STUDY OF TEMPERATURE EFFECT ON SINGLE-CELL FLUID-PHASE ENDOCYTOSIS USING MICRO CELL CHIPS AND THERMOELECTRIC DEVICES

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ABSTRACT

In this work, an array-type micro cell chip with micro temperature sensors (iMTS) and a thermoelectric device was fabricated to investigate temperature effect on fluid-phase endocytosis (FPED) of HeLa cells at the single-cell level. Using two different molecular probes, large-scale single-cell FPED data (more than 500 cells) demonstrated the existence of a critical endocytosis temperature of 16-17ºC for HeLa cells. Activation energy at higher temperature range (<6 kcal/mol) is significantly smaller than lower temperature range (>18 kcal/mol). This energy difference suggests that endocytosis can occur through different mechanisms at different temperatures.

KEYWORDS: Endocytosis, HeLa cell, FM4-64, Alexa Fluor 488 dextran, Temperature sensor, Thermoelectric device

INTRODUCTION

Cells internalize extracellular molecules through endocytosis (ED) (Fig. 1). The plasma membrane can invaginate and form submicron vesicles carrying molecules. Endocytosis has a crucial role in immune response, signal transduction, neurotransmission, intercellular communication, cellular homeostasis, etc [1]. In particular, fluid-phase endocytosis (FPED) is a type of non-receptor-mediated endocytosis for solutes and liquid uptake. Temperature can affect both physical & chemical properties of cell membranes [2], which further influences membrane protein activity, cell transport [3] and endocytosis [4]. It has been shown that there exists a critical temperature for endocytosis rate change (T_c), corresponding to (1) gel-to-liquid phase transition of lipid bilayer and (2) activity change of endocytosis-related proteins [2]. However, whether this temperature exists in specific cell types and how this temperature is related to these two phenomena are still under debate and the results reported in the literature are often contradictory [2,4–7] (Table 1), due to possible diverse endocytosis pathways [1]. Conventional ED experiments require bulky temperature control. Most of these experiments only provide statistical results from large cell population with large errors. Herein we fabricated an integrated thermal microsystem consisting of micro cell chip with iMTS and a thermoelectric device to conduct systematic study of temperature effect on FPED.

Table 1. Summary of contradictory temperature effects on endocytosis

Cell type	Molecules	T_c	E_a in two temperature ranges (L=lower than T_c ,H=higher than T_c)
E. Coli [2]	Proline	19 ° C	$E_{a,L} > E_{a,H}$
Hepatocyte [4]	Orosomucoid (ORM)	20 ° C	$E_{a,L} > E_{a,H}$
Macrophage [5]	Horseradish Peroxidase (HRP)	No	$E_{a,L} = E_{a,H}$
MDCK [6]	Lucifer Yellow (LY)	27 ° C	$E_{a,L} < E_{a,H}$
COS 5-7 [7]	BSA-FITC	15 ° C	$E_{a,L} < E_{a,H}$

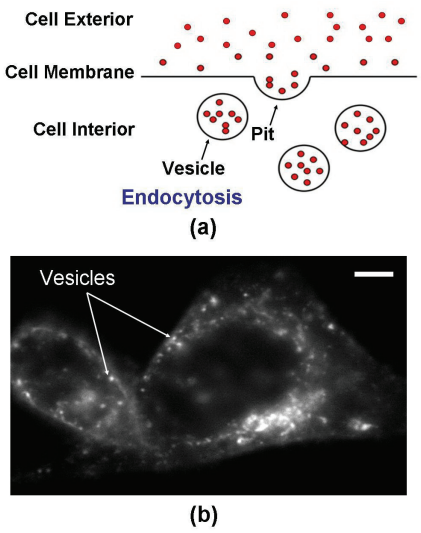

Figure 1: (a) Cell uptakes external molecules through fluid-phase endocytosis (FPED), (b) Fluorescence micrograph of endocytotic vesicles in a HeLa cell (scale bar: 5μm).

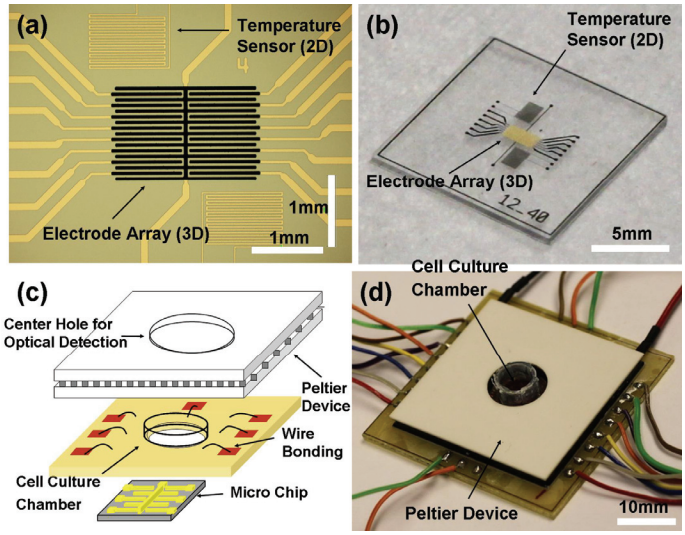

Figure 2: (a) Micrograph of on-chip iMTS and microelectrode array, (b) Photograph of a fabricated microchip, (c) Schematic drawing and (d) photograph of a Peltier thermoelectric device mounted on microchip-PCB assembly.

EXPERIMENTAL

An array-type microchip with integrated micro temperature sensors (iMTS) was fabricated on a glass wafer using two-mask MEMS technology at HKUST (Fig. 2). Two sets of 2D planar gold microelectrodes (width = 20μm, height = 0.1μm, length = 20mm) were sputtered to work as iMTS, with the temperature coefficient of resistance (TCR) around 2.01×10^{-3} K^{-1}. A Peltier thermoelectric device (TE Technology Inc, USA) was mounted on the microchip-PCB assembly, providing both cooling and heating modes. After the burn-in procedure, the iMTS showed good linearity and repeatability as shown in Fig. 3. Step response testing of the measured temperature on a microchip revealed that typical temperature time constant in the temperature range of 4-37ºC is less than 2 min, which is much smaller than 15-60min duration of FPED experiments. The steady-state error of the setpoint temperature is less than 0.4ºC.

Besides, an epi-fluorescence microscope (Eclipse TE2000-U, Nikon Corp., Japan) with a 40X objective (CFI Plan Fluor ELWD ADL 40X C; N.A. 0.60) was used to observe molecular uptake by HeLa cells on micro chips at single-cell level. A cooled digital CCD camera (SPOT™ RT-SE18 Monochrome, Diagnostic Instruments Inc., USA) mounted on the microscope and the control software package (SPOT™ Imaging Software Advanced Version, Diagnostic Instruments Inc., USA) were used to record 8-bit micrographs with spatial resolution of 1360 × 1024 pixels. Digital image processing software (AlphaDigiDoc®, Alpha Innotech Corp., USA) was used to quantify each single-cell endocytosis rate based on fluorescence micrographs.

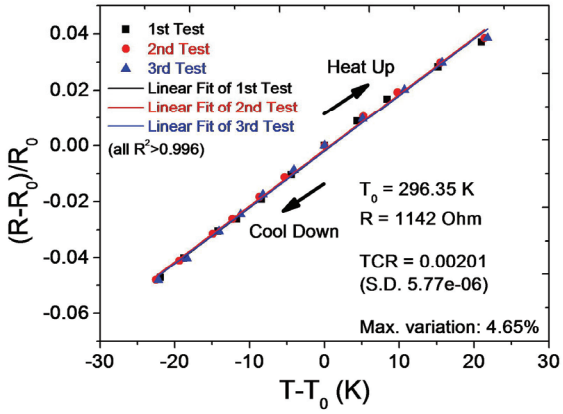

Figure 3: The TCR testing of the on-chip temperature sensor in both cooling mode (0-23ºC) and heating mode (23-45ºC).

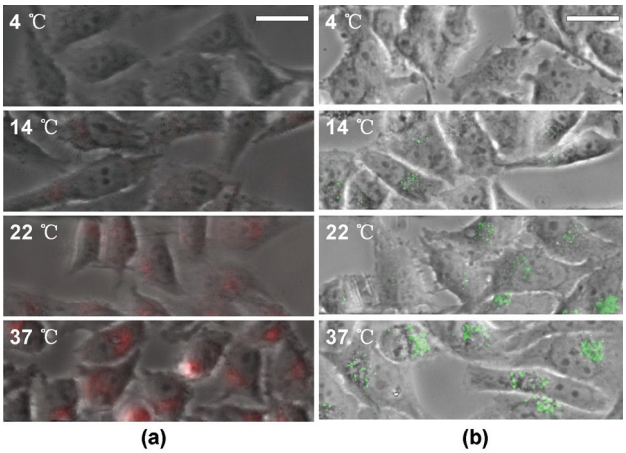

Figure 4: Fluorescence micrographs of the uptake of (a) FM4-64 and (b) Alexa Fluor 488 dextran by HeLa cells at four temperatures (scale bar: 20μm).

Cultured HeLa cells (CCL-2™, ATCC, USA) were harvested from a petri dish by trypsin/EDTA in the log phase of growth. These cells were then re-suspended at a concentration of 2×10^5 cells/mL on a micro chip for 10 hr, in the culture medium containing Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (Invitrogen Inc., USA). FM4-64 (Invitrogen Inc., USA; molecular weight = 607.51 Da), a lipophilic styryl fluorescence dye for lipid labeling, was used to characterize membrane internalization and endocytosis vesicles formation. In this experiment, FM4-64 was dissolved in the culture medium at a concentration of 4 μM, and incubated with HeLa cells for 15 min. In addition, a large molecular probe, Alexa Fluor 488 dextran (Invitrogen Inc., USA; molecular weight = 10 kDa), was used to characterize FPED rate. Dextran molecules were dissolved in the culture medium at a concentration of 50 μM for 60 min incubation with adherent HeLa cells.

RESULTS AND DISCUSSION

Fig. 4(a) shows the endocytosis of FM4-64 probes on a microchip at different temperatures. Obviously, within the range of 4-37ºC, adherent HeLa cells uptake more FM4-64 molecules when the temperature is higher. Endocytosis rate, k_E, characterized as the average fluorescent intensity (I_{avg}) from endocytosed FM4-64 probes in each single cell, increases with temperature. Fig. 5 illustrates the quantitative analysis from more than 200 single-cell endocytosis data. To further consolidate these temperature-dependent data, Arrhenius equation [4, 5, 6] was used to study $k_E(T)$:

$$\ln(k_E) = \ln(A) - E_a/(RT)$$

where E_a is the activation energy, R is the gas constant and T is the absolute temperature. Arrhenius plot of FM4-64 uptake in Fig. 5 shows biphasic characteristics with one inflection point between 15-23ºC. Using linear regression subrountine in Matlab™, we determined the critical temperature (T_c) to be 17.1±0.1ºC. Meanwhile, the activation energies (E_a) in the range of 4-17ºC and 17-37ºC are also determined to be 18.9±0.5 and 5.0±0.5 kcal/mol, respectively.

Furthermore, the FPED of HeLa cells using a larger molecular probe, Alexa Fluor 488 dextran, increases with temperature in the range of 4-37ºC. Fig. 4(b) shows that the number of fluorescent spots, which are endocytosis-induced vesicular structures carrying fluorescent dextran molecules, increase with temperature. Using image processing subrountine in Matlab™, we consistently completed contrast enhancement, edge detection and counted fluorescent spots number in each single cell. The quantitative results from more than 200 single-cell are illustrated in Fig. 6. We used

average fluorescent spots number (N_{avg}) to characterize endocytosis rate (k_E). Similarly, the Arrhenius plot for Alex Fluor 488 dextran uptake shows biphasic features with determined T_c=16.1±0.7ºC and the activation energies are 30.5±5.4 and 5.8±1.8 kcal/mol in the range of 4-16ºC and 16-37ºC, respectively.

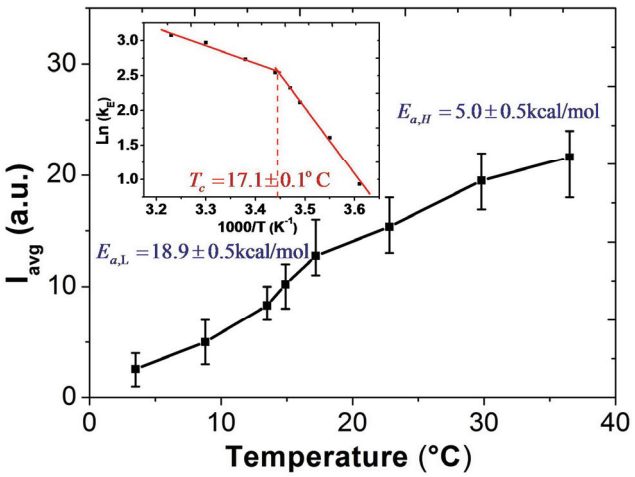

Figure 5: Normalized average fluorescent intensity (I_{avg}) of FM4-64 increases with temperature. Each data point is the average from 20 single cells. Inset: Arrhenius plot of HeLa cell endocytosis rate characterized by FM4-64 uptake.

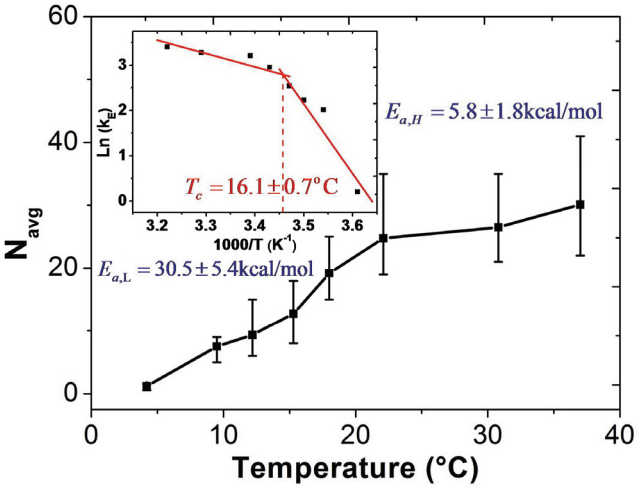

Figure 6: Average fluorescent spots number in single-cell (N_{avg}) increase with temperature. Each data point is the average from 20 single cells. Inset: Arrhenius plot of HeLa cell endocytosis rate characterized by AF488-dextran uptake.

Table 2. FPED's critical temperatures and activation energies for HeLa cells

Molecules	T_c (ºC)	E_a (kcal/mol)
FM4-64	17.1±0.1ºC	18.9±0.5 (4-17ºC)
		5.0±0.5 (17-37ºC)
Alexa Fluor 488 dextran	16.1±0.7ºC	30.5±5.4 (4-16ºC)
		5.8±1.8 (16-37ºC)

CONCLUSION

In summary, an array-type micro cell chip with iMTS and a Peltier thermoelectric device was successfully fabricated. This bio-MEMS device, for the first time, was used to study temperature effect on fluid-phase endocytosis (FPED) at the single-cell level. Using two different molecular probes, the measured large-scale single-cell data (~500 cells) illustrated biphasic features of FPED with temperature variation, and demonstrated the existence of a critical endocytosis temperature of 16-17ºC for HeLa cells. The activation energy at higher temperature range is significantly smaller. This suggests that fluid-phase endocytosis can occur through different mechanisms at different temperatures.

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