Review

Electrotransfer of RNAi-based Oligonucleotides for Oncology

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Abstract. *For more than a decade, there has been tremendous growth in our understanding of RNA interference (RNAi). The potent ability that small oligonucleotides have in gene silencing makes them desirable as novel cancer therapeutics, but many biological barriers exist for their efficient delivery into target cells or tissues. Electropulsation (EP) appears to be a promising method for cancer-associated gene therapy. EP is the direct application of electric pulses to cells or tissues that transiently permeabilize the plasma membranes, allowing efficient in vitro and in vivo cytoplasmic delivery of exogenous molecules. The present review reports on the type of therapeutic RNAi-based oligonucleotides that can be electrotransferred, the mechanism of their electrotransfer and the technical settings for pre-clinical purposes.*

Difficulties with blocking therapeutic targets using conventional approaches have prompted many to consider using RNA interference (RNAi) as a new antitumor strategy (1). RNAi offers the possibility of targeting and silencing any pathological protein in a specific way (2). RNAi is mediated endogenously by microRNAs (miRNAs) (3) and experimentally by small silencing RNAs (siRNAs) (4). Both are small (~22 nt) noncoding RNAs that, once loaded into the RNA-induced silencing complex (RISC), bind to their target messenger RNA (mRNA) impairing its translation. As a result, gene expression is suppressed (5, 6).

The development of therapeutic RNAi-based oligonucleotides is now moving to a new stage that involves efficient tumor delivery. In fact, their physicochemical characteristics (*i.e.* large molecular weight and anionic charge) prevent passive diffusion across the plasma membrane of most cell types. Therefore, enhanced delivery methods are required to allow therapeutic oligonucleotides to enter cells while being biocompatible, safe, and targeted.

In this context, electropulsation (EP) is a promising non-viral biophysical method for *in vitro* and *in vivo* delivery of various molecules such as drugs (7) and nucleic acids (8, 9). EP was introduced in the 1960s (10) and consists of the application of an external electric field to target cells or tissues. Under calibrated electric conditions, it transiently destabilizes the plasma membrane, causing its permeabilization (11). The efficiency and convenience (*i.e.* ease of the procedure, low cost and speed) of this technique lead to its extensive *in vivo* use for a large number of both internal and surface organs and tissues (12, 13). Moreover, only very few side-effects have been reported (mostly superficial burn), emphasizing the innocuousness of this method for clinical use. In addition, no change in the expression profile of major tumor suppressor genes or oncogenes, of genes involved in the stability of DNA and no promotion of tumor genesis were detected. The expression of metastasis promoting genes was not increased after electrochemotherapy (14, 15). To date, several preclinical and clinical studies using EP for cancer treatment showed encouraging results demonstrating antitumor effectiveness (16-21).

This article reviews the type of therapeutic RNAi-based oligonucleotides that can be electrotransferred, the mechanism of their electrotransfer and the technical settings for clinical purposes.

RNAi-based Oligonucleotides Suitable for Electrotransfer

RNAi is an mRNA-targeted therapeutic that specifically targets and degrades mRNA using natural cellular mechanism (2).

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Table I. *Current clinical trials for RNAi-based oligonucleotides.*

Company	Diseases	Chemistries	Status	References
Calando	Solid tumor	Cationic polymer for siRNA delivery	Phase I	http://www.calandopharma.com (85)
Silence therapeutics	Advanced solid tumors	Stabilized siRNA (AtuRNAi)	Phase I	http://www.silence-therapeutics.com (86)
Regulus therapeutics	Oncology, fibrosis, metabolic disease	miRNA inhibitor using 2'methoxyethyl, 2'fluoro RNA, bicyclic ribose modifications	Pre-clinical	http://www.regulusrx.com
Mirna therapeutics	Non-small cell lung and prostate cancer	miRNA replacement using siRNA	Pre-clinical	http://www.mirnatherapeutics.com

RNAi is a highly promising therapeutic approach for those tumors involving aberrant protein production. In fact, human clinical trials have been initiated to utilize RNAi potential for therapeutic purposes (Table I). However, these trials were dramatically hampered by difficulties in the delivery of RNAi-based oligonucleotides (22). EP appears to be well adapted for all kinds of RNAi-based oligonucleotides.

Small silencing RNA (siRNA). SiRNA is engineered to precisely match the protein-encoding nucleotide sequence of the target mRNA. It associates with the RISC complex and the siRNA-associated RISC binds to the target mRNA through a base-pairing interaction and degrades it. RNAi-based experiments can suffer from lack of specificity due to silencing of non targeted genes unless a well designed sequence is used (23).

Although siRNA *in vitro* efficiency is high, its *in vivo* delivery remains a critical issue for its therapeutic development. A safe approach requests a direct transfer to the cytoplasm to avoid unwanted effects associated with the delivery pathways (24). EP has been used successfully for *in vivo* siRNA electrotransfer in a wide variety of tissues such as skin (25), muscle (26, 27), joint tissue (28, 29), eyes (30), brain (28), kidney (31). EP has also proved its efficacy in siRNA tumor electrotransfer (9, 32-34). In fact, tumor siRNA electrotransfer is much more efficient than the use of plasmid DNA. EP, as compared with other delivery methods such as hydrodynamic transfection, needs much smaller amounts of siRNA to be effective (27, 35) and increases its duration of action (35, 36). In addition, no tissue damage (27) or immune response were observed with EP, in contrast to other delivery technics (37).

MicroRNA (miRNA)-based oligonucleotides. Targeting pathways of human disease with miRNA-based drugs represents a novel and potentially powerful therapeutic approach (38). miRNAs play a role in numerous biological processes, including the immune response, cell-cycle control, metabolism, viral replication, stem cell differentiation and human development (39). miRNA expression or function is significantly altered in many disease states, including cancer (40, 41), cardiovascular diseases (42) and diabetes (43). Since miRNAs do not require perfect complementarity for target recognition, a single miRNA is able to regulate multiple messenger RNAs, in contrast to siRNA. Therefore miRNA-based therapy is anticipated to be highly efficacious. Depending on miRNA function and its status in diseased tissues, there are two approaches to developing miRNA-based therapy: use of antagonists or mimics. In many cases, tumor-suppressor miRNAs reactivation by synthetic miRNA mimics (44) or oncogene inhibition by antagonists (45) leads to a significant antitumor response, as long as an efficient delivery method is present.

Most of the published reports used systemic delivery (46, 47), which implies repetitive high-dose injections, associated with non-specific targeting and toxic side-effects or direct intra-tumor injection alone without any delivery system (48), with associated poor tumor uptake and high degradation by tumor nucleases. Recently, tumor electrotransfer of miRNA mimics has also been described in mice (49). By rescuing *miR-143* expression with *in vivo* electrotransfer of mimic oligonucleotide, prostate cancer cell growth was abrogated. These data show that EP is effective in delivering therapeutic miRNA-based oligonucleotide to tumor tissue *in vivo*.

Chemically modified oligonucleotides. Due to its small size, RNAi-based therapy calls for robust and improved antisense oligonucleotide technology. Therefore, new generations of chemically modified oligonucleotides have been developed (50) including 2'-*O*-methyl, 2'-methoxyethyl, locked nucleic acids (LNA), and phosphorothiate linkages (51, 52). LNA oligonucleotide incorporation into a DNA or RNA oligomer improves the mismatch discrimination compared to unmodified reference oligonucleotide (53). In addition, LNA oligonucleotide is highly resistant to nuclease degradation and presents low toxicity for biological systems (54, 55). The

Table II. *Electropulsation overcomes barriers to RNAi-based oligonucleotide therapy.*

Obstacles to efficient RNAi-based therapeutic	EP advantages
Renal clearance	Local delivery
Non-specific targeting	
Ineffective transfer from blood to tumor	
Poor access to tumor	
Limited passage through tumor matrix	Migrate rapidly and efficiently through the extracellular matrix because of the electrophoretic forces
Degradation by tissue nucleases	Short delay between injection and targeted delivery
Inefficient uptake by tumor cell	Direct entry through the permeabilized membrane of tumor cell
Ineffective endosomal release	Immediate free access to the cytoplasm
Safety	Innocuity of the technique, naked materials
Cost-effective	Naked materials, reduced dose of oligonucleotides

most advanced miRNA-based oligonucleotide is an antagonist LNA specific to miR-122, which is currently in clinical phase II trial for patients infected with hepatitis C virus (56).

Little work has been performed on the delivery of these chemically modified oligonucleotides. Interestingly, we observed that LNA-DNA oligomer can be efficiently electrotransferred *in vitro* and *in vivo* (personal communication). The number or the position of LNA into the DNA sequence does not interfere with electrotransfer efficiency. However, electrotransferred phosphorothiate-modified LNAs spontaneously stacked to the membrane and strongly aggregated at the cell surface, rendering them less attractive for RNAi-based therapies (unpublished observation). Thus, if chemically modified oligonucleotides appear, in theory, to be promising for RNAi-based therapy, more work for their efficient delivery needs to be performed.

Mechanism of RNAi-based Oligonucleotide Electrotransfer

EP represents a very attractive delivery method (Table II) that has led to abundant literature. However, very little was known about the basic processes supporting the electrotransfer of RNAi-based oligonucleotides. Using a fluorescently labeled siRNA, we observed that after the electrotransfer, siRNA was distributed homogeneously throughout the cytoplasm of cultured tumor cells (57). Thus, upon EP, siRNA had immediate, free access to the cytoplasm allowing its direct interaction with the enzymatic machinery (RISC) and its mRNA target. In these experiments, no electrotransferred siRNA was observed in the nucleus of viable cells. Similar localization was observed *in vivo* in mice after intratumor injection of siRNA followed by EP (58).

It is well known that electrotransfer of plasmid DNA is a multistep process: electrophoretic migration towards the permeabilized membrane, insertion into the membrane, translocation across the membrane and migration towards the nucleus (59, 60) (Figure 1). Electrotransferred plasmid DNA is not spread into the cytoplasm as is observed for small molecules, such as anticancer drugs. Small molecules enter into the cell across permeabilized zones of the membrane facing both electrodes *via* the concentration gradient difference between the exterior and the cell interior by post pulse diffusion process (61) (Figure 1). In contrast, siRNA electrotransfer implies electrophoretic movements. However, contrary to plasmid DNA, where a complex between the DNA and the membrane results from pulse application (Figure 1), a direct transfer is present with siRNA. After pulse application no diffusion process, as occurs for drugs, occurs although the membrane is still permeabilized (57).

The mechanism of LNA electrotransfer appears to be closely similar to that for siRNA, meaning that LNA oligomer entry is driven by the electrophoretic forces (personal observation). Electrotransferred LNA is spread homogenously throughout the cytoplasm contrary to lipid-mediated transfection in which LNA oligomer is shown to be localized in the nuclear periphery in a punctate way, suggesting an endosomal distribution (55, 62, 63). More work is under way to characterize the mechanism and the potential of LNA electrotransfer for RNAi-based therapies.

Technical Designs

The basic instrumentation for EP comprises a pulse generator and specific electrodes. However, the definition of the electrical parameters and the design of the electrodes are crucial steps for efficient and safe electrotransfer into the tumor.

Electrical parameters. Electrical conditions are characterized by physical parameters: electric field intensity (E), number (N), duration (T) and frequency (F) of the electric pulses. These parameters are essential to achieve effective transfer while preserving cell viability and avoiding drastic effects on the

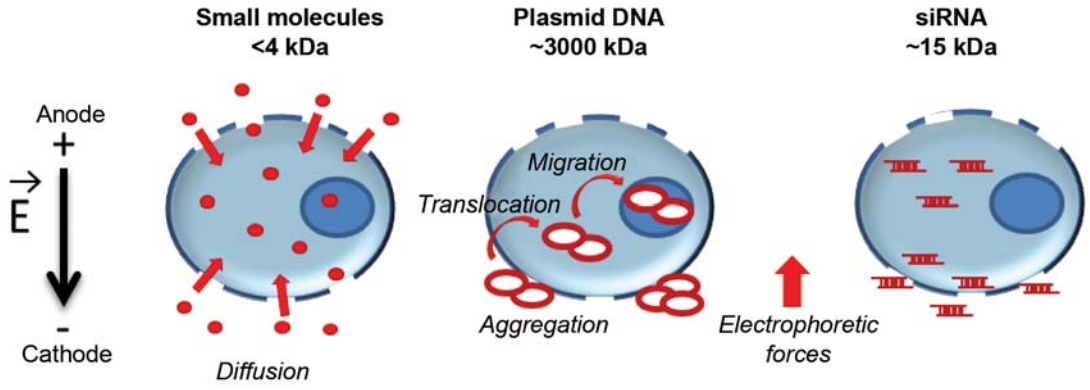

Figure 1. *Mechanisms of electrotransfer. Small molecules mostly enter the cells by diffusion through both sides of the permeabilized membrane facing the electrodes after the pulse. Plasmid DNA, dragged by the electrophoretic forces, interacts with the permeabilized membrane at the cathode side and remains on the membrane for a few minutes before its translocation into the cytoplasm. SiRNA migrates electrophoretically during the pulse through the membrane only at the cathode side, resulting in direct cytosolic localization.*

patient (essentially superficial burns and muscle contractions). Depending on the size and nature of the molecule to be transferred, there are two types of electrical parameter settings: electrochemotherapy (ECT) and electrogenotherapy (EGT).

ECT is a treatment where a cytotoxic drug, such as cisplatin or bleomycin (low molecular weight), is usually intravenously injected, directly followed by pulse application to the tumor. This method uses high electric field intensity (kV/cm) of short duration (microseconds). ECT protocols (8 pulses of 100 μs at 1300 V/cm; 1 Hz) have been tested in human clinics to treat malignant cutaneous and subcutaneous melanoma (64). ECT has also been used successfully in veterinary medicine for treatment of feline sarcoma (65) and equine sarcoids (66). Interestingly, these settings were proven to be efficient for a clinical trial for plasmid delivery (20).

The EGT parameters allow the electrotransfer of macromolecules (*e.g.* nucleic acids) to the tissues. Compared to ECT, this procedure uses lower field intensity (V/cm) with longer duration (milliseconds) to increase the electrophoretic movement of the electrotransferred macromolecule during the pulse. This procedure also allows simple and efficient transfer of siRNA both *in vitro* and *in vivo* (58). In animal models, EGT has been performed in many different tissues: skeletal (67, 68), cardiac muscle (69), liver (70, 71), skin (72, 73), spleen (74), kidney (75), brain (76), joints (77) and tumor (58).

Other electrical settings are reported. They consist of combinations of pulses of high voltage and short duration (HV, permeabilizing pulse) (78) followed by low voltage and long duration non-permeabilizing pulses (LV, electrophoretic pulse). Study on the skeletal muscle of mice shows that the (HV-LV) pulse sequence leads to an efficient gene transfer, similar to the one obtained by use of EGT described above (79).

As yet, no standardized EGT electrical parameter settings exist, partially due to the fact that the tissue response to the electric pulses depends on its origin, shape and environment, but also on the type of electrodes used.

Choice of electrodes. The success of the EP technique is linked to the proper distribution of the electric field in the tumor which is dependent on the type of electrodes used. Numerous configurations of electrodes have been developed for therapeutic purposes: parallel plate, needle, contact wire *etc*. (80).

Parallel plate electrodes are used most frequently for subcutaneous tumor electrotransfer. This consists of placing the electrodes on both sides of the tumor prior to electric pulse delivery (81). This simple design has produced high response rates (70 to 85%) in animal studies and in clinical anticancer trials (82).Their limitation is that the tumor should fit into the inter-electrode space and that the high field at the contact of the electrode with the skin can induce superficial burns if sharp angles are kept in their design.

If plate electrodes have been shown to be more suited for the treatment of cutaneous tumor, needle electrodes are more efficient in intraoperative settings or for treating the deepest regions of cutaneous tumors (83). Needle electrodes are inserted through the skin allowing deeper penetration of the electric field into the tumor. However, with these electrodes, the electric field is heterogeneous as it is confined to the immediate proximity of the needles. A strong burning of the tissue in contact with the needles was reported. Several configurations are in development such as linear and circular arrays (83).

Contact wire electrodes have been proven to be very efficient and convenient when large tissue surfaces (several square centimeters) must be treated due to the ease of their use at the cutaneous level (80).

New designs of electrodes are under development in order to adapt the field distribution to the geometry of the tumor, enabling cancer cell permeabilization with minimum tissue damage. These improvements are based on numerical modeling but the irregular shape of the tumor and the heterogeneity of the surrounding layers make it difficult (84).

Conclusion

Since the discovery of the RNAi pathway, there has been an explosion of interest and knowledge in using this technology for clinical application. Although highly attractive as a therapeutic approach, several hurdles must be overcome to successfully introduce RNAi-based therapies into clinical practice. An efficient, safe and localized delivery to diseased cells and tissues must be obtained. Progress is being made in developing new delivery approaches to address these issues. However, the vast majority is still only applicable *in vitro*. In this context, EP is a promising biophysical strategy to target RNAi-based oligonucleotides to the correct tissue, to facilitate their cellular uptake and to give direct access to their cytoplasmic target. The EP technique is already capable of overcoming many of the delivery hurdles, as proven by its successful translation to clinical practice.

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