様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

令和　２ 年　 ７ 月 １０ 日現在

機関番号：１７４０１
研究種目：基盤研究(C)（一般）
研究期間：2017～2019
課題番号：１７Ｋ０６１６３

研究課題名（和文）Microfluidic targeted drug delivery

研究課題名（英文）Microfluidic targeted drug delivery

研究代表者
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交付決定額（研究期間全体）：（直接経費）　　3,600,000円

研究成果の概要（和文）：本研究の目的は、治療と診断のための新しいマイクロフルイディクスの使用、および最小限の損傷または副作用で済む薬物・ワクチンの送達であった。
顕微鏡下でのインビトロリアルタイム高速可視化、細胞への薬物とナノ粒子送達のためのインビトロでの評価等、モデル実験の設計と構築を研究計画に沿って行った。カルシウムのエレクトロトランスファーの新しいメカニズム、皮膚への無針無痛薬物・ワクチン送達、ナノ粒子送達、及び脳への衝撃波マイクロフルイディクスのインビボでの効果が得られた。
研究成果は、Physics Life Reviews や Drug Delivery Transl Res 等の主要な科学雑誌に掲載された。

研究成果の学術的意義や社会的意義
本研究により、薬物・ナノ粒子の細胞・組織へのマイクロフルイディクスによる送達機構の解明がなされた。これらの研究成果の学術的意義は、国際的に主要な科学雑誌に掲載されたことから証明されている。
痛みを伴わず、無針で皮膚に薬物・ワクチンを送達することに成功し、今後、コロナウイルスワクチンの送達等、様々な場面で社会に貢献でき社会的意義も高い。また、カルシウムエレクトロトランスファーとナノ粒子マイクロフルイディクスの送達の実験結果は、費用対効果が高く実用的な癌治療の方法の開発に大きく貢献する。

研究成果の概要（英文）：The research aimed to investigate the use of novel microfluidics for therapy and diagnostic, and delivery of drug/vaccine with minimum damage/side effects.
According to the research plan, design and construction of model experiments, in-vitro under microscope real-time high-speed visualization; in-vitro evaluation of results for drug/nanoparticle delivery to cells; ex-vivo needle-free delivery of drug/vaccine; and in-vivo animal experiments, have been performed. Valuable results from the research have been obtained. The results include novel mechanisms of calcium electro-transfer, needle-free pain-free drug/vaccine delivery to skin, novel effects on cells membrane, nanoparticle delivery, and in-vivo effects of shock waves microfluidics to the brain. The research results have been published in highly reputed scientific journals including, Physics of Life Reviews, Technology and Health Care, Drug Delivery and Translational Research, and IEEE Transactions on Plasma Science.

研究分野：流体工学関連

キーワード：マイクロフルイディクス　ドラッグデリバリー　ワクチンデリバリー　衝撃波　超音波　バイオエレクトリックス

様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９（共通）

１．研究開始当初の背景

The targeted therapies have been recently performed for new diagnostic and therapeutic approaches that are based on understanding the molecular nature of the diseases. The targeted approaches are used to improve the efficiency and treatment outcomes while minimizing the side effects. Introduction of gene or drug into tissue and cells has been used for a wide range of therapies. The commonly used delivery techniques can be classified into three main categories; genetically engineered viruses, chemical methods, and physical methods. The first two methods have high efficiency, whereas they can cause serious complications. These issues can be prevented through the use of physical delivery of gene or drug into the targeted cells. Physical delivery methods are based on applying electrical or mechanical stresses to the cells. Their successes rely on the biological responses and the physical forces. The main physical delivery methods are categorized as electroporation, microinjection, micro/nano particle and carrier delivery including biolistic, laser focusing, cell squeezing, sonication, and shock waves. Among them, electroporation, sonoporation, and shock waves are shown to be appropriate for in-vivo deliveries and clinical applications.

Electric pulses were used to electroporate the mammalian cells for gene transfection. It has been recently used for calcium ion delivery, a cost-effective method with lower side effects for cancer treatment. New approaches have been made in Bioelectrics with ultrashort duration electric pulses to manipulate the subcellular structures and electroporate the nucleus membrane. Sonoporation or transfection by ultrasound, on the other hand, has been a promising new area for therapeutic ultrasound. Sonoporation is accomplished by combining ultrasound and contrast agent microbubbles. High negative amplitude pressure ultrasound can cause irreversible damage to the cells. Our recent studies have shown that low amplitude sonication causes linear oscillation of microbubbles to induce microstreaming and microfluidics to reversibly manipulate the cells membrane. We have further studied microfluidics to enhance the efficiency of the delivery.

Injection of liquid vaccines and drugs into the skin by microfluidic techniques has the advantages of minimal invasion, infection, contamination and trauma, as the vaccines are propelled into the target with a breach less than that caused by a needle. The microfluidic delivery enables preservation of the microcirculation system and blood vessels in the targets, which can speed up the drug uptake. Jet injectors were developed for the purpose of delivering liquid and colloidal vaccines into the skin. These injectors could be classified as mechanical, pneumatic, and shock wave driven, which could deliver a few microliters of vaccine, through a micro-nozzle in the form of a high speed jet. While the jets generated by mechanical and pneumatic injectors could penetrate through a few millimeters in the targets, we have shown that the shock wave driven microjet could be controlled to reach a few hundreds of micrometers, adapting their application for delivery into the human skin. Effective drug dissemination through skin requires preservation of its micro blood vessels and Langerhans cells; therefore, the delivery has to be conservative up to the depth of dermis. After confirming the advantages of microfluidic delivery device, we further investigate the veracity of the depth of penetration of the delivered liquid into the skin.

２．研究の目的

Purpose of the research has been to investigate the use of novel microfluidics for non-invasive/less-invasive therapy and diagnostic. We specifically aimed to study the use of the microfluidic for manipulating cells for effective delivery of nanoparticles, drug, and calcium to treat cancer tumors; and for needle-free pain-free vaccine/drug delivery.
We aimed to design and construct real-time ultra-high-speed monochrome and high-speed fluorescent microscopic visualization setups to observe microfluidics and the delivery mechanisms in-vitro. We observed the spatial and temporal extracellular calcium uptakes and cellular responses to microsecond order time duration pulsed electric fields. The setup made it possible to accurately observe the permeabilization of HeLa cancer cells during application of various levels of pulsed electric fields. The method was used to observe mechanisms of extraction of valuable materials from algae cells and plant after poration, as energy and food resources. For the microfluidic injector, its operation was investigated by delivering a dyed liquid into the skin samples and soft tissue models. The delivery mechanics and the depth of penetration were analyzed theoretically with an elastic model for the skin and a viscoelastic model for the soft tissue

targets.

３．研究の方法

The real-time high-speed microscopic visualization system was consisted of an inverted fluorescence microscope (Nikon Eclipse Ti-U, Japan) connected to two 3-D micromanipulators (MMO-202ND, Narishige, Japan). A high-speed color video camera (Memrecam Q1v, nac Image Technology, Japan) was used for the observations. The camera can capture images with 640 × 480 pixels resolution at 8,000 frames per second rate (125 μs interframe) and high 32,000 ISO light sensitivity. The camera was plugged to the inverted microscope and was synchronized with a pulse generator using the output trigger signal of the generator. Images were recorded with different frame rates. For the calcium uptake study, due to the low intensity of the green fluorescent light of Fluo-8 AM (excitation at 490 nm, emission at 514 nm), the camera was set at 10 ms inter-frame and maximum ISO.

To generate underwater shock waves with different pulse durations, we used a magnetic pulsed compression (MPC) modulator, developed by our group, as energy source for the shock wave streaming experiments. The circuits boost the voltage with a transformer after applying the charging voltage to a capacitor, then compresses the pulse with a magnetic switch/switches. As the pulse energy is compressed by the magnetic switch, the rise time of the voltage and current is reduced and the pulse duration becomes shorter. To experimentally evaluate the effect of the voltage and current rise times on the shock wave pressure, we first used an MPC circuit with a constant peaking capacitor (Cp) capacitance of 10 nF and changed the number of pulse compressions from two, to one and no compression; then we kept the number of magnetic switches to two compressions and changed the Cp from 10 nF to 6 and 2 nF. During each experiment, current and voltage waveforms were measured by a current monitor (Pearson, Model 3972, USA) and a high voltage probe (Tektronix P6015A, USA), respectively, and were recorded using a digital oscilloscope (Tektronix DPO4104, USA). The shock wave pressure was measured by a fiber optic probe hydrophone (FOPH 2000, RP acoustics, Germany). Spherical shock waves, produced by the underwater discharge, reflected from a reflector to generate uniform shock waves.

HeLa cells (DS Pharma Bio Medical Company, Japan) were used for experiments. The cells were cultured according to a standard procedure as detailed in the published articles from this proposal. After cells covered the culture dish surface, they were washed with phosphate buffered saline PBS(-) and harvested with ethylenediamine-tetra-acetic acid, 1 mM, 20 s (EDTA, Sigma-Aldrich, MO, USA). The dish was shaken to detach the cells. The cell suspension was transferred to a poly-L-lysine coated glass bottom dish (27 mm diameter, Matsunami Glass Ind., Japan) with fresh medium. The glass bottom dish was set at the microscope visualization and calcium electrotransfer and microstreaming experiments were conducted. For the calcium observations, cells were incubated for 30 minutes with 4 μM Fluo-8 AM (AAT Bioquest Inc., USA) diluted in Hank's balanced salt solution HBSS(-) and then washed three times. The dish was filled with Hank's balanced salt solution containing 1.26 mM Ca++ (HBSS(+); Wako Pure Chemical Industries, Japan). The applied energy and current and voltage waveforms were measured during each experiment. For in-vivo experiments, mice animal models were used.

４．研究成果

The effects of voltage and current pulse durations of underwater discharge on shock wave waveform, therefore duration of shock wave impulse and particles behind it, were obtained. The maximum pressure of the generated shock wave after the reflection was in the range of 40 MPa. The energies of the output shock waves were calculated from the pressure histories and the electrical to shock wave energy conversion efficiencies were calculated from the electrical energies used to generate the shock waves. The results indicated that the rise time of voltage and current significantly affected the electrical energy that could be delivered to generate the shock waves. For the applied range of the electric pulse durations of 100 to 500 ns, the shock waves pressures were independent of the pulse duration for the same input energy consumed during the rise and full width at half maximum times of the current. The method provided us with the possibility to select the shock wave pulse duration and its profile, which were applied to microstreaming studies.

The results of calcium electrotransfer study confirmed that the calcium uptake continued after the pulsed electric fields application, an evidence that permeabilization was maintained even after the 0.45 to 1.8 kV/cm treatment. HeLa cells subjected to such low levels of pulsed electric fields were transiently electro-permeabilized, as the membrane could recover and the majority of cells survived. The electric pulse destabilized the lipid bilayer and disrupted the interaction between the cortical actin filaments and the cell membrane. Damage to the cytoskeleton greatly affected the sealing process of the cell membrane and the stability of the electro-induced permeated structures. While the appearance of a pore in the bilayer lipid membrane could be a very fast process, it required relatively a longer period of time to seal the pores in the cell membrane. During this period, before the complete closing of the pores, the small extracellular Ca++ molecules could still cross the cell membrane. This continuous influx gave the late positive pick for the Ca++ shown in our results. According to numerical studies, electrophoresis played the main role for molecular uptake via electroporation. In our experiments, at high electric fields of 1.26 and 1.8 kV/cm, we observed a high rate of calcium uptake by electrophoresis, followed by an order of magnitude slower rate transport by diffusion. Interestingly, for low electric fields of 0.45 and 0.63 kV/cm, the rate of calcium uptake by electrophoresis and diffusion were almost the same. The mechanisms of molecular transport during electro-permeabilization greatly involved passive diffusion of small Ca++ molecules, through generated small pores in the lipid bilayer. This mechanism continued affecting the Ca++ influx into the cells after electrophoretic during the brief pulsing time, when the pores in the cell membrane were sealing. On the other hand, giving the transient nature of the electro-permeabilization and drastic changes in the electric surface charges, electro-osmosis was thought to be negligible factor. The results confirmed the importance of ionic diffusion and importance of forced microfluidics for minimally invasive low electric field calcium electroporation.

For needle-free drug/vaccine delivery to the skin, the velocity of the microjet leaving the nozzle was measured for different operating parameters of the device (such as, diaphragm thickness, compression tube fill pressure, and incident shock wave Mach number). The magnitudes of the velocity were obtained based on the real-time high-speed visualization of the microjet. The jet was observed to maintain a constant velocity over a distance of 5–6 mm, which offered a substantial stand-off distance from the nozzle for the operations. The velocity of the jet was a function of the shock strength, which in turn was a function of the initial fill pressure in the compression tube, across the diaphragm. The steady velocity of the jet ranged from 60–80 m/s for the diaphragm-stack-thickness range of 480–900 um. Methylene blue-dyed water was injected into excised human skin samples (n = 7). Multiple sections of these samples at the injection site were studied under microscope. Histology of the specimens revealed a clear breach through the epidermis in the injection spots, with the features consistent with deposition of methylene blue in the upper dermis. The penetration depth observed in the sectioned skin samples was 570 ± 150 um. The targets were placed in contact with the device's nozzle exit, without any standoff distance between the two, as the skin samples were fresh and highly elastic, requiring a higher jet velocity (80 m/s) to cause the failure. A higher jet velocity could also be obtained by increasing the diaphragm-stack-thickness. Impingement and penetration of the microjet into human skins were analyzed by considering a turbulent jet flow in a narrow, dead-end channel with a substantial backflow, due to the high elasticity of the skin. The backflow could reduce the progression of the hole in the target to a considerable extent. The decay of the jet velocity with respect to distance into the target in such cases was reported to be linear due to a high confinement. Considering an approximate critical stress of failure (τ_c) for the excised human skin, the theoretical penetration depth was calculated to be about 514 um, which was in good agreement with the experimental results.

We applied impulsive microfluidics in-vivo to mice animal models, the results are under preparation for publication. Also, we obtained interesting results from the evaluation of the microfluidic induced cell modification, which are under submission for publication.

５．主な発表論文等

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〔図書〕　計0件

〔産業財産権〕

〔その他〕

―

6．研究組織

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