Synergistic effect of electrical and chemical factors on endocytosis in micro-discharge plasma gene transfection

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Abstract

We have developed a new micro-discharge plasma (MDP)-based gene transfection method, which transfers genes into cells with high efficiency and low cytotoxicity; however, the mechanism underlying the method is still unknown. Studies revealed that the N-acetylcysteine-mediated inhibition of reactive oxygen species (ROS) activity completely abolished gene transfer. In this study, we used laser-produced plasma to demonstrate that gene transfer does not occur in the absence of electrical factors. Our results show that both electrical and chemical factors are necessary for gene transfer inside cells by microplasma irradiation. This indicates that plasma-mediated gene transfection utilizes the synergy between electrical and chemical factors. The electric field threshold required for transfection was approximately 1 kV m\(^{-1}\) in our MDP system. This indicates that MDP irradiation supplies sufficient concentrations of ROS, and the stimulation intensity of the electric field determines the transfection efficiency in our system. Gene transfer by plasma irradiation depends mainly on endocytosis, which accounts for at least 80% of the transfer, and clathrin-mediated endocytosis is a dominant endocytosis. In plasma-mediated gene transfection, alterations in electrical and chemical factors can independently regulate plasmid DNA adhesion and triggering of endocytosis, respectively. This implies that plasma characteristics can be adjusted according to target cell requirements, and the transfection process can be optimized with minimum damage to cells and maximum efficiency. This may explain how MDP simultaneously achieves high transfection efficiency with minimal cell damage.

Keywords: plasma medicine, plasma gene transfection, synergistic effect, endocytosis

1. Introduction

Gene transfection is a technique of introducing nucleic acids into cells for characterization of gene function. It is a fundamental tool used in medicine and biology, such as in gene therapy, regenerative medicine, drug development, and plant breeding [1–5], and therefore, it has to be efficient and practically feasible. Traditional gene transfection methods are of three major types, namely, physical, chemical, and biological. In electroporation, a physical method, genes are transferred into cells using high electric field pulse [6], and it is commonly used because of the short treatment time and high efficiency; however, this technique damages the target cells. Lipofection is a chemical method, in which a cationic lipid is employed to mediate the transfer of genes in the target cells [7]. This method requires no special equipment. However, the reagents are expensive and may be toxic to certain target cells.
Among biological methods, viral vectors are used for gene delivery with high transfection efficiency [8], and can be used for in vivo applications. However, this method is associated with the risk of pathogenic expression and neoplastic transformation [9]. Therefore, new methods for gene transfer, which are free from adverse side effects on target cells, are required for medical and biological applications.

The past decades have seen dramatic progress in the generation and regulation of atmospheric non-equilibrium plasmas (ANEP) and accumulation of data pertaining to the measurement and simulation of plasma parameters such as electron density, electron temperature, etc [10–19]. The application area of ANEP has expanded to include medical and biological fields. In 2002, Miyoshi et al invented a novel gene transfection method using plasma irradiation [20], which was later published by Ogawa et al [21] and Sakai et al [22]. Subsequently, gene/molecule transfection using a variety of plasma sources have been reported [23–31]. However, the mechanism of transfection is not yet clear. Similar to Ogawa et al, we examined various plasma sources, and found that micro-discharge plasma (MDP) can simultaneously achieve high transfection efficiency and cell viability [32].

Previously, we evaluated the role of clathrin-mediated endocytosis in plasma gene transfection, which was estimated to be 60% using the clathrin inhibitor (Pitstop 2-100: Abcam, Cambridge, UK) [33]. However, other clathrin-independent endocytosis pathways such as the caveolae-mediated and lipid raft-dependent endocytosis pathways exist. The contribution of these endocytosis pathways to plasma gene transfection has not yet been evaluated. We also found that there is a synergy between electrical and chemical factors during plasma gene transfection (figure 1) [33]. We also examined that transfection efficiency by plasma irradiation depends on the introduced molecular weight, and this suggests that the transfection mechanism changes according to the molecular weight [34].

In this study, we further investigated the above-mentioned synergistic effect and the endocytosis mechanism involved in MDP gene transfection. Specifically, we evaluated the roles of certain clathrin-independent endocytosis pathways and reactive oxygen species (ROS) (chemical factors) in microplasma-based gene transfer.

Figure 1. Schematic diagram of factors generated by micro-discharge plasma, their effects, and processes for gene/molecule transfection.

Figure 2. Schematic of the micro-discharge plasma-mediated gene transfection system.

2. Material and methods

Figure 2 shows the MDP irradiation system for plasma gene transfection. A thin copper capillary with 70 μm outer diameter was employed as a high voltage (HV) electrode. A grounded (GND) copper plate was used as the counter electrode and was placed under a 96-well microtiter plate. The target cells were seeded at the bottom of each well, and the buffer solution containing the plasmid DNA was placed in each well prior to plasma irradiation. The distance between the tip of the capillary electrode and the surface of the cell mixture was 1 mm. The applied voltage waveform was a 20 kHz sinusoidal wave with an inter-peak amplitude of 15 kV. A microplasma was generated at the tip of the capillary electrode. The plasma irradiation time was 5 ms.

The effect of reactive species (RS) (chemical factors) such as reactive nitrogen species (RNS) and ROS on gene transfection was evaluated after deactivating the electrical factors such as electric field, electric charge, and current. We introduced a laser-produced plasma (LPP) to supply similar concentrations of RS to the cells as obtained with MDP in the absence of electrical factors. An LPP was generated when an intense laser beam was focused in the air to trigger a breakdown.

In the case of MDP irradiation, the plasma column was attached to the buffer solution to ensure that electrical charges were supplied and the current flowed in the solution. On the contrary, the LPP was generated at a position that was outside the solution level. Since the mean free path of the electron is 0.1 μm under atmospheric pressure, electrons and ions disappeared before they could arrive at the buffer solution, and therefore, current did not flow in the solution. The plasma also shielded the electric field such that no electric field was applied to the solution by LPP irradiation. Therefore, in the absence of electrical factors, we used the LPP to obtain concentrations of chemical species that were similar to those obtained with MDP irradiation.

A schematic of the LPP irradiation system is shown in figure 3. A laser beam of the pulsed Nd:YAG laser (Quantra-Ray PRO 230: Spectra-Physics, Santa Clara, CA) at the
second harmonic wavelength of 532 nm was focused 5.2 mm above the target cell mixture. The diameter of the LPP was about 0.25 mm, which was estimated from the burn pattern on thermal paper exposed to the LPP. The pulse width and the repetition frequency of the laser beam were 10 ns and 10 Hz, respectively. The laser power was 33 mJ/pulse and the number of laser pulses was three.

The gap length and the treatment time for the LPP were determined to generate concentrations of RS in the buffer solution similar to those observed for MDP irradiation. Since more than half of the transferred genes were transported into the target cells long after the plasma irradiation [35], the contributions of long-lived RS were important in our gene transfection system. The concentrations of H₂O₂ (ROS with long life span) and NO₂⁻ (RNS with long life span) in the buffer solution were evaluated using an absorption spectrometer (V-670: Jasco, Hachioji, Japan) at 540 nm following reactions with 4-aminoantipyrine for H₂O₂ and with naphthyl ethylenediamine for NO₂⁻ using commercially available kits (WAK-H2O2 and WAK-NO2: Kyoritsu Chemical-Check Lab, Tokyo, Japan).

For LPP irradiation, the gap length was adjusted to 5.2 mm to obtain concentrations of H₂O₂ and NO₂⁻ similar to that obtained for MDP irradiation. Since the solution volume for gene transfection (6 μl) was not enough for measuring the absorbance, larger solution volumes and longer LPP treatment were required. Solutions of 2.00, 3.00 and 4.80 ml were subjected to 500 laser pulses with a gap length of 5.2 mm to confirm the link between solution volume and concentration. The concentrations of H₂O₂ (c₄H₈O₇) and NO₂⁻ (cNO₂⁻) were proportional to the inverse of the volume. Concentrations in a fixed volume (500 ml) were measured for different numbers of laser pulses (nLaser = 125, 250 and 500 pulses) to confirm the relationship between the number of laser pulses and concentration. The concentrations were proportional to the number of laser pulses. From these results experimental equations were derived as cH₂O₂ = 1.79 ng ⋅ nLaser/v and cNO₂⁻ = 2.20 ng ⋅ nLaser/v.

For MDP irradiation, 1.35, 2.70 and 5.40 ml solutions were exposed to the MDP, and the exposure times for each solution were proportional to the solution volume (75, 150 and 300 exposures of 5 ms each for 1.35, 2.70 and 5.40 ml, respectively). Almost similar concentrations were obtained under these three conditions and the experimental equations were derived as c₄H₈O₇ = 1.14 μg s⁻¹ ⋅ t/v and cNO₂⁻ = 1.11 μg s⁻¹ ⋅ t/v, where t is the plasma exposure time.

Finally, under the condition of MDP irradiation for maximum gene transfection efficiency, the concentrations of H₂O₂ and NO₂⁻ obtained were 0.95 mg l⁻¹ and 0.92 mg l⁻¹, respectively, which was similar to that obtained by LPP irradiation (0.90 mg l⁻¹ for H₂O₂ and 1.1 mg l⁻¹ for NO₂⁻) of a 6 μl solution by three laser pulses.

Mouse L-929 fibroblast cells (RCB1422: RIKEN BRC, Tsukuba, Japan) were seeded in a 96-well microtiter plate and a micro-slide chamber. The former was used for MDP irradiation and the latter for LPP irradiation. The cells were incubated with 100 μl culture medium for more than 24 h until the number of cells reached semi-confluence at the ambient temperature of 37 °C and a CO₂ concentration of 5%. Prior to plasma irradiation, the culture medium was aspirated and 6 μg of the pAcGFP1-N1 plasmid (Clontech, Mountain View, CA) harboring the AcGFP gene suspended in 6 μl TE/PBS buffer was added to each well. Each well was subjected to MDP irradiation for 5 ms with a discharge gap length of 1 mm (n = 6: six wells were irradiated for 5 ms per each identical experimental condition with or without inhibitors) or to LPP irradiation for three shots with a gap length of 5.2 mm (n = 4: four wells were irradiated per each identical experimental condition with or without inhibitors). Hundred microliters culture medium was added to the cells after the irradiation. After a 48 h incubation period, the number of green fluorescent cells and surviving cells stained with Hoechst 33342 (H1399: Life Technologies, Carlsbad, CA) were counted with an imaging cytomter (Cytell: GE Healthcare UK, Little Chalfont, UK).

The following three reagents were used to evaluate the contribution of endocytosis to transfection: Pitstop 2-100 (ab144650: Abcam plc, Cambridge, UK), Pitstop 2-100-negative control (ab144658) and methyl-β-cyclodextrin (M/CD, 320-84252: Wako, Osaka, Japan). Pitstop 2-100 inhibits clathrin-mediated endocytosis, while M/CD inhibits clathrin-mediated, caveolae-mediated, and lipid raft-dependent endocytosis [36]. Each reagent was dissolved in serum-free medium. The target cells were incubated with each of the reagents at 37 °C for 10 min before plasma treatment. The concentrations of Pitstop 2-100 and Pitstop 2-100-negative control were 25 μM each, and that of M/CD was 10 mM. After incubation, the medium was aspirated and 6 μg plasmid DNA was added to each well. Then, the target cells and plasmid DNA were subjected to MDP irradiation, following the protocol mentioned above. For ROS inhibition, 20 mM of N-acetylcysteine (NAC) was mixed with the plasmid DNA solution and added to each well before plasma irradiation.

To evaluate the effects of electrical factors in MDP irradiation (figure 1), the distribution of current flow at the bottom of the TE/PBS buffer solution (where the cells are present) was calculated using the finite element method of a commercial solver (COMSOL Multiphysics: COMSOL AB, Stockholm, Sweden). The electric field was determined from the voltage drop, as ascertained by measuring the solution conductivity (0.376 S m⁻¹). The geometry and boundary conditions used in the calculation are shown in figure 4. A sinusoidal voltage (amplitude of 7.5 kV and frequency of 20 kHz) was applied to the HV electrode and the bottom of the 96-well plate was GND. To simplify the model, the
plasma was substituted by a conductor with uniform conductivity of \( \sigma_p = 1.6 \, S \, m^{-1} \), which was derived from 
\[
\sigma_p = e n_e \mu_e,
\]
where \( e \) is electron charge, \( n_e \) is electron density of \( 10^{20} \, m^{-3} \) [19], \( \mu_e \) is electron mobility of \( 0.1 \, m^2 \, V^{-1} \, s^{-1} \). The electron mobility was derived from a typical electron drift 
velocity of \( 10^3 \, m \, s^{-1} \) [37] divided by the typical reduced electric field of the positive column of \( 10^{-7} \, V \, m^{-1} \, Torr^{-1} \) (here, \( 13.2 \, V \, m^{-1} \, Torr^{-1} \times 760 \, Torr = 10^4 \, V \, m^{-1} \) was used) [38]. The plasma was represented as a circular truncated cone shape, with a top radius of 35 \( \mu m \), contacting the HV electrode, and a bottom radius of 0.5 mm. Since the bottom edge was chamfered with a curvature radius of 0.1 mm to suppress divergence, the area of the buffer solution in contact with the plasma had a radius of approximately 0.4 mm. The permittivity of the buffer solution and the well-bottom and wall were set at 80.4 (water) and 2.4 (polystyrene), respectively.

3. Results

Figure 5 shows fluorescent dark-field microscopic views, which were captured 48 h after plasma treatment without inhibitor (A), with Pitstop 2-100 (B), with Pitstop 2-100-negative control (C), and with M/CD (D). Areas with maximum transfection efficiency in each well were captured as images. The gene transfection efficiency was calculated by averaging five values taken at five different points in the same well (figure 6) and the normalized gene transfection efficiencies are shown in figure 7. The gene transfection efficiency decreased by 60% with Pitstop 2-100, whereas the efficiency did not change with Pitstop 2-100 (negative control), which is similar to the results of our previous study [33]. The gene transfection efficiency decreased by 80% with M/CD. As mentioned above, M/CD also inhibits clathrin-mediated endocytosis, and therefore, this 80% reduction in efficiency included clathrin-mediated endocytosis, which contributed to 60% of the transfection events. Consequently, these results showed that at least 80% of the MDP-mediated gene transfection depended on endocytosis.

We have already reported that catalase-mediated inhibition of hydrogen peroxide (\( H_2O_2 \)) reduces the gene transfection efficiency by 60%. In this study, NAC also inhibited other ROS, and therefore, we investigated their involvement in the transfection process. The addition of 100 mM NAC before plasma irradiation resulted in zero transfection with the MDP irradiation.

Next, we used LPP instead of MDP as a source of chemical factors in the mixture of cells and plasmid DNA in order to analyze the necessity of electrical factors for efficient transfection. \( H_2O_2 \) (1 mg l\(^{-1} \)), a major chemical factor, was also supplied to the mixture of cells and plasmid DNA instead of plasma treatment [34].

The gap length of the LPP and the solution was set at 5.2 mm and the treatment time was set at 0.3 s to obtain similar concentrations of RS in the buffer solution by the LPP and MDP treatments. The absorption spectroscopic method was used to evaluate the concentrations of \( H_2O_2 \) and \( NO_2^- \), and similar concentrations were obtained for the LPP treatment and MDP irradiation. Duplicate measurements for each species and each plasma treatment showed that the concentration of \( H_2O_2 \) was 0.95 mg l\(^{-1} \) and that of \( NO_2^- \) was 0.92 mg l\(^{-1} \) for MDP irradiation, whereas the concentrations of \( H_2O_2 \) and \( NO_2^- \) were 0.90 mg l\(^{-1} \) and 1.1 mg l\(^{-1} \), respectively, for the LPP treatment. We also measured the temperature of the plate bottom with a thermocouple and confirmed that the same temperature (22 °C) was maintained after the MDP and LPP treatment.

Thus, neither \( H_2O_2 \) supply at 1 mg l\(^{-1} \) without plasma treatment [34] nor the LPP treatment induced transfection.

4. Discussion

We investigated the contribution of endocytosis to MDP-mediated gene transfection and the results are summarized in figure 7. A specific clathrin-mediated endocytosis inhibitor (Pitstop2) and a non-specific endocytosis inhibitor (M/CD) suppressed the gene transfection efficiency by 60% and 80%, respectively. We demonstrated that the main process of gene transfer across the cell membrane occurs via endocytosis after
MDP irradiation, and that clathrin-mediated endocytosis dominates among all other endocytosis pathways.

Next, the contributions of electrical and chemical factors to the gene transfection process were analyzed by partial suppression of these factors during MDP irradiation using four different methods. Table 1 summarizes the factors that were active in the buffer solution for each method along with the normalized gene transfection efficiencies. Since catalase-mediated inhibition of \( \text{H}_2\text{O}_2 \) suppressed the transfection efficiency by 0.4-fold than that of the MDP irradiation [34], and NAC-mediated ROS inhibition completely impeded the transfection, we concluded that the ROS are indispensable for inducing gene transfection by MDP irradiation. In contrast, there was no transfection with \( \text{H}_2\text{O}_2 \) supply (the main ROS affecting transfection) in the absence of plasma treatment [34] or upon LPP-mediated suppression of electrical factors. Thus, lack of transfection in the presence of only chemical factors indicated that the electrical factors were indispensable for

\[ \text{Figure 5.} \] Fluorescent microscopy images of the GFP plasmid-transfected L-929 cells. Plasma irradiation without inhibitor (A), with Pitstop 2-100 (B), with Pitstop 2-100 negative control (C), and with M\( \beta \)CD (D). These pictures were captured at the point of maximum efficiency. The gene transfection efficiency was evaluated at five points in each well.

\[ \text{Figure 6.} \] Fluorescent microscopy image of the GFP plasmid transfected-L-929 cells, transfected using plasma irradiation without inhibitor (left side). Measurement points of gene transfection efficiency are shown on the right side. The gene transfection efficiency was evaluated at five points in one well.
transfection. Therefore, both electrical and chemical factors are necessary (table 2), and they synergize to induce efficient transfection by MDP.

Next, we investigated the relationship between electric field and transfection efficiency. The radial distribution of the electric field at the bottom of the TE/PBS buffer solution was calculated using the finite element method. The radial distribution of transfection efficiency was determined from the fluorescent images (figure 6) by counting the number of green pixels. Figure 8(a) shows the radial distribution of transfection efficiency for MDP irradiation without the inhibitor (condition of figure 5(A)) and the calculated electric field. To clarify the relationship between transfection efficiency and electric field, transfection efficiency was plotted as a function of electric field (figure 8(b)). The decrease in electric field towards the outside of the well resulted in lower transfection efficiency, and the radial distribution of transfection efficiency roughly coincided with that of the electric field in the range of 0.4–1.7 mm of radial position. In addition, transfection occurred within the circular area of 1.7 mm radius, and the transfection efficiency reduced significantly at the edge of this circular area. This result shows that transfection occurs within the defined threshold of the electric field (approximately 1 kV m\(^{-1}\)) in this system. Furthermore, sufficient concentrations of ROS were supplied by the MDP irradiation, and the stimulation intensity of the electric field determined the transfection efficiency in our system. The transfection efficiency and electric field did not coincide in the area enclosed within 0.4 mm radius. Certain effects caused by the contact of plasma with solution, such as ion bombardment on cells and reduction in solution depth due to the pressure of plasma expansion etc, should be included in the future model to explain the phenomena observed in the area contacted by plasma.

We have summarized our previous and present data in figure 9. The electrical and chemical factors generated using microplasma might alter the physicochemical structure of the cell membrane and influence the intracellular signaling and/or biological functions of the cell. Several types of endocytosis pathways such as the clathrin-mediated and caveolae-mediated pathways exist, which might account for 80% of the gene transfer. The remaining 20% gene transfer might occur via other endocytosis pathways and/or other transmembrane pathways such as small pores similar to those generated by electroporation, channels, transporters, and so on.

ROS and RNS contribute to MDP-mediated gene transfection as chemical factors. The life spans of ROS vary widely. For example, the life span of H\(_2\)O\(_2\) is long. On the contrary, the superoxide ion (\(\text{O}_2^\cdot\)) survives for only few milliseconds [39] and the hydroxyl radical (\(\cdot\text{OH}\)) has several hundred microseconds lifetime [40]. These ions are generated from plasma, and the superoxide ion is rapidly converted to H\(_2\)O\(_2\) and hydroxyl radical in sequential chain reactions. Considering that transfection does not occur immediately after plasma irradiation [35] and that H\(_2\)O\(_2\) has a long life span, H\(_2\)O\(_2\) appears as the dominant ROS in the cell-plasmid DNA mixture after irradiation. Since endocytosis is the main process involved in microplasma-mediated gene uptake, it contributes to 60% of the transfection events (table 1) and H\(_2\)O\(_2\) possibly triggers the endocytosis.

Plasma also produces RNS. For example, NO is an RNS that plays important roles in cell signaling, anti-infection, and anti-oxidation [41]. Therefore, similar to ROS, RNS may also contribute to the gene transfection process. However, further investigations on the effect of RS such as ROS and RNS on transfection efficiency are required.

Repulsive Coulomb forces operate between the cell membrane and the plasmid DNA since both are negatively charged. This may reduce endocytosis as repulsive forces decrease the frequency of collision between the plasmid DNA and the cell membrane, which is critical for efficient endocytic uptake. In our previous study, we measured the electrostatic charge of the artificial cell membrane and found that plasma irradiation generates positive charge on the cell membrane [33]. In contrast, exogenous materials enclosed in positively charged lipid bilayer membranous vesicles (liposomes) are attracted and endocytosed by the cell membrane in lipofection [42]. This suggests that depolarization and subsequent reverse polarization of membrane potential occurs during plasma-mediated gene transfection, which enables movement of plasmid DNA towards the cell membrane via attractive Coulomb forces. Consequently, increasing the charge on the cell membrane may increase the frequency of collisions and subsequent attachment.

Since lipids such as lipofectamine do not envelope the plasmid DNA in the microplasma-mediated gene transfection method, membrane fusion does not occur by simple attachment of the plasmid DNA on the cell membrane, and a triggering signal is necessary for inducing endocytosis. Reports show that ROS influences intracellular signaling [43]. Yusupov et al performed a molecular dynamic simulation for analyzing the interactions of ROS with a lipid bilayer. They
reported that the oxidation of lipid molecules decreased the electric field threshold required for poration of the lipid bilayer. They also showed that the oxidation of lipid molecules decreased the permeation free energy barriers of ROS. In plasma-mediated gene transfection using MDP, we assumed that the ROS have a role in triggering endocytosis by stimulating an intracellular signaling pathway as described below (figure 9). In our system, similar to the results presented in reference [43], the electrical and chemical factors synergistically contributed to ROS permeation through the cell membrane, which enhanced gene transfection via endocytosis triggered by the permeated ROS.

Thus, we have considered the contributions of both electrical and chemical factors in understanding their synergistic effect on plasma-mediated gene transfection (figure 9). The electrical factors regulate the net positive charge on the cell membrane, which subsequently affects the frequency of plasmid DNA collision on the cell membrane. ROS is a chemical factor that triggers endocytosis. The synergistic effect of electric field and ROS causes permeation of ROS through the cell membrane. Conventional methods such as lipofection and electroporation consist of two steps. In lipofection, the first step is the collision/attachment of the

**Table 1.** Active factors in the buffer solution and the normalized gene transfection efficiency of each method.

| Active factors | MDP | MDP with catalase [34] (100 units) | MDP with NAC (100 nM) | LPP | H$_2$O$_2$ [34] (1 mg l$^{-1}$) |
|----------------|-----|----------------------------------|------------------------|-----|-----------------------------|
| Electrical factors | ✓   | ✓ | ✓ | ✓ | ✓ |
| Chemical factors  | ✓ | ✓ | ✓ | ✓ | ✓ |
| Normalized gene transfection efficiency | 1.0 | 0.4 | 0.0 | 0.0 | 0.0 |

✓: active factor, -: inhibited or not existing factor.
liposomes with the cell membrane and the second step is fusion/endocytosis. In electroporation, the first step is poration of the cell membrane via charge collisions and the second step is the transfer of exogenous material through the pores [45]. The critical parameter for lipofection is the density of the cationic lipid, whereas it is voltage-regulated current density for electroporation. In these conventional methods, the strength of the critical parameters are generally increased to increase transfection efficiency, which may cause cell damage and cell death. In plasma-mediated gene transfection, the steps of plasmid DNA attachment and triggering of endocytosis can be controlled independently by electrical and chemical factors, respectively. This indicates that adjustment of plasma characteristics per target cell requirements for introduction of exogenous material can minimize cell damage and maximize transfection efficiency. This may explain why MDP achieves high transfection efficiency with low cell damage.

5. Conclusion

This study revealed that both electrical and chemical factors are required for efficient gene transfer inside cells using plasma irradiation. This also indicates that the synergy between electrical and chemical factors affected the transfection efficiency of this method.

Our results show that MDP-mediated transfection requires an electric field threshold of approximately 1 kV m\(^{-1}\). This implies that the MDP irradiation supplied sufficient concentrations of ROS, and the stimulation intensity of the electric field determined the transfection efficiency in our system.

The process of gene transfer by plasma irradiation depends mainly on endocytosis, which accounts for at least 80% of the transfer, and clathrin-mediated endocytosis was the dominant endocytosis pathway used. Plasmid DNA adhesion and triggering of endocytosis are regulated independently by the electrical and chemical factors, respectively, during plasma-mediated gene transfer. This indicates that by adjusting the plasma characteristics per target cell requirements, the transfection process can be optimized with minimum damage to cells and maximum transfection efficiency. This may explain why MDP simultaneously achieves high transfection efficiency with minimal cell damage.

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