Suspending nanoliter droplet arrays for cell capture and copper ion stimulation

Hai-Fang Li, Yuan-Feng Pang, Jiang-Jiang Liu, Jin-Ming Lin*

The Key Laboratory of Bioorganic Phosphorus Chemistry & Chemical Biology, Department of Chemistry, Tsinghua University, Beijing 100084, China

1. Introduction

Researches on biological cells have developed from a large cell population to individual cells. In conventional experiments, one challenge is how to manipulate and separate numerous cells from a large population. The development of microchip technology allows capture, precise control and analysis of individual cells at the single-cell level. The microscale cell analysis in microchips allows investigating the function of microenvironmental change at the single-cell level. There is a growing interest for single-cell separation and analysis [1–4]. Different methods have been reported for cell or particle capture based on the miniaturization technology.

The physical restriction methods use variations of surface topography to separate particles from a flow and immobilize them on certain sites [5–7]. However, controlling precision deposition of particle seems to be very difficult. The dielectrophoretic trapping method depends mainly on differences of permittivity and conductivity between cells and the liquid medium, which vary as a function of the frequency of the applied field; the particle is either attracted toward the higher electric field or pushed away. Electric cages can be built that, irrespective of flow, enable single particles to remain freely suspended [8]. The energetic field capture methods include magnetic trapping [9], acoustic trapping [10] and laser tweezer [11,12]. Moreover, the long time exposure to energetic field may be detrimental to cells and destroy the activity of cells. There is no shearing effect which is inevitably induced by the liquid flow. For the retained single yeast cell stimulated by glucose and pH continuous Cu²⁺ stimulation was investigated and the two-step bioabsorption process was revealed clearly. The Cu²⁺ toxicity accumulation effect on the cell was also studied by monitoring the fluorescence of cellular chlorophyll.

In this paper, we developed a microdevice with raised cylinder arrays for capture of Chlorella vulgaris cells and kinetic analysis of stimulation. The cell solution was injected into the chip and held in the gaps between the opposite raised cylinders, forming 0.314 nl volume of suspending droplets. The number of the captured cells in each nanoliter suspending droplet could be controlled within five under our experiment conditions. When the stimulation reagent of copper ion (Cu²⁺) was injected into the chip and contacted with the suspending droplets, gradient concentration of Cu²⁺ stimulation to the captured C. vulgaris cells by free diffusion was formed. The bioabsorption kinetic process of C. vulgaris cells under continuous Cu²⁺ stimulation was investigated and the two-step bioabsorption process was revealed clearly. The Cu²⁺ toxicity accumulation effect on the cell was also studied by monitoring the fluorescence of cellular chlorophyll.

© 2010 Elsevier B.V. All rights reserved.
tum dots CdS labeled both on the membrane and interior of the cell, the Cu²⁺ toxicity accumulation to the chlorophyll in the *C. vulgaris* cell was also revealed by the fluorescence signal. This method integrated single-cell retention, gradient concentration of chemical stimulation, on-line qualitative analysis onto one microdevice.

2. Materials and methods

2.1. Microchip fabrication and cells retaining in the chip

Traditional photolithography and wet chemical etching techniques were used to fabricate the glass chip (shown in Fig. 1). The glass plates used to fabricate chips were purchased from Changsha Shaoguang Chrome Blank Co., Ltd. (Changsha, China). The process is described as follows. Firstly, the photomask with designed pattern was put onto a glass plate (1.0 mm × 1.5 mm) which was precoated with chromium and photoresist, then they were exposed under UV light for about 7 min. Following UV exposure, the glass plate was developed with 0.5% NaOH solution and then etched in 5% HF solution for 30 min to fabricate the protrudent circular plot arrays with the same dimension of 60 µm height and 100 µm diameter. Finally, a blank glass slide was used as a cover plate and assembled together with the etched glass plate by clamps. A piece of Teflon film (0.1 mm × 1.5 mm) was sandwiched between the two plates to form a confined gap. The inlet and outlet orifices for solution were mechanically drilled on the cover plate. As shown in Fig. 1a, the thickness of the Teflon film was 100 µm so the height of the gap between the protrudent circular plot and the cover plate was 40 µm (subtract 60 from 100 µm). The diameter of the columnar gap was 100 µm, the same with the glass protrudent circular plots. The distance between the circular plots in the array was 0.2 mm and a typical array of 8 plots was selected.

*C. vulgaris* cells and culture solution used in the experiments were provided by Ocean University of China (Qingdao, China). Firstly, the suspending cell solution was introduced into the chip from the inlet orifice by a micropump at a rate of 0.5 µL/min and the whole interspace between the two glass plates was filled as shown in Fig. 1b. Then the redundant solution was sucked out from the chip by vacuum pump at a rate of 0.1 µL/min and the suspending droplets were stored in the gap between the plot-cover plates as presented in Fig. 1c. The volume of the suspending droplet was calculated as 0.314 nL according to 100 µm diameter of the assay and 40 µm height of gap. The number of cells captured in each suspending droplet was counted under a microscope. We repeated this cell storage process three times under the same condition to test the reproducibility.

2.2. Trypan blue coloration to monitor the activity of the *C. vulgaris* cell in the chip under gradient Cu²⁺ stimulation

Trypan blue was purchased from Sigma (St. Louis, MO, USA) and sodium chloride was purchased from Beijing Chemical Plant (Beijing, China). The 0.05 g trypan blue, 0.18 g sodium chloride were added into 10 mL cell solution. Copper sulfate was purchased from...
Beijing Chemical Plant (Beijing, China) and 200 μg/L Cu²⁺ solution was prepared. The droplet of the cell solution containing trypan blue was retained in the chip after the storage process referred above. Then the Cu²⁺ solution was injected into the chip to saturate the space around the droplets at a velocity of 400 nL/min for 240 min. The laminar flow phenomenon and free interface diffusion in miniature induced a gradient Cu²⁺ concentration spreading from the edge to the center of the droplet. So the closer to the center the lower the Cu²⁺ concentration was.

2.3. The fluorescence monitoring of the chlorophyll in C. vulgaris cell with continuous Cu²⁺ stimulation

A fluorescence microscope (370 nm excitation light, Leica DMI 4000 B, Germany) was used to monitor the fluorescence of the cell chlorophyll during Cu²⁺ stimulation. After the cells were captured in the suspending droplet arrays, 200 μg/L Cu²⁺ solution was injected into the space around the droplet at a stable flow rate of 400 nL/min. At the same time, the alteration of fluorescence in C. vulgaris cell without Cu²⁺ stimulation was also observed for contrast.

2.4. CdS quantum dots labeling for monitoring Cu²⁺ stimulation kinetics in live and dead C. vulgaris cells

Water-soluble positive CdS quantum dots were purchased from Jiayuan Quantum Dots Chemical Industries (Wuhan, China). The excitation wavelength of the CdS quantum dot was 470 nm and the maximum emission was 589 nm. The diameter and the original concentration of the CdS quantum dots were 4 nm and 4 μmol/L, respectively. The CdS quantum dots solution of 0.04 μmol/L was prepared by diluting the original solution with 50 mmol/L borate buffer solution. Live and dead C. vulgaris cells (cells were killed by 100 °C water boiling) were incubated, respectively with the CdS quantum dots solution for 8 h at 37 °C. The redundant CdS quantum dots were removed after centrifuging with a TG16-W centrifuge (Changsha, China) in 4000 rpm/min and the cells were washed with deionized water. After that, CdS quantum dots labeled cells were retained in the suspending droplets in the chip, then 50 μg/L Cu²⁺ solution was injected into the chip at 400 and 600 nL/min, respectively. The attenuation of fluorescent signal was monitored from the beginning of Cu²⁺ stimulation with the 470 nm laser excitation.

3. Results and discussion

3.1. The cell capture efficiency in the microdevice

According to the Young–Laplace equation, the larger curvature radii produced the smaller surface pressure. Therefore, the cell solution nanodroplets can be retained in the narrower gap and will not escape from the 40 μm height of cylinder gap to the 100 μm height of space. The number of cells retaining on each plot was dependent on the original cell concentration in the solution. When the cell solution at concentration of 1 × 10⁷ cells/mL was injected into the microchip at a velocity of 500 nL/min and then was drawn out by vacuum pump at a rate of 100 nL/min. According to the 0.314 nL volume of droplet, the average number of cells in one droplet should be three. In fact, as shown in Fig. 2, the number of the cells in each droplet was within five for three repetitive experiments.

3.2. The effect of Cu²⁺ toxicity accumulation on the activity of the C. vulgaris cell

Many published reports have confirmed the toxicity of ionic copper and copper complex to alga [23,24]. The origin of toxicity may result from interacting with the chloroplast, the mitochondrion, or the cell membrane. To research the Cu²⁺ bioabsorption kinetic process and the toxicity accumulation effect of Cu²⁺ on the chlorophyll of the C. vulgaris cell, the activity of the cells should be monitored. We used trypan blue as an indicator to monitor the activity of the cell under Cu²⁺ stimulation. Trypan blue is a vital stain commonly used to selectively color dead cells in blue. Some previous researches have reported the toxicity of Cu²⁺ to the Chlorella cells [25–27] and mostly focused on the growth inhibition and cell densities. The trypan blue cannot traverse the intact membrane of living cell but can traverse the membrane of a dead cell. In our study, the whole Cu²⁺ toxicity accumulation process in the single C. vulgaris cell was monitored by staining with trypan blue. The trypan blue was directly added to the cell solution and was concomitant with the trapped cells in the droplet. The 200 μg/L Cu²⁺ solution was continuously injected into the microchip at 400 nL/min velocity. Photos of cell living states were taken every 30 min as shown in Fig. 3. The trapped cell did not flow out of the suspending droplet after a continuous Cu²⁺ solution injecting as long as 210 min. The stability of the suspending droplets held in the microchip can be explained by the Stokes flow – likeness phenomenon. When the Cu
solution was inputted into the microchip at 400 μL/min velocity, the calculated Reynolds number was about 5.0. So the advective inertial force was smaller compared with viscous forces. The cell was not stained after Cu^{2+} stimulating for 60 min, which indicated that Cu^{2+} did not destroy the activity of the cell membrane. After about 90 min, the trypan blue began to diffuse into the cell and there was an obvious plasmolysis in the cell while the color inside the cell turning to blue. It illustrated that the accumulation of the Cu^{2+} had reached to a deleterious grade and the integrity of the cell membrane was destroyed by the superfluous Cu^{2+}. The whole cell was dyed and displayed dark blue color after 180 min stimulation. The blue color intensity was sustained for additional 30 min Cu^{2+} stimulation. Therefore, it could be deduced that it took more than 60 min for Cu^{2+} (in a range of 50–200 μg/L) to accumulate to the cellular deleterious grade under our experiment condition.

3.3. The effect of Cu^{2+} toxicity to the chlorophyll of the C. vulgaris cell

The previous research showed that the different variations of fluorescence could reveal the circadian state of the algae cells [28]. However, their research was based on the large cell populations which neglected the diversities of physiological information between cells. In the Chlorella cell, red fluorescence is mainly produced by the cellular chlorophyll so that the diversification of the red fluorescence could reflect the circadian information of the chlorophyll. Here, through monitoring the change in the emission fluorescence in single C. vulgaris cell, the whole process of Cu^{2+} accumulation toxicity to chlorophyll was revealed. Fig. 4a–d shows the attenuation of red fluorescence in single C. vulgaris cell with Cu^{2+} injecting and the photos were taken every 3 min. Fig. 4e and f shows the numeric diversification of the cell fluorescence without and with Cu^{2+} injecting, respectively. From Fig. 4e we can see the fluorescence attenuates quickly at the beginning and becomes stable in the following time. Finally, the fluorescence keeps almost in a plain. In Fig. 4f, the attenuation of fluorescence also appears during the first several minutes, then the fluorescence intensity increases instead after 5 min Cu^{2+} stimulation and decreases again after in the following 4 min. The different fluorescence intensity trends may be induced by the chemical diversification of chlorophyll under Cu^{2+} stimulation. For a living C. vulgaris cell, chlorophyll absorbs the luminous energy and uses most proportion of it for photosynthesis and less proportion for red fluorescence emission. There is a competition between these two processes. Once the photosynthesis is restrained, the intensity of red fluorescence emission would be reinforced. Mg^{2+} in the chlorophyll is indispensable for photosynthesis. When the Cu^{2+} entered into the cell, Mg^{2+} in the chlorophyll can be replaced by the Cu^{2+} so that the photosynthesis would be restricted and fluorescence emission is enhanced. In the primary Cu^{2+} stimulation period, only thimbleful Cu^{2+} could contact with the cell and there is no Cu^{2+} enter into the C. vulgaris cell. Then, with the Cu^{2+} accumulation and concentration around the
Fig. 5. The fluorescence quenching process of the *C. vulgaris* cell labeled with CdS quantum dots. Photo (a) was taken before Cu\(^{2+}\) contact with the cell; photos (b–d) were taken per 3 min after forming a gradient solution of Cu\(^{2+}\) to contact with the cell. (e) and (f) the fluorescence quenching processes with 400 and 600 nL/min Cu\(^{2+}\) injecting velocities, respectively. (g) The fluorescence changing of the labeled cell without Cu\(^{2+}\) injecting. The fluorescence signals were repetitively detected for three times.
cell, some Cu$^{2+}$ entered inside the cell and substituted Mg$^{2+}$ in the chlorophyll complex. As shown in Fig. 4f, there is a clear enhancement of fluorescence intensity in cell after Cu$^{2+}$ accumulation for several minutes. It was a very interesting phenomenon which was different from former cell group experiments. In conventional fluorescence measurement experiment, C. vulgaris cells were cultured in the Cu$^{2+}$ solution for several hours and then the fluorescence of the cells was measured. The off-line detection could not reveal the fluctuation of the fluorescence intensity and just provided the ultimate attenuation fluorescence results.

3.4. The absorption kinetic process of Cu$^{2+}$ in the live single C. vulgaris cell

To observe the detailed Cu$^{2+}$ absorption dynamic process in the C. vulgaris cell, a suitable marker which is steady and nontoxic to the cell is necessary. CdS quantum dots (semiconductor nanocrystal) as a new fluorescence material present many advantages: (1) the stability and anti-photobleach of fluorescence is very strong; (2) the color of the fluorescence can be adjusted by changing the size of the quantum dots and surface modification [29]; (3) the quantum dots could attach to bio-moleculars such as proteins, DNA, or viruses. These nanoconjugates are biocompatible and suitable for use in cell biology and immunoassay [30]. Here, we used CdS quantum dots to monitor the Cu$^{2+}$ absorption kinetic process in cells because Cu$^{2+}$ could quench the fluorescence of CdS by forming either Cu$\text{S} (x = 1, 2)$ precipitate or isolating Cu$^+$ on CdS, which has been proposed by Isarov et al. [31]. There are two main advantages of using quantum dots CdS as label: firstly, the intensity of fluorescence of quantum dots is very stable; secondly, the quantum dots can be absorbed both in the membrane and inner of the cell, which is very suitable to observe the absorption kinetic process. Water-soluble positive CdS quantum dots firstly electrostatic interacted with the negative charges of the cell membrane and then were phagocytized into the cell.

In Fig. 5, the photos a–c were taken every 3 min to show the fluorescence diversification in the labeled C. vulgaris cell under continuous Cu$^{2+}$ stimulation. Before Cu$^{2+}$ stimulation, the whole cell looked bright as the spread of the quantum dots both in the membrane and inside the cell. Then, with the continuous Cu$^{2+}$ stimulation, the surface fluorescence of the C. vulgaris cell attenuated quickly, but the inner of the C. vulgaris cell was still bright, which illustrated that Cu$^{2+}$ was absorbed only by the cell membrane, those CdS quantum dots inside the cell were not be affected. It illustrated the two-step process of Cu$^{2+}$ bioabsorption in C. vulgaris cell clearly. At the beginning, Cu$^{2+}$ was just accumulated in the cell membrane. When Cu$^{2+}$ contacted with the quantum dots labeled in the cell membrane, fluorescence of the quantum dots was quenched. It took time and energy for Cu$^{2+}$ moving into the cell, so there would be a transitory stabilization in the fluorescence intensity. Finally, Cu$^{2+}$ penetrated the cell membrane and quenched the fluorescence of quantum dots inside the cell, and thus the decrease of the fluorescence began to accelerate again (Fig. 5e and f). The fluorescence of the quantum dots labeled cell without Cu$^{2+}$ stimulation was also monitored as a contrast (Fig. 5g).

To study the influence of Cu$^{2+}$ injection velocity to the absorption kinetics, different injection velocities of 400 and 600 nL/min were performed, respectively. Fig. 5e and f shows the contrast. The fluorescence quenched more quickly before the plain period when the injection velocity increased from 400 nL/min to 600 nL/min, but the difference in the fluorescence diversification was not obvious after the plain period.

It can be concluded that the injection velocity only changed the absorption in the cell membrane and did not affect the penetration of Cu$^{2+}$ into the cell. It showed that the C. vulgaris cell had a self-adjusting ability for Cu$^{2+}$ absorption. Cu$^{2+}$ was accumulated and must conquer the resistance of the cell membrane to enter the cell.

3.5. The absorption kinetic process of Cu$^{2+}$ in the dead single cell

In previous studies, the absorption kinetic process in the dead C. vulgaris cells was ignored. Here, we studied the kinetic process of Cu$^{2+}$ in a single dead C. vulgaris cell. The CdS quantum dots were used to label the dead cell. Fig. 6 shows the relationship between absorption time and intensity of fluorescence in a single dead cell. Comparing Fig. 5e with Fig. 6, there is evident difference in fluorescence attenuation between the live and dead cells. The fluorescence intensity platform disappeared when the cell was killed. It proved that the academic two-step process of absorption [32] in the live cell disappeared in the dead one. For the live cell, Cu$^{2+}$ was absorbed passively by the cell membrane and some cations were even transferred into the cell due to the metabolism of the cell. Once the cell was killed, the adjustable function of the cell for Cu$^{2+}$ absorption disappeared, the process of Cu$^{2+}$ entering into the cell turned to totally passive and no supererogatory energy was needed. Cu$^{2+}$ could enter into the cell directly to quench the quantum dots fluorescence. So the fluorescence attenuation platform disappeared and decreasing rate in a dead cell was quicker than in the live one.

4. Conclusions

The constructed array microdevice makes it possible to control the capture and location of single or numerable cells in nanoliter droplets. The cells stored in the suspending droplets by surface tension can be circled with atmosphere or another kind of reagent. So oxygen and stimulation substance were convenient to contact with the cell. In our study, with the Cu$^{2+}$ solution continuously injecting into the microdevice to encircle the droplet, the stimulation kinetic process of Cu$^{2+}$ and the effect of Cu$^{2+}$ toxicity accumulation to the single C. vulgaris cell were monitored by trypan blue staining, chlorophyll fluorescence and CdS quantum dots labeling. The design of array provides a possibility for study of interaction between cells and their microenvironment.

Acknowledgements

This work was supported by National Natural Science Foundation of China (Nos. 30772006, 20775042, 90813015) and National Basic Research Program of China (2007CB714507).