RESEARCH ARTICLE

A piezoelectric electroporator (Piezopen) for enhanced "naked" RNA vaccine delivery

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Abstract

Despite the success of COVID-19 mRNA vaccines, they still face challenges with high costs, complex manufacturing, off-target biodistribution, and systemic reactogenicity stemming from their inflammatory carriers: lipid nanoparticles (LNPs). While "naked" RNA delivery could in principle solve these issues, studies have suggested that it is infeasible due to rapid degradation by RNases and poor cellular entry, thereby necessitating formulations that enhance intracellular delivery and RNA stability. Now, we challenge this paradigm by showing that a simple and inexpensive (<$1), lighter-derived electroporator with microneedle electrodes (Piezopen) can augment gene expression and immunogenicity to naked mRNA leading to comparable responses to LNPs at low doses. We achieve robust responses in the absence of systemic inflammation and reactogenicity using skin-targeted delivery, administer diverse construct types (i.e., mRNA, self-amplifying RNA (saRNA), circular RNA (circRNA)), and demonstrate cross-species validation in live human skin to derisk subsequent clinical application. Our results introduce Piezopen as an inexpensive, well-tolerated, and efficacious alternative to LNPs for mRNA vaccine delivery, designed to facilitate routine vaccinations and pandemic response.

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Citation: Michalaki E, Van Zanten A, Najjar J, Byagathvalli G (2026) A piezoelectric electroporator (Piezopen) for enhanced "naked" RNA vaccine delivery. PLoS One 21(7): e0353214. https://doi.org/10.1371/journal.pone.0353214

Editor: Satish Rojekar, Icahn School of Medicine at Mount Sinai Department of Pharmacological Sciences, UNITED STATES OF AMERICA

Received: March 5, 2026

Accepted: June 19, 2026

Published: July 7, 2026

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Data availability statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by Piezo Therapeutics, Inc. and by the National Science

Introduction

mRNA vaccines have been revolutionary in terms of their rapid development and ability to prevent disease onset and severity, as exemplified during the COVID-19 pandemic [1,2]. Despite their efficacy, they face challenges with expensive and complex manufacturing, excessive inflammation, poor reactogenicity, and limited durability. LNPs possess immunostimulatory properties, which provide potent adjuvant activity but also lead to significant side effects [3]. In addition, these hurdles have impeded the uptake of mRNA vaccines in low- and middle-income countries, creating disparities in vaccine distribution. Addressing these challenges with LNPs and improving tolerability through an alternative delivery platform could facilitate further application of mRNA vaccines.

Foundation Small Business Innovation Research (NSF SBIR) Phase I Award #2437939 (PI: G.B.). NSF SBIR Phase I funds provided salary support for G.B., E.M., and J.N. A.V.Z. was supported by Piezo Therapeutics, Inc. during the conduct of this work. The funders provided financial and salary support as described but had no additional role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: All authors are employees of Piezo Therapeutics, Inc., a for-profit company developing the Piezopen platform described in this manuscript. E.M. and G.B. are named inventors on filed, granted, or licensed patents pertaining to the Piezopen technology. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

Localized delivery of "naked" RNA has been suggested as an approach to resolve issues with LNPs and other formulations while inducing immunity sufficient for protection [2]. However, rapid degradation by RNases and poor intracellular uptake have previously resulted in poor responses even when delivering high payload doses. Successful application likely requires minimizing degradation via rapid and efficient intracellular delivery while inducing innate stimulation to adjuvant responses at doses that are not cost-prohibitive.

We previously described a platform that combines piezoelectric electroporation (EP) with microneedle (MN) electrodes (called ePatch or Piezopen) to efficiently deliver nucleic acid vaccines in the skin [4]. This innovation precisely targets intracellular delivery in the immune-cell-rich epidermis while adjuvanting immune responses via localized cell death. The use of short, high-voltage electric pulses physically creates temporary pores in cell membranes enabling rapid uptake of nucleic acids. In previous studies, Piezopen improved gene expression and immune responses to naked DNA by >100-fold and >10-fold respectively, with superior tolerability compared to a hypodermic needle in humans [5].

Building on our prior success [4], we evaluated whether EP using Piezopen could deliver naked mRNA and induce immune responses comparable to LNPs at low doses. To do so, we investigated Piezopen's ability to drive intracellular delivery of naked RNA (including, mRNA, self-amplifying RNA (saRNA), and circular RNA (circRNA)) and boost immune responses to a naked SARS-CoV-2 mRNA vaccine. We benchmarked to state-of-the-art LNPs clinically approved for RNA therapies, namely LNPs containing SM102, ALC0315, or MC3 [6]. In addition, we evaluated our hypothesis around cross-species translatability. Our study challenges the paradigm that suggests vaccination with naked mRNA is not feasible, uncovering a new modality for the delivery of mRNA vaccines more broadly.

Results

Piezopen significantly augments gene expression and induces immune responses to naked mRNA with comparability to LNPs *in vivo*

To test our hypothesis that Piezopen (**Fig 1A**) leads to successful intracellular delivery of naked mRNA *in vivo* superior to intradermal (ID) injection alone and comparable to LNPs, we conducted reporter expression kinetics studies using luciferase. We benchmarked against two state-of-the-art LNP formulations containing SM102 and ALC0315 [6]. We demonstrated that Piezopen delivery of mRNA-Luc leads to comparable expression magnitude and improved durability/kinetics compared to LNPs in a dose-dependent manner (**Fig 1B**). Notably, gene expression after Piezopen administration persisted at a level 10- to 100-fold higher on Day 14 compared to LNPs, potentially improving antigen availability and vaccine durability. Together, Piezopen: (1) successfully delivered naked mRNA intracellularly and (2) mitigated mRNA degradation concerns.

After demonstrating we can augment intracellular delivery and gene expression to naked RNA with comparable magnitude to LNPs, we assessed whether Piezopen can induce immune responses to naked RNA vaccination. We benchmarked the

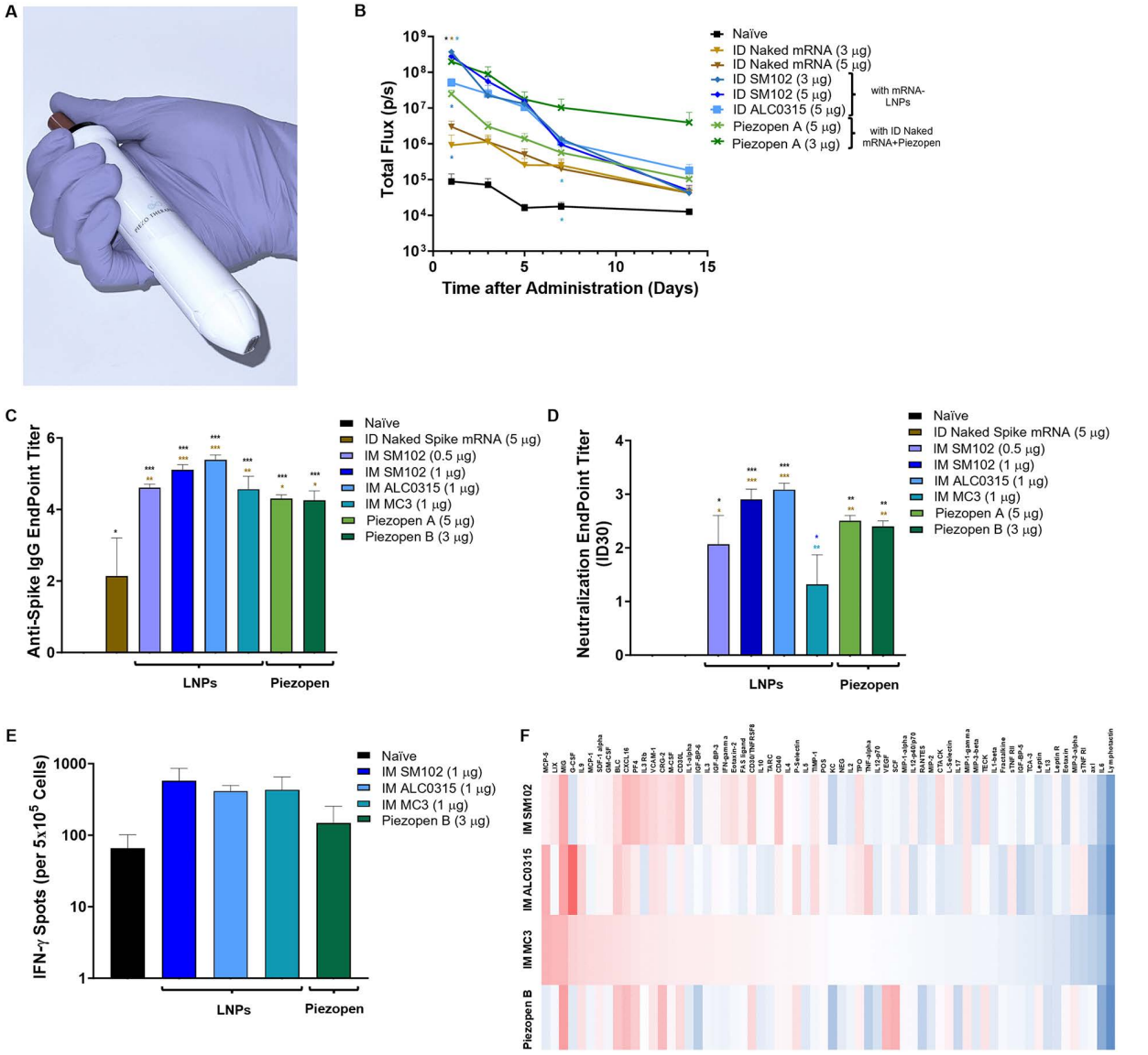


Fig 1. Piezopen augments gene expression and immune responses to naked RNA comparable to state-of-the-art LNPs. All panels: mice (female 6−8 wk BALB/c). **(A)** Photograph of the Piezopen – an inexpensive (<$1), battery-free, handheld, portable device that combines piezoelectric electroporation and microneedle electrodes. Original photograph, previously deposited on *bioRxiv* (doi:101101/2025.02.07.637103). **(B)** Luciferase reporter expression kinetics following ID administration of FLuc-mRNA (3 μg or 5 μg) with or without Piezopen A, or ID-delivered LNPs (SM102 at 3 or 5 μg; ALC0315 at 5 μg); total flux (p/s) vs. day post-administration by IVIS; $n = 4$ (Naïve), 3 (ID 3 μg), 5 (ID 5 μg), 3 (ID SM102 3 μg), 5 (ID SM102 5 μg), 5 (ID ALC0315 5 μg), 3 (Piezopen A 3 μg), 3 (Piezopen A 5 μg). **(C, D)** Anti-Spike IgG endpoint titers **(C)** and SARS-CoV-2 neutralizing antibody endpoint titers (sVNT, ID30) **(D)** in Day 35 serum; Spike mRNA delivered ID ± Piezopen (5 μg ID naked; Piezopen A 5 μg; Piezopen B 3 μg) or IM as LNP (SM102 0.5 or 1 μg; ALC0315 1 μg; MC3 1 μg); $n = 3$ (Naïve), 3 (ID 5 μg), 3 (IM SM102 0.5 μg), 5 (IM SM102 1 μg), 5 (IM ALC0315 1 μg), 5 (IM MC3 1 μg), 3 (Piezopen A 5 μg), 3 (Piezopen B 3 μg); ELISA IgG in technical duplicate, averaged per animal. **(E)** Spike-specific IFN-γ ELISpot spots per 5×10^5 splenocytes at Day 35; $n = 3$ (Naïve), 5 (IM SM102 1 μg), 5 (IM ALC0315 1 μg), 5 (IM MC3 1 μg), 3 (Piezopen B 3 μg). **(F)** Systemic reactogenicity heatmap of 62 serum cytokines, chemokines, and growth factors 24 h post-prime (RayBiotech G3 array); $n = 5$ (IM SM102 1 μg), 5 (IM ALC0315 1 μg), 5 (IM MC3 1 μg), 3 (Piezopen B 3 μg). Different doses or administration routes are depicted by different shades of the same color. Each data point = one biological replicate (one animal); mean ± SEM; 2-way **(B)** or 1-way **(C-E)** ANOVA with Tukey's post hoc. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; color-coordinated asterisks indicate pairwise comparisons.

https://doi.org/10.1371/journal.pone.0353214.g001

delivery of a SARS-CoV-2 mRNA vaccine using Piezopen compared to three FDA-approved LNP formulations, quantifying humoral (anti-spike IgG and neutralizing antibody) and cellular (spike-specific IFN-γ production) responses along with reactogenicity. We selected doses between 0.5 μg and 1 μg for LNP groups to align with preclinical studies of approved COVID-19 vaccines while beginning with a higher dose of naked mRNA as initial proof-of-concept [6].

We showed that Piezopen delivery of naked mRNA using Piezopen A (Piezopen design that led to the highest mRNA-Luc expression) induces robust immune responses significantly greater than the control conditions (both Naïve and ID injection alone) (Fig 1C-1E). In addition, manipulating EP field strength (Piezopen B) further augmented immune responses despite decreasing the mRNA dose by 40%, suggesting a viable path for optimizations to further reduce dosing while improving responses (Piezopen A: 5 μg vs Piezopen B: 3 μg). Delivery using both Piezopen A (5 μg) and Piezopen B (3 μg) led to comparable humoral and cellular immune responses to MC3 (1 μg) and slightly lower than SM102 (1 μg) and ALC0315 (1 μg). Importantly, neutralizing antibody titers induced by both Piezopen groups are similar to those reported in mRNA-1273 and BNT162b2 vaccines assessed using the same assay, suggesting clinical translatability [7,8].

Quantification of a panel of relevant cytokines demonstrated an excellent systemic reactogenicity profile for all Piezopen groups and LNPs tested (**Fig 1F**). Given the known inflammatory nature of LNPs, this is a rather surprising finding.

Taken together, we challenge conventional wisdom by showing that Piezopen delivery of naked mRNA: (1) achieves comparable gene expression kinetics to benchmark LNPs, (2) induces robust humoral and cellular immune responses comparable to best-in-class LNP formulations, and (3) produces minimal reactogenicity concerns.

Piezopen significantly augments gene expression to diverse array of naked RNA payloads with comparability to LNPs across species *in vivo* and in live human skin *ex vivo*

By leveraging Piezopen across RNA and its subtypes, we can enable a diverse array of applications. Here, we explored Piezopen delivery of saRNA and circRNA, which have either shown enhanced antigen expression at lower doses (saRNA) [9–13], or have been more stable and easier to store with lower innate immunogenicity, prolonged antigen-yielding capabilities, and durable immune responses (circRNA) [14,15] compared to canonical linear mRNA. We validated saRNA delivery using reporters (i.e., luciferase) showing comparable expression magnitude and kinetics to state-of-the-art LNP (SM102) at an identical 1 μg dose (**Fig 2A**). We showed that Piezopen delivery of naked circRNA leads to higher expression than ID injection alone, also at a low 1 μg dose (**Fig 2B**). Our demonstration of naked mRNA/saRNA/circRNA delivery illustrates Piezopen can achieve comparable intracellular delivery and effectiveness to state-of-the-art LNP formulations in a “payload-agnostic” manner.

To demonstrate our results are translatable across species, we evaluated mRNA delivery in rats and preserved human skin explants, the latter of which provides the closest available proxy to real-world human use. We show that Piezopen delivery significantly improves gene expression over injection alone in rats (**Fig 2C**) and is comparable to SM102 LNP in human skin *ex vivo* (**Fig 2D**), both at a low subtherapeutic dose (5 μg), indicating efficient intracellular delivery with minimal mRNA degradation. Since the Piezopen device used in the *ex vivo* human skin study is identical to what was used in mice, we confirm our results are translatable across species derisking clinical translation for human use. Lastly, comparable expression to LNPs at a low dose in human skin suggests Piezopen could potentially match or decrease clinical mRNA vaccine doses given the increased thickness and broader immune-cell repertoire of the human epidermis compared to mice.

Discussion

Our study reveals that naked RNA vaccination is feasible without LNPs or other formulation approaches currently considered necessary for cargo (RNA) protection and intracellular delivery [6–8], presumably because the EP-based mechanisms deliver RNA into cells within seconds [16]. Piezopen achieves comparable immunogenicity and improved expression kinetics compared to LNPs at low doses. We note that LNP groups were dosed at 0.5–1 μg to align with published preclinical studies of clinically approved mRNA vaccines, while naked mRNA was initially dosed at 3–5 μg,

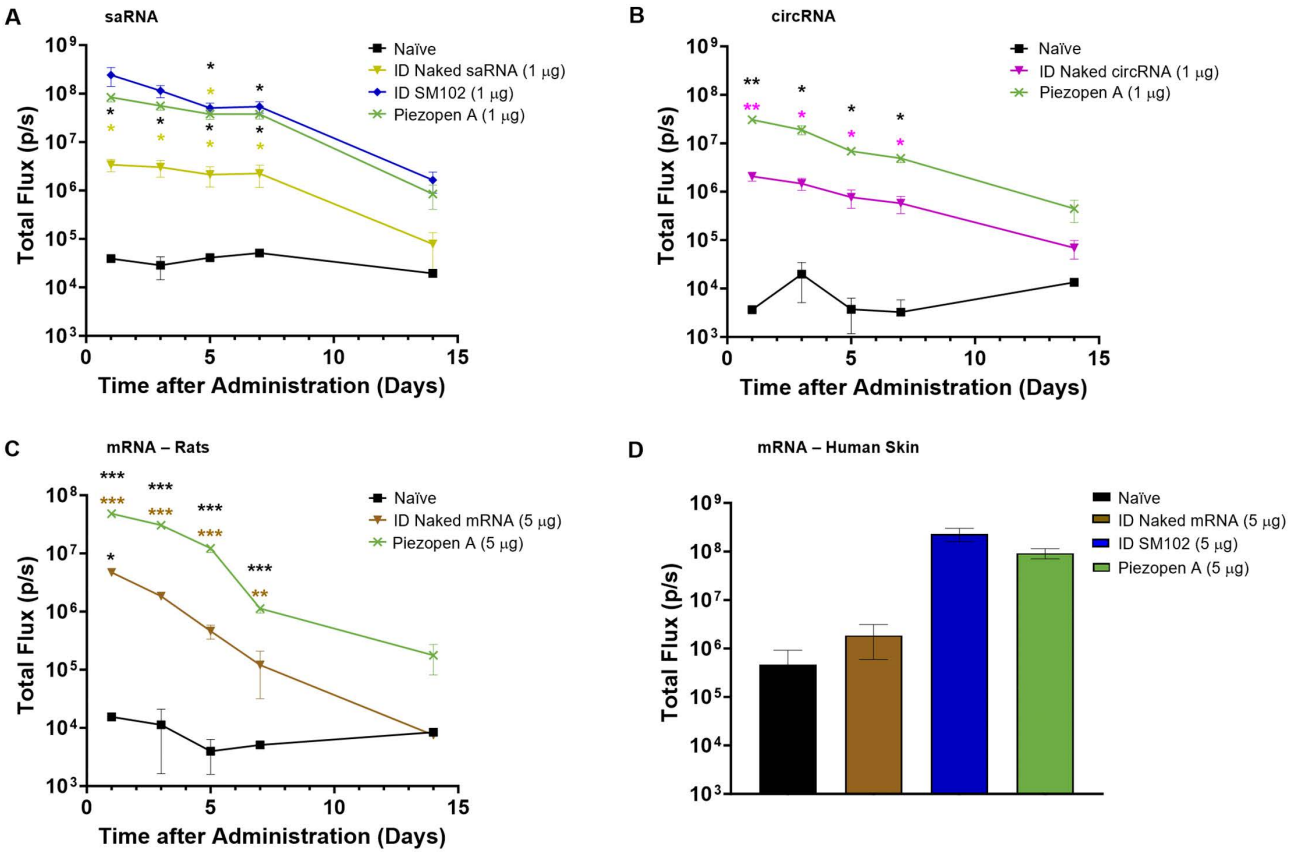


Fig 2. Piezopen drives intracellular delivery of naked RNA (mRNA, saRNA, circRNA) across species (mice and rats) *in vivo* and in live human skin *ex vivo*. **(A)** FLuc-saRNA reporter kinetics in mice (female 6–8 wk BALB/c); ID (1 μg naked, ± Piezopen A) vs. SM102 LNP (1 μg ID); $n = 3$ (Naïve), 3 (ID), 6 (ID SM102), 6 (Piezopen A). **(B)** FLuc-circRNA reporter kinetics in mice (female 6–8 wk BALB/c); ID (1 μg naked, ± Piezopen A); $n = 3$ (Naïve), 3 (ID), 6 (Piezopen A). **(C)** FLuc-mRNA reporter kinetics in rats (female ~8 wk Wistar); ID (5 μg naked, ± Piezopen A); $n = 3$ (Naïve), 3 (ID), 10 (Piezopen A). **(D)** FLuc-mRNA expression in *ex vivo* live human skin (Genoskin) 24 h post-administration; ID (5 μg naked, ± Piezopen A) vs. SM102 LNP (ID 5 μg); $n = 3$ (Naïve), 5 (ID), 3 (ID SM102), 6 (Piezopen A) technical replicates from one donor. Total flux (p/s) quantified by IVIS. For **(A-C)**, each data point represents one biological replicate; for **(D)**, each data point represents one technical replicate from a single skin donor. Mean ± SEM; 2-way **(A-C)** or 1-way **(D)** ANOVA with Tukey's post hoc. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$; color-coordinated asterisks indicate pairwise comparisons.

https://doi.org/10.1371/journal.pone.0353214.g002

reflecting the historically reported need for higher doses of unformulated RNA to overcome rapid degradation and inefficient cellular uptake. This study was therefore designed as a proof-of-concept for Piezopen-enabled naked RNA delivery rather than a dose-equivalence comparison. The 40% dose reduction achieved with Piezopen B (3 μg) over Piezopen A (5 μg) at comparable or improved immunogenicity outlines a viable path toward further dose reduction through EP parameter optimization. This is a key finding as antigen persistence has been shown to significantly impact vaccine durability [17,18]. Piezopen showed no reactogenicity concerns in this study despite using higher doses compared to LNPs, enabling multivalent or combination vaccines via increased tolerability/dosing thresholds. Piezopen delivery covers diverse payload sizes from ~1,000 bp mRNA to ~10 kbp saRNA/DNA, showcasing vast cargo capacities to deliver larger, complex, or multiple antigens. Notably, Piezopen induces robust immune responses to N1-methylpseudouridine (M1Ψ)-modified linear mRNA which is the least stable and immunogenic platform [19], indicating significant promise with circRNA and saRNA modalities. These findings uncover the benefits of Piezopen in improving mRNA vaccine durability via antigen persistence and adjuvantation, addressing a major limitation of mRNA vaccines.

The development of efficacious mRNA medicines is hindered by the limited understanding of the exact cell types that are necessary targets for effective protection. Studies have suggested that antigen-presenting cell (APC) transfection efficiency is associated with antigen-specific immune responses [20], while non-APCs have been shown to play a significant role in inducing broad and robust immune responses relative to expression in APCs alone with other physical delivery systems [21–24]. These studies suggest that cross-presentation by non-APCs to dendritic cells, rather than direct presentation by dendritic cells, is responsible for inducing the most robust humoral and cellular immune responses. LNP delivery targets immune cell types including T cells, natural killer (NK) cells, macrophages, and dendritic cells at the injection site (muscle) but also in the draining lymph nodes, spleen, and liver [25]. In contrast, Piezopen concentrates pulses (and antigen expression) to the epidermis, targeting resident APCs, keratinocytes, Langerhans cells, etc. to maximize immunogenicity without off-target biodistribution. Nonetheless, the observed differences in immune responses between Piezopen and LNPs could be attributed to the distinct cell populations and/or the total number of APCs transfected by each delivery mechanism.

While future research will further elucidate mechanism(s) by which Piezopen delivery achieves robust immune responses, we hypothesize a combination of efficient intracellular delivery, prolonged antigen expression, localized immune cell transfection, and adjuvantation via cell death [26] drive innate and adaptive immunity. This study serves as a proof-of-concept toward successful naked RNA intracellular delivery and vaccination, and our findings further uncover potential benefits of Piezopen in improving RNA vaccine: 1) durability via antigen persistence and adjuvantation, and 2) tolerability via localized administration vs systemic uptake, addressing major limitations of LNP-delivered RNA vaccines.

Several limitations should be considered when interpreting these findings. First, the head-to-head comparison between Piezopen and LNPs used different administration routes; Piezopen was administered intradermally, consistent with its skin-targeted design, while LNPs were administered intramuscularly to mirror their clinical administration. This was a deliberate choice reflecting each platform's intended use, but it introduces route-associated variables (tissue immunology, draining lymph node kinetics) that cannot be disentangled from platform-intrinsic effects. Future studies matching route would further isolate platform-specific contributions. Second, systemic reactogenicity was assessed at a single time point (24 h post-prime); earlier (4–6 h) and later (Day 22, post-booster) time points would provide a more complete temporal profile of cytokine kinetics and are planned for follow-up studies. Third, *ex vivo* human skin experiments were performed with samples from a single donor; multi-donor validation will be required to establish generalizability across skin phenotypes. Finally, while we observed robust immune responses across RNA modalities, the precise cell populations mediating antigen presentation and the contribution of electroporation-induced cell death to adjuvantation remain to be characterized in follow-up mechanistic studies.

Overall, our findings underscore Piezopen as a potent, inexpensive, and versatile delivery platform to facilitate increased acceptability and global translation of mRNA vaccines.

Materials and Methods

Animal Studies

Female 6–8-week-old Balb/c mice or ~8-week-old Wistar rats were used for the *in vivo* studies.

For immunogenicity studies, blood was collected via the lateral tail vein on Day −1 (pre-bleed, baseline) and via closed cardiac puncture under deep isoflurane anesthesia on Day 35 (terminal); spleens were harvested at terminal necropsy. The study duration was 35 days, with prime immunization on Day 0 and booster on Day 21. To alleviate suffering and minimize distress, isoflurane inhalation was used as the anesthetic throughout all procedures, including for terminal blood collection via closed cardiac puncture. The method of sacrifice was euthanasia by carbon dioxide inhalation at a chamber displacement rate of 30–70% per minute, followed by cervical dislocation or bilateral thoracotomy as a secondary confirmation method, in accordance with the AVMA Guidelines for the Euthanasia of

Animals and the Georgia State University (GSU) Institutional Animal Care and Use Committee (IACUC) Euthanasia Guideline.

All procedures were approved by the GSU IACUC (Protocol A23037). Studies were carried out at the Division of Animal Resources (DAR), GSU, Atlanta, GA.

Ex vivo human skin studies

Live human skin samples were purchased from Genoskin and maintained using the manufacturer's protocol. Genoskin utilizes donated surgical human skin biopsies and stabilizes them in a proprietary matrix to preserve tissue viability and live skin responses for 7 days. All *ex vivo* human skin experiments in this study were performed using samples from a single donor, with multiple technical replicates per condition.

Piezopen administration

Piezopen consists of a piezoelectric electroporator coupled with MN electrodes that localize electric fields to the epidermis, as described previously [4–5]. The piezoelectric energy source used in the present study is identical to the core reported in Xia et al. [4] and demonstrated to be well-tolerated in a first-in-human study [5]. For the present study, the device form factor was modified from the two-handed configuration used in prior work to a single-handed configuration to improve ergonomics and field-deployability. Two device configurations (Piezopen A and Piezopen B) were evaluated here. Briefly, Piezopen administration involves performing a 20 μL intradermal (ID) injection via the Mantoux method (insulin syringe) followed by Piezopen application of 5 or 10 pulses. Bleb formation served as visual confirmation of a successful ID injection. For naked RNA injections, payloads were prepared by adding an RNase inhibitor to Ca^{2+}-free PBS as described previously [27].

Reporter expression kinetics studies

For reporter studies, animals (mice and rats) and human skin samples were administered FLuc-mRNA (SC2325, GenScript; M1Ψ-modified) at 5 μg. For ID injections, a volume of 20 μL was used; for IM injections, 50 μL. We benchmarked to FDA-approved LNP formulations, namely SM102, ALC0315, and MC3, with LNP-formulated mRNA custom-formulated and supplied by GenScript. LNPs were administered ID for reporter studies. Mice were additionally administered 1 μg FLuc-saRNA (SC8888, GenScript; 5-methylcytidine (5meC)-modified) or 1 μg FLuc-circRNA (SC2339, GenScript; unmodified per manufacturer specification).

In Vivo imaging system (IVIS)

IVIS was performed 24 hrs after FLuc-RNA delivery. For animal studies, D-luciferin (122799, Revvity) was administered intraperitoneally at 150 mg/kg. For human skin samples, D-luciferin was administered intradermally at the injection site at a concentration of 15 mg/mL (volume matched to the original injection). Samples were incubated for 10–15 min before imaging. IVIS was performed using an IVIS® Spectrum Imaging System (Revvity). For animal reporter kinetics studies, animals were monitored for 14 days post-administration. For human skin reporter kinetics studies, human skin samples were imaged 1 day post-administration.

Vaccination studies

Spike mRNA (SC2346, GenScript; M1Ψ-modified) was delivered to mice using ID injections with or without Piezopen EP and IM injection of LNPs encapsulated with Spike mRNA (SM102, ALC0315, and MC3) (Day 0). Injections were performed on the left flank (for ID) or left quadricep (for IM) of each animal at different doses (0.5 μg, 1 μg, 3 μg, 5 μg) depending on the group. A booster dose was administered on Day 21.

Blood and tissue collection

A baseline blood collection was performed for serum processing a day prior to prime dose injections (pre-bleed, Day −1). On Day 35, a final blood collection and necropsy were performed, collecting blood for serum processing and harvesting the spleen for testing. Animal weight was monitored throughout the study.

ELISA IgG

Spike protein-specific IgG levels in Day 35 serum were measured using the Mouse Anti-SARS-CoV-2 Antibody IgG kit (RAS-T023, ACRO Biosystems) according to the manufacturer's protocol. Endpoint titers were calculated as the highest dilution emitting an optical density greater than 4x background.

Neutralizing Antibodies

Levels of neutralization antibodies in Day 35 serum were measured using the SARS-CoV-2 Surrogate Virus Neutralization Test (sVNT) Kit (L00847-A, GenScript) according to the manufacturer's protocol. Of note, not all replicates in SM102 (1 μg) and ALC0315 (1 μg) achieved endpoint titers due to limited assay availability to run additional dilutions. Endpoint titers were calculated as the highest dilution emitting an inhibition rate exceeding 30% based on the manufacturer's protocol.

ELISpot

IFN-γ ELISPOT assays were performed on Day 35 using a commercial kit Mouse IFN-γ Single-Color ELISPOT (96-well, precoated, strip, CTL) following the manufacturer's protocol. In brief, 500,000 cells (from spleens) were stimulated overnight (17−18 hrs) with 2 μg/mL Spike peptide (5823-45-01, InvivoGen). SARS-CoV-2 Spike Glycoprotein-crude (RP30020, GenScript) was used as a positive control at a concentration of 2 μg/mL.

Reactogenicity array

To profile systemic reactogenicity, a semi-quantitative membrane-based sandwich immunoassay (Mouse Cytokine Array G3 kit, AAM-CYT-G3-4, RayBiotech) was used to measure the relative levels of 62 mouse cytokines, chemokines, and growth factors simultaneously in serum collected 24 h post-prime dose. The array was run and analyzed by RayBiotech according to manufacturer's instructions.

Statistical Analysis

For animal reporter studies, a two-way ANOVA with Tukey's multiple comparisons test was performed. For human skin reporter studies and vaccination studies, ordinary one-way ANOVA with Tukey's multiple comparisons test was performed. For *in vivo* studies, each data point represents one biological replicate (i.e., one animal). ELISA IgG assays were run in technical duplicate per animal and averaged prior to plotting. For *ex vivo* human skin studies, data points represent independent technical replicates from a single donor, as indicated in the corresponding figure legend. Error bars indicate the corresponding standard error of the mean. Data were analyzed using GraphPad Prism 7 (GraphPad Software). Reported p values are multiplicity adjusted to account for multiple comparisons. For all cases, significance was defined as $p < 0.05$ (*) or $p < 0.01$ (**), or $p < 0.001$ (***).

Supporting information

S1 Data. Underlying data of Michalaki et al., PLOS ONE.
(XLSX)

Acknowledgments

We thank M.R. Prausnitz, M.S. Bhamla, P. Rohilla, and C.L. Sundell for their helpful suggestions. We thank Kensei Komatsu and Jian-Dong Li for generously allowing us to use their IVIS system.

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