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Masafumi Jinno, Yoshihisa Ikeda, Hideki Motomura, Yugo Kido, Susumu Satoh

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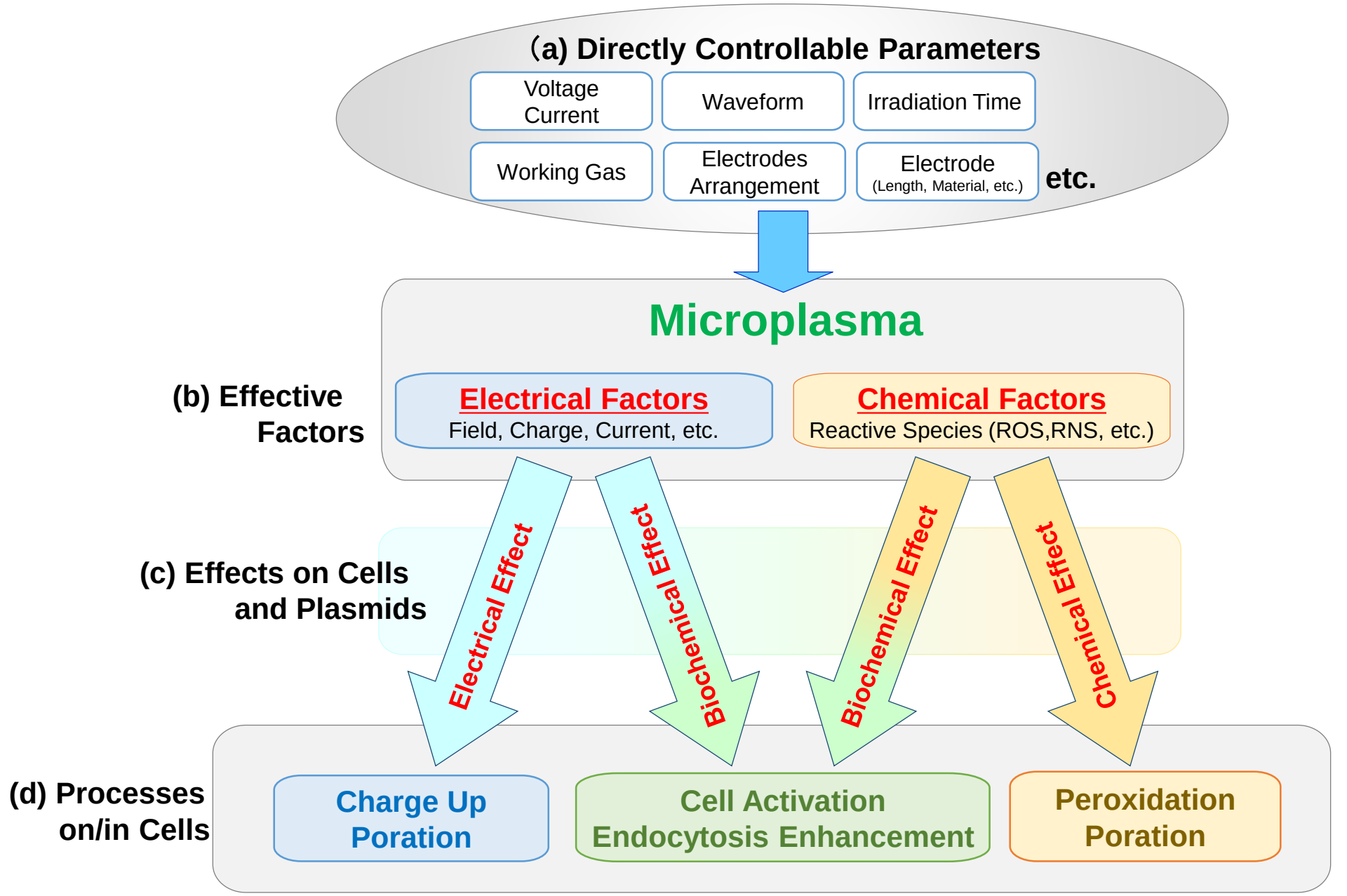
(a) Directly Controllable Parameters
Voltage
Current
Waveform
Irradiation Time
Working Gas
Electrodes
Arrangement
Electrode
(Length, Material, etc.)
etc.
Microplasma
(b) Effective
Factors
Electrical Factors
Field, Charge, Current, etc.
Chemical Factors
Reactive Species (ROS,RNS, etc.)
(c) Effects on Cells
and Plasmids
Electrical Effect
Biochemical Effect
Biochemical Effect
Chemical Effect
(d) Processes
on/in Cells
Charge Up
Poration
Cell Activation
Endocytosis Enhancement
Peroxidation
Poration

Investigation of plasma induced electrical and chemical factors and their contribution processes to plasma gene transfection

Masafumi Jinno[1*], Yoshihisa Ikeda[1], Hideki Motomura[1], Yugo Kido[1,2], Susumu Satoh[1,3]

[1]*Department of Electrical and Electronic Engineering, Ehime University, 3 Bunkyo-cho, Matsuyama 790-8577, Japan*

[2]*Pearl Kogyo Co., Ltd., 3-8-13 Minami-Kagaya, Suminoe, Osaka 559-0015, Japan*

[3]*Y's Corp., 2-31-17 Minamino, Tama, Tokyo 206-0032, Japan*

E-mail: mjin@mayu.ee.ehime-u.ac.jp

Abstract

This study have been done to know what kind of factors in plasmas and processes on cells induce plasma gene transfection. We evaluated the contribution weight of three groups of the effects and processes, i.e. electrical, chemical and biochemical ones, inducing gene transfection. First, the laser produced plasma (LPP) was employed to estimate the contribution of the chemical factors. Second, liposomes were fabricated and employed to evaluate the effects of plasma irradiation on membrane under the condition without biochemical reaction. Third, the clathrin-dependent endocytosis, one of the biochemical processes was suppressed. It becomes clear that chemical factors (radicals and reactive oxygen/nitrogen species) do not work by itself alone and electrical factors (electrical current, charge and field) are essential to plasma gene transfection. It turned out the clathrin-dependent endocytosis is the process of the transfection against the 60% in all the transfected cells. The endocytosis and electrical poration are dominant in plasma gene transfection, and neither permeation through ion channels nor chemical poration is dominant processes. The simultaneous achievement of high transfection efficiency and high cell survivability is attributed to the optimization of the contribution weight among three groups of processes by controlling the weight of electrical and chemical factors.

Key words

plasma gene transfection, membrane permeation, membrane poration, endocytosis, synergy

effect, plasma medicine

1. Introduction

For the last decades, drastic progresses have been done in generation and control of atmospheric non-equilibrium plasmas (ANEP) and in accumulations of data in measurement and simulation of plasma parameters, i.e. electron density, electron temperature, etc. [1–11]. It has become possible to supply effective stimuli such as ions, electrons, reactive species, electric field, etc., by ANEP to cells or living bodies with keeping them at room temperature. These facts have expanded application areas of plasmas in medical and biological fields [12–15] such as wound healing [16], blood coagulation [17], cell proliferation [18], cancer treatment [19], etc. In this stream, a novel gene transfection method by plasma irradiation was invented by Miyoshi *et al.* in 2002 [20] and was published by Ogawa *et al.* [21] and Sakai *et al.* [22]. After these publications, several gene/molecule transfection using variety of plasma sources have been reported. Leduc *et al.* used a pulsed RF-excited atmospheric pressure glow discharge torch and they showed dextran molecules up to 6.5 nm in radius are transferred into HeLa cells [23]. They also used a DBD (dielectric barrier discharge) source and evaluated negative effects of plasma treatment of HeLa cells and naked DNA [24]. They showed that HeLa cells demonstrated oxidative stress but the lipid was not peroxidized after direct contact with the plasma. They also showed that these plasma sources can fragment naked DNA and to cause DNA double-strand breaks. Chalberg *et al.* demonstrated gene transfer to rabbit retina with electron avalanche on a micro-electrode array [25]. Ramachandran *et al.* used corona discharge to produce ions deposited onto the liquid surface of media containing cultured media and demonstrated transfer of fluorescent dye calcein, drug bleomycin and nucleic acid stain SYTOX-green to murine B16 melanoma cells [26]. Connolly *et al*. used an atmospheric glow discharge and demonstrated transdermal administration of plasmids to mice [27]. Nakajima *et al.* used a DBD plasma jet and showed plasmid transfer into *E. coli.* [28]. Sasaki *et al.* also used a DBD plasma jet and demonstrated YOYO-1 dye transfer into 3T3-L1 cells [29] and they suggested that both electrical and chemical factors played important role for the transfection [30]. Edelblute *et al.* used a cathode-directed streamers generated by a shielded sliding discharge and demonstrated plasmids transfer into mouse melanoma cells (B16F10) and human keratinocyte cells (HaCaT) [31]. Although several reports on plasma transfection have been done mentioned above, the transfection mechanism is not clear yet.

Since an ANEP is generated as a result of a breakdown of discharge gases, the ANEP

supplies discharge current to target cells or living bodies. If the target materials are insulated from the ground (GND) electrode, ions and electrons are accumulated on the surface of the target materials. The accumulated surface charges generate local electric field besides the applied electric field for the plasma generation. These electrical factors give stimuli to the target materials. Besides, the ANEP generates chemically reactive species, such as excited molecules, radicals, reactive oxygen/nitrogen species (RONS), through electron impact reactions such as ionization, excitation, dissociation, etc. If the target is living tissue, as we describe here, the ANEP is generated in contact with water such as medium, buffer solution, saline, etc. Under this condition, many RONS are generated such as OH, O, H, O_3, HNO_3, HNO_2, etc. in both gas and liquid phases. Generation paths of these chemical factors are summarized in reviews by Samukawa *et al.* [32] and Bruggeman *et al.* [33] and the reactions are numerically analyzed by Sakiyama *et al.* [34] and Liu *et al.* [35]. Fig. 1 illustrates four steps of the top-down process from the microplasma generation to the processes on/in cells. The electrical and chemical factors are generated by a microplasma as described above. The ratio of generated amount of these factors (Fig. 1 (b)), i.e. electrical current, charge, electrical field, radicals or RONS, is determined by the directly controllable parameters (Fig. 1 (a)). However, contribution weight of these factors, of three effects (Fig. 1 (c)) and of three processes (Fig. 1 (d)) to plasma gene transfection is not clear.

The plasma source in Refs. 20–22 had a problem that the plasma flare fluttered so that reproducibility of the transfection efficiency was low. There was another problem that the gas flow dried up the target cells. We have established a special plasma source using a microcapillary electrode (capillary discharge plasma source: CDP source) to obtain high transfection rate and high cell survivability simultaneously [36–38]. In this system, spatiotemporally stable microplasma is generated from the tip of the capillary electrode. Moreover, the target cells are placed between the capillary electrode and the counter grounded electrode so that stable and appropriate amount of the electric current is supplied to cells. More detailed feature of the CDP source is described in Ref. 37. However, the contribution weight of three groups of effects/processes to the transfection shown in Fig. 1 is still unknown in our systems. To improve the performance of the plasma gene transfection method with the CDP source, precise understanding of the contribution weight of three groups of effects/processes is essential. As a first trial to clarify the processes in plasma gene transfection, we removed plasmid suspension just after plasma irradiation, and we have found that about half of the transfected cells by the plasma irradiation are transfected after plasma irradiation [36].

In this study we try elucidating the contribution weight of three groups of effects/processes shown in Fig. 1 by inhibiting some of the factors, the effects and the processes through the following three experiments: (i) a laser produced plasma (LPP) is employed to estimate the contribution of the chemical factors, (ii) artificial cells are employed to evaluate the gene transfer processes induced by plasma irradiation, (iii) clathrin-dependent endocytosis is inhibited and its contribution on the transfection is evaluated.

By optimizing the contribution weight of three groups of processes according to the target cells and transferred gene, it will be possible to achieve high transfection rate and high cell survivability simultaneously.

2. Materials and methods

2.1 Evaluation of effect of chemical factors in plasma gene transfection

Fig. 2 (a) shows the CDP irradiation system for plasma gene transfection. A thin copper capillary was employed as a high voltage (HV) electrode on which high voltage was applied by a high voltage power supply. The outer and inner diameters of the capillary were 70 and 20 μm respectively. A grounded copper plate was used as the counter electrode and was placed under a 96 well titer plate. The distance between the tip of the capillary electrode and the surface of the cell suspension was set at 1 mm. The applied voltage waveform was 20 kHz sinusoidal and the amplitude was 15 kV (peak to peak). A microplasma was generated at the tip of the capillary electrode. The plasma irradiation time was set at 5 ms.

The effects of the chemical factors such as reactive species like RNS or ROS were evaluated by deactivating the electrical factors such as electric field, electric charge and current, etc. A laser produced plasma (LPP) is one of the suitable plasma sources for this purpose. An LPP is a plasma generated by the breakdown of the air which is caused by focusing and irradiating an intense laser beam to the air. Since the plasma generated point is apart from the cells, almost ions and electros disappear through recombination each other before they arrive at the cells. Consequently only the chemical factors have effects on the cells. A schematic of the LPP irradiation system is shown in Fig. 2 (b). A laser beam of the pulsed Nd:YAG laser (Spectra-Physics, Quantra-Ray PRO 230) at the second harmonic wavelength of 532 nm was focused 0.5 mm above the target cell suspension. The diameter of the LPP was about 0.25 mm which was estimated by a burn pattern of thermal paper exposed to the LPP. The pulse width and the repetition frequency of the laser were 10 ns and 10 Hz respectively. The laser power was 0.3 W (30 mJ/pulse) and the irradiation time was 3 s.

The target adherent cells (L-929, mouse fibroblast cell, IFO50089: JCRB) were seeded

on a 96 well titer plate for the capillary discharge plasma irradiation or a micro-slide chamber for the LPP irradiation. The cells were incubated with 100 μL of culture medium for more than 24 h until the number of cells reached the semi-confluent under the ambient temperature of 37°C and the CO_2 concentration of 5%. For plasma irradiation, the culture medium was aspirated and 6 μg of the EGFP gene encoded plasmid pCX-EGFP was placed in each well. Each of the well was irradiated by the CDP or the LPP. After plasma irradiation, 100 μl of culture medium was added. After 48 h incubation, fluorescence expression was quantitatively measured with imaging cytometer Cytell (GE Healthcare Bioscience).

2.2 Evaluation system using artificial cell for effect of plasma irradiation on membrane

To exclude the biochemical effects on the cell induced by plasma irradiation, we introduced an experimental system using artificial cell (liposome), which is a spherical vesicle consisting of a lipid bilayer. In this study, we employed dioleoyl phosphatidylcholine (DOPC) as phospholipid molecules forming the lipid bilayer. The liposomes were prepared by the natural swelling method from a dry lipid film [39]. Phospholipid molecules was dissolved in 25 μl of methanol at 2 mM concentration. In this solution, 25 μl of 10 mM glucose aqueous solution and 2.5 μl of 2 mM fluorescence dye (Nanocs, MPEG Fluorescein, MW = 1000, excited at 490 nm and fluorescence at 520 nm) aqueous solution was added. This mixture was dried with nitrogen gas flow to form thin lipid film. The film was hydrated with 250 μl distilled water. Finally, dye molecule encapsulated liposomes were generated.

First, dye molecule permeation through the lipid bilayer in the CDP and LPP irradiations was investigated. The plasma sources and the irradiation condition were identical to those shown in Sect. 2.1. After the plasma irradiation, fluorescence microscope image was observed. From the fluorescence microscope image, the diameter of each liposome was measured and green pixel value at each liposome and at the background were obtained using an image processing software (ImageJ) in order to estimate the dye molecule flowed out through the lipid bilayer.

Second, charge up on the lipid bilayer by the CDP irradiation was investigated. The plasma sources and the irradiation condition were identical to those shown in Sect. 2.1 but the irradiation time was varied from 0.05 to 20 ms. In this experiment, the lipid bilayer was stained using Rhodamine DHPE (excited at 560 nm and fluoresces at 581 nm), instead of the water soluble MPEG Fluorescein. Before and after plasma irradiation, the liposome suspension was sampled and set on a handmade electrophoresis chamber (10 mm in width × 20 mm in depth × 1 mm in height) and DC voltage of 15 V was applied across the 10 mm gap

as shown in Fig. 3. The electrophoresis chamber was placed on a microscope (Olympus, IX-71) and liposome mobility was observed. From the observed electrophoretic velocity *v*, the amount of the electrical charge *Q* on each liposome was evaluated from the equilibrium between the electrostatic and viscous force,

$$Q = \frac{6\pi\eta a v}{E}, \quad (1)$$

where *a* is the radius of the liposome observed with the microscope, η is the viscosity of the solution (1.002 mPa·s) and *E* is the applied electric field (15 V/cm).

2.3 Evaluation of biochemical effects in plasma gene transfection

To evaluate endocytosis effect in the CDP irradiation, the clathrin inhibitor Pitstop 2-100 (ab144650: Abcam plc, Cambridge, UK) was used. Pitstop 2-100-negative control (ab144658) is a reagent which has a similar chemical structure but does not inhibit clathrin formation. This reagent was used to evaluate side effects of the clathrin inhibitor.

The target cell (L-929) and the standard experimental protocol, i.e. CDP irradiation, cell incubation and fluorescence observation, was identical to that described in Sect. 2.1. To evaluate the effects of predosing with inhibitor, 25 μM of clathrin inhibitor and of negative control inhibitor in a serum-free medium were added respectively to L-929 cells in a 96 well titer plate, and they were incubated at 37°C for 10 min. After aspiration of the serum-free medium with inhibitor or negative control inhibitor, 6.0 μg of plasmid DNA was added and then the CDP irradiation was performed according to the standard protocol.

3. Results

3.1 Evaluation of effect of chemical factors in plasma gene transfection

As long life chemically reactive species, concentrations of hydrogen peroxide (H_2O_2), nitrate ion (NO_3^-) and nitrite ion (NO_2^-) were evaluated from the absorbance by 4-aminoantipyrine method for H_2O_2 (Kyoritsu Chemical Check Lab., WAK-H2O2) and naphthylethylenediamine method for NO_3^- and NO_2^- (WAK-NO3, WAK-NO2). The obtained concentrations are summarized in Table 1. Similar or higher concentrations of these reactive species were obtained with the LPP compared with the capillary discharge plasma. As one of the short life chemically reactive species, production of superoxide anion (O_2^-) in the cell suspension by plasma irradiation was examined. Nitro blue tetrazolium (NBT) was added in the solution and absorption of NBT-diformazan at 550 nm was measured with a spectrophotometer (Jasco, V-670). Absorption coefficient of 0.08 cm^{-1} was detected after the LPP irradiation so that the

production of superoxide anion by the LPP irradiation was confirmed. In the case of the CDP irradiation, absorption at 550 nm was not detected so that the production of superoxide anion was not confirmed. Fig. 4 shows the gene transfection efficiency by the capillary discharge plasma and the LPP. The transfection efficiency of 24% was achieved by the capillary discharge plasma, whereas the LPP did not cause any gene transfection. As described in Sec. 2.1, since the electrical factors such as electric field, charge and current by the LPP are negligible compared to those by the capillary discharge plasma, this result shows that the electrical factors are indispensable to the plasma gene transfection. The specific electrical factors are analyzed in the next section.

3.2 Evaluation system using artificial cell for effect of plasma irradiation on membrane

Figs. 5 (a) and (b) show fluorescence microscope images observed before and after the capillary dishcarge plasma irradation for 5 ms. The photographs show that large liposomes reduce their size with decreasing in fluorescence intensity after the CDP irradiation, which were not observed for the LPP irradiation. From these photographs, the diameter of each liposome was picked up and average and standard deviation of the liposome diameters were (15.5±16.2) μm before the CDP irradiation and (13.1±12.5) μm after the CDP irradiation. From the result of the decrease in the fluorescence intensity, the outflow of the dye molecules by poration on the membrane is suggested. Fig. 5 (c) shows temporal change of the normalized fluorescence intensities in the liposomes after the CDP and the LPP irradiations. In the capillary discharge plasma irradiation, 10–20% of the dye molecules permeated the membrane toward the outside of the liposome just after plasma irradiation and reached steady state. On the other hand, the LPP irradiation did not cause any permeation of the dye molecules.

Fig. 6 shows temporal change of the charge amount on a liposome after the CDP irradiation. The liposomes were negatively charged before plasma irradiation and the charge amount on one liposome was estimated as –(106 ± 65) nC. The negative charge on the liposome decreased and became almost zero by 1 ms CDP irradiation. The decrease in the negative charge saturated and the charge amount did not change by longer plasma irradiation.

3.3 Evaluation of biochemical effect in plasma gene transfection

To evaluate the contribution of the clathrin-dependent endocytosis on plasma gene transfection clathrin inhibitor was dosed before the CDP irradiation. The result is shown in Fig. 7. In this figure, gene transfection efficiency by the CDP irradiation decreased by 60%

with dose of clathrin inhibitor, whereas dose of the negative control inhibitor did not change the transfection efficiency. The result of the negative control inhibitor shows that the effect of the clathrin inhibitor was only inhibition of endocytosis: such as scavenge of plasma-induced chemically effective species did not occur with the dose of the clathrin inhibitor. It turned out that in the plasma gene transfection the clathrin-dependent endocytosis which is caused by biochemical effects is the process of the transfection against the 60% in all the transfected cells.

4. Discussion

In this paper, we started to work out the contribution ratio among three groups of effects shown in Fig. 1, i.e. electrical, chemical and biochemical effects, for plasma gene transfection.

To estimate the effectiveness of only the chemical factors working without the electrical factors for plasma gene transfection, we used LPP which suppresses electrical factors in plasma. From the results of LPP gene transfection, it is suggested that the electrical factors are indispensable for chemical factors to transfer gene into cell. Our previous experiments in terms of H_2O_2 showed same phenomena [38]. Though H_2O_2 did not induce any transfection without plasma, the combination of plasma and H_2O_2 induced transfection. From these results it becomes clear that chemical factors do not work by itself alone and electrical factors are essential to plasma gene transfection.

We suppressed the biochemical processes by using liposome and evaluated the contribution of the electrical and the chemical processes to the transfection. The LPP irradiation to liposomes, which gives only chemical effect on liposomes, did not cause the outflow of encapsulated dye molecules as shown in Fig. 5 (c). This result shows the chemical effect leads to the transfection process only in synergy effect with the electrical effect. The electrical factors of the capillary discharge plasma caused the outflow of encapsulated dye molecules as shown also in Fig. 5 (c). This result shows the membrane poration induced by the electrical effect such as electrical current, charge and electrical field is one of the important processes causing plasma gene transfection. Tero *et al.* observed membrane poration caused by plasma irradiation [40]. Zerrouki *et al.* suggested numerically that ions and electrons bombardment (electrical factor), as well as radical bombardment (chemical factor), cause membrane poration [41]. Since the fluorescent intensity in the liposomes decreases within a few minutes and is saturated at normalized intensity 0.8, membrane poration is not permanent, but the pores are closed by the lateral mobility of lipid molecules

which form the membrane. Pore closure phenomenon in a liposome was reported by Hamada *et al.* [42]. We think that size reduction of the liposomes shown in Fig. 5 (b) was caused by electrical effect, especially, electric field or charge accumulation on the surface of the liposomes (see Fig. 6). Yamamoto *et al.* reported the liposome deformation by the electric field application [43]. Segmentation of the liposome after deformation of the membrane is assumed as another process of the outflow of the dye molecules. Since obvious membrane permeation of the dye molecule was detected using liposomes, which have no ion channels, these results suggest that the contribution of ion channels to plasma gene transfection is relatively low.

The experiment using liposome also reveals the negative charge neutralization on the liposome by plasma irradiation (Fig. 6). Since plasmid DNAs are also negatively charged, the charge neutralization gets rid of the Coulomb repulsive force and increases the approach of plasmid DNAs to cells. This enhancement may leads to endocytosis followed by gene transfection.

Fig. 7 shows the contribution of the clathrin-dependent endocytosis is dominant transfection process. We have reported the contribution of H_2O_2 on gene transfection [38]. The result shows 60% decrease in the transfection efficiency by decomposition of H_2O_2 using catalase. Thus, at least 20%, which is the overwrap of the H_2O_2 contribution (60%) and the clathrin-dependent endocytosis (60%), of all the transfection processes is induced by the process of the clathrin-dependent endocytosis which requires H_2O_2. Moreover, this clathrin-dependent endocytosis also requires synergetic effect with electrical factors because H_2O_2 supply by the LPP irradiation showed no transfection.

In summary, it is shown that the endocytosis and electrical poration are dominant in plasma gene transfection, and that neither permeation through ion channels nor chemical poration is dominant processes. The simultaneous achievement of high transfection efficiency and high cell survivability is attributed to the optimization of the contribution weight among three groups of processes by controlling the weight of electrical and chemical factors.

5. Conclusion

We evaluated the contribution ratio of three groups of the effects and processes shown in Fig. 1, i.e. electrical, chemical and biochemical ones, inducing gene transfection. First, the LPP was employed to estimate the contribution of the chemical factors. Second, liposomes were fabricated and employed to evaluate the effects of plasma irradiation on membrane under the condition without biochemical reaction. Third, the clathrin-dependent endocytosis,

one of the biomedical processes was suppressed. It becomes clear that chemical factors do not work by itself alone and electrical factors are essential to plasma gene transfection. It turned out that in the plasma gene transfection the clathrin-dependent endocytosis is the process of the transfection against the 60% in all the transfected cells. The endocytosis and electrical poration are dominant in plasma gene transfection, and neither permeation through ion channels nor chemical poration is dominant processes.

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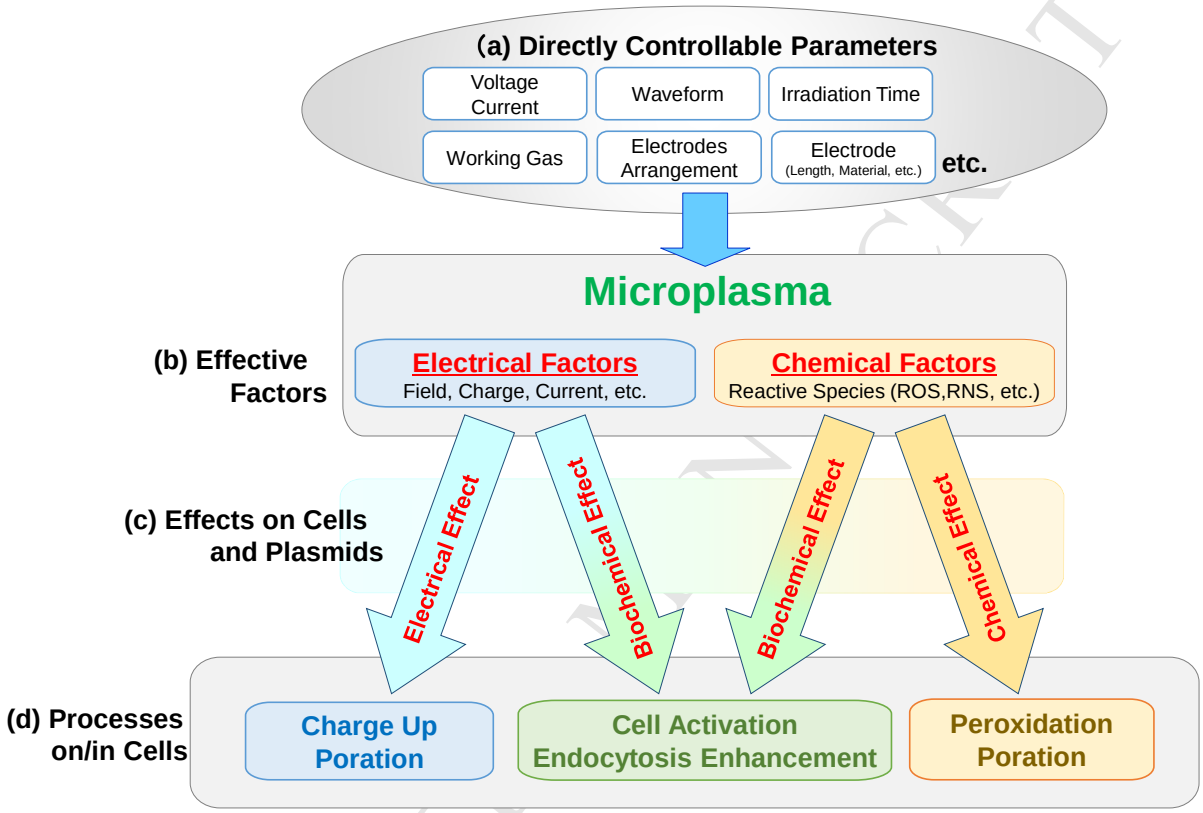

Fig. 1. Conceptual diagram of the mechanism structure in plasma gene transfection / molecular introduction. It consists of four steps: directly controllable parameters such as voltage, current, waveform, irradiation time, working gas, electrodes arrangement, electrode structure, etc. (a), plasma and effective factors generated by plasma such as electrical factors and chemical factors (b), effects on cells and plasmids such as electrical, biochemical and chemical effects (c), each processes on/in cells (d). By changing the directly controllable parameters the weight among electrical factors and chemical factors and among electrical, biochemical and chemical effects are controlled.

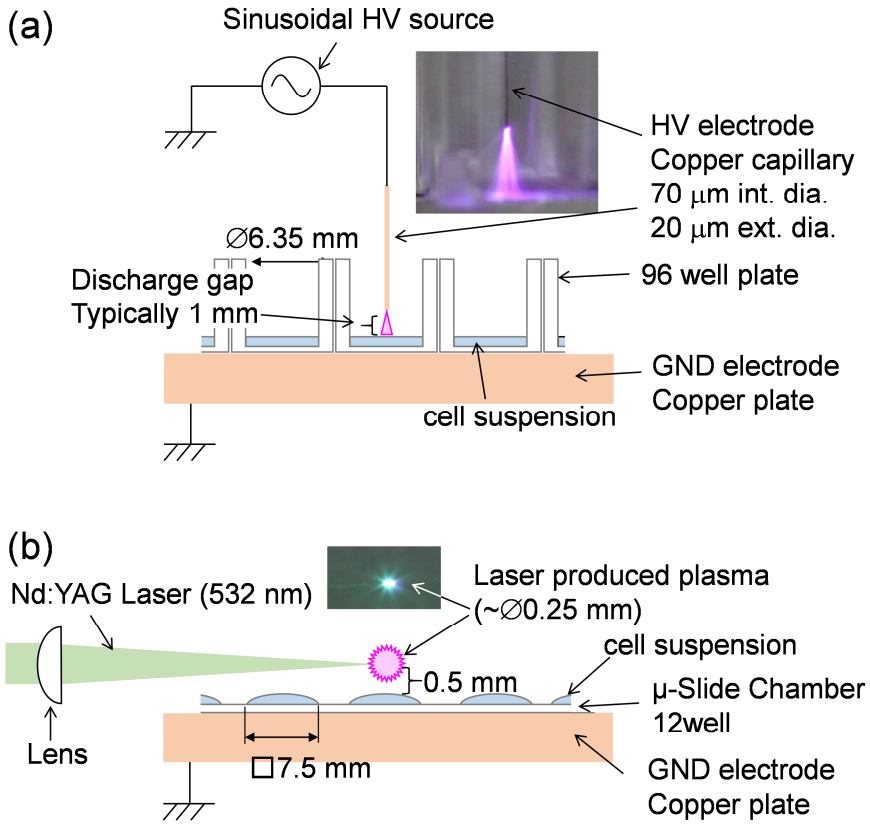

Fig. 2. Schematic of irradiation systems for plasma gene transfection. The capillary discharge plasma: CDP (a). The laser produced plasma: LPP (b).

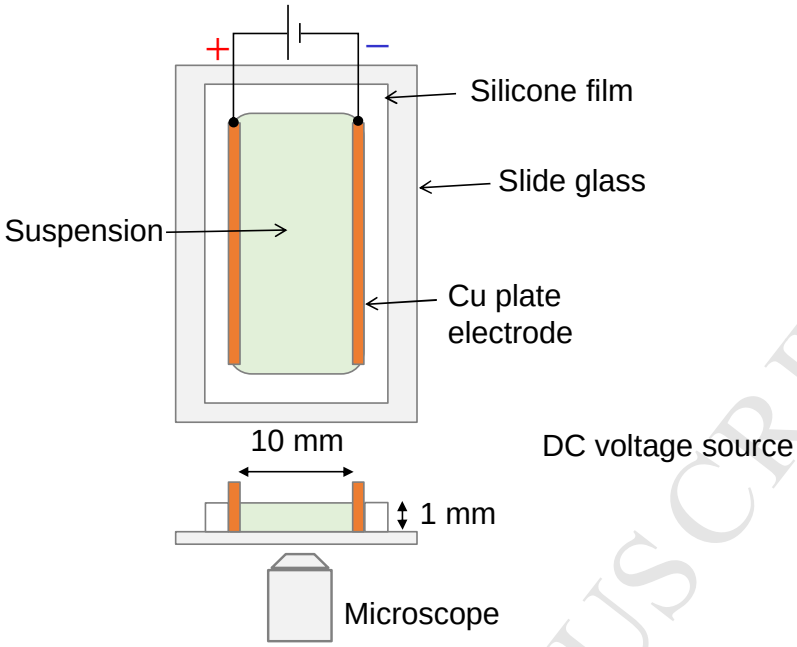

Fig. 3. Schematic of electrophoresis chamber.

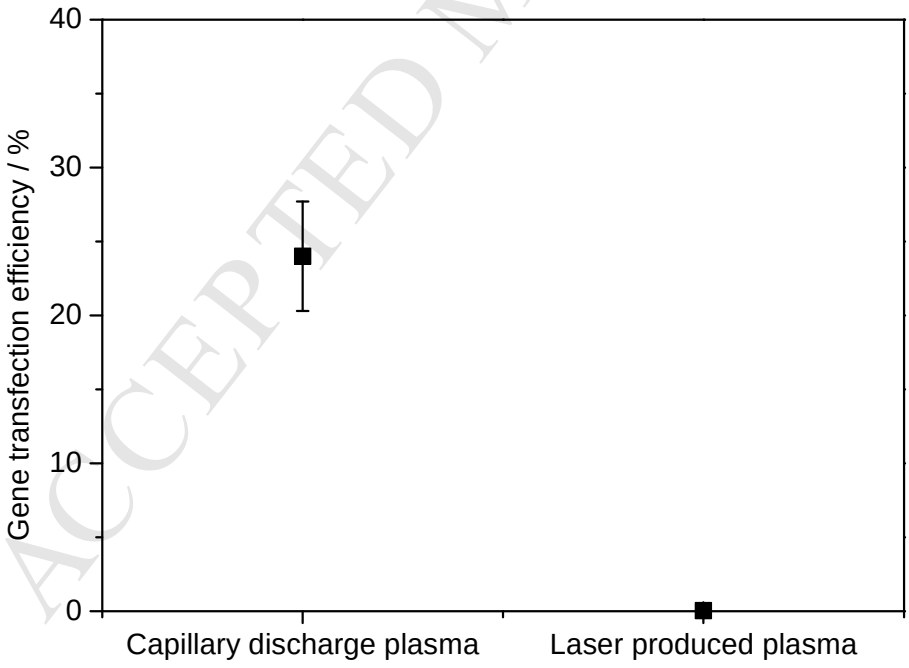

Fig. 4. Gene transfection efficiency by the CDP and the LPP irradiations.

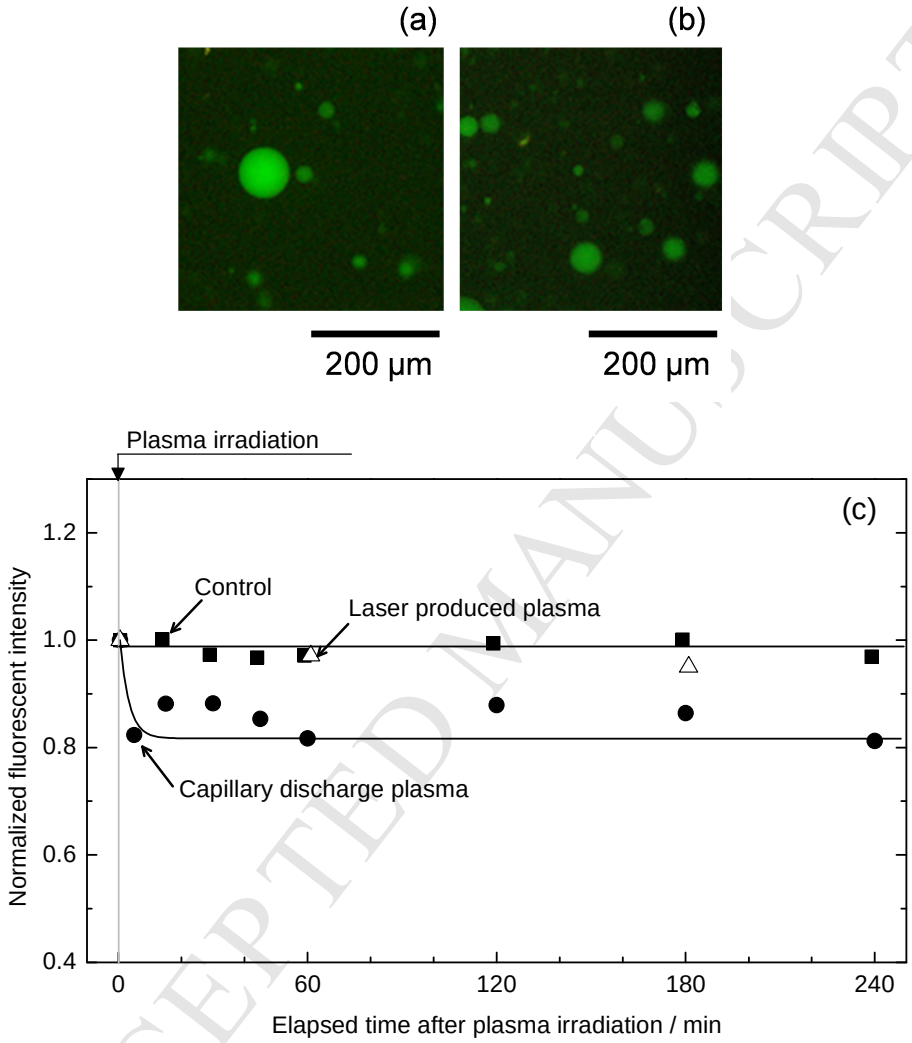

Fig. 5. Fluorescence microscope images before (a) and after (b) the CDP irradation for 5 ms and temporal change of the normalized fluorescence intensities in the liposomes before and after the CDP and the LPP irradiations (c).

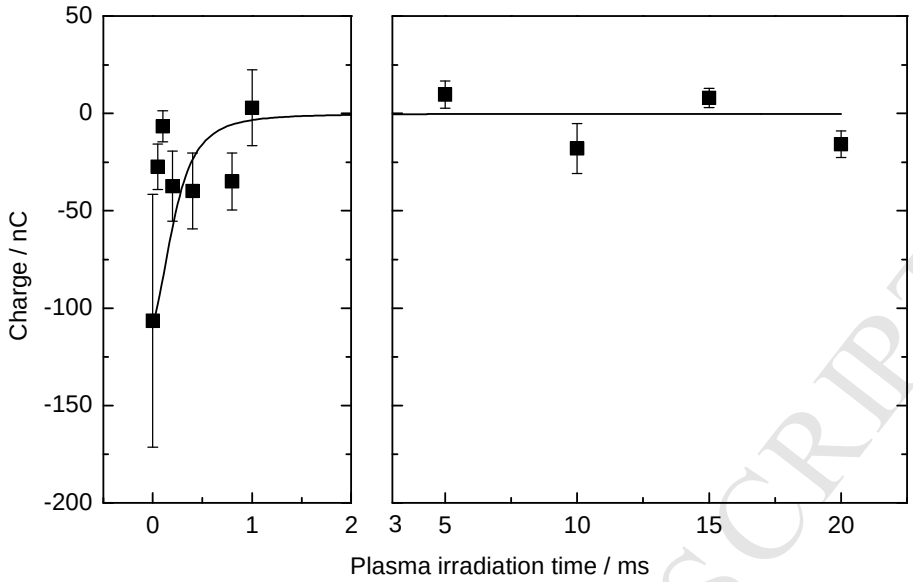

Fig. 6. Temporal change of the charge amount on a liposome after the capillary discharge plasma irradiation.

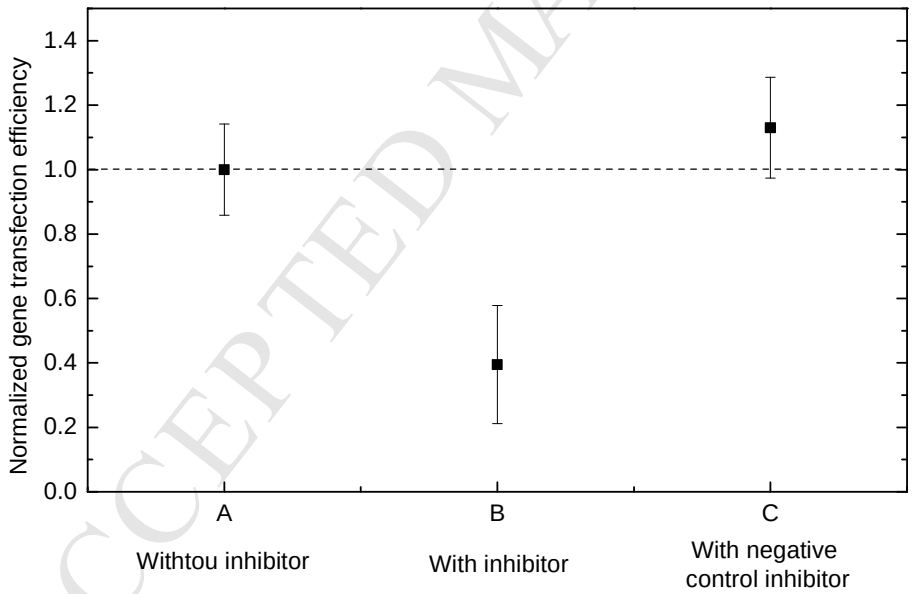

Fig. 7. Normalized gene transfection efficiency by plasma irradiation without clathrin inhibitor (A), with inhibitor (B) and with negative control inhibitor (C).

Table 1. Concentrations of long life chemically reactive species, hydrogen peroxide, nitrate ion and nitrite ion, in the buffer solution after exposure to the capillary and the laser produced plasmas. Results of detection of short life chemically reactive species, superoxide anion, is also shown.

	Capillary discharge plasma	Laser produced plasma
Long life species		
H_2O_2	1.0 mg/L	4.0 mg/L
NO_3^-	1.9 mg/L	3.1 mg/L
NO_2^-	1.2 mg/L	0.95 mg/L
Short life species		
O_2^-	Not detected	Detected (0.08 cm^{-1} absorption for NBT-diformazan at 550 nm)

Highlights

ABBI-16-6R1

- In the plasma gene transfection the clathrin-dependent endocytosis is the process of the transfection against the 60% in all the transfected cells.
- The endocytosis and electrical poration are dominant in plasma gene transfection, and neither permeation through ion channels nor chemical poration is dominant processes.
- The simultaneous achievement of high transfection efficiency and high cell survivability is attributed to the optimization of the contribution weight among three groups of processes by controlling the weight of electrical and chemical factors.