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科学研究費助成事業（科学研究費補助金）研究成果報告書

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研究課題名（和文）　電子スプレーで生細胞にあらゆるナノ分子を定量的・非損傷的に注入する技術の開発

研究課題名（英文）　Development of a quantitative and non-traumatic strategy to insert any nano-molecules into living cells with electrospray

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研究成果の概要（和文）：
我々は分子や粒子をイオン化・加速して特定の神経細胞に穿通させるナノ電子スプレー微小管を設計、２本の同心の表面疎水処理ガラス管内部の溶液を電子スプレーにして標的に局所的に到達させた。液滴の安定噴射には管配置と電圧波形が重要で、20-250nA のパルスピーク電流が得られた。しかし陰イオン色素と蛍光顕微鏡で観察すると粒子は管先端-培養液面境界のほか外管内壁にも急速に蓄積、終には管内部への引水と溶液流出が生じ、菅形状の調整を重ねたが不可避だった。静電的性質のシミュレーションによる改良型を提示する。

研究成果の概要（英文）：
We designed a nanoelectrospray micronozzle to ionize, accelerate and focus molecules/particles toward a single neuron for injection. The two concentric capillaries with hydrophobic coating produced an electrospray of solutions, with localized deposition on solid target. Precise nozzle alignment and voltage wave form were both critical to achieve stable droplet production with peak current reaching 50-250nA. With anionic dye and epifluorescence microscopy, we observed particles reaching the gas-medium interface but also run-away droplets accumulation on the inside of the outer pipette, until an abrupt flooding of the tip and leak of solution. Various nozzle morphologies were tested but this limitation is intrinsic to the current nozzle. Improved designs based on simulations of electrostatic properties are presented.

交付決定額

（金額単位：円）

	直接経費	間接経費	合　計
2008 年度	0	0	0
2009 年度	0	0	0
2010 年度	2,200,000	660,000	2,860,000
2011 年度	800,000	240,000	1,040,000
2012 年度	500,000	150,000	650,000
総　計	3,500,000	1,050,000	4,550,000

研究分野：総合領域
科研費の分科・細目：脳神経科学・神経科学一般
キーワード：Electrospray, Transfection, Neuron

１．研究開始当初の背景・２．研究の目的・３．研究の方法

(1) Original design of nanospray-based particle delivery system.

Numerous methods based on lipid encapsulation, viral vectors or coated particles can be used to insert molecules/particles into living cells. These methods do not allow to target a single cell nor to deliver a reproducible amount of material into the cell. In this project, our goal was to introduce solid particles (colloidal quantum dots), organic dyes and biological polymers (peptides, plasmid) into fragile mammalian cells, in a controlled and minimally invasive manner and with single-cell accuracy. Our main focus was on the primary culture of neurons that are either resistant (no introduction of material) or intolerant (unacceptably high cell death) to conventional methods based on lipid encapsulation or virus. Electrospray ionization is considered a "soft ionization" method that does not fragment even kiloDalton-range molecules such as DNA and proteins. This is important for mass spectrometry analysis and even more critical for our purpose since we want to insert functional gene/protein into living cells. In addition, contrary to nanoelectrospray protocols for mass spectrometry, our aim was to achieve only partial desolvation (evaporation of solvent) of the polymer to avoid denaturation as well as to alleviate surface tension obstacle at the air-saline interface. In addition, the solvent used had to be non-denaturing to polymers and harmless to cells, precluding the use of methanol, surfactant and strong organic acids commonly used for ESI-mass spectrometry. Finally, a critical parameter for efficient electrospray generation is the electrical conductivity of the solvent, which affects the Taylor cone formation and the initial size of the droplets. Furthermore, since DNA and quantum dots intrinsically bear a net negative electrical charge, we decided to operate the nanoelectrospray at negative voltage. These considerations guided the formulation of several alternative solvents (see below).

The formation of droplets at the tip of the Taylor cone represents only the first step of the ion cloud formation. As mentioned above, the solvent in the (negatively) charged droplets will tend to evaporate, increasing charge density up to the Rayleigh limit, at which point repulsion exceed surface tension and the droplets fragment into smaller droplets, producing a cascade of droplet fission events. Since these droplets also repel each others (Coulombic repulsion), the cloud of droplets tend to expand into a cone (see scheme). Thus, some of the droplets will accumulate on the inner wall of the outer pipette, before reaching the aperture. To avoid the formation of a continuous liquid film, we coated the surface of the outer pipette with a <10µm layer of paraffin, a highly hydrophobic wax. Since the deposited droplets are negatively charged and will tend to repel incoming droplets, theoretical considerations suggest that the deposition should reach equilibrium when the surface charge becomes high enough to prevent further deposition of droplets. This layer of immobilized negative charges should create an ion funnel, focusing droplets/ions toward the aperture of the outer pipette.

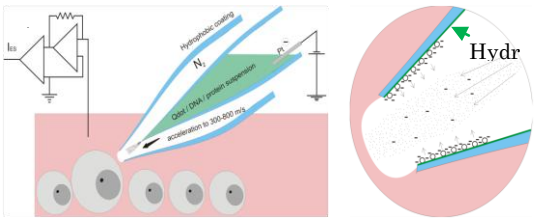

Figure 1: (Left) Concentric nanoelectrospray design and measurement setup. The inner pipette contains the solute to be ionized (Qdot/DNA/protein suspension). (Right) Coulombic repulsion causes droplet fragmentation into smaller droplets, accelerated in the electrical field. Initial immobilization of charged droplets on the hydrophobic surface of the outer pipette creates repulsion and focusing of the ion plume toward the exit.

(2) Construction of high voltage holder for centering of concentric glass capillaries.

Borosilicate glass capillaries (Ragnotti star bore, 1mm OD, 0.48 ID and Warner 2mm OD, 1.16 ID) were selected for minimal gap and thick wall low dielectric loss factor, minimizing capacitive coupling. The star-bore lumen of the inner pipette creates an air-gap between the solution and the pipette wall, allowing a low-resistance flow. This renders unnecessary the use of a pump connected to a high voltage connector, increasing safety and simplicity. Precision-machined polycarbonate was

utilized for the body and caps of the holder, in a configuration similar to the one found in low noise pipette holders for patch-clamp recording. We tested that no arcing was generated even when operating the holder at the highest voltage delivered by our generator (3000V).

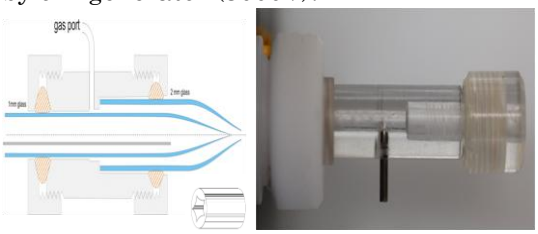

Figure 2: Design and micro-fabrication of a holder for concentric glass capillaries. Note the presence of a gas port allowing the creation of an internal pressure in the gap between the two capillaries. High voltage is applied to the solution through a platinum wire inserted to the back of the inner pipette. Materials (Teflon, polycarbonate) were selected for their high quality as insulator.

4．研究成果 _ research results

(1) Pulsed nanoelectrospray for stable low-flow droplet generation.

To evaluate the optimal conditions, a test rig was constructed consisting of a polished copper plate placed perpendicular to the axis of the capillary, 0.5-2mm from the aperture (60µm diameter). To measure the electrospray current, the copper plate potential was connected to a low-noise current-to-voltage converter circuit (see scheme above), thus measuring the charges reaching the plate (i.e.: electrospray current). In addition, we observed the deposition of a fluorescent polymer (Sulfo-Rhodamine Lysine dextran) under a microscope and measured the weight of the pipette before and after electrospray. Thus we could quantify the flow rate as well as the plume dispersion.

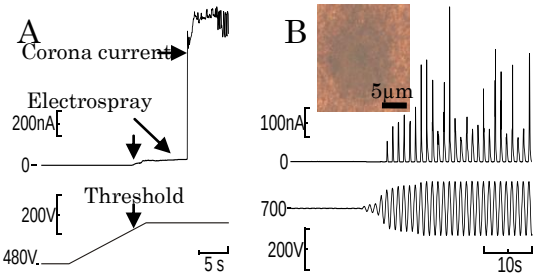

Figure 3: Pulsed electrospray by combination of DC voltage and low frequency sine oscillation yield higher stability than DC voltage alone. (A) Threshold voltage for electrospray initiation, determined by ramp stimulation. Note that the electrospray (~90nA) remained stable for less than 10s before a short-circuit appeared due to solution deposition on the outer pipette (leading to saturation of the measurement amplifier). (B) Adding a low frequency oscillation to DC voltage results in pulsed electrospray, with a phase lag of 150-200ms. Note that current amplitude shows fluctuations corresponding to different amount of droplets generated during each pulse. Pulsed nanospray can deliver solutes very locally (Sulfo-Rhodamine Lysine dextran) on a copper target plate (tip to plate distance: 0.5mm). The diameter of the circular spot is comparable to cell soma diameter (5-20µm).

Initially, we measured the electrospray current while gradually increasing the negative voltage from a high voltage source (Trek PZD2000, Trek Japan KK, Tokyo). Note that on all graphs presented in this report, the voltage polarity is omitted and only amplitude is reported. We observed the initiation of electrospray for voltage in the 600-900V range (see figure 3A, "threshold"), with a resulting electrospray current of 50-200nA, consistent with previous reports. Note however that the spray was found to be highly unstable, with apparition of a corona discharge at the tip of the capillary. This appeared as a sudden increase of the current reaching the µA range (Fig 3A). The corona discharge results from the ionization of air around the tip, an effect compounded by the humidity resulting from the electrospray. Consequently, we could not achieve more than a few second of continuous electrospray. Furthermore, upon interruption of the voltage to stop the corona discharge, hysteresis prevented the spontaneous re-initiation of the electrospray once the voltage was re-established. To solve both difficulties, we superimposed a 0.5-5Hz AC voltage of 100-300V on a DC bias voltage. The DC amplitude was chosen to be 50-100V below the threshold to initiate electrospray when used alone, and then we increased the amplitude of the AC component until each cycle of the periodic voltage resulted in a transient current (Fig 3B). When aimed at the copper plate target, this "pulsed electrospray" remained stable, without any appearance of corona discharge (tested up to 30min). In this mode, the electrospray current returned to its zero baseline between each pulse, suggesting that droplets reached the ground plate as separate clouds or jet. The deposition of electrosprayed material on the target was restricted to circular areas (Fig 3B, inset) with diameter in the 5-20µm range, below the average soma diameter of mammalian cells.

Weighting the pipette before and after electrospray was used to estimate the flow rate. Note however that the low flow rate characteristic of nanoelectrospray requires long spray time (>30min) to create a measurable decrease of pipette weight. Considering these limitations, the figures given should be considered as estimates. For the optimal conditions presented above, we observed some wide variation of the flow rate, from 0.15µl/min to 0.40µl/min,

corresponding to a volume per pulse of 2.5-6nl.

(2) Electrostatic properties of the pipette assembly immersed in saline/culture medium.

Since nanoelectrospray has never been reported to be used in the configuration proposed here, with its outer tip immersed in saline, we conducted additional simulations to clarify the behavior of the saline at the outer aperture. These simulations were done in collaboration with Dr. Y. Yamagata (UHF Team, ASI, RIKEN) using Comsol Multiphysics (Comsol, Palo Alto, CA).

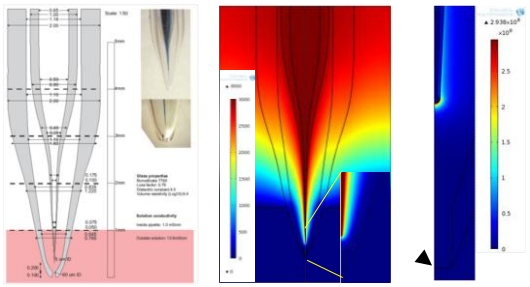

Figure 4: Simulation of electrostatic properties of the concentric pipette assembly. (A) Geometry from actual pipette (inset). (B) Electric potential when 3000V is applied to the inner pipette solution. (C) Simulation of electric field. In the presented configuration, field strength reaches 300 10^6V/m at the tip of the inner pipette, enough to produce a Taylor cone and electrospray. The force exerted at the aperture (arrow-head) is below surface tension, preventing entry of outer saline.

The material properties and nanospray dimension were accurately modeled from the actual capillaries used in our tests, to create realistic models of electrical fields and forces within the nanospray assembly (Fig 4). The simulation confirmed the conditions of electrospray generation. More importantly, we could estimate the force exerted on the gas-saline interface, at the outer aperture. Our concern was that while the electrical field propels droplets toward this aperture, the same force "pulls" the liquid interface into the nanospray outer tip. The simulation demonstrated that even at the highest voltage applied to the inner pipette, the applied force remained several order-of-magnitudes below the force resulting from saline surface tension, ensuring that the liquid would not enter into the tip.

(3) Nanoelectrospray performance when immersed in saline/culture medium.

A test bench was constructed to evaluate our nano-electrospray configuration in actual experimental conditions (with culture medium and an electrolyte containing organic fluorescent dyes, Fig 5A). The glass coverslip serving as growth substrate for primary neuronal culture also formed the bottom of the sealed recording chamber, placed on the stage of a fluorescence microscope for direct observation of the electrospray under epifluorescence illumination. The electrospray tip was attached to a micromanipulator for precise positioning in the vicinity of target cells. With application of moderate pressure in the gap between inner pipette and outer pipette, the outer saline solution could be prevented from filling the tip when immersed in saline, in the absence of any voltage applied to the inner pipette.

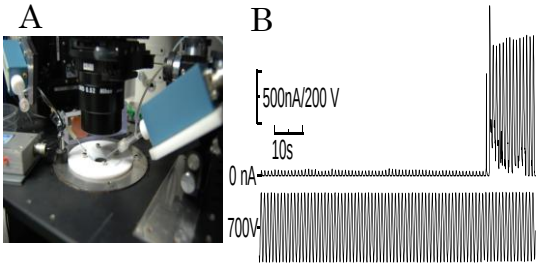

Figure 5 : (A) Nanoelectrospray (right) attached to a high-voltage source and immersed in saline. The recording chamber is insulated (Teflon) and connected to ground through a current-to-voltage converter (left). The nanospray and chamber are attached to the stage of a microscope, allowing epifluorescence observation during operation. Note the presence of a gas inlet on the side of the capillary holder, connected to a regulated pressure supply. (B) When immersed in saline, the pulsed electrospray eventually failed after 30-90sec. This failure mode abruptly filled the tip of the outer pipette with solution, and resulted in massive leakage of the dye/plasmid to the culture medium, through electrophoresis.

Application of a DC biased AC voltage initially consistently produced a weak but visible fluorescence at the aperture, and a pulsed electrospray current of 50-250nA peak current (Fig 5B). This was indicative of electrospray generation within the gap between the inner pipette tip and saline at the outer aperture. With fluorescent dye mixed in the spray solution, we also observed that the initially uncolored outer pipette was quickly becoming lightly fluorescent (compared to the very bright inner pipette).

When placed in close vicinity of a neuron (without contact), we could see that the cell rapidly became colored. However, within 1-2 minutes of operation, we observed an abrupt increase of the current (Fig 5B), concomitant with the tip of the outer pipette becoming filled with brightly fluorescent dye and the dye itself being strongly electrophoresed into the saline. Once this condition was reached, stopping voltage or moving the nanoelectrospray out

of the solution did not restore the nominal conditions and the whole assembly had to be replaced. We interpreted this observation as the sudden coalescence of the charged droplets deposited on the inner wall of the outer capillary, abruptly forming a continuous electrical path to the outside saline. The droplets that are initially kept separate by the hydrophobic surface and mutual repulsion merge into a continuous film once a critical droplet density is reached. The presence of this film abolishes the hydrophobic barrier and allows the outer saline to fill the tip, causing a resistance drop and thus a current increase to microampere level.
When plasmids coding for fluorescent reporter proteins were present in the solution, we did not see expression even 24h after exposure to nanospray while independent electroporation (Neon, Invitrogen) showed robust expression in sister cultures. Note that because of the abrupt nanospray failure observed, we limited the duration and amplitude of accelerating voltage to a minimum (10-20s). Since only a minute amount of plasmid may be inserted in cells, these results are inconclusive as to whether molecules bigger than fluorescent dye can be inserted into neuron with this method.
Modification of the outer pipette geometry to create a wider aperture did not alleviate the problem and made more difficult the establishment of a stable gas-saline interface. In addition, the diameter of the outer capillary could not be increased beyond 100µm as this prevented the approach of the tip to the targeted cells.

Conclusions and perspectives.

While we could observe nanospray generation with the pipette tip immersed in saline, our current design suffers from insufficient focusing of the droplets. Gas-phase collisions and Coulombic repulsion inevitably result in expansion of the ion cloud as it leaves the tip. For macroscopic electrospray, focusing of the ion plume can be robustly achieved using ion funnels (a stack of thin, alternatively charged plates) but this form of ion optic would be difficult to scale down to the dimensions required here. An alternative would be to use a sheath gas layer, flowing around the tip of the pipette (Fig 6). This configuration would contribute to focus the plume as well as to prevent any contact with the outer saline. The limitation however is that such a design would require the precise alignment of three concentric capillaries. Our experience shows that maintaining the alignment can be challenging once pressure is applied to the nanospray assembly.

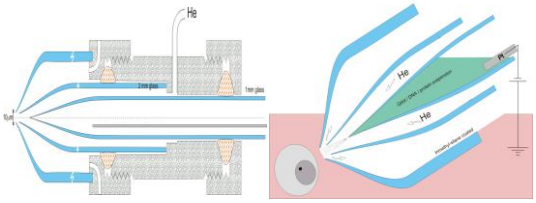

Figure 6: Proposed alternative design for sheath flow-assisted electrospray. Three concentric tapered capillaries allow a continuous flow of dry gas, drying droplets deposited on pipette surfaces and providing additional velocity to the electrosprayed polymers. Gas escape and gas pressure are calibrated to regulate sheath flow and keep a stable interface between the saline and the aperture of the outer pipette.

Microfabrication of planar electrospray using microfluidic engineering approach would produce rigid assembly but this project would require investments that are beyond the scope of the present trial.

While nanoelectrospray can deliver droplets of solute onto living cells *in vitro*, we could not achieve a stable generation and the tip became irreversibly compromised after less than 2min of operation. The focusing effect obtained through initial deposition of charged droplets is currently insufficient. Additional focusing would be obtained by the addition of a sheath gas (helium) and this would prevent the occurrence of corona discharge. This would require complex microfabrication setting. We envision that a complex approach may have niche applications in cell micromanipulation but this may not address the need for efficient and cost-effective delivery of molecules/particles into single living cell.

５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計 0 件）

〔学会発表〕（計 0 件）

〔その他〕
ホームページ等

６．研究組織

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