cases, patient experience improved during subsequent vaccinations, suggesting that familiarity with the microneedles was an important factor. Serum-based antibody response was comparable between the intramuscular injection and microneedle groups. Further, there was no difference in the immune response and antigen delivery between self-administered and healthcare worker-administered microneedles.

Microneedle-mediated dose sparing has been observed in the clinic with different vaccines administered via dissolving microneedles [37], surface-coated microneedles [38], and hollow microneedles [26,39–41]. For example, an inactivated virus vaccine containing the Japanese encephalitis virus was loaded into the tip of dissolving microneedles [37]. Microneedle patches were loaded with only 10% of the dose used for subcutaneous vaccination and of that, 60% of the dose was delivered into the skin. Despite the low vaccine dose, antibody responses in the microneedle-treated group were comparable to those observed in the subcutaneous vaccine group.

As discussed before, dose-sparing arises because microneedles introduce the vaccine in the epidermis, allowing greater access to immune cells. This was excellently showcased using the MicronJet600™ device. The MicronJet600 is a 600 µm hollow microneedle device that is attached on top of a syringe and ensures targeted delivery into the skin (not to be confused with 'microjets', which are discussed in a later section). MicronJet600-based inactivated virus vaccines [39] and virosomes (viral proteins encapsulated in lipid vesicles) [41] produced higher antibody responses than the intramuscular vaccine. Interestingly, intradermal injection using the Mantoux technique also produced stronger antibody titers than intramuscular vaccination—supporting the importance of the depth of administration. The authors argue that intradermal injections using the Mantoux technique may be less reproducible as compared to the microneedle-based administration.

Despite the significant success of microneedle-based vaccination in clinical studies, some limitations remain. Routinely, the transfer of vaccine into the tissue is less than 100%. This suggests that there is a loss of vaccine components due to retention in the microneedles. This may present a significant barrier for expensive modalities such as mRNA vaccines. Further, this suggests that dosing may not be as consistent as injections. Second, some microneedle-based vaccinations may need complex setups and prolonged application times. This may be unsuitable for mass vaccination campaigns, especially in low socio-demographic index countries. Prolonged application may not be appropriate for young children who may lack the patience to keep the microneedles in place. Finally, in studies where the microneedles are self-administered, patient training is provided. This brings into question whether microneedles can be self-administered in populations with limited literacy, and where there are limited resources to train people.

Needle-free jet injector

Needle-free jet injectors are a promising technology for delivering vaccines in a minimally invasive manner. The needle-free jet injector was developed in the 1930s, and later (1956) tested for vaccination against infectious diseases such as smallpox and cholera [42]. In the early 1990s, the repeat use of needle-free jet injector systems was banned by WHO due to the cross-contamination originating from the splash-back on the injection nozzle [42]. Consequently, single-use nozzles were introduced. In jet injectors, the injector system accelerates a solution of the vaccine to produce a high-velocity fluidic jet stream (Figure 3). As the stream passes through a narrow orifice at high pressure, it penetrates the tissue at the application site [42–44]. To produce the jet stream, the device mainly uses springs or compressed gas as an energy source [42]. Hand-held jet devices are available for convenient administration.

Comparison to needle-based administration in preclinical studies

Jet injector-based DNA vaccines yielded stronger immune responses than needle-based vaccination in non-human primates. Plasmid DNAs encoding proteins found in Nombre and Andes viruses were delivered using a spring-powered disposable syringe jet injection device in the intramuscular and intradermal space [45]. Intramuscular administration produced seroconversion after a single dose, while two doses were required for the intradermal vaccination. Importantly, intramuscular administration using the jet injector showed improved antibody responses compared to the intramuscular injection. Similar results were obtained in hamsters [46].

Independent studies by Inoue *et al.* [47] and Chang *et al.* [48] propose that the improved immune responses with jet injectors are due to enhanced cell uptake of the DNA vaccine. Interestingly, jet-injector-based delivery of plasmid DNA was found to be more immunogenic than lipid nanoparticle-based DNA vaccination delivered using a needle [49]. These studies motivate the testing of nucleic acid-based vaccines made without the use of excipients such as lipids, which may, in some cases, elicit adverse effects [50,51].

Mucosal application

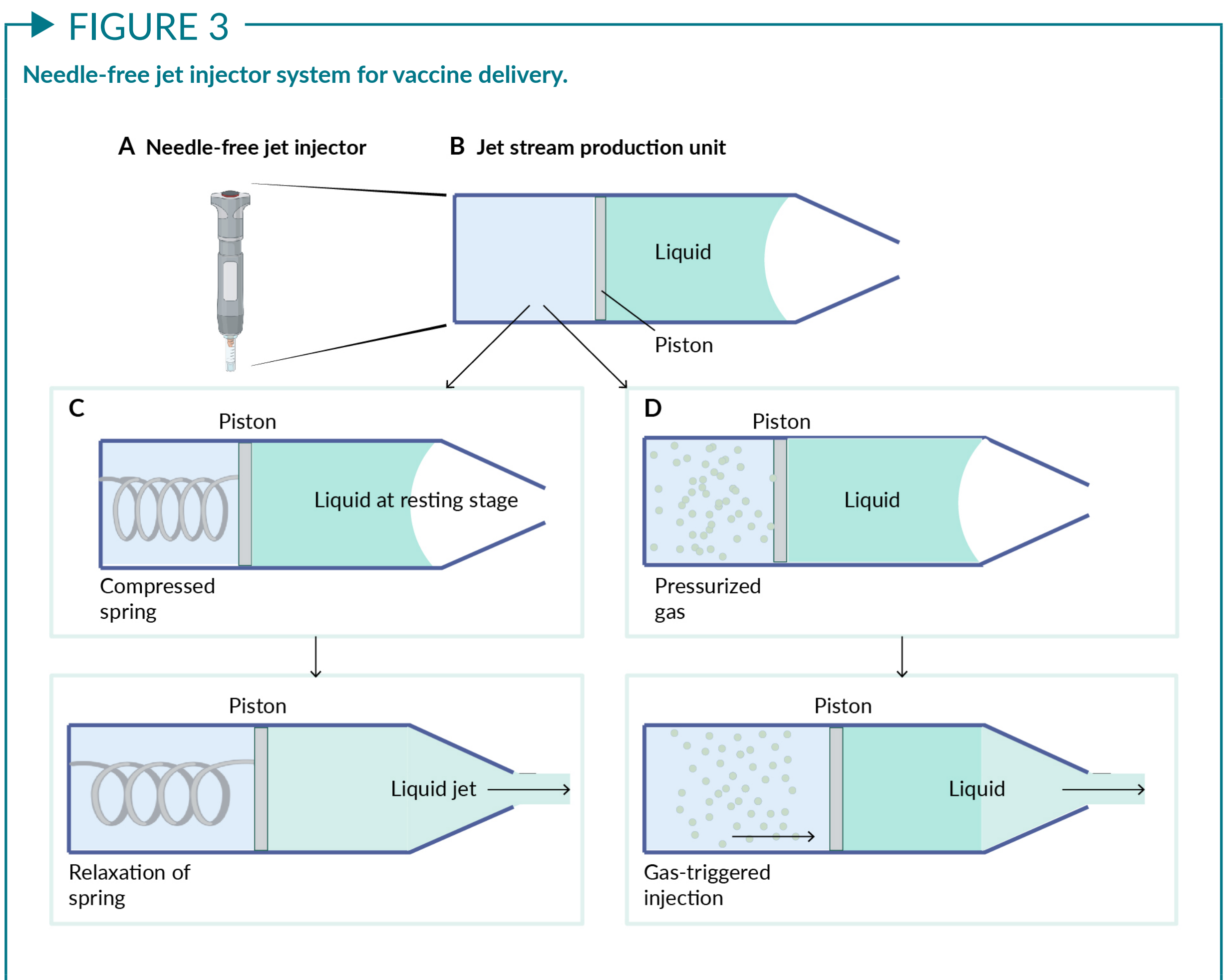
Intranasal microjet vaccines have been evaluated in the context of veterinary medicine using a live porcine reproductive and respiratory syndrome virus [52]. The vaccine was administered intranasally using an atomization device and a high-pressure nasal jet system, and intramuscularly via a needle. All three modalities generated comparable protection against the viral challenge. The authors preferred jetting to atomization as it could be rapidly administered without restraining the animal.

Jones *et al.* compared jet injector and topical administrations of Ankara virus vaccine

DOI: 10.18609/vac/2023.050

FIGURE 3

Needle-free jet injector system for vaccine delivery.



(A) Most commonly used needle-free jet injector e.g., microjet device. **(B)** Different components of a jet injector device- jet stream production unit, piston, and the vaccine liquid. **(C)** Compressed spring and **(D)** gas-actuated jet injector, which propel the piston and jet the vaccine liquid. Parts of the figure are adapted from [42]. Created with [88].

and protein-based vaccines targeting HIV in the sublingual/buccal space in non-human primates [53]. The jet injector-based application in the oral mucosa yielded stronger serum antibody responses than topical administration at that site. The serum antibody response after microjet vaccination was comparable to systemic vaccination. Akin to the results discussed in the buccal microneedle section, buccal microjets elicited stronger vaginal IgA responses as compared to the intradermal vaccine.

Aran and colleagues devised a pill capable of producing fluid jets for sublingual administration—termed MucoJet [54]. Specifically, the pill contained two parts—a vaccine compartment and a propellant compartment—separated by a movable piston. Immediately before administration, the two compartments were clicked together, initiating a chemical reaction in the propellant compartment. Carbon dioxide gas generated from the chemical reaction propelled the piston forward. The pill allowed for the sublingual dosing of ovalbumin protein and yielded stronger antibody responses than topical administration.

Clinical experience

The tolerability of needle-free jet injector-based vaccines has varied across studies. Jet injector-based administrations of a DNA vaccine produced twice as many local adverse events as compared to intramuscular injection. Despite this, the study population preferred jet-based administration to the intramuscular injection [55]. Others have also

reported a higher frequency of local adverse events associated with jet injectors [56,57]. In another study, a spring-powered jet was used to vaccinate infants (average age: 6.9 weeks) [58]. This study was discontinued due to moderate-severe injection site reactions associated with the jet injector.

Needle-free jet injectors have yielded comparable, if not superior, immune responses to needle-based vaccines. Jackson and colleagues compared the efficacy of a gas and spring-powered jet injector (VitaJet™) used for the delivery of an inactivated trivalent flu vaccine [57]. The gas-powered system was expected to deliver the vaccine intramuscularly, while the spring-powered system delivered subcutaneously. As compared to subcutaneous microjet and intramuscular injection, the intramuscular jet injector yielded higher serum IgG titers against the H1N1 strain of the flu virus. Jet injector-based delivery of an alum-adjuvanted hepatitis B vaccine was more immunogenic than injection-based delivery [59]. However, we note that not all studies have found jet injectors to be superior to injections [56]. The precise mechanisms underlying differences across the studies are unknown.

In summary, jet injectors provide an efficacious means of delivering vaccines; however, patient acceptability is questionable. Factors such as differences in the type of vaccine/jet injector device, differential tolerance to pain amongst different populations, and variability between methods of application may play a part in the acceptability of jet injectors. Small-volume jet injectors, also known as microjets, have the potential to reduce bruising and pain [60]. Microjets dispense multiple small doses of vaccine continuously, in contrast to conventional jet injectors that dispense a single large dose. Further jet injectors that are driven by electromagnetic Lorentz force (instead of spring/gas) may provide greater control over the force with which the liquid is dispensed [61,62] and may improve delivery consistency and tolerability.

Electroporation

Electroporation was initially reported by Neumann and co-workers in 1982 to deliver plasmid DNA into cells *in vitro* [63,64]. Since then, electroporation has been widely used to deliver genes, macromolecules, and drugs to the skin, muscles, liver, and other organs [65]. Electrodes are inserted into the target site (e.g., muscle), and millisecond electric pulses are applied to the electrode to generate an electric field, creating a transient opening in the cell membrane that leads to the entry of the cargo into the cells (Figure 4) [66]. Additionally, the lipid bilayer of the plasma membrane can be considered as a capacitor that stores charge and can act as a dielectric between the extracellular medium and cytoplasm [65,67]. Upon application of an external electric field, the cell membrane becomes partially conductive and builds charge as transmembrane potential. The electric field induces orientation of macromolecular dipoles within and outside of cells and leads to the accumulation of charges across the membrane. Once the electric field-induced transmembrane potential surpasses the dielectric strength of the membrane, a permeation event occurs, leading to the formation of hydrophobic pores that allow the entry of the cargo into the cells [67].

Comparison to needle-based administration in preclinical studies

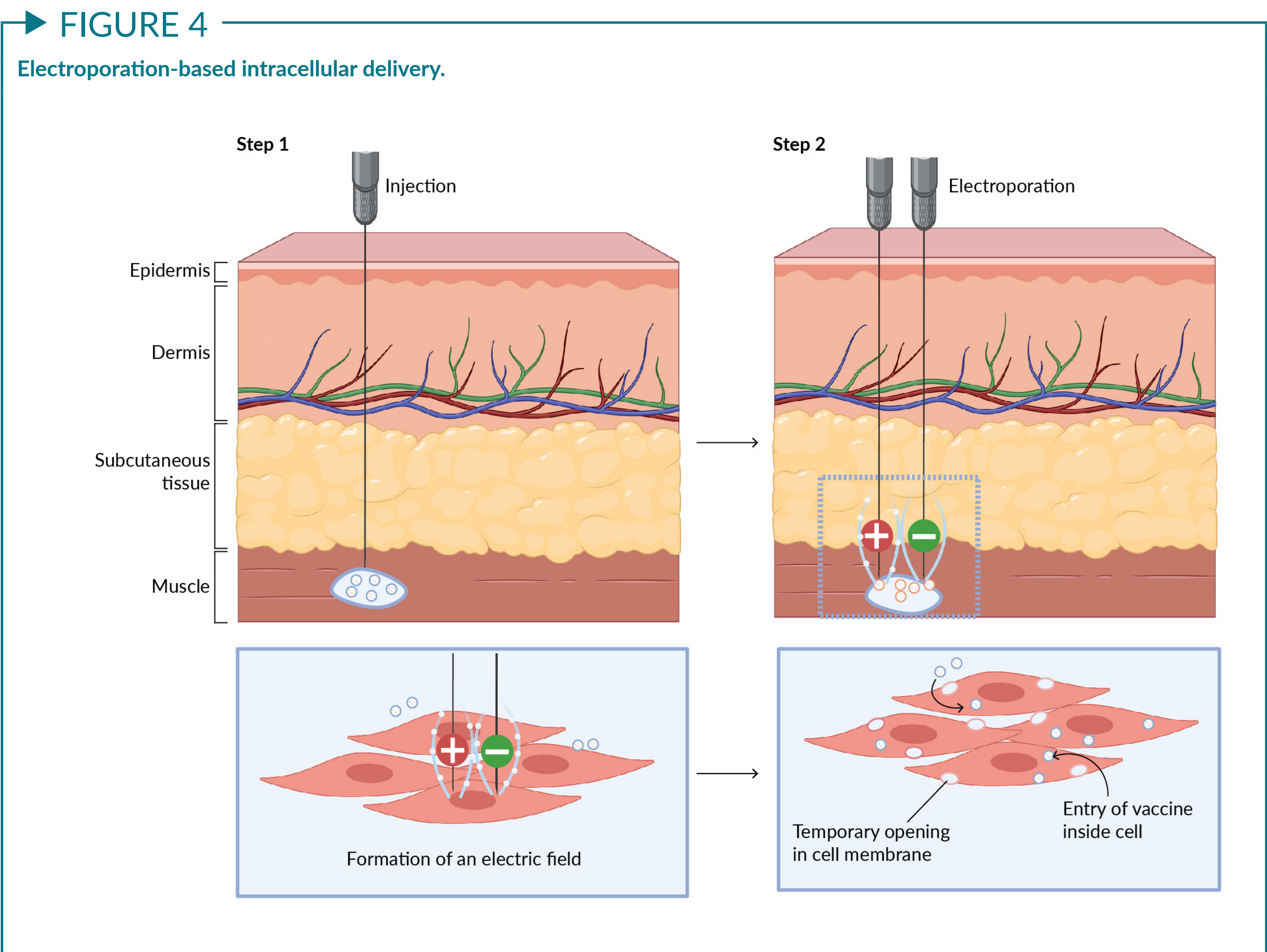
As electroporation aids in the intracellular delivery of the vaccine, and not in overcoming epithelial barriers, it is typically employed in conjunction with needle-based application. We identified papers that compared the efficacy of plasmid DNA vaccines in large mammals in the presence and absence of electroporation.

In pigs, plasmid DNA encoding a pseudorabies virus glycoprotein was injected with and without electroporation [68]. Injection of naked plasmid DNA was effective at producing an antibody response; however, antibody

 DOI: 10.18609/vac/2023.050

FIGURE 4

Electroporation-based intracellular delivery.



In step 1, the vaccines are injected into the muscle layer. In step 2, the electrodes are inserted and millisecond electric pulses are applied. This induces a partial opening in the plasma membrane and allows the vaccines to enter the cells. Created with [88].

titers increased two- to three-fold when using electroporation in conjunction with the injection. In a separate study, the efficacy of a DNA vaccine encoding an antigen found in canine leishmania was tested [69]. DNA was administered via electroporation or encapsulated in solid-lipid nanoparticles. Both treatments yielded only weak antibody titers. Levels of interferon γ in the peripheral blood mononuclear cells were elevated in both vaccination groups. Importantly, both vaccination groups provided comparable protection against a pathogen challenge in dogs.

These studies suggest that electroporation may enable intracellular delivery of complex cargoes such as plasmid DNA. More work is needed to adequately compare the intracellular delivery efficiency and efficacy of formulation-based (e.g., nanoparticles) and electroporation-based intracellular delivery.

Mucosal application

Our literature search showed no reports of electroporation-based vaccine delivery at mucosal surfaces.

Clinical experience

Electroporation has been clinically tested for the delivery of vaccines targeting multiple diseases such as hepatitis [70], human papillomavirus [71–73], malaria [74], HIV, and cancer. Specifically, a recent study evaluated electroporation-based administration of a DNA vaccine against the Zika virus in

approximately 4000 participants [75]. The vaccine was well tolerated and effective in producing an antibody response in this large trial—motivating further evaluation of this modality. We highlight below a few clinical studies around electroporation-based vaccination. We note that there are numerous clinical studies where electroporation was used to transform immune cells *ex vivo* before introduction into the patient. For brevity, we will not discuss these studies.

Vassan and colleagues designed a DNA vaccine against HIV infection [76,77] and administered it using a Trigrid® electroporation delivery system. This method allows for intramuscular (needle-based) or intradermal (jet-based) delivery of the vaccine followed by an electric pulse to enable cell uptake. Intramuscular injection of the plasmid DNA vaccine (without electroporation) yielded weak responses on an interferon γ ELISpot assay. However, immune responses were significantly higher when using the electroporation system. 100% of the participants reported that the discomfort related to electroporation was acceptable when vaccinating against a life-threatening condition such as HIV. However, participant acceptability dropped to 80% for other vaccines such as the flu.

Electroporation-mediated DNA vaccines have been tested in prostate cancer patients [78,79]. The DNA vaccine encoded a fusion protein containing the antigenic fragment of the prostate-specific antigen linked to tetanus toxoid. Injection of the DNA vaccine yielded a weak antibody response, while electroporation following injection produced a significantly greater antibody response. CD4 and CD8 T cell responses were comparable across the two delivery methods. Importantly, following treatment with an electroporation-based DNA vaccine, prostate-specific antigen doubling time increased, suggesting that the vaccine was effective at slowing down tumor growth [78,79].

The Shattock group has evaluated immune responses to DNA vaccines given via the combined intradermal and intramuscular routes with electroporation [80,81]. Their data suggests that there are only subtle differences between the T cell responses across the different routes of administration. This suggests that electroporation in conjunction with the more convenient injection route (i.e., intramuscular route) could be adopted without compromising efficacy.

In summary, much work has been done using electroporation for the delivery of DNA vaccines. In most studies, electroporation was found to be more efficacious than injection alone. More careful studies may be needed to draw comparisons between electroporation and chemical strategies for intracellular delivery.

Ultrasound

Ultrasound-based delivery involves the cyclic application of compressional and rarefactional pressure with a frequency of over 20 KHz (Figure 5A). This leads to the formation of gaseous cavities in the surrounding medium—a phenomenon known as cavitation (Figure 5B). Stable cavitation (oscillation of bubble size around a certain radius) causes shear forces on adjacent tissue and microstreaming. Inertial cavitation (the rapid growth and collapse of the bubbles) can cause shock waves that disrupt surrounding tissue and microjet formation. Through these tissue-disruptive and streaming effects, ultrasound-mediated stable and inertial cavitation improve the uptake of cell- and tissue-impermeable cargoes [82,83]. Ultrasound has been widely used for drug delivery and has found some application in the delivery of vaccines, although this mode of physical vaccination is relatively less explored.

Comparison to needle-based administration

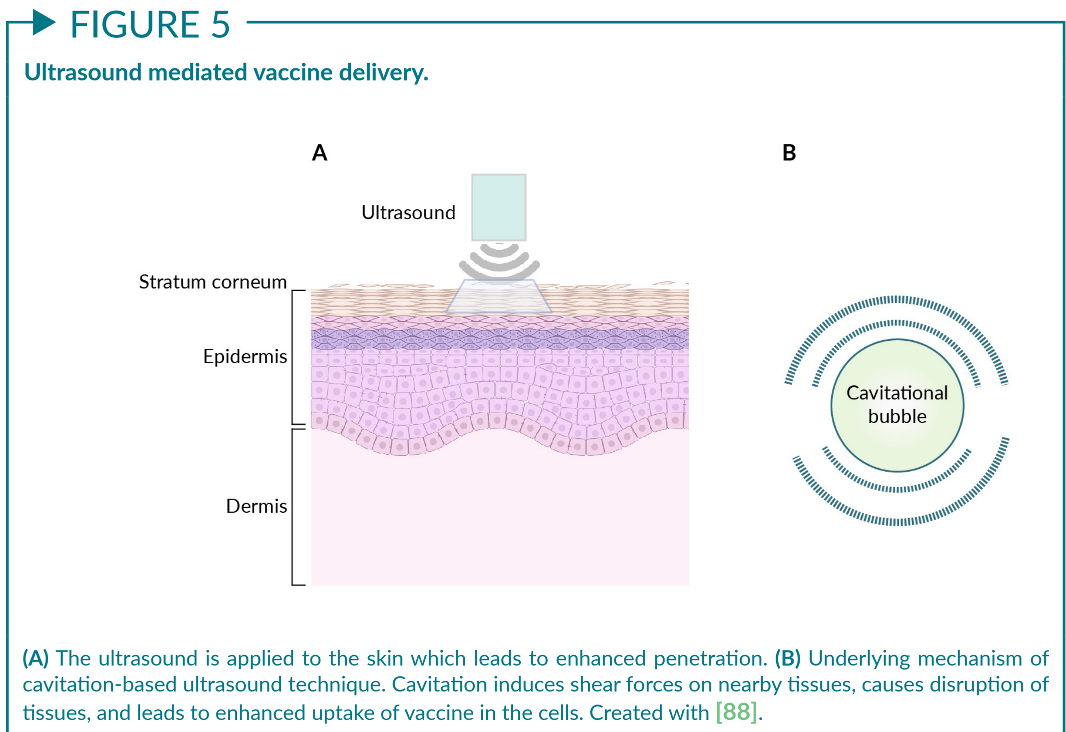
Reports surrounding the use of ultrasound for vaccination are limited to studies in rodents and are discussed here.

The Mitragotri group evaluated transcutaneous vaccination with tetanus toxoid [84].

 DOI: 10.18609/vac/2023.050

FIGURE 5

Ultrasound mediated vaccine delivery.



(A) The ultrasound is applied to the skin which leads to enhanced penetration. **(B)** Underlying mechanism of cavitation-based ultrasound technique. Cavitation induces shear forces on nearby tissues, causes disruption of tissues, and leads to enhanced uptake of vaccine in the cells. Created with [88].

Transcutaneous vaccination is used to target Langerhans cells in the epidermis without the use of needles. Ultrasound-based transcutaneous vaccination, and not the topical application, led to a serum antigen-specific IgG response. However, subcutaneous vaccination with a tenth of the dose produced a comparable antibody response. Low antibody responses to ultrasound could be because of the low antigen delivery efficacy of ultrasound. Specifically, only approximately 1% of the applied dose was taken up in the skin after ultrasound treatment.

The depth of penetration and uptake of topically applied vaccines could be enhanced with uniquely engineered nanocups [85]. Nanocups are sub-micron structures containing an air bubble, which expands and bursts upon the application of ultrasound pushing the formulation into the skin. Despite this elegant strategy, ultrasound-mediated vaccination yielded a weaker immune response than subcutaneous injection at the same dose. More recent work has suggested that high-frequency ultrasound (instead of the low-frequency ultrasound used in previous studies) could improve uptake. The focused application of high-frequency ultrasound was claimed to be safe in rodents [86]. Evaluation of the acceptability of this approach in a clinical setting may be insightful.

Mucosal application

We did not find reports of mucosal application of ultrasound for vaccine delivery. However, recent work from the Traverso lab has shown that ultrasound could enable the delivery of reporter mRNA nanoparticles into colonic tissue in mice [87]. Ultrasound application following the luminal application of the mRNA nanoparticles enabled the production of the reporter protein in mice. Luminal delivery alone was ineffective. This work suggests that ultrasound could be useful for mucosal delivery of modalities such as mRNA vaccines. However, its application to tissues more relevant for vaccination (e.g., nose, cheek, tongue) needs further investigation.

Clinical experience

The literature search did not produce any report of clinical testing of ultrasound-based vaccination methods.

TABLE 1

Comparison between different physical methods-based vaccine delivery systems.

Category	Microneedle	Needle-free jet injector	Electroporation	Ultrasound
Definition and mechanism	Micron-sized needles are made to pierce through the cell layers and deliver cargo; microneedle delivers vaccine to a shallower depth into the skin than intramuscular injections, thereby accessing a distinct subset of immune cells; minimally invasive technique	The injector system generates a high-velocity fluidic jet stream that passes through a narrow orifice at high pressure and penetrates the tissue to deliver the vaccine; most commonly used devices have had spring or compressed gas as an energy source to generate jet-stream	This technique involves an electric-pulse-based permeabilization of the cell membrane; when an external electric field is applied, the cell membrane becomes partially conductive and accumulates charge as transmembrane potential; this field aligns macromolecular dipoles inside and outside cells, resulting in charge accumulation across the membrane; once electric field-induced transmembrane potential surpasses the dielectric strength of the membrane, a permeation event occurs. This leads to the formation of hydrophobic pores that allow the entry of the cargo into the cells	This method uses a cyclic application of compressional and rarefactional pressure which leads to the formation of cavitation. Cavitation induces disruption in the surrounding tissue and improves the uptake of cells and tissue-impermeable cargoes
Loaded therapeutic agents/cargo for *in vivo* delivery	Peptide subunit, attenuated organism, whole protein	Attenuated organism, DNA, whole protein, live organism	DNA	Nanoparticles, subunit peptide
Application sites explored	Skin, cheek	Skin, cheek	Skin	Skin
Advantages	Prevents contact with the nerve and provides a painless vaccination option; targets immune cells in the intradermal space, thereby producing dose sparing; vaccines can be stored in solid form, thereby enhancing long-term storage	Targets immune cells in the intradermal space, thereby producing dose sparing; may enable intracellular delivery of DNA vaccines	Enables intracellular delivery of large cargoes such as DNA without the need for chemical enhancers	May be used to target Langerhans cells in the epidermis, thereby accessing a distinct immune cell population than intramuscular injections
Challenges	Loss of vaccine components due to retention on the microneedles; need for complex set-up and prolonged application time; need for patient/staff training	Pain related to application	Limited utility for tissue penetration	Delivery efficiency is limited

TRANSLATION INSIGHT

Needle-based delivery is a convenient and highly effective means of administering vaccines. However, challenges associated with adherence and efficacy motivate the exploration of other methods. Herein, we reviewed physical methods for overcoming biological barriers to vaccinating at the skin and mucosal tissues (summarized in Table 1). We find that physical methods for vaccine delivery are an excellent alternative to needle-based administration,

DOI: 10.18609/vac/2023.050

albeit several challenges remain. Pain during administration, patient acceptability, and the need for training healthcare workers are some of the critical challenges. Specifically, pain during vaccination may be difficult to assess in preclinical studies. These effects become evident only during clinical studies, which require significant resources to undertake and allow for the testing of only select designs. Additionally, the need for training personnel to use these advanced delivery systems can be a major barrier to their use in low sociodemographic index countries. The need for training adds a significant financial burden on healthcare systems and may be heavily restricted by the number of local experienced personnel who can provide this training. Additionally, the unfamiliarity of patients with novel delivery systems may engender fear. Hence, patient education is critical for acceptance of these systems, which may add further limitations on their use in resource-limited settings.

Despite several challenges, physical modes of vaccination provide significant advantages. Improved immunogenicity with select physical methods is highly attractive. Improved immunogenicity may allow for reducing the dose and cost, which has significant benefits for low sociodemographic index countries. A platform like microneedles allows storage of the vaccine in solid form and application without reconstitution. Storage in solid form may enable improved stability. Circumventing reconstitution reduces the risk of contamination. Finally, avoiding liquids in the formulation leads to a reduction in its weight, which allows for easier transport. These benefits may be highly attractive for vaccinating in low-resource settings.

In summary, physical modes of vaccine delivery hold significant promise. With concerted efforts on the research and clinical side, these systems may have a significant impact on vaccination campaigns worldwide.

REFERENCES

1. Small Pox: A Great and Terrible Scourge. US National Library of Medicine 2002 (accessed Aug 2023).
2. Anderson A. What is Variolation. WebMD 2022 (accessed Aug 2023).
3. Korsman SNJ, van Zyl GU, Nutt L, Andersson MI, Preiser W. Immunotherapy and immunoprophylaxis– passive and active immunity. In: *Virology* (Editors: Korsman SNJ, van Zyl GU, Nutt L, Andersson MI, Preiser W) 2012; 46–47. Churchill Livingstone.
4. Defeating the “Speckled Monster”: The Fight against Smallpox from Inoculation to Vaccination. Massachusetts Historical Society 2021 (accessed Aug 7).
5. Love AS, Love RJ. Considering needle phobia among adult patients during mass COVID-19 vaccinations. *J. Prim. Care Community Health* 2021; 12, 21501327211007390.
6. Freeman D, Lambe S, Yu LM, *et al.* Injection fears and COVID-19 vaccine hesitancy. *Psychol. Med.* 2023; 53(4), 1185–1195.
7. Moran-González JD, Padilla-Orozco M, Guzman-Lopez A, Ochoa-Bayona HC, Camacho-Ortiz A. Frequency of needle stick injuries among healthcare providers during large-scale SARS-CoV-2 vaccination brigades. *Front. Public Health* 2023; 11, 1084812.
8. Chaudhary N, Weissman D, Whitehead KA. mRNA vaccines for infectious diseases: principles, delivery and clinical translation. *Nat. Rev. Drug Discov.* 2021; 20(11), 817–838.
9. Lavelle EC, Ward RW. Mucosal vaccines—fortifying the frontiers. *Nat. Rev. Immunol.* 2021; 22(4), 236–250.
10. 10. Kirtane AR, Tang C, Freitas D, Bernstock JD, Traverso G. Challenges and opportunities in the development of mucosal mRNA vaccines. *Curr. Opin. Immunol.* 2023; 85, 102388.
11. Sallam MA, Prakash S, Kumbhojkar N, Shields CW, Mitragotri S. Formulation-based approaches for dermal delivery of vaccines and therapeutic nucleic acids: Recent advances and future perspectives. *Bioeng. Transl. Med.* 2021; 6(3), e10215.
12. Huang M, Zhang M, Zhu H, Du X, Wang J. Mucosal vaccine delivery: a focus on the breakthrough of specific barriers. *Acta Pharm. Sin. B* 2022; 12(9), 3456–3474.

13. Menon I, Bagwe P, Gomes KB, *et al.* Microneedles: A New Generation Vaccine Delivery System. *Micromachines (Basel)* 2021; 12(4), 435.

14. Sheng T, Luo B, Zhang W, *et al.* Microneedle-mediated vaccination: innovation and translation. *Adv. Drug Deliv. Rev.* 2021; 179, 113919.

15. Hossain MK, Ahmed T, Bhusal P, *et al.* Microneedle systems for vaccine delivery: the story so far. *Expert Rev. Vaccines* 2020; 19(12), 1153–1166.

16. Escobar-Chávez JJ, Bonilla-Martínez D, Angélica M, *et al.* Microneedles: a valuable physical enhancer to increase transdermal drug delivery. *J. Clin. Pharmacol.* 2011; 51(7), 964–977.

17. Donnelly RF, Raj Singh TR, Woolfson AD. Microneedle-based drug delivery systems: microfabrication, drug delivery, and safety. *Drug Deliv.* 2010; 17(4), 187–207.

18. Kim Y-C. *Skin Vaccination Methods: Gene Gun, Jet Injector, Tattoo Vaccine, and Microneedle. In: Percutaneous Penetration Enhancers Physical Methods in Penetration Enhancement* (Editors: Dragicevic NI, Maibach H). 2017; 485–499, Springer.

19. Weldon WC, Zarnitsyn VG, Esser ES, *et al.* Effect of adjuvants on responses to skin immunization by microneedles coated with influenza subunit vaccine. *PLoS One* 2012; 7(7), e41501.

20. Esser ES, Romanyuk A, Vassilieva EV, *et al.* Tetanus vaccination with a dissolving microneedle patch confers protective immune responses in pregnancy. *J. Control. Release* 2016; 236, 47–56.

21. vander Straeten A, Sarmadi M, Daristotle JL, *et al.* A microneedle vaccine printer for thermostable COVID-19 mRNA vaccines. *Nat. Biotechnol.* 2023.

22. Gill HS, Söderholm J, Prausnitz MR, Sällberg M. Cutaneous vaccination using microneedles coated with hepatitis C DNA vaccine. *Gene Ther.* 2010; 17(6), 811–814.

23. Kim Y-C, Quan F-S, Compans RW, Kang S-M, Prausnitz MR. Formulation and coating of microneedles with inactivated influenza virus to improve vaccine stability and immunogenicity. *J. Control. Release* 2010; 142(2), 187–195.

24. Choi Y, Lee GS, Li S, *et al.* Hepatitis B vaccine delivered by microneedle patch: Immunogenicity in mice and rhesus macaques. *Vaccine* 2023; 41(24), 3663–3672.

25. Ito S, Hirobe S, Yamashita R, *et al.* Analysis of immune response induction mechanisms implicating the dose-sparing effect of transcutaneous immunization using a self-dissolving microneedle patch. *Vaccine* 2022; 40(6), 862–872.

26. Van Damme P, Oosterhuis-Kafeja F, Van der Wielen M, Almagor Y, Sharon O, Levin Y. Safety and efficacy of a novel microneedle device for dose sparing intradermal influenza vaccination in healthy adults. *Vaccine* 2009; 27(3), 454–459.

27. Ray S, Wirth DM, Ortega-Rivera OA, Steinmetz NF, Pokorski JK. Dissolving microneedle delivery of a prophylactic HPV vaccine. *Biomacromolecules* 2022; 23(3), 903–912.

28. Edens C, Collins ML, Goodson JL, Rota PA, Prausnitz MR. A microneedle patch containing measles vaccine is immunogenic in non-human primates. *Vaccine* 2015; 33(37), 4712–4718.

29. Edens C, Dybdahl-Sissoko NC, Weldon WC, Oberste MS, Prausnitz MR. Inactivated polio vaccination using a microneedle patch is immunogenic in the rhesus macaque. *Vaccine* 2015; 33(37), 4683–4690.

30. Petrovsky N. Comparative safety of vaccine adjuvants: a summary of current evidence and future needs. *Drug Saf.* 2015; 38(11), 1059–1074.

31. Ma Y, Tao W, Krebs SJ, Sutton WF, Haigwood NL, Gill HS. Vaccine delivery to the oral cavity using coated microneedles induces systemic and mucosal immunity. *Pharm. Res.* 2014; 31(9), 2393–2403.

32. Kim Y, Park IH, Shin J, *et al.* Sublingual dissolving microneedle (SLDMN)-based vaccine For inducing mucosal immunity against SARS-CoV-2. *Adv. Healthc. Mater.* 2023; 19, e2300889

33. Caffarel-Salvador E, Kim S, Soares V, *et al.* A microneedle platform for buccal macromolecule delivery. *Sci. Adv.* 2021; 7(4), eabe2620.

34. Abramson A, Kirtane AR, Shi Y, *et al.* Oral mRNA delivery using capsule-mediated gastrointestinal tissue injections. *Matter* 2022; 5(3), 975–987.

35. Frew PM, Paine MB, Rouphael N, *et al.* Acceptability of an inactivated influenza vaccine delivered by microneedle patch: Results from a phase I clinical trial of safety, reactogenicity, and immunogenicity. *Vaccine* 2020; 38(45), 7175–7181.

36. Rouphael NG, Paine M, Mosley R, *et al.* The safety, immunogenicity, and acceptability of inactivated influenza vaccine delivered by microneedle patch (TIV-MNP 2015): a randomised, partly blinded,

 DOI: 10.18609/vac/2023.050

placebo-controlled, phase 1 trial. *Lancet* 2017; 390(10095), 649–658.

37. Iwata H, Kakita K, Imafuku K, *et al.* Safety and dose-sparing effect of Japanese encephalitis vaccine administered by microneedle patch in uninfected, healthy adults (MNA-J): a randomised, partly blinded, active-controlled, phase 1 trial. *Lancet Microbe* 2022; 3(2). e96–104.

38. Fernando GJP, Hickling J, Jayashi Flores CM, *et al.* Safety, tolerability, acceptability and immunogenicity of an influenza vaccine delivered to human skin by a novel high-density microprojection array patch (Nanopatch™). Vaccine 2018; 36(26), 3779–3788.

39. Levin Y, Kochba E, Kenney R. Clinical evaluation of a novel microneedle device for intradermal delivery of an influenza vaccine: are all delivery methods the same? *Vaccine* 2014; 32(34), 4249–4252.

40. Troy SB, Kouiavskaia D, Siik J, *et al.* Comparison of the immunogenicity of various booster doses of inactivated polio vaccine delivered intradermally versus intramuscularly to HIV-infected adults. *J. Infect Dis*. 2015; 211(12), 1969–1976.

41. Levin Y, Kochba E, Shukarev G, Rusch S, Herrera-Taracena G, van Damme P. A phase 1, open-label, randomized study to compare the immunogenicity and safety of different administration routes and doses of virosomal influenza vaccine in elderly. *Vaccine* 2016; 34(44), 5262–5272.

42. Schoppink J, Fernandez Rivas D. Jet injectors: perspectives for small volume delivery with lasers. *Adv. Drug Deliv. Rev.* 2022; 182, 114109.

43. Ledesma-Feliciano C, Chapman R, Hooper JW, *et al.* Improved DNA vaccine delivery with needle-free injection systems. *Vaccines* 2023; 11(2), 280.

44. Giacca M. Gene Therapy. In: *Introduction to Gene Therapy* (Editor: Giacca M). 2010; 1–7. Springer.

45. Kwilas S, Kishimori JM, Josleyn M, *et al.* A hantavirus pulmonary syndrome (HPS) DNA vaccine delivered using a spring-powered jet injector elicits a potent neutralizing antibody response in rabbits and nonhuman primates. *Curr. Gene Ther.* 2014; 14(3), 200–210.

46. Brocato RL, Kwilas SA, Josleyn MD, *et al.* Small animal jet injection technique results in enhanced immunogenicity of hantavirus DNA vaccines. *Vaccine* 2021; 39(7), 1101–1110.

47. Inoue S, Mizoguchi I, Sonoda J, *et al.* Induction of potent antitumor immunity by intradermal DNA injection using a novel needle-free pyro-drive jet injector. *Cancer Sci.* 2023; 114(1), 34–47.

48. Chang C, Sun J, Hayashi H, *et al.* Stable immune response induced by intradermal DNA vaccination by a novel needleless pyro-drive jet injector. *AAPS PharmSciTech* 2019; 21(1), 19.

49. Omori-Urabe Y, Yoshii K, Ikawa-Yoshida A, Kariwa H, Takashima I. Needle-free jet injection of DNA and protein vaccine of the Far-Eastern subtype of tick-borne encephalitis virus induces protective immunity in mice. *Microbiol. Immunol.* 2011; 55(12), 893–897.

50. Ndeupen S, Qin Z, Jacobsen S, Bouteau A, Estanbouli H, Igyártó BZ. The mRNA-LNP platform's lipid nanoparticle component used in preclinical vaccine studies is highly inflammatory. *iScience* 2021; 24(12), 103479.

51. Tahtinen S, Tong AJ, Himmels P, *et al.* IL-1 and IL-1ra are key regulators of the inflammatory response to RNA vaccines. *Nat. Immunol.* 2022; 23(4), 532–542.

52. Opriessnig T, Rawal G, McKeen L, Filippsen Favaro P, Halbur PG, Gauger PC. Evaluation of the intranasal route for porcine reproductive and respiratory disease modified-live virus vaccination. *Vaccine* 2021; 39(47), 6852–6859.

53. Jones AT, Shen X, Walter KL, *et al.* HIV-1 vaccination by needle-free oral injection induces strong mucosal immunity and protects against SHIV challenge. *Nat. Commun.* 2019; 10(1), 798.

54. Aran K, Chooljian M, Paredes J, *et al.* An oral microjet vaccination system elicits antibody production in rabbits. *Sci. Transl. Med.* 2017; 9(380), eaaf6413.

55. Epstein JE, Gorak EJ, Charoenvit Y, *et al.* Safety, tolerability, and lack of Antibody responses after administration of a Pf CSP DNA malaria vaccine via needle or needle-free jet injection, and comparison of intramuscular and combination intramuscular/intradermal routes. *Hum. Gene Ther.* 2002; 13(13). 1551–1560.

56. Simon JK, Carter M, Pasetti MF, *et al.* Safety, tolerability, and immunogenicity of inactivated trivalent seasonal influenza vaccine administered with a needle-free disposable-syringe jet injector. *Vaccine* 2011; 29(51), 9544–9550.

57. Jackson LA, Austin G, Chen RT, *et al.* Safety and immunogenicity of varying dosages of trivalent inactivated influenza vaccine administered by needle-free jet injectors. *Vaccine* 2001; 19, 4703–4709.

58. Bavdekar A, Malshe N, Ravichandran L, *et al.* Clinical study of safety and immunogenicity of pentavalent DTP-HB-Hib vaccine administered by disposable-syringe jet injector in India. *Contemp. Clin. Trials Commun.* 2019; 14, 100321.

59. Hoke CH, Egan JE, Sjogren MH, *et al.* Administration of hepatitis A vaccine to a military population by needle and jet injector and with hepatitis B vaccine D. *J. Infect. Dis.* 1995; 171, S53–S60.

60. Arora A, Hakim I, Baxter J, *et al.* Needle-free delivery of macromolecules across the skin by nanoliter-volume pulsed microjets. *Proc. Natl. Acad. Sci.* 2007; 104(11), 4255–4260.

61. Hogan NC, Anahtar MN, Taberner AJ, Hunter IW. Delivery of immunoreactive antigen using a controllable needle-free jet injector. *J. Control. Release* 2017; 258: 73–80.

62. Taberner A, Hogan NC, Hunter IW. Needle-free jet injection using real-time controlled linear Lorentz-force actuators. *Med. Eng. Phys.* 2012; 34(9), 1228–1235.

63. Wong T-K, Neumann E. Electric field mediated gene transfer. *Biochem. Biophys. Res. Commun.* 1982; 107(2): 584–587.

64. Neumann E, Schaefer-Ridder M, Wang Y, Hofschneider PH. Gene transfer into mouse lyoma cells by electroporation in high electric fields. *EMBO J.* 1982; 1(7), 841–845.

65. Campelo SN, Huang P-H, Buie CR, Davalos R V. Recent Advancements in Electroporation Technologies: From Bench to Clinic. *Annu. Rev. Biomed. Eng.* 2023; 25(1), 77–100.

66. Sardesai NY, Weiner DB. Electroporation delivery of DNA vaccines: prospects for success. *Curr. Opin. Immunol.* 2011; 23(3): 421–429.

67. Young JL, Dean DA. Electroporation-mediated gene delivery. *Adv. Genet.* 2015; 89, 49–88.

68. Le Moigne V, Cariolet R, Béven V, Keranflec'h A, Jestin A, Dory D. Electroporation improves the immune response induced by a DNA vaccine against pseudorabies virus glycoprotein B in pigs. *Res. Vet. Sci.* 2012; 93(2), 1032–1035.

69. Shahbazi M, Zahedifard F, Saljoughian N, *et al.* Immunological comparison of DNA vaccination using two delivery systems against canine leishmaniasis. *Vet. Parasitol.* 2015; 212(3–4), 130–139.

70. Weiland O, Ahlén G, Diepolder H, *et al.* Therapeutic DNA vaccination using in vivo electroporation followed by standard of care therapy in patients with genotype 1 chronic hepatitis C. *Mol. Ther.* 2013; 21(9), 1796–1805.

71. Bagarazzi ML, Yan J, Morrow MP, *et al.* Immunotherapy against HPV16/18 generates potent TH1 and cytotoxic cellular immune responses. *Sci. Transl. Med.* 2012; 4(155), 1–15.

72. Kim TJ, Jin HT, Hur SY, *et al.* Clearance of persistent HPV infection and cervical lesion by therapeutic DNA vaccine in CIN3 patients. *Nat. Commun.* 2014; 5, 5317.

73. Trimble CL, Morrow MP, Kraynyak KA, *et al.* Safety, efficacy, and immunogenicity of VGX-3100, a therapeutic synthetic DNA vaccine targeting human papillomavirus 16 and 18 E6 and E7 proteins for cervical intraepithelial neoplasia 2/3: a randomised, double-blind, placebo-controlled phase 2b trial. *Lancet* 2015; 386(10008), 2078–2088.

74. Spearman P, Mulligan M, Anderson EJ, *et al.* A phase 1, randomized, controlled dose-escalation study of EP-1300 polyepitope DNA vaccine against Plasmodium falciparum malaria administered via electroporation. *Vaccine* 2016; 34(46), 5571–5578.

75. Tebas P, Roberts CC, Muthumani K, *et al.* Safety and Immunogenicity of an Anti–Zika Virus DNA Vaccine. *N. Engl. J. Med.* 2021; 385(12), e35.

76. Vasan S, Hurley A, Schlesinger SJ, *et al.* In vivo electroporation enhances the immunogenicity of an HIV-1 DNA vaccine candidate in healthy volunteers. *PLoS One* 2011; 6(5), 1–10.

77. Kopycinski J, Cheeseman H, Ashraf A, *et al.* A DNA-based candidate HIV vaccine delivered via in vivo electroporation induces CD4 responses toward the α4β7-binding V2 loop of HIV gp120 in healthy volunteers. *Clin. Vaccine Immunol.* 2012; 19(9), 1557–1559.

78. Chudley L, McCann K, Mander A, *et al.* DNA fusion-gene vaccination in patients with prostate cancer induces high-frequency CD8+ T-cell responses and increases PSA doubling time. *Cancer Immunol. Immunother.* 2012; 61(11), 2161–2170.

79. Low L, Mander A, Mccann K, *et al.* Vaccination with Electroporation Induces Increased Antibody Responses in Patients with Prostate Cancer. *Hum. Gene Ther.* 2009; 20, 1269–1278.

80. Cheeseman HM, Day S, McFarlane LR, *et al.* Combined skin and muscle DNA priming provides enhanced humoral responses to a human immunodeficency virus type 1 clade C envelope vaccine. *Hum. Gene Ther.* 2018; 29(9), 1011–1028.

 DOI: 10.18609/vac/2023.050

81. Haidari G, Cope A, Miller A, *et al.* Combined skin and muscle vaccination differentially impact the quality of effector T cell functions: The CUTHIVAC-001 randomized trial. *Sci. Rep.* 2017; 7(1), 1–11.

82. Mo S, Coussios C-C, Seymour L, Carlisle R. Ultrasound-enhanced drug delivery for cancer. *Expert Opin. Drug Deliv.* 2012; 9(12), 1525–1538.

83. Mitragotri S. Healing sound: the use of ultrasound in drug delivery and other therapeutic applications. *Nat. Rev. Drug Discov*. 2005; 4(3), 255–260.

84. Tezel A, Paliwal S, Shen Z, Mitragotri S. Low-frequency ultrasound as a transcutaneous immunization adjuvant. *Vaccine* 2005; 23(29), 3800–3807.

85. Bhatnagar S, Kwan JJ, Shah AR, Coussios CC, Carlisle RC. Exploitation of sub-micron cavitation nuclei to enhance ultrasound-mediated transdermal transport and penetration of vaccines. *J. Control. Release* 2016; 238, 22–30.

86. Hu Y, Mo Y, Wei J, Yang M, Zhang X, Chen X. Programmable and monitorable intradermal vaccine delivery using ultrasound perforation array. *Int. J. Pharm.* 2022; 617, 121595.

87. Schoellhammer CM, Lauwers GY, Goettel JA, *et al.*. Ultrasound-mediated delivery of RNA to colonic mucosa of live mice. *Gastroenterology* 2017; 152(5), 1151–1160.

88. BioRender.

AFFILIATIONS

Taksim Ahmed
Division of Gastroenterology, Hepatology and Endoscopy, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, USA

Dylan Freitas
Division of Gastroenterology, Hepatology and Endoscopy, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, USA

Xisha Huang
Division of Gastroenterology, Hepatology and Endoscopy & Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, USA

Qing Rui Simon Qu
Division of Gastroenterology, Hepatology and Endoscopy & Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, USA

Giovanni Traverso
Author for correspondence
Division of Gastroenterology, Hepatology and Endoscopy & Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, USA
and
Department of Mechanical Engineering & David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, USA

Ameya R Kirtane
Author for correspondence
Division of Gastroenterology, Hepatology and Endoscopy & Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, USA
and
David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, USA

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: Dr Taksim Ahmed and Mr Dylan Freitas are the co-first authors and contributed equally to this work. All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.

Acknowledgements: None.

Disclosure and potential conflicts of interest: Dr Kirtane has received royalties from Lyndra and Syntis Bio, and consulting fees from Celero Bio and Guidepoint Global Advisors. He also holds patents with the MIT Technology Licensing Office and Mass General Brigham Licensing Office. Dr Traverso has received royalties from Exact Sciences, Horizon, Lyndra, Bilayer Therapeutics Inc., Johns Hopkins University / Technology Transfer Office, Massachusetts Institute of Technology / Technology Licensing Office, Massachusetts Institute of Technology / Technology Licensing Office, Suono Bio, Vivtex, Celero and Teal Bio, Inc. He has received consulting fees from Novo Nordisk, Eagle Pharmaceuticals, Inc., Wired Consulting, Avadel Pharmaceuticals, Moderna, Syntis Bio and Vitakey. He holds patents with Johns Hopkins University / Technology Transfer Office, Massachusetts Institute of Technology / Technology Licensing Office and Massachusetts Institute of Technology / Technology Licensing Office. He is on boards for Lyndra, Novo Nordisk, Suono Bio, Vivtex, Celero, Teal Bio, Inc., Bilayer Therapeutics, Inc., Syntis Bio and Vitakey; and is a stockholder in Lyndra, Suono Bio, Vivtex, Celero, Teal Bio, Inc., Bilayer Therapeutics, Inc., Syntis Bio and Vitakey.

Funding declaration: Dr Kirtane has received grants from MIT Global Oncology Nano Program. Dr Traverso has received grants from Oracle, CSL Vifor, NIH/NCI, Karl van Tassel (1925) Career Development Professorship, MIT, The Leona M. and Harry B. Helmsley Charitable Trust, NIH/NIBIB, Bill and Melinda Gates Foundation and Defense Advanced Research Projects Agency.

ARTICLE & COPYRIGHT INFORMATION

Copyright: Published by *Vaccine Insights* under Creative Commons License Deed CC BY NC ND 4.0 which allows anyone to copy, distribute, and transmit the article provided it is properly attributed in the manner specified below. No commercial use without permission.

Attribution: Copyright © 2023 Ahmed T, Freita D, Huang X, Qu QRS, Traverso G & Kirtane AR. Published by *Vaccine Insights* under Creative Commons License Deed CC BY NC ND 4.0.

Article source: Invited; externally peer reviewed.

Submitted for peer review: Aug 8, 2023; **Revised manuscript received:** Oct 11, 2023; **Publication date:** Oct 25, 2023.

 DOI: 10.18609/vac/2023.050