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Micro-/Nano-Electroporation for Active Gene Delivery

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Abstract: Gene delivery, a process of introducing foreign functional nucleic acids into target cells, has proven to be a very promising tool for inducing specific gene expression in host cells. Many different technologies have been developed for efficient gene delivery. Among them, electroporation has been adopted in gene delivery for decades, and it is currently widely used for transfection of different types of cells. Despite of the success achieved by bulk electroporation (BEP) for gene delivery *in vitro* and *in vivo,* it has significant drawbacks such as unstable transfection efficacy and low cell viability. In recent years, there is an emerging interest in understanding how individual cell accepts and responds to exogenous gene materials using single cell based micro-/nano-electroporation (MEP/NEP) technologies. In this review, the authors provide an overview of the recent development of MEP/NEP and their advantages in gene delivery. Additionally, the future perspectives of gene delivery with the application of electroporation are discussed.

Keywords: Electroporation, gene delivery, nano-electroporation, nanocarriers, microfluidics.

1. INTRODUCTION

Gene delivery, a process of introducing target cells with foreign functional nucleic acids, including plasmid DNAs (pDNAs), small interfering RNAs (siRNAs), antisense oligonucleotides (ASOs), messenger RNAs (mRNAs) and microRNAs (miRNAs), has proven to be a promising tool for regulating specific gene expression in host cells [1]. Various techniques have been developed for the delivery of gene materials in different types of cells, from bacterial to mammalian cells [2-5]. In 1990, the world first FDA-approved gene therapy was performed in a severe immune system deficient boy, and achieved temporary but successful result. Since then, a variety of gene delivery trials have been performed *in vivo* for the treatment of different diseases such as cancer, amyloidosis, and hypercholesterolemia [6-8].

In spite of the great promise of gene delivery, great gaps still remain in applying such technologies in broad clinic use due to their poor pharmacological performance. One of the major hurdles is the lack of highly efficient and safe gene delivery methods. Current gene delivery methods may be divided into viral and non-viral approaches. Viral gene delivery uses certain viruses or viral functional domains as the carrier to transfer the gene material into the target cells. Commonly used virus includes adenovirus, adeno-associated virus, retrovirus, and herpes simplex virus [9-12]. Though it has achieved high transfection efficiency in many studies, its application in human is limited due to its safety issues [9]. The non-viral approach for gene delivery includes chemical methods and physical methods. In chemical methods, gene materials are packaged into particles, such as liposomes and nanoparticles [3, 10]. Physical methods for gene delivery include electroporation, gene gun, sonoporation, laser ablation, and microinjection [15-18]. These methods utilize either physical or mechanical forces to transiently penetrate the cell membrane to introduce the gene materials.

Among these physical methods, electroporation has been adopted into gene delivery for decades, and it is currently widely used for transfection in different types of cells [11-13]. Electroporation, also termed as electro-permeabilization, is a physical method where cell membrane permeability is transiently compromised with the application of an electrical field to the cells, thus allowing the perfusion or injection of impermeable cargo into the cells. Generally, the commercialized electroporation system (*i.e.* bulk electroporation, BEP) is consisted of two parts: one narrow cuvette, and a pair of planner electrodes which are usually parallel, as shown in Fig. **1**. With the application of the electric pulse to the electrodes, the cells between the two electrodes are exposed to the strong electric field. This strong electric pulse induces the sudden change in the cell transmembrane potential, resulting in the formation of many hydrophilic pores on the cell membrane. Thus, the cell membrane permeability to otherwise low or non-permeable molecules,

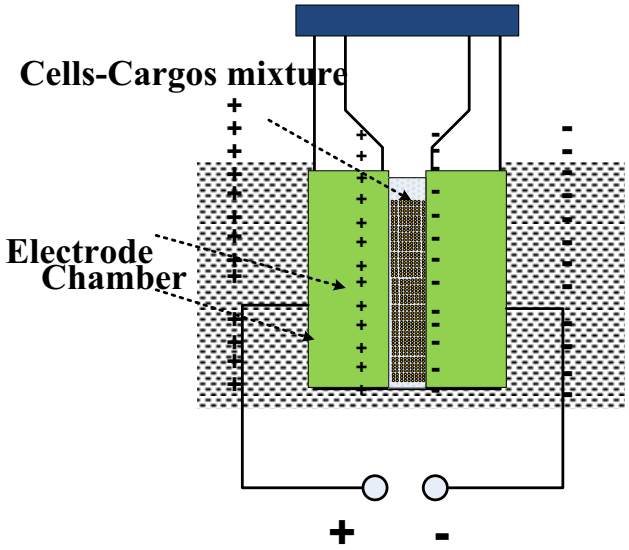

Fig. (1). The basic setup of bulk electroporation systems (BEP), which is consisted of a narrow-shaped cuvette made of plastics or glass, a couple of electrodes connecting with cell buffers, and a high-voltage power supply. Cells and cargos are concentrated in the cuvette chamber. High-voltage pulses are applied between the two electrodes where cells undergo random and harsh shock.

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such as DNA and large size drugs, is dramatically increased, allowing the perfusion of such molecules into the cells through these pores. Though the bulk electroporation has been widely used for gene delivery for many years, there are several drawbacks that limit its applications in clinic. First, the low transfection quality, either the transfection efficiency or the transfection uniformity, remains an important unsolved problem in bulk electroporation. Due to the non-uniform electric field created by electrodes, bulk electroporation can only transfect part of cells in the cuvette with low efficiency. Furthermore, the serious cell damage caused by electric shock leads to the low cell viability after transfection, particularly for primary cells with low cell population. The transfected dose significantly varies between cells, which is regarded as a key downside of this technique for applications of cell reprogramming and adoptive immunotherapy where the cellular transfection quality is extremely important. Therefore, new electroporation methods with excellent transfection efficiency, uniform dose control, and high cell viability are needed. These new methods would not only benefit basic scientific research, but also bring the gene delivery from bench to bedside.

Attempts have been made to address these practical issues for long term. While some of them focus on optimization the elements in BEP [14-16], such as pH value, ion composition and concentration in BEP buffers, the applied voltage, *etc.*, many others paid attention on miniaturizing the cellular environment to micro-scale where a single cell could be safely and efficiently porated by a strengthen electric field, namely micro-electroporation. The implementation of these devices is mainly based on the fast development of micro-fabrication. Particularly, there is an emerging interest in understanding how individual cell accepts and responds to exogenous gene materials by using single cell based nanoscale electroporation technologies recently [17-19]. Many excellent review works have been published to notice these events. Early on 2006, S. Mehier-Humbert and R.H. Guy compared the current available physical approaches for gene delivery and have recognized electroporation would be one of the best techniques with high potential of optimization between transfection efficiency and cell viability [20]. T. Geng and C. Lu contributed a broad but deep summary of recent progress on microfluidic platforms for electroporation scenario [21]. The basics of electroporation, including the transmembrane potential in correspondence to the applied electric field, as well as the pores formation on cell membrane were analyzed. Various strategies on microfluidic electroporation were presented and discussed. S. Wang focused on micro-/nano-fluidic based single-cell electroporation, while taking the major effort on introducing the advantages of these designs over the conventional bulk electroporation [22]. Chen showed a broad picture on the status of both chemical and physical based methods for intracellular delivery. Some of micro-/nano-techniques with their applications for gene delivery were briefly introduced [23]. In this review, we focused on the recent development of MEP / NEP. The characteristics of representative MEP devices, as well as their advantages in gene delivery were presented. The rationale of miniaturizing the size of electroporation environment from MEP to NEP was discussed. Several special NEP devices, based on various fabrication and designs, were mainly focused, while their advantages and disadvantages were analyzed with regards to the transfection efficacy and cell damage. Finally, the future perspectives of gene delivery with the application of electroporation were discussed.

2. MICRO-ELECTROPORATION

In terms of the scale of the physical environment where cells are electroporated, current electroporation devices can be divided into three categories: bulk electroporation (BEP), micro-electroporation (MEP), and nano-electroporation (NEP). The common definition of BEP, MEP and NEP is based on the local electric field under a micro-/nano-engineered environment where a single-cell is electroporated, which is relevant to the size of the elements (such as, electrodes, channels, pores, *etc.*) involved in electroporation in comparison with the size of the cell (typically in the range of 1 μm to 100 μm) [22, 24]. For example, when a cell is aligned to a microchannel with ~ 5 μm in diameters, the area of cell membrane that have the chance of successful permeabilization is of μm^2. As compared to the BEP where millions of cells are placed under a large electrode with the dimensions of mm^2, the electric field strength of MEP will be increased with several orders of magnitude, which allows a low-voltage (*e.g.* less than 10 V applied over the microchannel-cell) to sufficiently porate the cell for the diffusion of cargos considering the transmembrane potential of cells, with the typical value in the range of 200 mV to 1 V [25]. In a previous work, 1.5 V has shown successful electroporation in a MEP system with microchannels of 2 μm in dimension. However, the intracellular delivery is mainly dependent on diffusion because the low-voltage cannot supply significant momentum to the cargo with surface charges. Increasing the system voltage may cause irreversible damage to the cell membrane since most parts of the potential drop were applied over the cell due to the relatively low resistance of the microchannel. Therefore, MEP offers the advantage of strengthen electric field which leads to safe electroporation at single-cell level over BEP systems, though both of them depend on the diffusion for gene delivery. In contrast, when the cell is positioned against a nanochannel with 100 nm in diameter, most part of potential (high than 99%) will drop across the nanochannel with a high resistance, which promises a safe cell poration even under a high voltage [26]. For example, when a high voltage (*e.g.* 100 V) is applied over the nanochannel and the cell, 99V will be distributed across the nanochannel, while a safe and enough biased voltage, 1V, will be loaded on the cell for electroporation. Meanwhile, the electric field applied over the nanochannel will accelerate the cargos with surface charges within nanochannel, and thus propelling the cargos into the cells upon electroporation. Obviously, the behavior of gene delivery in NEP is significantly different from that in either BEP or MEP. 'Deterministic injection' could be achieved in NEP devices.

While classic BEP is based on a large randomly distributed cell population, *i.e.* millions of cells suspended in a chamber, an emerging interest of electroporation focuses on understanding how individual cell accepts and responds to exogenous gene materials, and the single cell based micro-/nanoscale electroporation technologies have gained attention rapidly in the area of gene delivery. Micro-electroporation (MEP), an advanced technique developed by several research labs, is able to provide single cell based transfection by introducing gene material into the cell via micron-sized channels [27]. Compared to the BEP which is a stochastic process due to the random distribution of cells in a non-uniform electric field between the electrodes, MEP enables the implementation of single-cell electroporation to study the heterogeneity of cells. According to their special configuration and approach, they are further classified into following groups: microfluidics with electrodes and microchannel electroporation.

2.1. Microfluidics Based MEP

In the microfluidics based MEP approach, the electrodes used are in parallel, similar to those in the commercialized BEP system. The major difference is that the large cuvette chamber is replaced by a long microscale flow channel. The cells inside the electroporation solution are forced to flow through the microchannel sequentially, instead of being suspended in the cuvette chamber. Under such a microfluidics design, individual cells "sandwiched" by two parallel electrodes on the inner surface of the microchannel are electroporated under a uniformly distributed electric field.

In 2001, Lin *et al.* first used a poly(methyl methacrylate) (PMMA) microchip system for continuous gene transfection [28]. In this type of microfluidic electroporation system, the two parallel gold electrode plates were placed on the top and bottom of a straight microchannel, as shown in Fig. **2A**. The cell suspension

with the exogenous gene material flowed through the microchannel continuously subjected to electrical pulses delivered by the electrodes at a relatively low voltage. This microchip electroporation system can achieve continuous gene transfection with high efficiency and cell viability. Since then, several other groups also introduced various microfluidics electroporation concepts into gene transfection. For example, Zhan *et al.* [29] designed a simple microfluidic device for the flow-throw electroporation using Chinese hamster ovary (CHO) cells as the model. The suspension solution containing the individual cells and gene was continuously passed through the electrode zone inside the microchannel. The electroporation process was triggered with a transit time of 1.8 ms under the voltage of 5 V. A layer-by-layer method and micro-spiked electrodes were used for nucleotide delivery including plasmid and siRNA [30, 31]. The application of this microchip electroporation was also extended to the transfection of zebrafish embryo using quantum dots and green fluorescence protein gene [26, 27]. Recently, using the 96-well plate based array electroporation as the platform, Xu *et al.* [32] introduced printed circuit board (PCB) technique into electroporation. In their design, an array of 96 through-holes were fixed on the printed circuit board, and in each through-hole, two electrodes were located on the wall. The cell and gene material suspension was loaded on top, got transfected when passing through the hole, and was collected under the PCB. In this way, it achieved high throughput while maintained high transfection efficiency and cell viability for gene delivery.

In all these cases, the parallel plate electrodes design in conjunction with either microfluidics or microelectrodes electroporation could provide a uniform electric field inside the microfluidic channel for continuous cell transfection. Further structure modification on the electrodes, *i.e.* incorporation of silicon nanowires or nanoribbons on the two electrodes, could focus the condensed electric field on target cells, thus achieving the enhanced gene transfection efficiency in a single cell level [33].

While the two-dimensional plate electrodes are widely used in microfluidics based electroporation due to their simple and reliable design, they suffer several drawbacks. First, the distance between the two parallel electrodes, typically in the range of several hundred micrometers, is much larger than the cell size which is usually around 10-20 micrometers. The position of the cell inside the electric field during electroporation process may greatly determine the transfection efficiency, leading to the non-uniformity of electroporation effect among different cells [34]. In addition, the transfection uniformity of each cell is further affected by the synchronization of cell position and electroporation pulse duration. Thus a precise cell flow rate control is necessary. For cell suspension, the non-homogenous distribution of the cells in the suspension must be taken into consideration. Another practical problem is the unstable characteristics of the thin metal layer used as electrodes. The continuous water electrolysis during the electroporation process would result in fast erosion of the electrodes, which could reduce the transfection uniformity. Furthermore, the considerable bubble formation and Joule heating produced in the microchannel may lead to severe cell damage. Therefore, alternative 3D electrode designs and platform structure that can precisely control the cell position during electroporation is under intensive investigation, though there are some practical issues due to the complexity in microfabrication [35-37].

2.2. Microchannel Based MEP

The microchannel based electroporation approach utilizes the microscale device design for cell localization and electroporation. In microchannel based MEP, the cells are trapped into a restricted structure such as a microchannel array before electroporation (see Fig. **2B** and **C**). Because a high electric field could be generated in the microchannel, a very low voltage (1-5 volts) is sufficient for cell poration [38]. Consequently, the cell viability is significantly improved compared to BEP. In BEP, a voltage in the range of 1, 000 volts is applied to the whole system. The strong Joule heating, water electrolysis and bubble formation caused by the very high electric field would result in poor cell viability after electroporation. More than half of the cells are often killed by such lethal effects. The non-uniform electrical field distribution among cells also would lead to severe cell apoptosis [39]. In contrast, these negative effects are largely reduced in microchannel based electroporation. In most cases, microchannel based MEP is capable of achieving both high transfection efficiency and high cell viability in gene delivery.

Microchannel based electroporation has been widely used for single cell or multiple-cell gene delivery. In 2001, Huang *et al.* [40] first reported a microfabricated electroporation chip for single cell transfection (Fig. **2B**). Two small chambers are connected with each other by a microhole with a diameter of 2-4 micrometer. The pressure gradient between these two chambers can automatically push the suspended cells into the microhole. Once trapped in the microhole, the cells can easily get permeabilized at a very low voltage and then the cargos are diffused into the cells. A systematic platform with an array design can treat numerous cells simultaneously for gene delivery. By using a 96 well formatted microfluidic device, Khine *et al.* [41] demonstrated that a microchannel array based electroporation system could achieve a real-time feedback at the "array" level. This device provided an affordable and practical platform for electroporation array. Instead of using photolithography based microfabrication to make MEP chips, Fei *et al.* developed a membrane sandwich electroporation (MSE) method [42, 43]. As shown in Fig. **2C**, this MSE design provides a sandwich microenvironment with an enhanced electric field which can facilitate better gene delivery into the cells. Compared with devices without such modification, MSE can achieve a higher transfection efficiency with a comparable cell viability. However, in spite of the high transfection efficiency obtained in gene delivery, these devices are not able to achieve dosage control at the single cell level.

3. NANO-ELECTROPORATION

Nanotechnology has been extensively used in gene delivery and it has shown great promise for improving the efficacy of exogenous gene transfection in different studies [37, 44-46]. It not only fundamentally changed the pharmacokinetics of gene materials, but also altered its pharmacodynamics and extended its application in clinic [47]. For example, with the recently fast development in nanotechnology, various types of nanoscale materials, such as liposomes, polymer nanoparticles, and micelles, are widely used for targeted gene delivery [48, 49]. As a powerful physical method for gene delivery, electroporation technology has been dramatically improved with the introduction of nanotechnology in recent years. Compared with traditional BEP and MEP, the nanoscale electroporation can achieve better performance in gene delivery such as enhanced gene delivery efficiency, precise dosage control, high transfection uniformity, and excellent cell viability.

In this part, we will discuss four types of representative nanoscale electroporation devices that are developed in recent years for gene delivery. The characteristics of these devices, including fabrication techniques and nano-structure for electroporation are highlighted. Their advantages and limitations for cell transfection and gene delivery are also briefly discussed.

3.1. Nanoporous and Nanostraw Electroporation

Surface patterning and texturing, such as nano-materials modified membrane, has been widely adopted in a wide variety of fields [50, 51], especially for the exploration of cell behavior including cell proliferation, signaling pathways, and apoptosis. In nanoscale electroporation, nano-porous membranes, such as track-etched films, have been used as a user-friendly and affordable device for gene delivery [43]. This electroporation method provides improved

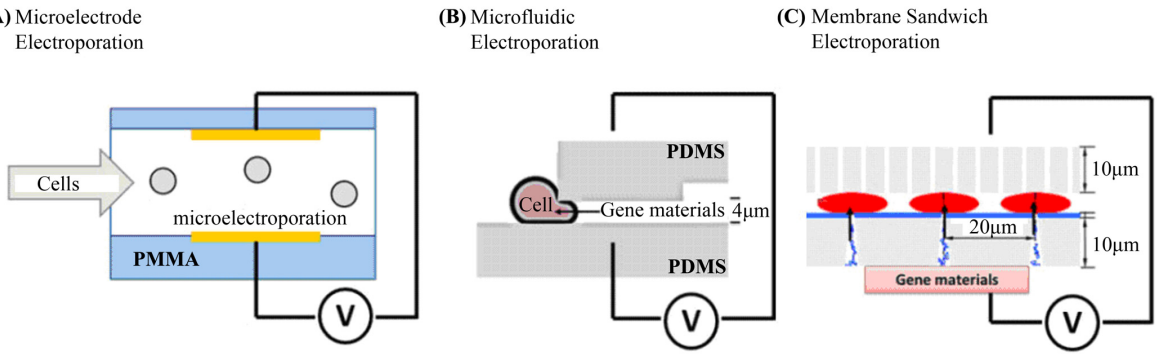

Fig. (2). Schematics of microelectrodes, membrane sandwich, and microfluidics electroporation. (**A**) Cell suspension is aligned and passed through the region between microelectrodes where the electroporation occurred. (**B**) The target cell is brought to the 4 μm trapping gap where an added electric field was applied to deliver the gene materials. (**C**) The membrane sandwich structure holds cells and locally delivers the gene materials through the attachment of trapping membrane and plasma membrane.

transgenic expression compared to other techniques. As illustrated in Fig. **3**(**A**), when cells are loaded on the membrane in the top chamber, some of them have the chance to be positioned in the vicinity of nanopores. Gene materials to be electroporated are located in the bottom chamber. For example, Z. Fei *et al.* [42] successfully used track-etched poly-ethylene terephthalate membrane sandwich for the gene transfection in NIH 3T3 fibroblasts cell model. With such nanopore devices, a much lower voltage is required to "penetrate" the cell membrane for the gene delivery compared to traditional BEP, which ultimately results in high cell viability after transfection. By confocal microscopy, it was shown that the negative charged nucleic acid molecules were driven towards the cell membrane by the electric field during electroporation, which may further promote the entry of cargo across the negative charged cell membrane. As evidenced from the results showing successful delivery of large molecular weight cargos, such as gWiz GFP (5.7 kbp), the reported device has demonstrated the usefulness for genetic modification of genome in the nucleus.

Based on this concept, Xie *et al.* [44] designed a nanostraw electroporation system for the intracellular gene delivery. The system schematic is shown in Fig. **3**(**B**). Using the alumina atomic layer deposition (ALD) and directional reactive ion etch (RIE) technique, the alumina nanostraws were fabricated on the track-etched polycarbonate surface. The diameter of the nanostraw was determined by the nanopores of the membrane, and the length of the nanostraw was controlled by etching duration. The primary difference between this nanostraw design and other nanoporous devices lies in that it can penetrate and deliver the cargo into the cells through the nanostraw without any electric field. The nanostraw was directly inserted into the cells under the non-electroporation condition due to the tight contact to the cell membrane. Study showed that the nanostraws could automatically deliver the fluorescence dyes into the cells, though with low efficiency, when it was in close contact with the cells [42]. With the application of a low electric field (<20V), the gene materials could be easily delivered into the cells with high transfection efficiency through these nanostraws. For example, the transfection efficiency was dramatically increased from 20% to around 71% when the electric field was applied in plasmid electroporation. Besides of the high transfection efficiency, other advantages of this nanostraw electroporation method include the relatively high cell viability and basic dosage control. On the other hand, this method is dampened by the following drawbacks. Since the nanostraws are randomly distributed, it is impossible to locate individual cells to the same number of nanostraws, leading to the non-uniform transfection among cells. Moreover, it is difficult to manipulate suspended cells for transfection because the cells can not be attached to the nanostraws when they are moving in the solution.

3.2. Nanopillar Electroporation

Recently, Xie *et al.* [52] reported a nanopillar electroporation method which is able to detect the subtle changes in action potentials by electroporation-based drug delivery. For the fabrication of the nanopillar device, they passivated the Si wafer substrate surface with a 350 nm Si_3N_4/SiO_2 layer deposited by plasma-enhanced chemical vapor deposition. The nanoscale holes were created by a focused gallium ion beam through the insulation layer. The vertical nanopillar electrodes were grown inside these nano-scale holes from the bottom which is an FIB-assisted platinum. The HL-1 cells, a mouse cardiac muscle cell line, were cultured on the nanopillar electrodes with spontaneous beating, suggesting the good biocompatibility of the electrodes. Because of the tiny tip of the electrodes

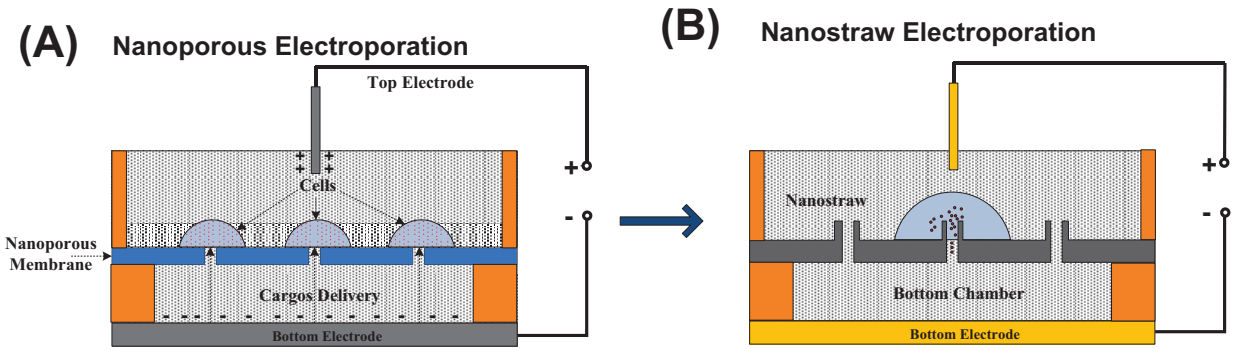

Fig. (3). Cross-section schematics of (**A**) nanoporous electroporation and (**B**) nanostraw electroporation. Both concepts are realized on commercial track-etched membrane with randomly distributed pores, while the nanostraw promotes the contact between the nanochannel and cell membrane by protruding for nanopore formation.

and its close contact with the cell membrane, a small voltage was good enough to produce a strong electric field near the electrode-cell connection, thus increased the cell membrane permeability to the gene materials. It was confirmed when they successfully delivered the membrane-impermeable calcein dye into the cells. Compared with other nanoscale electroporation methods, the nanopillar method provides a high throughput with minimal invasiveness for biomolecular delivery. Combined with its long-term measurement, high sensitivity, and array character, it would be of great help for drug screening.

Later on, based on the similar concept, the same group reported a nanosponge electroporation device to achieve high efficiency and affordable water disinfection [53]. The nanosponge was made of polyurethane sponge coated with carbon nanotube and silver nanowire. The electroporation magnitude was 2-3 orders lower than the BEP while the transfection efficiency was much higher in nanosponge electroporation. In their experiment, PI dye was efficient targeted into four different bacteria. This nanosponge electroporation system shares the similar performance to nanopillar electroporation in that it is still limited to the single cell level electroporation for certain adhesive cells. More powerful and efficient cell loading techniques are required for positioning more cells onto pillars.

3.3. Nanofountain Probe Electroporation

Almost all the micro-/nano-electroporation systems need to position the cells precisely inside the electric field before electroporation. During the cell positioning process, the microenvironment around the cells would be affected. For MEP, the electroporation solution with cells is injected into microchannels, and the cells inside are located into the microchamber by either a flow force or other cell manipulation techniques [54]. In nanostraw and nanopillar electroporation, the adherent cells have to be trypsinized from their original culture environment. Few studies have evaluated the effect of sudden change in the extracellular environment on cellular behavior, which may eventually affect the outcome of transfection [55]. Espinosa *et al.* [56] designed a new tool for single cell transfection using nanofountain probe electroporation (NFP-E). As shown in Fig. **4**, a cantilevered probe, which was connected to a micro-reservoir full of gene materials to be delivered, was used as the tip for local electroporation. While the hollow atomic force microscope (AFM) tip is usually equipped in dip-pen nanolithography for patterning proteins and other materials with submicron resolution, it was controlled by a micromanipulator or by the AFM stage to localize the cell in this NFP-E device. With the application of a voltage between the tip and the cell, the cell close to the tip was permeabilized by the electric field, and the cargo inside the tip was delivered into the cell. The size range of the cargo delivered by this method was broad, ranging from the GAPDH molecular beacon which is only 30 base pairs to GFP pDNA which is around 5 kbp. The ability of precise and close positioning of the tip to the cell

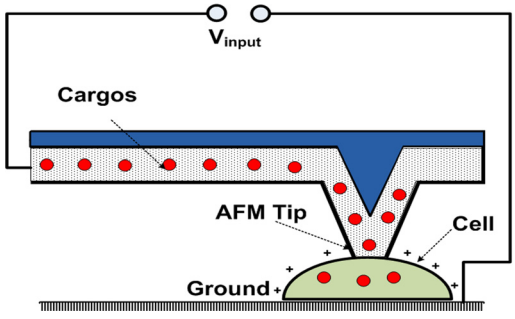

Fig. (4). The simplified schematics of nanofountain probe electroporation. In principle, a hollow micro-tip with a nanoscale mouth is moved by AFM stage. By directly contacting single cell, cargos filled up in the microchannel inside the tip can be delivered into the cell through the tip-electroporation pathway.

resulted in a low input voltage needed for cell transfection. Meanwhile, the constant microenvironment around the cell was not affected during the electroporation process. Compared to the variable efficiency and low cell viability achieved by BEP/MEP, NFP-E could offer high transfection efficiency and cell viability (>95% and >92%, respectively). Moreover, it also allowed sing cell selectivity and qualitative dosage control for gene delivery. However, there are some drawbacks compared to nanopillar or nanostraw electroporation methods. The major concern is that it is only suitable for adhesive cell transfection where the cells are not moving. This greatly limits its application in suspension cells. Secondly, it is currently incapable of handling multiple cells at the same time. Gaps still remain before its application into practical use.

3.4. Nanochannel Electroporation

As been discussed above, the micro-electroporation typically positions and handles single cell within microenvironment in the microscale. It offers a satisfied balance between transfection efficiency and cell viability by confining the electric field to the small micro-chamber/channel while the total voltage applied to the system is dramatically reduced (usually less than 10 V compared to ~1, 000 V in BEP). However, similar to BEP, MEP is difficult to achieve high uniformity and efficiency due to its channel size which is comparable to the cell size. For large molecules, such as plasmids with molecular weight greater than 7-8 kbp, their delivery into the target cells by low-voltage electroporation would be ineffective. Because the delivery is based on molecular diffusion, neither BEP nor MEP can achieve the precise dose control, which may be critical for the application of gene delivery in clinic uses.

Recently, our group invented a novel nanochannel electroporation (NCEP) device that is able to deliver the cargo (including pDNAs, siRNAs, and ODNs) into living cells with precise dose control [50]. Fig. **5** shows the basic phase contrast image of the NCEP chip (Fig. **5**(**A**)) and an evidence of efficient and uniform transfection of individual cells with GFP plasmids via different nanochannels (Fig. **5**(**B**)). The fabricated 2D NCEP device has a micro-nano-micro-channel configuration. It starts with the combing of a DNA nanowire over two micro-ridges, followed by contact imprinting. The last step for the fabrication is the connection of a nanochannel (~100 nm in diameter and 3-5 μm in length) to two microchannels (30 μm in height and 20-30 μm in width). Before electroporation, one single cell in the physiological medium is attached against one side of the nanochannel by optical tweezers. The other side connected with this nanochannel is a microchannel that is loaded with cargo to be delivered into the cell. Due to the high resistance of the nanochannel, a relatively high voltage pulse, typically a 100-250 V square pulse with several milliseconds dura-

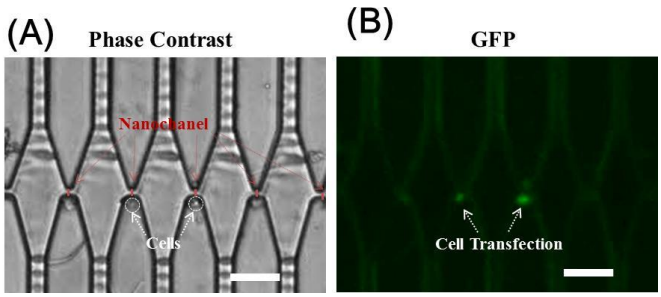

Fig. (5). Nanochannel electroporation for precise delivery of GFP into cells. (**A**) The phase contrast image shows the nanochannel electroporation with micro-nano-micro-channel design. Cells (labeled with white circle) were localized in the proximity of one end of nanochannels (labeled with red lines). GFP plasmids were placed at the other end. (**B**) Cells were successfully delivered with GFP 12 hours after NCEP, showing green fluorescence with uniform intensity. Scale bar: 25 μm.

tion, is required to generate strong enough electric field for electroporation. Compared to BEP or MEP where the cells are completely exposed to a strong electric field, only a very small area of the entire cell surface is affected by the electric field in NCEP. The tiny pore on the cell membrane generated by the strong electric field allows the instantaneous injection of the gene material into the cells by electrophoresis during the electroporation process. Our data shows that the "active injection" process was completed during cell poration when we performed the NCEP in K562 cells using propidium iodide as the model cargo. In contrast, we observed a gradual increase of fluorescence inside the cell over a 150 second period in both BEP and MEP experiments, suggesting the diffusion of the cargo into the cells in a long time duration after electroporation in the latter experiments.

The major advantage of NCEP is the capability for precise dosage control in gene delivery. Using Mcl-1 siRNA as the model cargo, a clear threshold of apoptosis by fluorescence staining was observed in K562 cells by NCEP while the pulse duration was increasing from 220V/1ms to 220V/5ms. By changing the pulse duration, NCEP could deliver the exact amount of gene material into the target cells. This inspires the feasibility of employing the NCEP to manipulate the gene expression at the single cell level. Furthermore, the large size cargo, such as plasmids with molecular weight greater than 7-8 kbp, can also be easily delivered into the cells by NCEP. With NCEP, the OSKM plasmid, with a molecular size of 13 kbp, was successful delivered into mouse embryonic fibroblast cells for reprogramming the somatic cells into induced pluripotent stem cells (iPSCs) with minimal cell damage [57]. Uniformity study by fluorescence intensity also showed that NCEP can provide quite homogeneous transfection efficiency for handling large size cargo. Overall, our NCEP technique provides a highly effective and cell benign platform over BEP or NCEP techniques for gene transfection with high efficiency.

4. FUTURE PERSPECTIVES

The detailed Pros and Cons of different electroporation methods mentioned in this review are summarized in Table **1**. One of the major difficulties encountered in applying micro-/nano-electroporation systems from bench to bedside lies in their inability to handle a large number of cells. While current commercialized BEP systems are able to transfect millions of cells at the same time, most micro-/nano-electroporation systems are limited to single cell to hundreds cells. For rare cells or highly proliferative cells such as circulating tumor cells, pluripotent stem cells and tumor cells, MEP/NEP for intracellular biomarker detection or gene therapy could be beneficial to reveal the fundamentals in lab, yet in most cases, a much larger cell population is needed for practical use. Hence, a high throughput MEP/NEP system is of great interest for future gene delivery. Comparing to viral transduction with potential bio-hazard, the combination of high throughput and transfection efficiency would make MEP/NEP ideal for gene therapy.

Over the past 10 years, numerous efforts have been made to achieve the high-throughput MEP/NEP for gene delivery, and most of which are based on following two prototypes: a) sequential flow-through and b) synchronic 3D electroporation. Generally, the flow-through devices for electroporation have microfluidic and planar microchannel patterns. A module for positioning the cells at precise regions, including magnetic tweezers [58], optical tweezers, mechanical/hydrodynamics [59], among many others, is required for the electroporation system [13, 47, 48]. Compared to the widely used BEP which can transfect about millions of cells by one shot, the MEP/NEP methods need the integration of each functional module. Different from flow-through concept which applies electroporation sequentially, the synchronic 3D electroporation parallelly transfect thousands of cells on the micro-/nano-porous chip. The key of achieving synchronic 3D electroporation, the fabrication of nanoporous membrane electroporation and nanostraw electroporation, have been applied in gene delivery by several groups. However, there are some unsolved problems with current 3D electroporation models. The primary challenge in applying the 3D models to electroporation is its relatively low transfection efficiency. The difficulty for fabricating well-distributed pores in nano scale on the membrane causes an inefficient and insufficient alignment of cells to the pores which highly decrease the transfection efficiency. This

Table 1. The Pros and Cons of different electroporation method.

Method		Description	Pros	Cons
Bulk Electroporation		A large number of cells were applied with high-voltage electric field causing cell membrane temporal leakage.	Stochastic transfection Significant cell damage	Stochastic transfection Significant cell damage
MEP Electroporation		Place each single-cell near to microchannel, and add electric field across the cell and microchannel	Focused electric field allowing membrane poration under a low-voltage High cell viability	Limited dosage control Low efficiency for delivering large weight molecules.
Nano Electroporation	Nanostraw	Nanostraws directly contact cells. With electric field, gene materials are delivered into cytosol.	High viability and basic dosage control	Low sealing for suspension cells, non-uniformity due to random distribution
	Nanopillar	A strong electric field near the electrode-cell connection on nanopillar delivers gene materials.	High viability, high throughput and measurement for cellular potential.	Complicated fabrication.
	Nanofountain	A hollow tip with a nanoscale opening touch to single cell and transfer the cargo into cell by electric field.	High viability and high efficiency.	Low throughput, only suitable for adhesion cells.
	Nanochannel	By a high resistance of nanochannel connecting to cell, a strong electric field was applied directly for electroporation.	High viability, dosage control and high efficiency.	Limited throughput, and Complicated fabrication.

also leads to the difficulty in precise dosage control for gene delivery. Thus, new cell manipulation technologies for large cell population on 3D electroporation chips are essential.

Based on its excellent dosage control for gene delivery, scaling the current 2D NCEP up to 3D NCEP will be of great significance for high-throughput cell transfection [2, 59]. In addition to the cell loading issues, another major challenge is how to fabricate a large number of nanochannels with high quality and low-cost for precise dose control and clinical use. The application of MEP/NEP in gene delivery is also hindered due to the difficulty of gene drugs to reach the target at the cellular lever after electroporation. Upon entering into the cytosol after MEP/NEP, the gene materials must be further transported to specific targets. For example, only less than 1% of the pDNAs delivered into cytosol are further moved into nucleus while more than 99% are destroyed by enzymatic degradation in the cytosol. Even after its successful delivery to specific target, another problem is that how long and stable the delivered gene material could function in the target cells. Current gene delivery and expression by electroporation is transient, which only lasts for a short period of time and diminishes quickly during cell division. In comparison, gene delivery by viral transduction has a much longer duration due to its higher percentage of chromosomal insertion. It can largely bypass the latticework of the cytoskeleton and endoplasmic reticulum, and escape the enzymatic degradation in the cytosol. For example, adeno-associated viruses can produce moderate gene regulation in both non-dividing and dividing cells, and the retrovirus-mediated gene transfection can produce long-lasting gene regulation in dividing cells due to its strong incorporation ability into host cell genome [60, 61]. Moreover, gene delivery by lentivirus can insert its viral RNA into the host cell genome by reverse transcription in both non-dividing and dividing cells [62]. Therefore, one of the future tasks for the MEP/NEP based gene therapy is to obtain highly stable nucleus targeting plasmid cargos with minimum cytosol degradation, thus to result in a robust gene regulation effect.

In summary, non-viral transfection technologies such as MEP and NEP for gene delivery are developing rapidly. Further improvement of MEP/NEP methods for specific target transfection into cellular nucleus will not only benefit the stable clone generating, but also broad the application of MEP/NEP in gene therapy from bench to bedside.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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REFERENCES

[1] Miake J, Marban E, Nuss HB. Gene therapy - Biological pacemaker created by gene transfer. Nature 2002; 419: 132-3.

[2] Chang L, Bertani P, Gallego-Perez D, *et al*. 3D nanochannel electroporation for high-throughput cell transfection with dosage control and high uniformity. Nanoscale 2015; DOI: 10.1039/C5NR03187G.

[3] Xie J, Teng L, Yang Z, *et al.*. A polyethylenimine-linoleic acid conjugate for antisense oligonucleotide delivery. Biomed Res Int 2013; 2013: 710502.

[4] Kanazawa T, Akiyama F, Kakizaki S, Takashima Y, Seta Y. Delivery of siRNA to the brain using a combination of nose-to-brain delivery and cell-penetrating peptide-modified nano-micelles. Biomaterials 2013; 34: 9220-6.

[5] Ragelle H, Vandermeulen G, Preat V. Chitosan-based siRNA delivery systems. J Control Release 2013; 172: 207-18.

[6] Kanasty R, Dorkin JR, Vegas A, Anderson D. Delivery materials for siRNA therapeutics. Nat Mater 2013; 12: 967-77.

[7] Tabernero J, Shapiro GI, LoRusso PM, *et al*. First-in-humans trial of an RNA interference therapeutic targeting VEGF and KSP in cancer patients with liver involvement. Cancer Discov 2013; 3: 406-17.

[8] Maguire AM, Simonelli F, Pierce EA, *et al*. Safety and efficacy of gene transfer for Leber's congenital amaurosis. N Engl J Med 2008; 358: 2240-8.

[9] Palfi S, Gurruchaga JM, Ralph GS, *et al*. Long-term safety and tolerability of ProSavin, a lentiviral vector-based gene therapy for Parkinson's disease: a dose escalation, open-label, phase 1/2 trial. Lancet 2014; 383: 1138-46.

[10] Yang Z, Yu B, Zhu J, *et al*. A microfluidic method to synthesize transferrin-lipid nanoparticles loaded with siRNA LOR-1284 for therapy of acute myeloid leukemia. Nanoscale 2014; 6: 9742-51.

[11] Young JL, Dean DA. Electroporation-mediated gene delivery. Adv Genet 2015; 89: 49-88.

[12] Sallberg M, Frelin L, Ahlen G, Sallberg-Chen M. Electroporation for therapeutic DNA vaccination in patients. Med Microbiol Immunol 2015; 204: 131-5.

[13] Sioud M. Cytoplasmic delivery of siRNAs to monocytes and dendritic cells via electroporation. Methods Mol Biol 2015; 1218: 107-15.

[14] Fox MB, Esveld DC, Valero A, *et al*. Electroporation of cells in microfluidic devices: a review. Anal Bioanal Chem 2006; 385: 474-85.

[15] Lee WG, Demirci U, Khademhosseini A. Microscale electroporation: challenges and perspectives for clinical applications. Integr Biol (Camb) 2009; 1: 242-51.

[16] Gabriel B, Teissie J. Direct observation in the millisecond time range of fluorescent molecule asymmetrical interaction with the electropermeabilized cell membrane. Biophys J 1997; 73: 2630-7.

[17] Bertani P, Chang L, Gallego-Perez D, *et al*. Bosch etching for the creation of a 3D nano-electroporation system. J Vacuum Sci Technol B 2015; 33: 06F903.

[18] Movahed S, Bazargan-Lari Y, Daneshmad F, Mashhoodi M. Numerical modeling of bi-polar (AC) pulse electroporation of single cell in microchannel to create nanopores on its membrane. J Membr Biol 2014; 247: 1229-37.

[19] Nishikawa S, Hirashima N, Tanaka M. Optimization of single-cell electroporation protocol for forced gene expression in primary neuronal cultures. Mol Biotechnol 2014; 56: 824-32.

[20] Mehier-Humbert S, Guy RH. Physical methods for gene transfer: improving the kinetics of gene delivery into cells. Adv Drug Deliv Rev 2005; 57: 733-53.

[21] Geng T, Lu C. Microfluidic electroporation for cellular analysis and delivery. Lab on a Chip 2013; 13: 3803-21.

[22] Wang S, Lee LJ. Micro-/nanofluidics based cell electroporation. Biomicrofluidics 2013; 7: 11301.

[23] Yan L, Zhang J, Lee CS, Chen X. Micro- and nanotechnologies for intracellular delivery. Small 2014; 10: 4487-504.

[24] Huang Y, Rubinsky B. Micro-Electroporation: Improving the Efficiency and Understanding of Electrical Permeabilization of Cells. Biomed Microdevices 1999; 2: 145-50.

[25] Valero A, Post JN, van Nieuwkasteele JW, Ter Braak PM, Kruijer W, van den Berg A. Gene transfer and protein dynamics in stem cells using single cell electroporation in a microfluidic device. Lab on a Chip 2008; 8: 62-7.

[26] Boukany PE, Morss A, Liao WC, *et al*. Nanochannel electroporation delivers precise amounts of biomolecules into living cells. Nat Nanotechnol 2011; 6: 747-54.

[27] Bamford RA, Zhao ZY, Hotchin NA, *et al*. Electroporation and microinjection successfully deliver single-stranded and duplex DNA into live cells as detected by FRET measurements. PLoS One 2014; 9: e95097.

[28] Lin YC, Jen CM, Huang MY, Wu CY, Lin XZ. Electroporation microchips for continuous gene transfection. Sens Actuat B-Chem 2001; 79: 137-43.

[29] Zhan Y, Wang J, Bao N, Lu C. Electroporation of cells in microfluidic droplets. Anal Chem 2009; 81: 2027-31.

[30] Fujimoto H, Kato K, Iwata H. Layer-by-layer assembly of small interfering RNA and poly(ethyleneimine) for substrate-mediated electroporation with high efficiency. Anal Bioanal Chem 2010; 397: 571-8.

[31] Miyano N, Inoue Y, Teramura Y, *et al*. Gene transfer device utilizing micron-spiked electrodes produced by the self-organization phenomenon of Fe-alloy. Lab Chip 2008; 8: 1104-9.

[32] Xu Y, Lu Y, Xing W. An individually addressable suspended-drop electroporation system for high-throughput cell transfection. Lab Chip 2014; 14: 686-90.

[33] Jokilaakso N, Salm E, Chen A, *et al*. Ultra-localized single cell electroporation using silicon nanowires. Lab Chip 2013; 13: 336-9.

[34] Park S, Bassat DB, Yossifon G. Individually addressable multi-chamber electroporation platform with dielectrophoresis and alternating-current-electro-osmosis assisted cell positioning. Biomicrofluidics 2014; 8: 024117.

[35] Adeyanju OO, Al-Angari HM, Sahakian AV. The optimization of needle electrode number and placement for irreversible electroporation of hepatocellular carcinoma. Radiol Oncol 2012; 46: 126-35.

[36] Wasungu L, Escoffre JM, Valette A, Teissie J, Rols MP. A 3D *in vitro* spheroid model as a way to study the mechanisms of electroporation. Int J Pharm 2009; 379: 278-84.

[37] Koester PJ, Tautorat C, Beikirch H, Gimsa J, Baumann W. Recording electric potentials from single adherent cells with 3D microelectrode arrays after local electroporation. Biosens Bioelectron 2010; 26: 1731-5.

[38] Movahed S, Li D. A theoretical study of single-cell electroporation in a microchannel. J Membr Biol 2013; 246: 151-60.

[39] Zhou W, Xiong Z, Liu Y, Yao C, Li C. Low voltage irreversible electroporation induced apoptosis in HeLa cells. J Cancer Res Ther 2012; 8: 80-5.

[40] Huang Y, Rubinsky B. Microfabricated electroporation chip for single cell membrane permeabilization. Sens Actuat A 2001; 89: 242-9.

[41] Khine M, Ionescu-Zanetti C, Blatz A, Wang LP, Lee LP. Single-cell electroporation arrays with real-time monitoring and feedback control. Lab Chip 2007; 7: 457-62.

[42] Fei Z, Wang S, Xie Y, Henslee BE, Koh CG, Lee LJ. Gene transfection of mammalian cells using membrane sandwich electroporation. Anal Chem 2007; 79: 5719-22.

[43] Fei Z, Hu X, Choi HW, Wang S, Farson D, Lee LJ. Micronozzle array enhanced sandwich electroporation of embryonic stem cells. Anal Chem 2010; 82: 353-8.

[44] Wu Y, Li L, Mao Y, Lee LJ. Static micromixer-coaxial electrospray synthesis of theranostic lipoplexes. ACS Nano 2012; 6: 2245-52.

[45] Varkouhi AK, Foillard S, Lammers T, *et al*. SiRNA delivery with functionalized carbon nanotubes. Int J Pharm 2011; 416: 419-25.

[46] Neagoe IB, Braicu C, Matea C, *et al*. Efficient siRNA delivery system using carboxilated single-wall carbon nanotubes in cancer treatment. J Biomed Nanotechnol 2012; 8: 567-74.

[47] Wong IY, Bhatia SN, Toner M. Nanotechnology: emerging tools for biology and medicine. Genes Dev 2013; 27: 2397-408.

[48] Chen F, Jiang X, Kuang T, *et al*. Effect of nanoporous structure and polymer brushes on the ionic conductivity of poly(methacrylic acid)/anode aluminum oxide hybrid membrane. RSC Adv 2015; 5: 70204-10.

[49] Zhou C, Yang Z, Teng L. Nanomedicine based on nucleic acids: pharmacokinetic and pharmacodynamic perspectives. Curr Pharm Biotechnol 2014; 15: 829-38.

[50] Wang J, Su MY, Qi JQ, Chang LQ. Sensitivity and complex impedance of nanometer zirconia thick film humidity sensors. Sens Actuat B-Chem 2009; 139: 418-24.

[51] Chang LQ, Liu CX, He YZ, Xiao HH, Cai XX. `Small-volume solution current-time behavior study for application in reverse iontophoresis-based non-invasive blood glucose monitoring. Sci China-Chem 2011; 54: 223-230.

[52] Xie C, Lin Z, Hanson L, Cui Y, Cui B. Intracellular recording of action potentials by nanopillar electroporation. Nat Nanotechnol 2012; 7: 185-90.

[53] Liu C, Xie X, Zhao W, *et al*. Conducting nanosponge electroporation for affordable and high-efficiency disinfection of bacteria and viruses in water. Nano Lett 2013; 13: 4288-93.

[54] Lindstrom S, Andersson-Svahn H. Overview of single-cell analyses: microdevices and applications. Lab Chip 2010; 10: 3363-72.

[55] Przybyla L, Voldman J. Probing embryonic stem cell autocrine and paracrine signaling using microfluidics. Annu Rev Anal Chem (Palo Alto Calif) 2012; 5: 293-315.

[56] Kang W, Yavari F, Minary-Jolandan M, *et al*. Nanofountain probe electroporation (NFP-E) of single cells. Nano Lett 2013; 13: 2448-57.

[57] Gao K, Li L, He L, *et al*. Design of a microchannel-nanochannel-microchannel array based nanoelectroporation system for precise gene transfection. Small 2014; 10: 1015-23.

[58] Chang L, Howdyshell M, Liao WC, *et al*. Magnetic tweezers-based 3D microchannel electroporation for high-throughput gene transfection in living cells. Small 2015; 11: 1818-28.

[59] Chang L, Gallego-Perez D, Zhao X, *et al*. Dielectrophoresis-assisted 3D nanoelectroporation for non-viral cell transfection in adoptive immunotherapy. Lab Chip 2015; 15: 3147-53.

[60] Liu Y, Joo KI, Wang P. Endocytic processing of adeno-associated virus type 8 vectors for transduction of target cells. Gene Ther 2013; 20: 308-17.

[61] Liu Y, Fang Y, Zhou Y, *et al*. Site-specific modification of adeno-associated viruses via a genetically engineered aldehyde tag. Small 2013; 9: 421-9.

[62] Schagen FH, Rademaker HJ, Fallaux FJ, Hoeben RC. Insertion vectors for gene therapy. Gene Ther 2000; 7: 271-2.

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