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研究課題名（和文）A novel design methodology for the construction of functionalized human cell monolayer on an egg

研究課題名（英文）A novel design methodology for the construction of functionalized human cell monolayer on an egg

研究代表者

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研究成果の概要（和文）：ニワトリ胚のしょう尿膜上の細胞を部分的に除去し、その場所にヒト由来細胞を播種することで、ヒトの環境により近い状態で生体医工学の実験を行うことができる。本研究では、しょう尿膜上の表層細胞を部分的に除去する手法として、電界誘起気泡を用いた物理的手法を提案した。気泡の崩壊によって発生する力を制御するため、液体中にあるしょう尿膜の表面からの距離を正確に計測する新しい距離センサを開発した。さらに、気泡発生デバイスと膜の距離を200umにし、500Vのパルス電圧を印加することで、直径約350umの範囲でしょう尿膜上の細胞を除去できることを確認した。本研究は、生体膜を微細加工する新しい技術を確立した。

研究成果の学術的意義や社会的意義
Chick chorioallantoic membrane (CAM) is used as a model for ophthalmic research. For example, during corneal surface excision, it is necessary to precisely remove the epithelial cells on the surface. I have established a method to remove cells on the CAM without damaging the underlying structures.

研究成果の概要（英文）：By locally removing cells from the chorioallantoic membrane (CAM) of a chick embryo and culturing human-derived cells on the CAM, biomedical experiments can be conducted in an environment more similar to that of humans. In this study, we propose a physical method using electrically induced bubbles to locally remove the epithelial cells on the CAM. To control the forces generated by the collapse of the microbubbles, we developed a new distance sensor that precisely measures the distance from the surface of the CAM in liquid (egg white) to the tip of the injector for bubble generation. In cell removal experiments, a pulse voltage of 500V was applied to generate the bubbles. We confirmed that cells within a circular region with a diameter of approximately 350 micrometers on the CAM can be removed when the distance between the bubble generation device and the membrane is set to 200 micrometers. This study establishes a new technique for the microfabrication of biological membranes.

研究分野：バイオメカニクス、医工学

キーワード：biomembrane fabrication　Biomembrane positioning　precise cell removal

1. 背景

A porous membrane is used to separate two distinct areas of a microphysiological system (MPS) (1). A monolayer of human epithelial/endothelial cells is cultured on it to form a cell barrier (1). The porous membrane with a functional cell monolayer is essential for the observation of drug diffusion. However, the porous polymer membrane has low transparency/porosity. Further, the composition and mechanical properties are different from the native membrane, resulting in different cell functions and responses.

Chick chorioallantoic membrane (CAM) is a porous and transparent membrane with an epithelial cell monolayer adhering on top (Fig. 1). CAM is a cost-effective membrane (less than one US dollar) and the chick embryo is not a protected animal before embryonic day 10 (2). However, to culture a human cell monolayer on the CAM, the chick epithelial cells should be removed before transplantation (Fig. 1). There is a basic question. Is there a method to remove epithelial cells on CAM without damaging the porous membrane of the chick embryo?

2. 目的

I hypothesize that by removing the cells attached to the biological membrane (CAM) of chick embryos, researchers can conduct experiments using a highly porous biological membrane with human-derived cells attached (Fig. 1). This approach is expected to facilitate research on drug diffusion in an environment more similar to that of humans. Consequently, it is essential to establish methodologies for the microfabrication of the CAM. This study aims to develop microsystems with high-speed microbubble generation/collapse (Fig. 2) that enable the microfabrication of biological membranes.

3. 方法

3.1 Electrically induced microbubbles

Two electrodes are inserted into a conductive liquid (egg white) (Fig. 2). One electrode serves as the bubble injector, which consists of a tungsten wire inserted into a Teflon tube. The other electrode is a tungsten opposite electrode. When a high-voltage pulse is applied between these two electrodes, microbubbles are generated. These microbubbles subsequently collapse, creating localized forces (Fig. 2) (3).

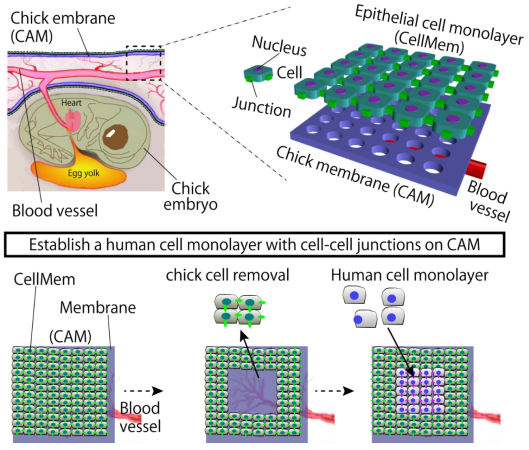

Fig. 1 The conceptual diagram of the CAM structure (upper) and the removal of chick epithelial cells

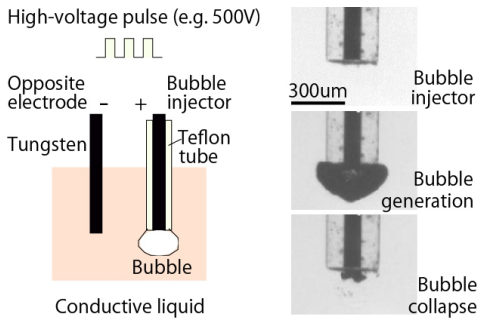

Fig. 2 The bubble injector for the generation of microbubbles

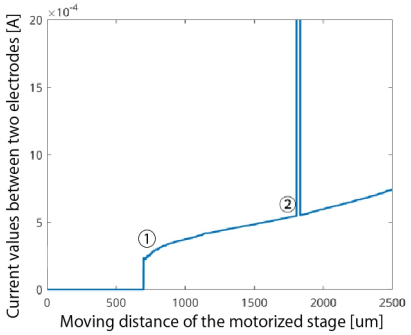

Fig. 3 The tip of the bubble injector is sequentially moved from the air to the surface of the egg white ① and then to the surface of the (CAM) ②

3.2 Sensing the distance between the bubble injector and the CAM

Before the application of a high-voltage pulse for bubble generation, a voltage of 5V is applied between two electrodes for the CAM position. The current flowing between the electrodes is measured using an ammeter. The opposite electrode (Fig. 2) is immersed in the egg white, while the other is lowered towards the surface of CAM. The moment the bubble injector touches the membrane, the current will increase sharply, and the position of the injector at this moment can be considered the position of the membrane. Measurement was conducted on the CAM of a chicken embryo (Fig. 3). Since the current increases sharply at the surface of CAM, the distance between the tip of the bubble injector and CAM can determined by moving the injector to a desired position using a high-precision motor.

3.3 Cell Removal Procedure

By moving the bubble injector over the CAM of the chicken embryo and applying a pulse voltage, electrically induced bubbles are generated for cell removal. The magnitude of the physical stimuli of the microjet varies depending on the power supply conditions and the distance between the device tip and the target. Therefore, by adjusting various experimental conditions, it is possible to control the cell removal process without damaging the membrane structure underlying the chick epithelial cells.

The experimental procedure is as follows:

1) Culturing the Chicken Embryo: The eggs were placed in an incubator set at 39° C to begin cultivation. After 2 days, the eggs were cracked open and transferred to a dish for an additional 3 days of cultivation. To maintain consistent humidity in the incubator, a tray filled with water was placed inside.

2) Bubble treatment and cell removal using the bubble injector.

3) Verification of cell removal through Hematoxylin-Eosin (HE) Stain or fluorescence staining. The cells of the chicken embryo were stained with CellTracker™ Green and observed under a confocal microscope. The presence or absence of cells on the blood vessels treated with electrically induced bubbles was checked to confirm the success of the cell removal.

4. 結果

4.1 Results of HE Stain

Fig. 4 shows the results of cell removal with or without applied voltages of 500 V, followed by HE staining. In the sample without the treatment with bubbles (non-treatd), areas stained with eosin and areas stained with hematoxylin can be observed. On the other hand, experiments using electrically induced bubbles generated with applied voltages of 500 V do not show areas stained with hematoxylin. This demonstrates that using electrically induced bubbles with applied voltages of 500 V can successfully remove cells on the CAM. Additionally, when examining the structure of the internal tissues, no significant differences are observed.

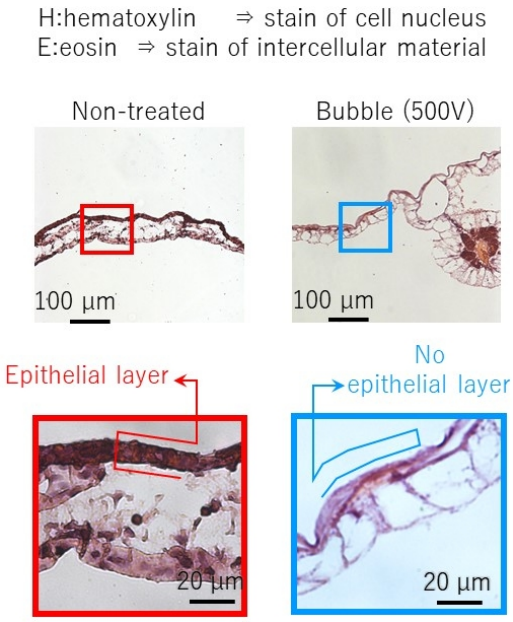

Fig. 4 HE stain of CAM. This figure shows the cross-section of CAM. The lower images are the enlarged view of the corresponding upper image.

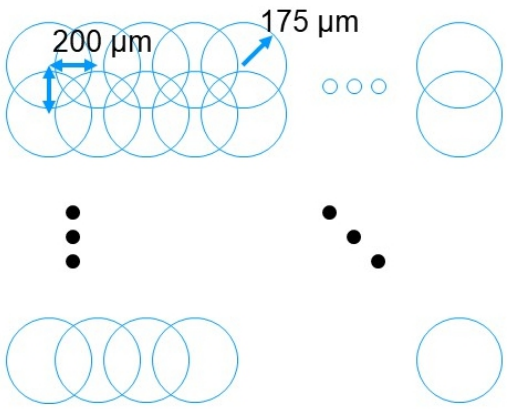

Fig. 5 Formation of a large area with cell removal on CAM. Each time the stepper motor moves 200 μm to the right or downward, bubbles are generated to treat the CAM, ultimately creating a large area of cell removal on the CAM

4.2 Results of fluorescence staining

Since the area with cell removal in each exposure to electrically induced bubbles, generated with an applied voltage of 500 V, was confirmed to be approximately 350 μm in diameter (data not shown), we considered

that setting the bubble treatment interval to 200 μm, as shown in Fig. 5, would allow us to completely remove cells in an area of several square millimeters. Therefore, with an applied voltage of 500 V, the position of the device tip was moved in 200 μm increments using a manipulator to remove cells within a desired area using electrically induced bubbles (Fig. 5).

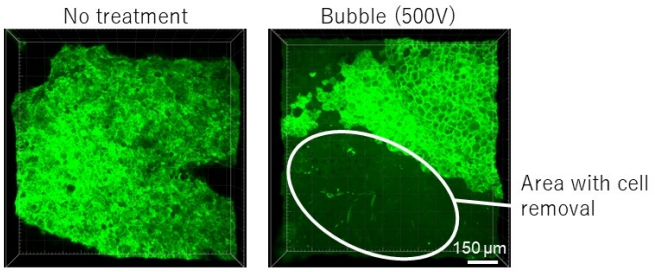

Fig. 6 The monolayer of epithelial cells on CAM with or without the treatment with electrically induced microbubbles

The results are shown in Fig. 6. Fig. 6 (left) shows the results of observing a chicken embryo without bubble treatment under a confocal microscope, where cells stained with CellTracker can be seen. Fig. 6 (right) shows the results of cell removal using electrically induced bubbles generated with an applied voltage of 500 V. It can be confirmed that cells were removed in the observed area.

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５．主な発表論文等

〔雑誌論文〕　計3件（うち査読付論文　3件／うち国際共著　1件／うちオープンアクセス　0件）

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２．論文標題 Viscosity-aided electromechanical poration of cells for transfecting molecules	５．発行年 2022年
３．雑誌名 Lab on a chip	６．最初と最後の頁 4276-4291
掲載論文のDOI（デジタルオブジェクト識別子） 10.1039/D2LC00628F	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

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オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

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〔図書〕　計0件

〔出願〕　計1件

産業財産権の名称	発明者	権利者
導入装置及びデリバリー方法	佐久間　臣耶，山西陽子，黄　文敬，木村　笑	同左
産業財産権の種類、番号	出願年	国内・外国の別
特許、特願2022-137072	2022年	国内

〔取得〕　計0件

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考
研究分担者	張　宏 (ZHANG Hong) (80774257)	東海大学・工学部・特定研究員 (32644)	2021年9月末まで

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関