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Review

Electrotransfer for nucleic acid and protein delivery

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Electrotransfer of nucleic acids and proteins has become crucial in biotechnology for gene augmentation and genome editing. This review explores the applications of electrotransfer in both *ex vivo* and *in vivo* scenarios, emphasizing biomedical uses. We provide insights into completed clinical trials and successful instances of nucleic acid and protein electrotransfer into therapeutically relevant cells such as immune cells and stem and progenitor cells. In addition, we delve into emerging areas of electrotransfer where nanotechnology and deep learning techniques overcome the limitations of traditional electroporation.

Why do we need electrotransfer?

A crucial step in vaccination, gene therapy, and genome editing is the successful delivery of exogenous cargo such as nucleic acids or proteins into the intracellular space. The method chosen for this purpose is often influenced by three main factors: the characteristics of the cargo, including size, charge, and stability; the cell type involved, whether it is a primary cell or an immortalized cell; and the specific situation in which the delivery takes place, whether it is *in vitro*, *ex vivo*, or *in vivo*.

One approach to achieve intracellular carrier-free cargo delivery is through the transient permeabilization of the cell membrane by the application of strong pulsed electric fields. When electric fields are applied, an immediate effect is the induction of a **transmembrane voltage** (see Glossary) across the cell membrane [1]. If the transmembrane voltage is sufficiently strong, the cell membrane becomes transiently permeable, allowing the entry of exogenous cargo into the cells (Figure 1A). The terms **electroporation** and electropermeabilization are often used interchangeably in literature to describe this physical delivery process. The strength of the transmembrane voltage induced during the process can lead to either irreversible or reversible permeabilization of the cell. When aiming to deliver molecules that induce functional changes in the cell such as transient gene expression or genome edits, reversible cell permeabilization is preferred. Throughout this review we use the term electrotransfer to describe the transfer of molecules across the cell membrane (extracellular to intracellular, or vice versa) by the application of electric pulses.

This review aims to highlight the prospects for engineers and biotechnologists in innovating safe, efficient, and economical (adjectives in no particular order of importance) electrotransfer methods to advance the field of protein and nucleic acid delivery. We illustrate significant applications of electrotransfer in *ex vivo* manipulation of therapeutically relevant cells such as immune cells, *in utero* implementations to create genetically modified model organisms, and clinical *in vivo* implementations in gene therapy and vaccination. We offer a comprehensive overview of the field with the aim of educating the developers of electrotransfer techniques about the therapeutically relevant cargo and cell types, and we highlight notable progress in electrotransfer techniques to excite biomedical researchers to consider adopting the new electrotransfer techniques. We also

Highlights

Electrotransfer is an effective non-viral strategy to deliver exogenous cargo such as nucleic acids and proteins into living cells in *ex vivo* and *in vivo* scenarios.

Next-generation electrotransfer strategies aim at enhancing efficiency through localized electroporation and hybrid methods involving microfluidics, mechanoporation, and sonoporation. In addition, there is a focus on creating affordable, single-use electroporation devices that will potentially expand the global reach of DNA vaccination.

Continuous advances in electrotransfer, and the integration of these next-generation electroporation techniques into *in utero* and *in vivo* applications, hold the promise of significantly improving gene-editing efficiencies in these scenarios.

Promising results from clinical trials utilizing DNA electrotransfer highlight its favorable safety profile and indicate encouraging prospects for its broader application.

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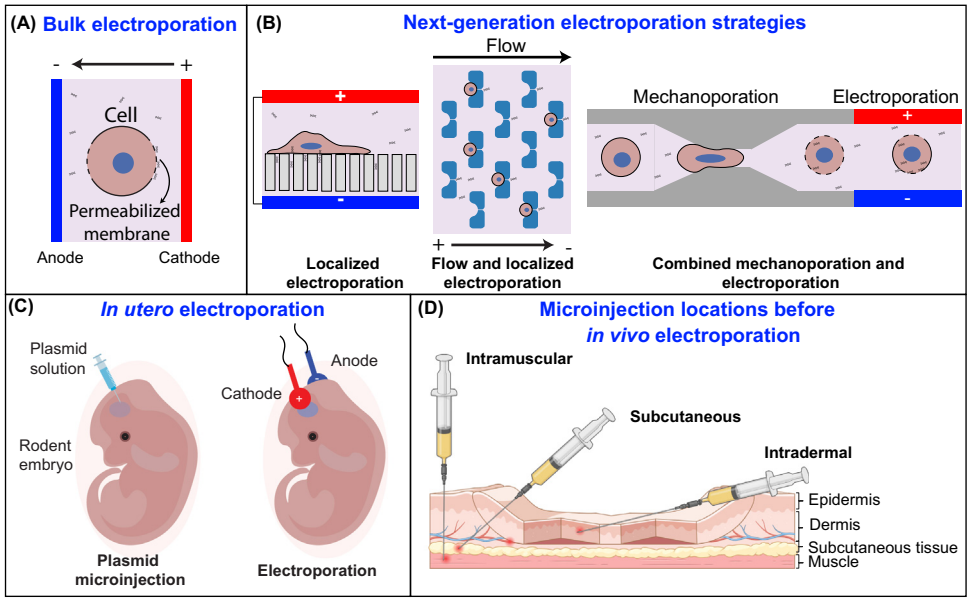

Figure 1. Electrotransfer from *ex vivo* to *in vivo* scenarios. (A) Bulk electroporation is a technique in which a cell suspension in a specialized electroporation buffer is exposed to pulsed electric fields. The electrodes are connected to a pulse generator and the cargo to be delivered is also dissolved in the electroporation buffer. The cell suspension is often loaded into cuvettes where the electrodes are spaced apart with standard dimensions. In this configuration, the electric field experienced by a cell is usually defined as the ratio of the applied voltage to the distance between the electrodes. (B) Different next-generation electroporation strategies. For localized electroporation (left), the cells must be in close contact to miniaturized structures fabricated to locally enhance the electric field. In this configuration, adhered cells can either be plated or suspension cells can be sedimented onto the miniaturized structures by centrifugal forces [35]. Another option is to flow the cells into the localized electric fields by use of microfluidics which embed nanofabricated structures capable of manipulating flow and electric fields (middle) [40]. Electroporation can also be combined with other cell-permeabilization techniques such as mechanoporation to achieve a high efficiency of functional cargo delivery while maintaining cell viability (right) [52]. (C) *In utero* electrotransfer of plasmid DNA involves microinjection of plasmid DNA into the required location followed by electroporation. This technique involves surgical exposure of the embryo to enable delivery of the plasmid DNA. (D) *In vivo* electrotransfer requires injection of the required cargo such as plasmid DNA to a delivery site. The common injection sites for DNA vaccination are shown for intradermal, subcutaneous, and intramuscular routes of administration. Parts of the figure were drawn with BioRender.

delve into the outstanding challenges and invite readers to propose strategies to address them. For the basic principles and mechanisms of electroporation or electrogene transfer, we refer the reader to prior reviews [2–4].

What is the best option for *ex vivo* genome engineering using electrotransfer: RNA, DNA, or ribonucleoprotein?

In recent years, several novel nucleases have been discovered, such as **zinc-finger nucleases (ZFNs)**, **transcription activator-like effector nucleases (TALENs)**, and RNA-guided nucleases including **CRISPR-Cas** endonucleases such as Cas9 or Cas12a/Cpf1. These tools have been effectively utilized for genome editing in various cell types [e.g., **T cells**, dendritic cells, or natural killer (NK) cells] through the delivery of appropriate cargo – RNA, DNA, or **ribonucleoprotein (RNP)** – via electrotransfer [5–8]. For example, by utilizing electrotransfer to deliver mRNA encoding ZFNs and viral vectors to deliver donor templates for **homology-directed repair (HDR)**, targeted genome editing was successfully achieved in T cells and **hematopoietic stem and progenitor cells (HSPCs)** [5,7]. A major challenge in the delivery of CRISPR-Cas components is the large size of the protein, and the DNA encoding such large proteins struggles to fit into viral vectors.

Glossary

Adoptive cell therapy: also known as cellular immunotherapy, this utilizes the immune cells of the body to combat cancer. Various approaches include tumor-infiltrating lymphocyte therapy, engineered T cell receptor therapy, CAR therapy, and natural killer (NK) cell therapy.

Base editing: the introduction of a chemical change to a specific nucleotide at the target site. For example, cytidine deaminase can convert a C–G base pair into a T–A base pair by changing cytosine (C) to uracil (U). The natural repair processes of the cell recognize the edited base as an error and correct it to the desired base pair. Base editing is performed by fusing a nucleotide deaminase to a DNA-binding protein such as Cas9 or Cas12a for single-nucleotide replacement.

Chimeric antigen receptor (CAR): a type of genetically engineered receptor that can be expressed on the surface of particular immune cells such as T cells. CARs are designed to recognize specific antigens such as those found on the surface of cancer cells or infected cells.

Cre recombinase: an enzyme derived from P1 bacteriophage that is widely used in genetic engineering and molecular biology research. Cre recombinase recognizes and acts on specific DNA sequences called *loxP* sites. When two *loxP* sites are present on the same DNA molecule in the same orientation, Cre recombinase catalyzes recombination between these sites.

CRISPR-Cas: specific DNA sequences found in the genomes of bacteria and other microorganisms. Cas (CRISPR-associated) proteins are enzymes that can cut DNA or RNA directed by gRNAs. The CRISPR-Cas system makes up the adaptive immune system of bacteria [107].

Deep convolutional network: also known as a convolutional neural network (CNN), a class of deep learning algorithms commonly applied to analyze visual imagery. CNNs are particularly effective for tasks such as image recognition and classification.

Double-stranded break (DSB): DNA damage where both strands of the DNA molecule are severed.

Duchenne muscular dystrophy (DMD): a genetic disorder characterized by progressive muscle degeneration and weakness due to the absence of a functional dystrophin protein.

Among the available technologies, CRISPR-Cas based systems have gathered significant attention recently in the field of genome engineering [9]. The minimal components that need to be delivered for CRISPR-Cas-based genome editing are a **guide RNA (gRNA)** [~100 nt single guide RNA (sgRNA) or a combination of CRISPR RNA (crRNA) and transactivating crRNA for Cas9 and ~42 nt crRNA for Cas12a], the CRISPR-associated (Cas) protein, and, in case HDR is needed, an exogenous single-stranded (ss)DNA template [10,11]. In some cases a combination of viral and electrotransfer methods has been used. The large size of the Cas proteins (Cas9 gene length, ~4 kb) is a major challenge in efficient delivery to mammalian cells [12]. A second challenge when delivering the components separately is that, to be functional, the gRNA should find and interact strongly with the Cas protein inside the cell. The third challenge is that these large RNA–protein complexes should then be successfully imported into the cell nucleus to perform genome editing. In situations where *ex vivo* genome engineering is relevant, particularly in the engineering of patient-derived immune cells where a high yield is crucial because of limited expansion capabilities, addressing these challenges becomes exceptionally critical.

All the individual components for CRISPR-Cas-based genome engineering can be delivered in mRNA, DNA, or RNP formats, but often require codelivery of multiple components. An important question is 'which delivery format of CRISPR-Cas-based genome engineering components is the most efficient for electrotransfer' (Box 1). For instance, electrotransfer of Cas9 mRNA and sgRNA to HSPCs showed poor gene editing, potentially due to the instability of unmodified sgRNA [13]. The use of partially chemically modified [such as 2′-O-methyl 3′-phosphorothioate or 2′-O-methyl 3′-thiophosphonoacetate (thioPACE) incorporation at the three terminal nucleotides at the 5′ and 3′ ends] sgRNAs, along with Cas9 mRNA or protein, or using Cas9 RNPs, effectively addressed this challenge in primary human T cells and HSPCs [13–16]. This approach has been crucial in generating knock-in primary human T cells using Cas9, and initial studies reported a genome modification efficiency of ~20% [14]. Another major goal in *ex vivo* genome engineering for immunotherapy is to maintain high cell viability of the expensive immune cells. The co-electrotransfer of T cells with Cas9 RNPs and long linear double-stranded (ds)DNA templates demonstrated reduced toxicity [17].

Similar strategies were also used in genome engineering of mammalian cells without introducing **double-stranded breaks (DSBs)**. Electrotransfer of plasmid DNA encoding a **base editing enzyme** and Cas9 gRNA to embryonic kidney cells (HEK293T) and mouse embryonic fibroblasts (MEFs) achieved ~20% base editing efficiency [18]. However, by delivering the base editor in mRNA format together with sgRNA to human T cells, a base editing efficiency of up to ~98% could be achieved [19]. **Prime editing** was successfully accomplished in HEK293T cells by codelivering mRNA encoding a prime editor together with gRNAs for nicking and prime editing via electrotransfer [20]. In primary fibroblast cells, efficient base editing and the generation of **human induced pluripotent stem cell (hiPSC)** monoclonal colonies were achieved by electrotransferring adenine base editor (ABE) RNA and its corresponding sgRNA [21].

RNPs are currently the most common format for the delivery of base editors [22,23]. Two cycles of electrotransfer separated by 24 h of cytosine base editor RNPs into HSPCs successfully mutated (with efficiencies of up to 80%) a critical transcription factor binding site within the repressor of fetal hemoglobin, resulting in the activation of fetal hemoglobin [23]. Electrotransfer of base editor RNPs demonstrated significantly higher effectiveness in comparison to codelivery of chemically modified sgRNAs with base editor mRNA to T cells [24].

Electroporation: increased cell membrane permeability when exposed to electric fields. The widely accepted theory is that electric fields form transient transmembrane pores through which hydrophilic molecules can pass.
Exosome: small, membrane-bound vesicles that are released by cells into the extracellular environment and are involved in cell-to-cell communication. Exosomes are being explored as potential drug delivery vehicles and therapeutic agents for various diseases including cancer.
Good manufacturing practice (GMP): a set of guidelines and regulations that ensure the quality, safety, and efficacy of pharmaceuticals, medical devices, and food products.
Guide RNA (gRNA): a synthetic RNA molecule that is designed to be complementary to a specific DNA sequence in the target gene. In the context of CRISPR-Cas systems, gRNA acts as a guide, leading the Cas protein to the exact location in the genome that needs to be modified.
Hematopoietic stem and progenitor cells (HSPCs): multipotent stem cells found in the bone marrow and umbilical cord blood. They can differentiate into various types of blood cells including red blood cells (erythrocytes), white blood cells (leukocytes), and platelets (thrombocytes).
Homology-directed repair (HDR): a mechanism to repair DSBs in DNA. Unlike NHEJ, which directly ligates broken ends together, HDR utilizes a homologous DNA template to repair the break.
Human induced pluripotent stem cells (hiPSCs): a type of stem cell that is created by reprogramming adult cells, usually skin or blood cells, to return to a pluripotent state. Pluripotent stem cells have the ability to differentiate into any cell type in the body.
Interleukin-12 (IL-12): a cytokine signaling molecule that plays a crucial role in the immune system. It is produced by particular immune cells, including dendritic cells, macrophages, and B cells, in response to infection or stimulation by antigens.
Laparotomy: a surgical procedure involving a large incision made through the abdominal wall to access the abdominal cavity.
***loxP site*:** the locus of crossing over in bacteriophage P1, a specific DNA sequence that is widely used in genetics and molecular biology research. It is a

An alternative approach is to infect primary HSPCs with lentiviruses carrying sgRNAs and subsequently electroporate the appropriate base editor protein after 2 days [25]. In this case, the efficiency of base editing was dependent on the protein concentration and an editing efficiency up to 65% was achieved. It was observed that this approach exhibited higher editing efficiencies compared to the delivery of RNPs. The variations in observations among various studies regarding the optimal method for electrotransferring proteins underscore the significance of assessing the effectiveness of protein electrotransfer into cells. This evaluation should consider factors including protein concentration, delivery format (RNA, DNA, or RNP), and consistent electrotransfer conditions for diverse cell types such as T cells, HSPCs, and hiPSCs.

Box 1. What are the rate-limiting steps in the delivery of different macromolecular cargoes?

RNA

Therapeutic applications of RNA encompass a wide range of possibilities, including the use of small interfering RNAs (siRNAs) to decrease gene expression, transient expression of non-native proteins such as nucleases for DNA or RNA editing, antigens to confer pathogen immunity, and the translation of delivered mRNA to replace dysfunctional proteins [98]. Owing to their substantial size, anionic charge, and vulnerability to RNases, the efficient cellular uptake and autonomous functionality of RNA pose challenges without assistance from a carrier or cell permeabilization. Given their anionic nature, RNA molecules are highly suitable for delivery via electrotransfer techniques. Currently, electroporation is commonly used as a non-viral delivery strategy for mRNA in *ex vivo* applications.

Smaller RNA molecules such as siRNA (~20 nt) freely enter the cell (*in vitro*) when electrotransferred, and electrophoresis is the main driving force for molecular transport (Figure I) [81]. For larger mRNA molecules (~2000–4000 nt for COVID-19 mRNA vaccine and Cas9) encoding functional proteins, the mechanism of transport and rate-limiting steps during electrotransfer are not yet elucidated. Based on mechanistic insights from DNA electrotransfer, it can be hypothesized that mRNA molecules above a particular size threshold (~25 nt) form diffusion-limited mRNA–cell membrane complexes which are later internalized through endocytosis [80].

DNA

Compared to RNA, DNA molecules offer advantages in terms of stability, making them more suitable for storage and transportation. DNA delivery is essential for achieving stable and heritable gene integration leading to permanent genetic modifications. DNA electrotransfer has already been used in several clinical trials in human patients for vaccination and cancer immunotherapy.

DNA molecules need to overcome the barriers of the cytoplasm and nucleus, and must undergo transcription and translation, which can result in slower efficacy as a therapeutic cargo. Like RNA, DNA molecules are anionic and are highly suitable for electrotransfer techniques. During electrotransfer, DNA molecules larger than 25 bp form DNA–cell membrane complexes with limited diffusive mobility (*in vitro*) before internalization by endocytosis [80,83]. Similarly to mRNA molecules, the substantial size of DNA molecule poses delivery limitations without the assistance of carries or cell permeabilization. This process involves the cytoskeleton as a regulator of the electropermeabilization and intracellular mobility of the electrotransferred molecules [82,99].

Proteins

Protein delivery offers distinct advantages compared to RNA or DNA delivery methods. Unlike RNA or DNA, proteins do not require transcription and translation, and can directly provide the desired functionality. In addition, protein delivery allows precise control over the dosage within cells and eliminates the risk of genomic integration. However, delivering proteins can be challenging owing to their complex 3D structures, diverse electrostatic and surface properties, and varying sizes (~1000–1600 amino acids for Cas9). For systems such as CRISPR-Cas9 or Cas12a, proteins pre-assembled with gRNAs as RNPs are often delivered. Despite these challenges, several therapeutically relevant proteins can be delivered with electrotransfer, including fluorescent proteins, enzymes, cytokines, hormones, and antibodies.

The functionalization of protein cores with a dense shell of radially oriented nucleic acids results in the formation of protein spherical nucleic acids [100]. These structures possess a high charge density, which can significantly amplify the electrophoretic drive and thereby enhance the efficiency of electrotransfer [44].

The mechanistic details of protein electrotransfer are still missing. Owing to their significant size, protein molecules could have limited diffusive mobility inside the cell as a result of the cytoskeleton, and could potentially rely on endocytosis for intracellular transport to their target site following electrotransfer.

recognition site for Cre recombinase which is derived from P1.

Mass spectrometry (MS): an analytical technique to measure the mass-to-charge ratio (*m/z*) of ions and identify and quantify complex mixtures of compounds. It is widely used in proteomics, metabolomics, and drug discovery.

Non-homologous end-joining (NHEJ): a mechanism used by cells to repair DSBs in DNA. In NHEJ, the broken DNA ends are directly ligated back together without the need for a homologous template, making it a rapid but error-prone repair process. NHEJ can result in small insertions or deletions (indels) at the repair site, which can lead to gene mutations.

Prime editing: offers precise modifications that allow all 12 potential base-to-base conversions, as well as insertions and deletions. Notably, it achieves these changes without the need for DSBs or donor DNA. Prime editing is performed by fusing a reverse transcriptase to a DNA-binding protein such as Cas9 for genome editing without inducing a DSB.

Ribonucleoprotein (RNP): a complex formed by RNA molecules and proteins.

T cells: a category of white blood cells identified by the presence of a T cell receptor on their cell surface. Their primary role involves immune-mediated cell death executed by $CD8^+$ cytotoxic and $CD4^+$ helper T cells. The T cell receptor can be engineered to target a specific population of cells and is the basis of many cell therapies.

Transcription activator-like effector nucleases (TALENs): sequence-specific restriction enzymes designed to target specific DNA sequences. They are constructed by combining a TAL effector DNA-binding domain with a DNA-cleavage domain, which is a nuclease responsible for cutting DNA strands.

Transmembrane voltage: the potential difference across the membrane between the intracellular and extracellular space of a cell. In eukaryotic cells, the typical resting transmembrane voltage ranges between –40 mV and –70 mV. Upon exposure to external fields, an increase in transmembrane voltage known as induced transmembrane voltage is observed.

Zinc-finger nucleases (ZFNs): sequence-specific restriction enzymes formed by combining a zinc-finger DNA-binding domain with a DNA-cleavage domain. These ZFNs usually consist of

3–6 zinc-finger repeats in their DNA-binding domains, allowing them to recognize sequences spanning 9–18 bp.

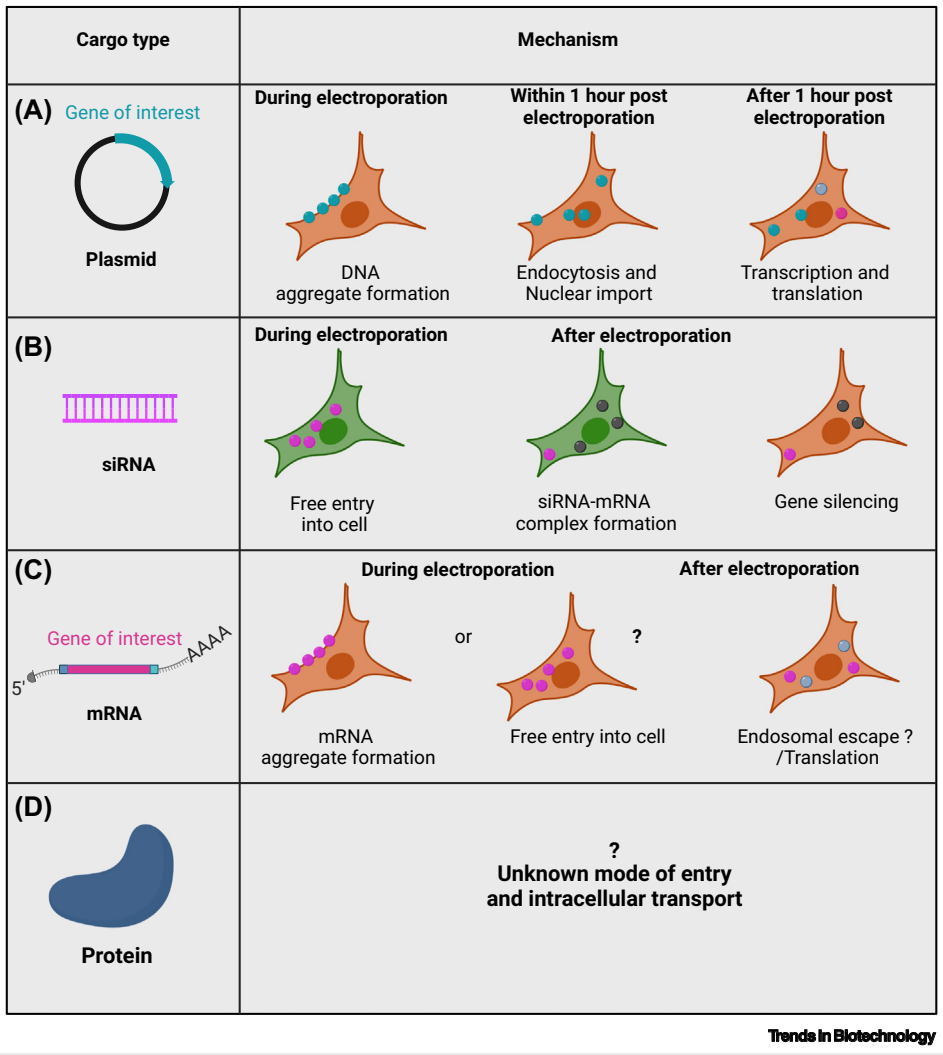

Figure I. Rate-limiting steps for different macromolecular cargoes delivered by electrotransfer techniques. (A) Exogenous plasmid DNA forms DNA–cell membrane complexes during electroporation which are subsequently internalized by endocytosis [80,83]. These DNA molecules must then be released from the endosomes and be imported into the nucleus for transcription and translation to form functional proteins. (B) siRNAs are able to freely enter the cell during electrotransfer and form siRNA–mRNA complexes to initiate gene silencing [81]. (C) The exact mechanisms of mRNA electrotransfer are not yet resolved. Based on studies on plasmid DNA and siRNA delivery, it can be hypothesized that that mRNA delivery could either involve mRNA–cell membrane complex formation or free entry to the cell. If the mRNA forms mRNA–cell membrane complexes, the possible route of internalization is endocytosis, which would require endosomal escape of the mRNA molecules before translation. If the mRNA freely enters the cell during electrotransfer, it is expected that these mRNA molecules can be directly translated into proteins in the cytoplasm. It is highly likely that this process is mRNA size-dependent. (D) There is no current theoretical model for protein entry into the cells upon electrotransfer. It is expected that the exact mode of protein entry would depend on the properties of the protein, such as charge, size, and solubility. Figure drawn with BioRender.

'Does the delivery format have an impact on genetic off-target effects?' Conflicting reports exist regarding the use of base editor RNPs, and higher rates of nontarget editing and indel formation were reported in T cells compared to the base editor expressed from transfected mRNA, while

the opposite trend was observed in HEK293T cells [24,26]. This discrepancy was hypothesized (but not tested) to be related to the intracellular half-life of the purified protein versus its expression from plasmid DNA. RNPs with covalently fused crRNA and Cas12a mutant protein showed a genome editing efficiency of ~67% compared to ~20% in the wild-type system in HEK293 cells, and showed increased on-target genome editing efficiency [27]. It cannot hence be excluded that the delivery format of the electrotransferred molecule (and not only the type of protein) influences genetic off-target effects.

The mechanistic understanding of electrotransfer for most cargoes used in genome editing is still limited, hindering the rational optimization of delivery strategies. It remains an open question whether the kinetics of guide RNA assembly with the expressed protein inside the cell, when the guide RNA and the protein/expression vector are electrotransferred separately, is a rate-limiting step.

Ex vivo electrotransfer in clinical settings

Owing to its carrier-free delivery nature, cells transformed by electrotransfer are often considered to be less immunogenic in patients compared to those transformed by viral vectors. Hence, there is substantial clinical interest in using *ex vivo* electrotransfer for engineering cells such as immune cells for treating diseases such as cancer (Table 1). For example, monotherapy utilizing autologous monocyte-derived dendritic cells transfected via electrotransfer of mRNA encoding CD40L, CD70, constitutively activated (ca)TLR4, and a melanoma-associated antigen has been used in patients with advanced melanoma, and resulted in a ~50% rate of disease control [28]. A case study on the use of cells engineered by electrotransfer approaches to treat hematological malignancies is presented in Box 2.

A paramount goal within the cell therapy industry is now to develop automated electrotransfer processes to generate engineered cells while adhering to clinically required **good manufacturing practice (GMP)** standards and ensuring scalability. Different versions of automated and closed platforms for T cell and HSPC engineering demonstrate GMP-compliant electroporation processes [29–31]. These systems facilitate the delivery of diverse cargoes, including TALENs, CRISPR-Cas, ZFNs, and transposons, in mRNA or RNP format. Viral transduction is commonly used for stable genomic insertion. The entire process takes place within a closed tubing system to preserve cell integrity and minimize contamination risks. It integrates into the overall T cell, NK cell, and HSPC engineering workflow, which includes cell separation and activation. By optimizing buffer conditions at each step, near-perfect cellular composition and viability have been achieved, with a throughput of ~1 billion cells per day while the required throughput is 1 billion cells per patient [32]. Optimizing the process with approaches to shorten the time and cost of the manufacturing process should be critically considered, especially in the conditions that require autologous cell therapy.

Next-generation *ex vivo* electrotransfer platforms

For reversible electroporation, the specific conditions such as applied voltage, pulse duration, number of pulses, and buffer composition are often optimized to strike a balance between cell viability, the efficiency of molecule transfer, and preservation of the functionality of the molecule. However, the traditional approach of applying a uniform electric field across a population of cells has drawbacks including reduced cell viability, suboptimal electrotransfer efficiency, and limitations in achieving desired outcomes such as effective gene expression or preserved protein functionality. Furthermore, traditional electrotransfer methods cause significant gene expression and metabolic changes, and abnormal cytokine secretion (IL-2, IFN-γ) in therapeutically relevant primary T cells and HSPCs, which negatively impact the long-term function of these cells [16,33]. Consequently, the electrotransfer field has witnessed the emergence of next-generation electrotransfer techniques aimed at overcoming these limitations (Figure 1B).

Table 1. *Ex vivo* electrotransfer methods for genomically manipulating HSPCs, hiPSCs, and T cells

Aim	Cell type	Cargo	Electroporation conditions	Biological outcome	Refs
Correction of *IL2RG* gene in HSPCs from a subject with severe combined immunodeficiency	Human cord blood- or bone marrow-derived $CD34^+$ cells	mRNA encoding a ZFN. The donor DNA template was delivered using an integrase-defective lentiviral vector	Electroporation used a Lonza P3 primary cell 4D nucleofector X Kit, program E0-100; 175 μg/ml ZFNs encoding mRNAs was used. One day after electroporation, cells were seeded in a methylcellulose-based medium	Edited HSPCs maintained normal hematopoiesis and generated functional lymphoid cells. The efficiency of gene editing in primitive cells could be enhanced twofold (reaching a maximum of ~18% HDR efficiency) through the addition of aryl hydrocarbon receptor and/or 16,16-dimethyl-prostaglandin E2. These additions promoted differentiation by extending the duration of cell culture	[5]
Massively parallel base editing of HSPCs	Primary HSPCs	Adenine base editor protein and pooled sgRNA	Primary HSPCs were infected with lentiviruses expressing sgRNA. Two days later the cells were electroporated. Base editor protein was reconstituted in Lonza electroporation buffer P3 such that final amount of protein per electroporation was in the range of 20–40 μg. Cells were electroporated using 4D Nucleofector X unit with pulse code DZ-100	The efficiency of editing was dependent on the concentration of electrotransferred protein. A maximum of 65% base-editing efficiency was achieved	[25]
Therapeutic base editing of human HSPC	Human peripheral blood mobilized $CD34^+$ HSPC	Cytosine base editor RNP complexed with five chemically modified sgRNAs	Electroporation used a Lonza 4D Nucleofector; 800 pmol of base editor protein was mixed with 800 pmol of sgRNA to produce the RNP	The level of base editing was found to be dependent on the dose, and 50 μM RNP resulted in ~80% base edits. In healthy donor HSPCs, one cycle of electroporation achieved a base editing efficiency of ~70%, which increased to ~90% with two cycles of electroporation	[87]
Simultaneous base editing and reprogramming to generate gene-edited hiPSCs	Patient-derived primary fibroblasts and hiPSCs	Reprogramming plasmids, single-stranded adenine base editor RNA, sgRNA	Fibroblasts were electroporated with three reprogramming plasmids (1.5 μg each in 100 μl buffer). The electroporation settings were three pulses, pulse width 10 ms, and 1650 V. To this electroporation solution, 23 μg of base editor RNA and 10 μg of sgRNA were added and the same conditions were used for electroporation. Electroporation used a Neon transfection system	This approach enables both base editing and the generation of hiPSCs from a patient biopsy in a single monoclonal expansion step, greatly reducing the overall process time. It achieved an editing efficiency of 96%	[21]
Deletion of genes encoding endogenous T cell receptor chains	Patient-derived T cells	CRISPR-Cas9 RNP loaded with three sgRNAs	RNP complexes containing wild-type Cas9 and *in vitro* transcribed gRNA sequences for *TRAC*, *TRBC*, and *PDCD1* were added to the cells and the cell mixture was electroporated using a Maxcyte electroporation system. Cells were then transduced with a lentiviral vector to express a T cell receptor specific for cancer antigens. Electroporation conditions were not specified	The endogenous T cell receptors and the immune checkpoint molecule programmed cell death protein 1 (PD-1) were knocked out in T cells derived from a patient. The results showed that ~30% of the cells had no mutations, while ~40% of the cells had a single mutation. In addition, ~20% of the cells had double mutations, and ~10% had triple mutations. A synthetic, cancer-specific T cell receptor transgene was introduced to enable the recognition of tumor	[15]

Table 1. (continued)

Aim	Cell type	Cargo	Electroporation conditions	Biological outcome	Refs
				cells. These engineered T cells could be successfully transplanted into patients without causing any clinical toxicities	
Site-specific genome editing in T cells	$CD8^+$ and $CD4^+$ T cells	mRNA which encodes a ZFN. Donor template was delivered using an adeno-associated virus serotype 6 (AAV6).	T cells were transduced with AAV6 donor vectors. After this, electroporation of the T cells was performed after resuspending them in BTXpress high--performance electroporation solution; 40–60 μg/ml of *in vitro* transcribed ZFN mRNA were electroporated in a BTX ECM830 square wave electroporator in a 2 mm cuvette using a single pulse of 250V for 5 ms	More than 45% of the $CD8^+$ T cells and 40% of $CD4^+$ T cells had a site-specific transgene addition	[7]
Site-specific genome insertion of large DNA sequences in human T cells	Primary human T cells from healthy human donors	Cas9 RNP complexed with crRNA and transactivating crRNA (tracrRNA) and HDR templates	RNPs and HDR templates were electroporated 2 days after initial T cell stimulation and resuspension in Lonza electroporation buffer P3. A Lonza 4D 96-well electroporation system with pulse code EH115 was used. 50 pmol of RNPs along with 4 μg HDR template was added	The editing efficiency ranged from ~30% to 40% for both $CD4^+$ and $CD8^+$ T cells. Cell viability varied between ~40% and 100%. Notably, a pathogenic *IL2RA* mutation found in cells from patients with monogenic autoimmune disease was successfully corrected. Furthermore, the endogenous T cell receptor locus was replaced by a new receptor that targets a cancer antigen	[17]
Inactivation of endogenous T cell receptors with base editing to generate universal off-the-shelf **chimeric antigen receptor (CAR)** T cells	Healthy volunteer T cells	mRNA encoding base editor and sgRNA targeting *TRBC*, *CD7*, and *CD52*	Healthy donor T cells were transduced with the use of lentivirus to express a CAR with specificity for CD7 (CAR7). Base editing was performed to inactivate three genes encoding CD52 and CD7 receptors and the β chain of the αβ T cell receptor by delivering base editor mRNA using a Lonza 4D nucleofector	Following base editing and a single round of exposure to a lentiviral vector, ~60% of cells expressed CAR7. Moreover, 60–100% of cells exhibited editing at the specific cytosine positions, without any observed off-target effects. These edited cells were successfully transplanted into patients with relapsed leukemia and demonstrated significant antileukemic effects	[88]

Localized electrotransfer has emerged as one such promising approach for improving electrotransfer efficiency while preserving cell viability. Through the manipulation of electric fields via miniaturized structures, the exposure of cells to voltages capable of electroporation is confined, ensuring that only a small portion of the cell is precisely electroporated [34]. This localization of electric fields provides enhanced control over biomolecule delivery compared to conventional bulk electrotransfer methods.

Nanochannel electrotransfer is a localized electrotransfer technique that involves positioning the cargo and the target cell in separate microfluidic chambers connected by a nanochannel [34]. This concept has been further developed to enhance throughput by utilizing porous substrates, nanostraws or nanoprobes, or microtraps to deliver mRNA, plasmid DNA, RNPs and functional

Box 2. Case study: *ex vivo* electrotransferred cells as therapeutics for hematological malignancies

An emerging modality of therapy for particular hematological malignancies is adoptive transfer of genetically engineered T or natural killer (NK) cells. One key consideration in optimizing widespread adoption of such cell therapy is the development of cheap, safe, and efficient manufacturing process [101]. Electrotransfer of plasmid DNA containing Sleeping Beauty transposon/transposase and a chimeric antigen receptor (CAR) construct demonstrated successful stable expression of a CD19-specific CAR (84% CAR expression) in activated T cells, providing clinically adequate yield for the majority of patients with non-Hodgkin lymphoma and acute lymphoblastic leukemia [102]. The $CD3^+$ CAR-T cells engineered using Sleeping Beauty transposase and prepared through electrotransfer of plasmid DNA to donor peripheral blood (43% CAR expression) demonstrated antileukemic activities in 13 patients, and no severe toxicities were observed for up to 10 months after infusion [103]. CAR-T cells generated using CRISPR-Cas9 electrotransfer technology are currently being deployed in the clinic. To evade lymphodepleting serotherapy and graft-versus-host disease, clinical grade universal off-the-shelf anti-CD7 CAR-T cells were subjected to base editing by using electrotransfer of mRNA encoding the base editor and sgRNA to inactivate specific genes, and were tested in three children with relapsed leukemia [88]. Single-cell analysis demonstrated that electrotransfer of CRISPR-Cas9 RNPs and linear dsDNA HDR template to T cells from healthy donors led to a significant proportion of memory T cells in the infusion products and solved the disadvantages of virus usage and random integration [104]. These non-viral anti-CD19 CAR-T cells were programmed to perform PD1 interference led to 87.5% complete remission without serious adverse events in eight patients with relapsed/refractory B cell non-Hodgkin lymphoma. These studies show that electrotransfer represents a promising and safe approach for engineering immune cells to target hematological malignancies.

proteins to model cell lines and mRNA to HSPCs (>10^5 cells per run) [35–40]. In this approach, cells are plated onto a porous substrate (or nanostraws) or are directed to microtraps, creating an electrically insulating barrier between the cells and the target molecule. However, these techniques require additional steps, such as centrifugation or flow, to attach the cells (especially for suspension cell lines such as T cells) to the regions of high electric field created at the conductive zones.

Automated versions of nanofountain probe electrotransfer systems, using a **deep convolutional network** to identify cell locations and a cell nanopipette contact algorithm for precise positioning of the nanopipette over each cell during electrotransfer, have resulted in significantly higher transfection efficiency and improved cell viability, particularly for difficult-to-transfect cells such as hiPSCs [41,42]. However, these systems are still in their early stages of development, exhibit throughputs of ~12–15 cells per minute, and have a capacity to handle ~70 cells in a single run.

Scaling up localized electrotransfer is a crucial consideration in the field. At the time of writing this review, there is currently no continuous localized electrotransfer system, limiting the process to batch mode of operation. Hence it is natural to wonder – 'what knowledge and potential applications have been provided by localized electrotransfer devices?'

Accumulating evidence supports the notion that localized electrotransfer provides superior cell viability and greater control over electrotransfer efficiency. Localized electrotransfer of stem cells does not activate cell stress-response pathways, unlike bulk electrotransfer, thus minimizing long-term cellular physiological impact [43]. Localized electrotransfer has facilitated the efficient intracellular delivery of proteins of various molecular weights such as Cas9 RNP (160 kDa), β-galactosidase (472 kDa), and protein spherical nucleic acids (ProSNAs, 668 kDa) [44]. Importantly, these delivered proteins have demonstrated functional integrity post-delivery. Apart from facilitating molecule delivery, localized electrotransfer enables non-destructive sampling of cytosolic and nuclear contents from living cells which can be combined with analytical techniques such as **mass spectrometry (MS)** for studying cellular responses [45–49]. Nanochannel-based localized electrotransfer has been used to transfect diverse source cells with plasmid DNA, resulting in the

generation of **exosomes** that carry transcribed mRNAs and targeting peptides [50]. These exosomes were utilized both as carriers and for diagnostic purposes. As an example, a nanochannel electrotransfer device was coupled to catalytic hairpin DNA circuits and reporters in lipid polymer hybrid nanoparticles, tethered to indium-tin-oxide (ITO) and gold-coated chips [51]. This enabled *in situ* RNA expression analysis upon fusion with exosomes.

Localized electrotransfer has proved to be successful in achieving desired levels of efficiency, precise dosage control, applicability across different cell and cargo types, and minimal cell perturbation, but upscaling for relevant *ex vivo* applications such as **adoptive cell therapy** remains a key challenge [32]. Further advances will be necessary to optimize scalability, ensure compliance with regulatory standards, and unlock the full potential of localized electrotransfer for large-scale *ex vivo* applications such as T cell therapy. Despite the existing challenges, localized electrotransfer holds great potential for applications that necessitate non-destructive sampling, such as transcriptomics and proteomics in fundamental biological research and drug discovery.

To improve throughput and enable scalability in electrotransfer, flow electrotransfer systems have been developed. These systems are often designed to operate within microfluidic chips and have been further enhanced by incorporating additional mechanical perturbations. A notable example involved the combination of hydrodynamic squeezing with electrotransfer, which facilitated the efficient nuclear delivery of plasmid DNA within a 1 hour treatment timeframe [52]. However, systems that utilize cell squeezing are inherently susceptible to potential issues of clogging. Microfluidic devices using hydrodynamic stretching alone have demonstrated significant delivery efficiency for various macromolecules, such as plasmid DNA, mRNA, siRNA, and large nanoparticles, across diverse cell types, including challenging primary stem and immune cells [53]. These devices operate at a high scale and can process ~10^6 cells/minute while maintaining excellent cell viability [54]. Unlike squeezing, microfluidic devices that utilize hydrodynamic stretching do not encounter problems associated with clogging. In a similar vein to squeezing, the synergistic combination of hydrodynamic stretching with electrotransfer has the potential to further advance the delivery efficiency of a wide range of macromolecules, pushing the boundaries of what can be achieved in terms of efficient and effective delivery.

'What stops us from reaching the desired throughput for transforming cells using precision electrotransfer methods?' A typical microfluidic flowthrough electrotransfer device has a processing capacity of ~1 ml/minute. Assuming a cell concentration of 10^6 cells/ml in the feed to the microfluidic chip, it allows the processing of 10^6 cells/minute. However, a major challenge lies in scaling up the process to exceed a 10^8 cells/patient, which is crucial for clinical applications of cell therapy. One possible solution is to increase the number of microfluidic devices to operate in parallel to achieve the desired scale, while also developing methods to ensure uniform voltage distribution across a large number of channels. In addition, exploring ways to process higher cell concentrations or flow rates without compromising efficiency and effectiveness is another approach to address this challenge [55,56]. In the absence of mechanical perturbation or electric field amplification, a minimum electric field strength of ~400 V/cm (detailed threshold electric field conditions for electropermeabilization are given in [57]) is required for successful transfection using plasmid DNA. Scaling up the process by increasing volumetric flow rates poses a challenge in generating such electric fields within larger channels that may be necessary for higher flow rates. Finding ways to address these challenges and the development of scalable approaches for continuous flow electrotransfer is crucial for advancing the field.

In utero and *ex utero* electrotransfer of genome engineering components for creating model organisms

Biomedical research involves the use of genetically engineered model organisms to study human disease or developmental cell biology. Producing these organisms can often take >5 months, hence safe and efficient genome engineering methods are needed and electrotransfer is often used for this. For instance, *in utero* electrotransfer of microinjected plasmids to mouse embryos after **laparotomy** has been used to produce neonatal mice with manipulated gene expression in the brain (Figure 1C). This advance has facilitated investigations into various aspects of neuronal biology including neuronal migration, axonal transport, synapse development, and interference with protein function [58,59]. During this procedure, laparotomy is performed to access the embryos, after which plasmids are microinjected into them. Subsequently, electrotransfer is applied, followed by stitching. Typically, the anode is positioned on the side where the plasmid DNA is injected, while the cathode is placed on the opposite side of the head of the embryo. For certain brain areas such as hippocampus, visual cortex, motor cortex, prefrontal cortex, and cerebellum, a three-electrode configuration rather than the conventional two-electrode configuration gives a higher transfection efficiency [59]. However, this comes at a cost of needing additional highly skilled personnel in some situations to perform the protocol. The success of *in utero* electrotransfer depends on the expertise of the personnel involved, with a reported success rate of >80% in producing live births. Most of the births (50–90%) express the electrotransferred vector. This method has also been used to introduce plasmids encoding the CRISPR-Cas9 system for generating DSBs and achieving HDR-mediated gene knock-ins in neural progenitors within developing ferret and mouse brains [60].

A notable drawback of *in utero* electrotransfer lies in the labor-intensive nature of individually injecting components into the embryos. To address this challenge, *ex utero* electrotransfer of CRISPR-Cas9 components in mRNA format directly into mouse zygotes has emerged as a solution. This approach has facilitated the generation of mice with targeted genetic modifications, with a live birth rate of ~60% for 20–50 embryos in a single run [61]. However, it is important to note that the efficiency of targeting can vary significantly, and **non-homologous end-joining (NHEJ)** mutation rates ranged from ~30% to 100% in a single study. A case study presented in Box 3 shows how electrotransfer is used to insert ***loxP* sites** into model organisms.

The next-generation of *ex vivo* electrotransfer technologies described in the previous section presents a compelling case for widespread adoption for highly efficient genome engineering of model organisms, with the potential of significantly accelerating progress in biomedical research. The scale of localized *ex vivo* electrotransfer currently matches the required scales of animal mutagenesis laboratories (hundreds of embryos per run) with efficiencies far exceeding those of

Box 3. Case study: electrotransfer for integrating *loxP* sites

In some scenarios the creation of model organisms with multiple genome edits is required. For instance, the development of animal models with two *loxP* sites flanking a specific exon or critical DNA sequence of interest is a valuable approach for studying conditional gene regulation because precise deletion or inversion of a gene segment can be achieved by the use of **Cre recombinase**. Generating these floxed (*loxP*-flanked) alleles is a complex and labor-intensive process because it necessitates the precise integration of two *loxP* sites simultaneously within the same chromosomal region. Electrotransfer of single target embryos with two gRNA/Cas9 RNP and two ssDNA templates (each gRNA/ssDNA set delivering one *loxP* site) has been used to flox ~70 target genes where the distance between individual *loxP* insertion sites can range from ~450 bp to 160 kb [105]. Electrotransfer of gene-editing cocktails containing Cas9 protein, gRNA, and ssDNA into mouse zygotes through oviduct electrotransfer allows multiple mouse zygotes to be handled together and transferred to pseudo-pregnant mice (~10% in the F0 generation and 37% in the F1 generation) [106].

traditional electrotransfer techniques. Moreover, it is currently unclear whether the classical bulk electroporation strategy used for *in utero* electrotransfer into zygotes and embryos is associated with long-term gene expression changes such as those observed following *ex vivo* electrotransfer into stem cells.

In vivo electrotransfer for DNA vaccination and therapeutics

Compared to *ex vivo* electrotransfer, *in vivo* electrotransfer poses additional barriers to cellular delivery of macromolecular cargo. A key question that arises in *in vivo* electrotransfer is – 'where should the cargo be delivered for optimal therapeutic effects and minimal impact on patient convenience?' (Figure 1D). In the context of DNA vaccination, the cargo, often a solution containing plasmid DNA, is typically microinjected either intramuscularly (IM) or intradermally (ID), followed by electrotransfer at the injection site to enhance the immune response.

ID administration is considered to be a less invasive procedure than the IM route because it requires shallower penetration and uses smaller-gauge needles. It delivers ~50–100 µl of the cargo to target tissues that are rich in resident immune cells. The predominant transfected cells for ID DNA electrotransfer are in the dermis and epidermis, but some adipocytes within the hypodermis are also transfected. Conversely, IM administration is capable of delivering larger volumes of DNA, typically ~1 ml, and can lead to long-term gene expression. Subcutaneous fat has also been identified as a suitable target site for the administration of DNA vaccines where adipocytes within subcutaneous fat tissue are targeted [62]. Both ID routes and IM routes have been used in clinical trials (Table 2) for DNA vaccinations against infectious diseases in human subjects. The ID route was better tolerated in patients for DNA vaccinations against Ebola virus [63]. In clinical trials for DNA vaccination against HIV, ID administration induced similar immune responses compared to IM route while being dose sparing [64].

For electrotransfer of plasmid DNA or RNA to treat solid tumors, the most common route is to administer the cargo intratumorally. This procedure entails surgical access to the tumors, followed by the injection of DNA/RNA cargoes and subsequent electrotransfer. However, it should be noted that this approach is often highly invasive, depending on the tumor site and its microenvironment. A therapeutically relevant payload for *in vivo* electrotransfer is plasmid DNA encoding proinflammatory cytokines such as **interleukin 12 (IL-12)**. Clinical trials are currently underway to explore its potential as a gene therapy for treating melanoma in humans [65–68]. In tumors subjected to intratumoral electrotransfer-mediated IL-12 gene therapy, there was rapid initiation of IL-12-controlled pathways and upregulation of the antigen-presentation machinery. This led to heightened infiltration of lymphocytes and induced gene expression alterations suggestive of immune responses, even in remote tumors [69]. Intratumoral electrotransfer of self-amplifying RNA which generates high and transient levels of IL-12 expression has demonstrated close to 90% survival rates and tumor volume control in mouse models of hepatocellular carcinoma and colon cancer [70]. To further enhance the antitumor effects in mouse models by enhancing the diffusivity of the cargo, the degradation of hyaluronan, a key component of the extracellular matrix, has been explored. For example, this can be achieved by injecting hyaluronidase [70]. The combination of IL-12 plasmid electrotransfer with intratumoral anti-CD3 plasmids, which stimulate T cell responses, amplified the production of cytokine, enhanced T cell cytotoxicity and proliferation, and mitigated the suppressive influence of the tumor microenvironment. These cumulative antitumor effects led to improved regression of treated tumors and the development of systemic immunity, resulting in the control of untreated contralateral tumors in mice and restored the function of tumor-infiltrating lymphocytes from a melanoma patient [71]. Overall, *in vivo* electrotransfer of nucleic acids has shown favorable safety profiles for vaccination and gene therapy with limited reports of adverse events.

Table 2. Completed clinical trials using electrotransfer of nucleic acids for therapeutic applications

Medical condition	Cargo	Clinical trial ID	Trial stage	Electroporation protocol	Trial outcome	Refs
Stage III/IV melanoma	Plasmid DNA encoding IL-12	NCT 01502293	Phase 2, 30 participants	Intratumoral injection of plasmid DNA at 0.5 mg/ml concentration followed by electroporation. Six pulses of 1500 V/cm, 100 μs each, with 1 s intervals. The procedure was performed on days 1, 5, and 8 every 90 days	Tumor regression in at least one lesion was observed in 46% of patients. 25% of patients had a net regression of all untreated lesions. Increased adaptive immune resistance was observed	[66,89]
Merkel cell carcinoma (MCC)	Plasmid DNA encoding IL-12	NCT 01440816	Pilot trial, 15 participants	The plasmid was injected at the treatment zone at a concentration of 0.5 mg/ml (0.5 ml or 0.25 mg). Electric field of 1300 V/cm and pulse width of 100 us at 400 ms intervals were administered. A sterile electroporation applicator comprising six stainless steel electrodes 1.5 cm long and arranged in a circular array ~1 cm in diameter was used	Sustained local expression of IL-12 protein achieved facilitating local and systemic immune responses in a subset of patients. Overall response rate of 25 % in patients with metastatic MCC was observed	[65]
Advanced inoperable human papilloma virus HPV-16 or HPV-18 cervical cancer	Plasmid DNA vaccine encoding HPV-16 and HPV-18 E6 and E7 tumor antigens	NCT 03444376	Phase 2, 36 participants	Intramuscular injection of 2 mg of plasmid DNA followed by electroporation. The procedure was performed at weeks 1, 2, 4, 7, 13, and 19. The study was a combination therapy with pembrolizumab. The TriGrid delivery system was used but electric field conditions were not provided	Preliminary antitumor activity observed. HPV E6-and E7-specific T cell responses were induced	[90]
HPV-associated recurrent/metastatic head and neck squamous cell carcinoma	Plasmid DNA encoding E6 and E7 of HPV-16 and HPV-18 with IL-12 adjuvant	NCT 03162224	Phase 1b/2a, 35 participants	7 mg of the plasmid was injected intramuscularly (IM) followed by electroporation at weeks 1, 3, 7, and 12. PD-L1 inhibitor durvalumab was intravenously delivered at weeks 4, 8, and 12, and then every 4 weeks. A CELLECTRA 5P device was used but electroporation conditions were not described	Primary efficacy endpoint was not reached; 80% of patients had treatment-related adverse events (two discontinued). Four of eight evaluable patients had a >twofold increase in tumor-infiltrating $CD8^+$ T cells	[91]
Middle East respiratory syndrome (MERS) coronavirus vaccination	Plasmid DNA encoding spike glycoprotein	NCT 02670187	Phase 1, 75 participants	Single IM injection of 0.67 mg, 2 mg, or 6 mg of plasmid DNA (1 ml) followed by electroporation. Three pulses at an interval of 1 s with 0.5 A current and 1–200 V per pulse were applied using a CELLECTRA 5P electroporation device. The procedure was performed at baseline, week 4, and week 12	MERS coronavirus DNA vaccine was tolerable and immunogenic in humans. The vaccine induced both antibody and cellular MERS coronavirus-specific immune responses	[92]
Cervical dysplasia	Plasmid DNA encoding E6 and E7 of HPV-16 and HPV-18	NCT 01304524	Phase 2, 167 participants	IM injection of 6 mg of plasmid (1 ml volume) followed by electroporation. A CELLECTRA constant current device was used which outputs 52 ms controlled electric pulses; other electric field details were not provided	Efficacy against CIN2/3 associated with HPV-16 and HPV-18 shown by eliciting adaptive immune response	[93–95]

Table 2. (continued)

Medical condition	Cargo	Clinical trial ID	Trial stage	Electroporation protocol	Trial outcome	Refs
Human immunodeficiency virus	Plasmid DNA encoding HIV-1 env/gag/pol	NCT 02431767	Phase 1, 94 participants	Plasmid DNA was administered with or without plasmid encoding IL-12 ID or IM at different concentrations. CELLECTRA 3P and 5P systems were used. Electric field conditions were not described	The immunogenicity of the DNA vaccine was enhanced by electroporation and inclusion of plasmid encoding IL-12. Intradermal electroporation was dose-sparing and induced immune responses equivalent to IM electroporation	[64]
Ebola virus (EBOV)	Plasmid DNA encoding EBOV glycoprotein	NCT 02464670	Phase 1, 240 participants	Plasmid DNA were administered at 0, 4, and 12 weeks via the IM (1 ml) or ID (0.1 ml) routes immediately followed by electroporation with a CELLECTRA 5P (IM: 13–19 mm electrodes, three pulses, max current 0.5A, ~2 s for full treatment) or CELLECTRA 3P device (ID: 3 mm electrodes, 4 pulses, max current 0.2 A, ~5 s for full treatment)	The ID route was better tolerated and antibodies to EBOV glycoprotein were generated. Cellular immune responses were activated in >70% of subjects	[63]
Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)	Plasmid DNA encoding SARS-CoV-2 S protein	NCT 04336410	Phase 1, 120 participants	Plasmid DNA (0.5 mg, 1 mg, or 2 mg) was delivered ID followed by electroporation at 0 and 4 weeks. Electroporation used the CELLECTRA 2000 device to deliver a total of four electrical pulses per electroporation, each 52 ms in duration, at a current of 0.2 A and a voltage of 40–200 V per pulse	A durable antibody response was observed 6 months following the second dose and the immune response was significantly increased with a homologous booster dose. Cytokine-producing T cells and activated $CD8^+$ T cells with lytic potential were significantly increased in the 2 mg dose group	[96]
Zika virus (ZIKV)	Plasmid DNA encoding ZIKV pre-membrane and envelope proteins	NCT 02809443	Phase 1, 40 participants	Plasmid DNA (1 mg or 2 mg dose, 0.1 ml) was administered as ID injections. Electroporation used a CELLECTRA 3P device to deliver four 52 ms pulses at 0.2 A (40–200 V, depending on tissue resistance) per session. The first two pulses were spaced 0.2 s apart, followed by a 3 s pause before the final two pulses, which were spaced 0.2 s apart. Electroporation needle arrays were 3 mm in length. The procedure was performed at baseline, 4 weeks, and 12 weeks	The DNA vaccine elicited anti-ZIKV immune responses. Side effects included local reactions at the vaccination site such as injection site pain, redness, swelling, and itching in 50% of the participants	[97]

In vivo electrotransfer for genome modulation

In vivo electrotransfer has been utilized for permanent genome corrections in various body regions to treat diseases resulting from genetic mutations. For instance, electrotransferred Cas9-mediated excision of a 23 kb genomic region on the X chromosome, covering the mutant exon 23 in a mouse model of **Duchenne muscular dystrophy (DMD)**, reinstated dystrophin expression in live mice [72]. In this study, plasmids encoding gRNA and Cas9 were locally electroporated into muscle fibers. Although such localized genetic corrections might enhance the quality of life of DMD patients by potentially restoring muscle function in specific areas such as the arms or legs, a

significant limitation of using electrotransfer for permanent *in vivo* genome modulation is evident – 'current electrotransfer strategies cannot be used for global genome modulation across the entire body because the technique is limited to specific administration areas'.

One key aspect to consider while using *in vivo* electrotransfer to treat genetic illnesses concerns whether local correction of the genome will be sufficient to treat the disease. One such area of potential for *in vivo* electrotransfer is for treating retinal diseases. For instance, injection of plasmid DNA encoding hyper-efficient Cas12a and gRNA into the subretinal space of neonatal mice followed by application of electrical pulses enabled simultaneous activation of endogenous targets in postnatal retina and altered the differentiation of retinal precursor cells [73]. Similarly, delivery of a single plasmid containing CRISPR-Cas9 and two sgRNAs via electroporation into the retina of transgenic mice with the P23H mutant allele resulted in ~20% of cells achieving the intended knockdown of the P23H rhodopsin gene through NHEJ [74].

The choice of the cargo between DNA or RNP can influence which cell types in the body are targeted, perhaps owing to differences in how they distribute within the body post-microinjection. For example, ID injection of plasmid DNA encoding Cas9 followed by electroporation in mouse tail skin showed protein expression only in the stratum corneum cells, with minimal effect on the skin stem cells in the basal epidermal layer. To overcome this limitation, Cas9/sgRNA RNPs were delivered via electroporation, resulting in efficient and precise DNA editing in skin stem cells within 2 days [75]. The edited cells continued to proliferate for up to 120 days. However, this method is confined to local skin areas, and the percentage of targeted cells after a single treatment is still limited.

Next-generation electrotransfer devices are now being used for *in vivo* genome modulation. For instance, nanopore systems that enable localized electroporation can release plasmid DNA with precision and control in the skin of mouse models, similarly to *ex vivo* techniques [76,77]. The method was further developed to deliver a DNA demethylation cocktail, comprising dCas9 and targeted sgRNAs, to an ischemic wound in a mouse model. The aim was to activate the *Tp53* promoter and facilitate wound closure by removing its silencing [78]. In addition, this technique was utilized to deliver a cocktail comprising developmental transcription factor genes, thus enabling the induction of endothelial cells and the generation of new vascular tissue through reprogramming of stromal tissue [79]. This approach aimed to counter tissue degeneration in a mouse model of limb ischemia.

Concluding remarks and future perspectives

The rapid advances in nucleic acid- and protein-based therapies necessitate the development of safe, affordable, and efficient delivery methods to unlock their full potential. In this overview we have outlined recent progress in *ex vivo* and *in vivo* electrotransfer techniques, highlighting key milestones and potentials in this field. Key takeaways include: (i) localized electroporation has achieved nearly perfect efficiency in model cell lines *ex vivo*, but currently lacks the throughput needed for adoptive cell therapy. (ii) There is a lack of consensus on whether to use RNA, DNA, or RNP formats for CRISPR-Cas systems during electrotransfer for optimal gene editing efficiency. (iii) Most *in vivo* and *in utero* studies still rely on traditional bulk electroporation, and unintended post-electrotransfer gene expression changes remain to be investigated. (iv) *In vivo* electrotransfer has demonstrated favorable safety profiles in DNA vaccination and gene therapy contexts.

Where is the field of electrotransfer heading?

The carrier-free and nucleic acid size agnostic nature of electrotransfer is poised to play a vital role in manufacturing facilities for *ex vivo* immune cell engineering (Figure 2 for a roadmap). A major

Outstanding questions

Can localized electroporation systems evolve into efficient and resilient cell engineering platforms operating at the throughputs required by adoptive cell therapy while being compliant with GMP?

Is the post-electrotransfer kinetics of gRNA assembly with the translated Cas protein a rate-limiting step in the delivery of CRISPR-Cas systems via nucleic acid electrotransfer?

What is the mechanism of intracellular delivery during protein electrotransfer? How do the electrotransfer efficiency and post-electrotransfer function of the protein depend on protein properties?

Do the efficiencies observed in *ex vivo* localized electroporation translate to *in utero* and *in vivo* electrotransfer scenarios?

Can artificial intelligence improve electrotransfer predictive models *ex vivo* and *in vivo*?

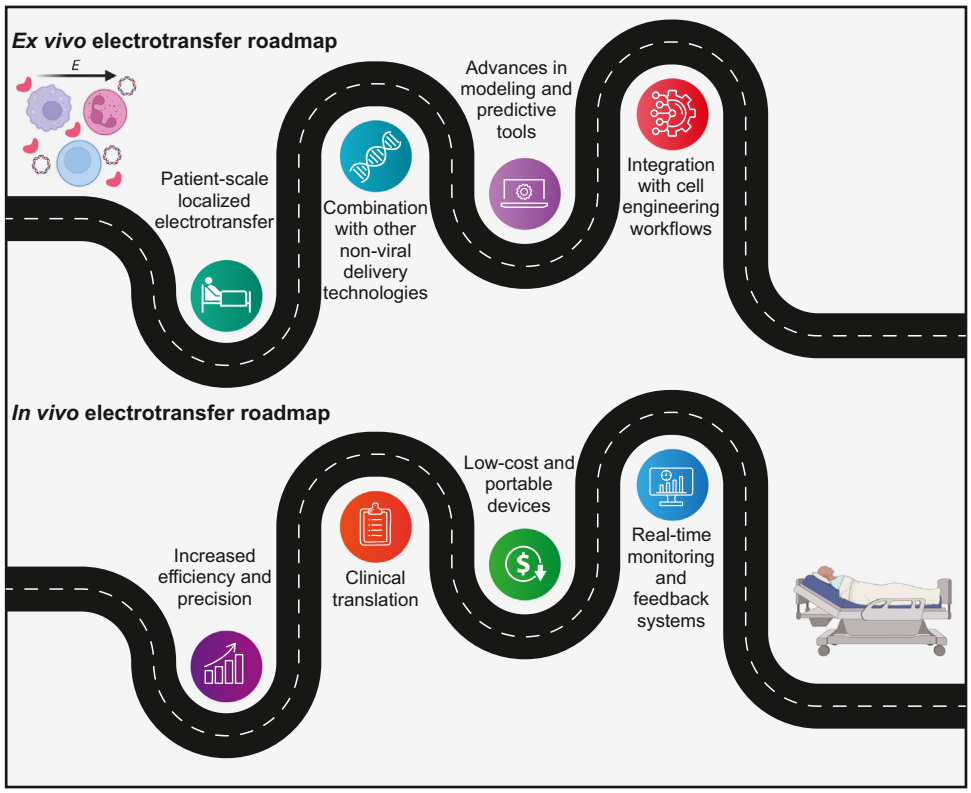

Figure 2. Roadmap for *ex vivo and in vivo* electrotransfer. For *ex vivo* electrotransfer, upscaling localized electrotransfer to reach desirable scales for patients (10^8 cells per patient) can boost the cell therapy industry. To achieve this scale, it is important to combine electrotransfer with other non-viral gene delivery techniques such as mechanoporation or sonoporation. We foresee advances in predictive modeling to improve the engineering and design of these devices, and predict that artificial intelligence will play a role in this development. Finally, the integration of electrotransfer with other steps within the cell engineering pipelines could potentially move to decentralized manufacturing facilities. For *in vivo* electrotransfer, possibilities to increase efficiency and precision should be investigated further. This would improve the success of many ongoing clinical trials and their translation to actual therapies. To make the technique globally accessible, R&D into reducing the cost and making the electroporation equipment more portable should be intensified. Finally, developments in real-time monitoring and feedback systems, for example assisted by image guidance, can assist in improving the safety profiles further.

bottleneck in optimizing the engineering of electrotransfer platforms lies in the limitations of existing predictive analytical and numerical models which struggle to precisely depict molecular transport [1]. Thorough investigations into the molecular transport facilitated by electrotransfer, its influence on cellular processes, and subsequent effects on single-cell transcriptomes, proteomes, and viability will be essential for accurate model development. Currently, there is a lack of studies that have clarified the operational mechanisms of protein electrotransfer, an increasingly relevant method in biotechnology for delivering CRISPR-Cas proteins to cells (see Outstanding questions). Readers can draw inspiration from previous investigations using fluorescence microscopy, synthetic membrane models, and 3D tumor mimics to advance our understanding of protein electrotransfer [80–85]. On the technology development side, localized electroporation devices offer significant efficiency advantages, while flow electroporation devices operate at high throughputs and are easily scalable. Combining these techniques has the potential to create efficient, high-throughput cell engineering platforms. The exploration of artificial intelligence to enhance electrotransfer workflows is anticipated to emerge as a novel research avenue

in the next decade. Ongoing efforts aim to make precise electrotransfer platforms accessible worldwide through the development of easy-to-fabricate and cost-efficient devices. For example, ultra-low-cost handheld, battery-free, single-use electroporation systems have been created to deliver DNA vaccinations in mice [86]. A significant challenge for *in vivo* electrotransfer lies in the requirement for invasive electrodes, which could hinder widespread adoption of the technology, even with optimal efficiencies and therapeutic results. Whether this limitation can be overcome remains uncertain, and will perhaps require the development of innovative non-invasive strategies to design painless electrodes such as microneedles with minimal contact with nerve endings in the skin and that are more patient-friendly. What is definite is that the progress of electrotransfer technology demands collaborative efforts from electrical engineers, biotechnologists, biomedical scientists, microfluidics technologists, and physicians, with valuable input from patients, to boost cutting-edge localized electroporation for clinical studies and translational medicine.

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Author contributions

A.M. conceptualized, prepared, and wrote the original draft. A.M. and P.E.B reviewed, edited and confirmed the final manuscript.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT to improve language and readability. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of interests

The authors declare no conflicts of interest.

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