Original

Red Blood Cell Lysis at the Single Cell Level by Using a Mini Electrophoresis Apparatus

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Abstract

Observation of the lysis phenomenon of human erythrocyte at the single cell level under a mini electrophoresis apparatus was studied. The lysis-inducing reagent (hydrogen peroxide in saline) was introduced to the uptake ferrous red blood cells at the end tip of the rectangular capillary by electroosmotic flow, generating hydroxyl free radical (Fenton reaction). Red blood cell exposed to hydroxyl free radical underwent progressive lysis, determined by observation through a microscope-CCD camera recording system. The erythrocyte lysis time of several single red blood cells varied between 450 sec and 510 sec. Distinct lysis behavior was delineated by monitoring the apparent light density of each single erythrocyte through time. The time dependence apparent light density curve of single red blood cell was used to assess the lysis phenomenon. It was found that the rate of red blood cell alteration dramatically increased after some initial period leading to eventually complete lysis. The half-lives of the initial step and the second step were estimated as kinetic parameters. The proposed technique stabilized the lysis mechanism by minimizing the depletion effect of the enveloped solvent layer of red blood cell. Moreover, electric field strength provided more homogeneity of the enveloped solvent layer and enabled the erythrocyte to maintain at the observed position.

Keywords: red blood cell, lysis, single cell, electric field, hydroxyl free radical

Introduction

Electric field application in separation science has recently emerged as a powerful separation technique in applying to single cell studies [1-13]. Electric field was applied as a sample handling technique to manipulate the single cell [2]. Manipulation of a single cell could be performed in a capillary tube under electric field such as the manipulation of rat clonol pleochromocytoma, snail neurons or adrenal medullar cells [1, 6-9]. Several researchers have mentioned the use of capillary electrophoresis as a good tool for studying the components in single cell [9-13]. Recently, the neurotransmitters in single neuron cell [9] or the components inside the single erythrocyte [13] were analyzed and perceived as a progress in the single cell studies. Most of the research works on the analysis of components inside a single cell were performed through lysing the cell immediately after led into the capillary for further separation and determination of the inner components. However, the lysis phenomenon of the cell was not well observed.

The main purpose of this study was to develop a new method for the investigation of lysis phenomenon of single cell in detail. The study was designed in order to observe the lysis phenomenon visually from the time the cell is in contact with the lysis induced reagent, carried through the electroosmotic flow (EOF) into the capillary within the simple homemade electrophoresis apparatus, until it is lysed completely. A new approach to follow the lysis process was invented. Then we further observed and analyzed the lysis phenomenon into different stages according to the behavior of the cell lysis. The half-life of each alternation stage was also determined. In response to these objectives, the red blood cell lysis was selected as a study model. The erythrocyte damage in this experiment was induced by the hydroxyl radical formation in the presence of hydrogen peroxide and ferrous ion (Fenton reaction) [14-16]. Hydroxyl radical had been shown to cause hemolysis in sev-

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eral studies [14-16], but the behavior of lysis had not been well established. The experiment was designed to obtain the ferrous-tagged red blood cells utilizing the simple in vitro method. Red blood cell lysis was studied as a macroscopic parameter for membrane damage and red blood cell deformability which are microscopic parameters. In the current study, the influence of the hydroxyl radical generating system on erythrocyte lysis observed through the microscope-CCD camera was monitored in order to obtain further insights into the behavior of the lysis process of this system.

Experimental

Apparatus, column and materials

The schematic diagram of an apparatus for the observation of red blood cell lysis was shown in Figure 1. The apparatus consisted of a rectangular capillary column (0.05 mm × 0.5 mm, 2 cm long, 500 nl), two polyethylene reservoirs (approximate volume of each: 250 μl) on a slide glass (Matsunami, Osaka, Type S-1214), two platinum wire electrodes inserted to the two reservoirs, a voltage

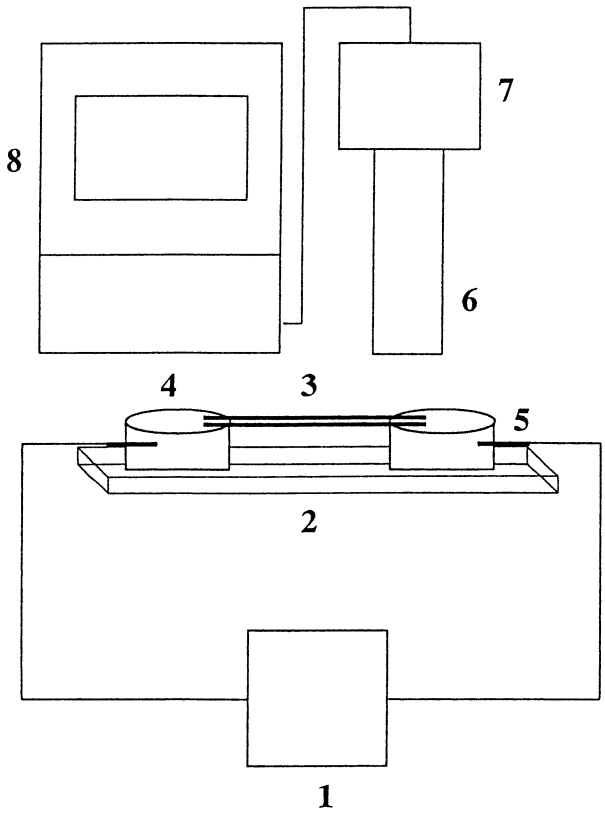

Figure 1. Schematic diagram of apparatus for observation of erythrocyte lysis. (1) dc power supply (2) slide glass (3) short rectangular capillary (0.05 mm × 0.5 mm, 2 cm long) (4) small polyethylene reservoirs (volume, 250 μl) (5) platinum wire electrode (6) microscope (7) CCD camera (8) video timer and video recorder with CRT monitor.

power supply (Kikusui Electronics Corp., Kawasaki, Type PMC 500), a microscope (Shimadzu, Kyoto), a CCD camera (Shimadzu, Kyoto, Type CCD-X2), a video recorder-CRT monitor (Aiwa, Type VX-T14G3) and a video timer (minimum time resolution 10 ms, ForA, Tokyo, Type VTG-33).

The column used for the observation of red blood cell lysis was the rectangular column (0.05 mm × 0.5 mm) purchased from Wilmad Glass (Buena, NJ, USA).

Ferrous sulfate, sodium chloride and 30% hydrogen peroxide were purchased from Wako Pure Chemical Industries (Osaka, Japan). One percent agarose gel (Nakarai Chemicals Ltd.) was prepared by dissolving in 0.9% saline.

Erythrocyte separation

Blood was obtained from healthy subject and diluted with 0.9% saline. After centrifugation at 500 xg for 5 min at room temperature, the supernatant, buffy coat, and upper 15% of the packed red blood cell were removed by aspiration. The remaining erythrocytes were washed three times with 0.9% saline at room temperature. Packed erythrocyte 40 μl was then diluted with 0.9% saline 960 μl and stored in refrigerator until used. Incubation of the red blood cell suspension with ferrous sulfate (0.1 mM final concentration of incubation suspension) for 5 min was freshly prepared before observing the lysis phenomenon.

Procedure

The mini electrophoresis apparatus as shown in Figure 1 was used. Red blood cells in precubation mixtures of 0.9% saline and ferrous sulfate are led in the short rectangular capillary column by capillarity. Then, both ends were sealed with 1% agarose gel so as to prevent the pressurized flow in the capillary generated by the difference of the level of mediums between the two reservoirs. The capillary was then placed in the homemade mini electrophoresis apparatus.

The precubation mixture of 0.9% saline and ferrous sulfate was filled into the polyethylene reservoir at cathodic end side of the capillary. Then, the 0.03% hydrogen peroxide in saline was filled into anodic reservoir. The concentration used in this experiment was adjusted in order to generate the adequate slow process for the observation of the lysis behavior. After focusing the microscope (magnification of 1,000 at the Cathode Ray Tube screen, 14 inches size) on red blood cells at the anodic end side of the capillary, electroosmotic flow of hydrogen peroxide in saline was introduced to the column by applying dc 100 V (50 volt/cm) along the capillary column. The images of the alteration of red cells were first recorded on videotape via the microscope-CCD camera video recording system. The video timer counting, superimposed on the alteration of the red blood cells, was also recorded. Then the im-

ages at small different time progress were captured by using a FDMCAP software (Photron Ltd.) and printed with a canon BJF 300 printer. The lysis of red blood cell was, therefore, observed at a single cell level through time. Lysis time of each single red blood cell under free radical generating system was finally estimated through the direct observation method. From the captured photographs of red blood cells, the color intensity of each erythrocyte within a constant limited area was determined by using the Adobe Photoshop software (Adobe Systems Incorporated). Within the constant limited area of single red blood cell, the average red color intensity, green color intensity, and blue color intensity of the selected single red blood cell were 168.39, 44.40 and 8.77, respectively. Then the summation of these three main colors (RGB color mode) became 221.56 unit, defined as the color intensity of single red cell in this experiment. For each red blood cell, the subtraction of the color intensity at each time from the maximum color intensity obtained from the whole lysis process gave the values introduced by the term “apparent light density of red blood cell”.

Results and Discussion

In order to clearly observe each red blood cell, the rectangular capillary column was selected. The transparency property of the rectangular capillary is the benefaction for the clearer observation of the particles inside the capillary column [17]. Furthermore, in comparing to the circular type column, the geometrical dimension of the rectangular column allowed the easier focussing and observation on each red blood cell.

In capillary, since the absolute value of the electrophoretic mobility of the red blood cell ($\approx 4.3 \times 10^{-4}$ cm^2 V^{-1} s^{-1} [18]) was greater than the absolute value of electroosmotic mobility of the saline medium (2.0×10^{-4} cm^2 V^{-1} s^{-1}), the red blood cell was able to maintain around the agarose gel frit without moving to the cathode. Eventhough for the red blood cell incubated with ferrous ion for 5 min in the system without hydrogen peroxide, it moved toward the anode against the electroosmotic flow once applying the electrovoltage. This implied that the net charge of the ferrous uptake red blood cell was still negative. This gave the contribution to the stable position of red blood cell at the observed chamber, enabling precise observation of lysis behavior under the microscope.

Starting time of lysis reaction

It was found that the starting time of the lysis reaction of the red blood cell at the end tip of the column next to the agarose gel frit, was approximate to be the same as the starting time of the application of electrovoltage. This was due to that once the electrovoltage was applied, electroosmotic flow had carried hydrogen peroxide solution to the capillary and surrounded the observed red blood cell at the end of the column immediately. The difference of the starting time of the lysis reaction among all the observed red blood cells was less than 1 sec calculated from the electroosmotic mobility (which is 2.0×10^{-4} cm^2 V^{-1} s^{-1}) of the unidentified neutral particles toward the cathode with a microscope. The starting time of the lysis reaction of each red blood cell was immediately after applying voltage.

Observation of erythrocyte lysis at a single cell level

Observation of the visual cell lysis was performed with a microscope equipped with a CCD camera. Figure 2 shows visual images of the treated red blood cells in the rectangular capillary at different time. Figure 2 a shows the visual images of red blood cells at the starting of the electric field application. While Figure 2 b shows the change in image of red blood cells after the first half observation period. Then in Figure 2 c, shows the image of several red blood cells completely lysed.

Red blood cell lysis as determined by monitoring under a microscope was achieved within 510 sec. The erythrocyte lysis time for several observed single red cells varied from 450 to 510 sec in this experiment as shown in Table 1.

For more distinct lysis phenomenon observation, the light absorption property of red blood cell structure and components was used and determined as the color intensity of red blood cell. Light absorption property of the hemoglobin, which is the main component (33.5%) of the inner compounds of erythrocyte, was used as a detection method in separation of hemoglobin variants in single human erythrocyte [13]. The red blood cell membrane, which is constituted roughly half (49.2%) by protein and half (43.6%) by lipid, can also absorb the light [19]. When the erythrocyte lysis took place, remarkably known from the release of hemoglobin from the cell and cell membrane deformability, red blood cell would absorb less light leading to higher color intensity of the single cell photograph. Thus, the color intensity of single red blood cell photograph is correlated to the red blood cell lysis phenomenon. In this report, the inversion of the color intensity was observed as the apparent light density of red blood cell.

The more apparent light density value the cell has, the more stable the cell is presumed. The less apparent light density value the cell possesses, corresponds to the cell that experiences partial lysis and lesser dense cell existed more lysis action. Lysis was shown to increase with a decreasing apparent light density value of cell. The apparent light density of red blood cell - time curve could be assumed to correlate with lysis curve.

The time dependence of the erythrocyte lysis phenomenon after applying voltage of six red blood cells was shown as the curves in Figures 3 and 4. Each curve represents each single red blood cell. Similar pattern of these curves was observed.

Three different curve patterns were observed from Figure 3

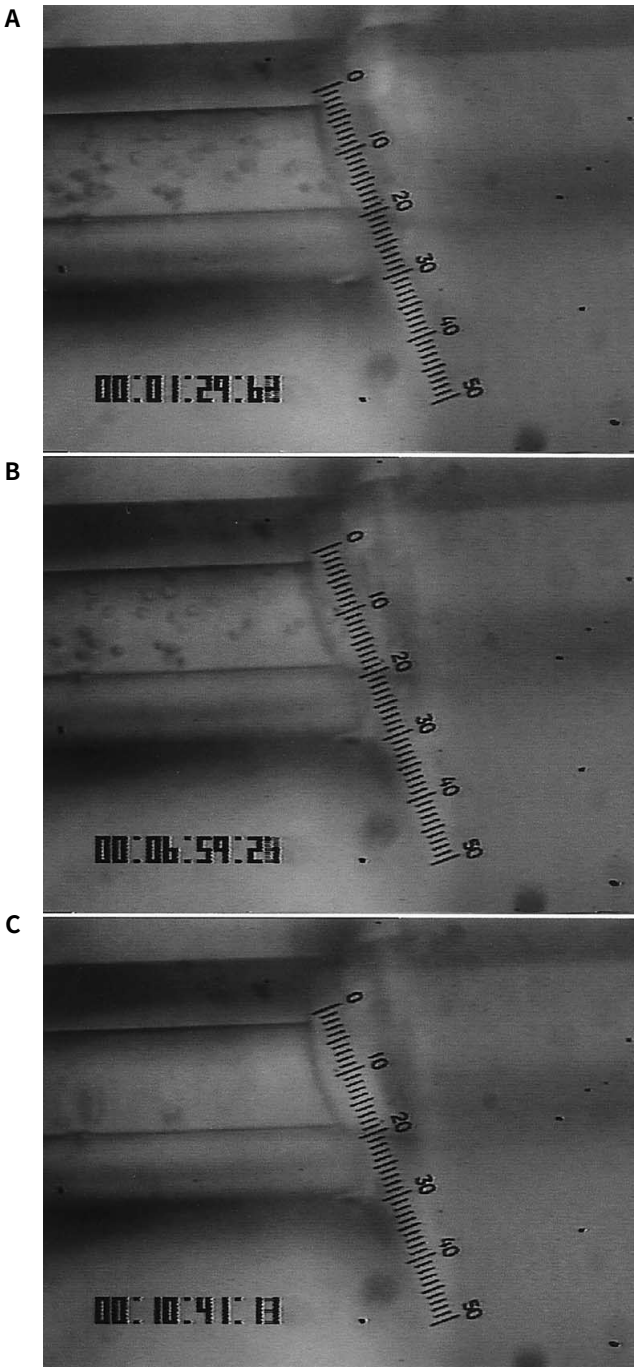

Figure 2. Visual images of the red blood cell confronting the hydroxyl free radical generating system at different time. (a) at the starting of the reaction (b) at the first half observation period (c) at the latter observation period. Images captured by FDMCAP software from the recorded visual images under the microscope-CCD camera system. The lysis phenomenon was performed in the rectangular capillary within homemade mini electrophoresis apparatus.

Table 1. Erythrocyte lysis time and half-life of several single red blood cells obtained from the red blood cell lysis phenomenon by using the mini electrophoresis apparatus.

Lysis time (sec) by visual observation method	Lysis time (sec) by apparent light density monitoring method	Complete lysis time (sec) by apparent light density monitoring method	Half-life for the initial step (sec)	Half-life for the second step (sec)
450	451	490	172	54
464	467	496	172	62
469	466	505	165	68
453	451	495	165	60
468	467	510	165	68
450	451	490	172	54

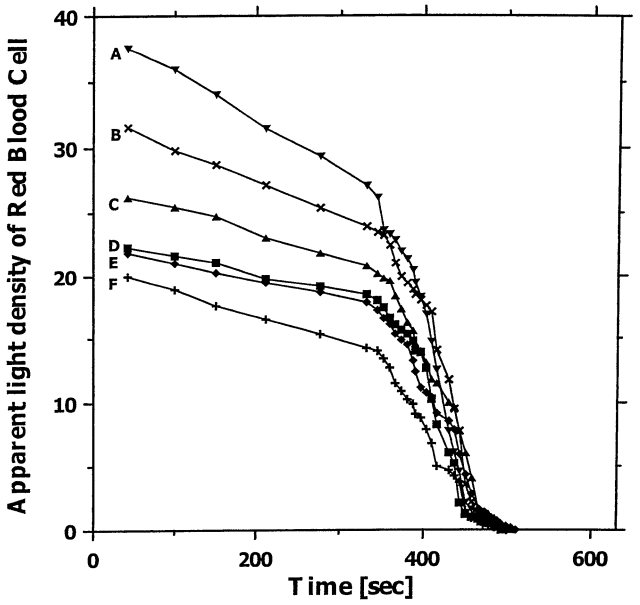

Figure 3. Relationship between apparent light density of red blood cell and time for the red blood cell lysis phenomenon observation. Each curve represented each observed single red blood cell. Other experiment conditions were the same as in Figure 2.

expressed as the three steps of cell lysis process : initial step, second step and third or final step. The apparent light density values of the curves in Figure 3 dramatically changed at the end of each step. This implied the difference in the lysis phenomena of the three steps. Distinct observation could be seen from Figure 4 which is the enlargement of the final step of the curves in Figure 3. Accord-

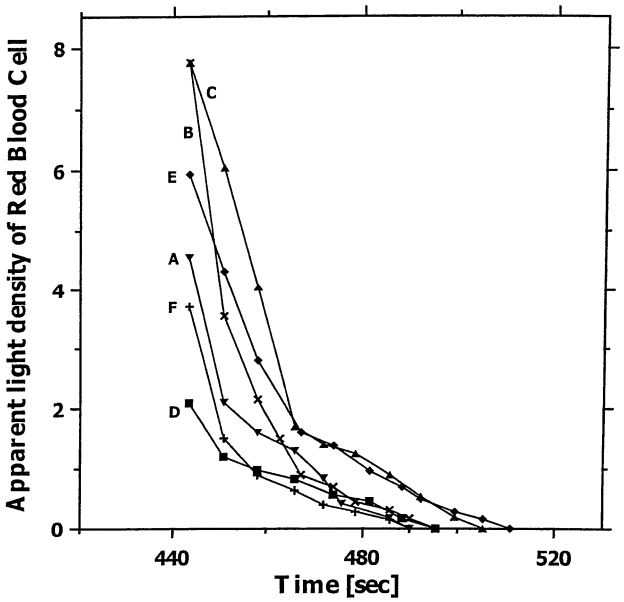

Figure 4. Relationship between apparent light density of red blood cell and time for the red blood cell lysis phenomenon observation. Enlargement from Figure 3. All conditions are the same as in Figure 3.

ing to Figure 3, apparent light density values of red blood cell were gradually decreased in the initial step, following a period in which the profile was dramatically changed until complete erythrocyte lysis finally occurred. The initial step of the curve in Figure 3 is expected to be associated with partial cell lysis. While the second step, the hydroxyl free radical caused a progressive change in the deformability, and ultimately led to complete cell lysis in the last step. From Figure 4, the apparent light density curve pattern suddenly changed at the point whose the image of single erythrocyte disappeared from the observed position. The rate of apparent light density alteration of red blood cell was alleviated in the last step comparing to the second step as clearly shown in Figure 4.

The single erythrocyte lysis time observed under microscope-CCD camera and through apparent light density of cell were compared in Table 1. These referred to the time that the single red blood cell image disappeared from the observed position and their values corresponded with each other very well. Whereas, the complete erythrocyte lysis time obtained by apparent light density monitoring method indicates the time that the color intensity of the observed single red cell photograph become approximate constant. This might be due to the total lysis of red blood cell fragments or the complete washing the components of the red blood cell out of the observed position.

To see the kinetics of cell lysis process, the half-lives of the initial step and the second step of the apparent light density - time curve were estimated and shown in Table 1. The half-lives of the initial step were between 165 and 172 sec. The starting time of lysis process of the second step, which was marked by obvious change in curve pattern from the initial step to the second step, were almost the same among all observed erythrocytes with the difference within 15 sec range. The half-lives of the second step were varied between 54 and 68 sec in this experiment.

According to Figure 3, in the initial step, the linear relationships between the apparent light density of red blood cell and time were observed with the correlation factors between 0.999 and 0.994 among all the observed curves. It implied that the alteration of apparent light density value by time at several observed points in the initial step was approximately constant. For instance, the relationship between apparent light density of observed erythrocyte and time in the initial step of curve B could be expressed as the linear relationship whose the slope value, Y-intercept and correlation factor are 2.60×10^{-2}, 32.55 and 0.999, respectively. Therefore, over all range of initial step of curve B, the differential of apparent light density value by time, d[apparent light density]/dt, is approximated to be a constant value of 2.60×10^{-2} unit/sec. The same assumption was introduced to initial step of curve A to F. With the mentioned reasons, the alteration of apparent light density of red blood cell of the initial period by time was probably analogous to zero order. However, the lysis processes of the second and third steps were too complicated for simple estimation. Further work is required for unequivocal identification.

It is interesting to note that three different processes of erythrocyte lysis induced by Fenton reaction was observed. In the light of these studies, it corresponded very well with the known reaction of lipid peroxidation induced by active hydroxyl radical. The lipid of the erythrocyte membrane can be oxidized by a free radical chain mechanism consisting of three steps: initiation, propagation and termination steps [14]. There are several parameters that may be studied for the quantitative mechanism of this lysis process. We hope that the proposed technique after elaboration would be useful for further reaction mechanism study.

Conclusion

The using of mini electrophoresis apparatus has been proposed to observe the behavior of erythrocyte lysis. Our experiment shows that hydrogen peroxide reacted with ferrous loading red cell altering red cell deformability. This led to inevitable and complete lysis through time. By using the erythrocyte apparent light density value monitoring method, the results in estimation of red cell lysis time shows an extremely good correlation to the observation under microscope-CCD camera method.

By using this proposed technique, the starting time of the lysis reaction on each single red blood cell could be observed. In addition, each single red blood cell was surrounded with the freshly hy-

drogen peroxide in saline medium by the rinsing effect of the electroosmotic flow of the medium. Therefore, the surrounding solution of each red blood cell was always nearly the same as of the bulk solutions. We believed that it stabilized the lysis reaction mechanism owing to the minimizing of the depletion effect of enveloped solvent layer of cell. The flat flow profile of electroosmotic flow of lysis induced reagent also increased homogeneity of the enveloped solvent layer of the cells especially the cell that is adjacent to the capillary wall comparing to the introduction of pressurized flow.

The technique described in this paper is not limited to the study of erythrocyte lysis alone. The proposed technique might be very fruitful for the study of other phenomena, behaviors, metabolisms, and reaction mechanisms at the single cell level. The development for the investigation in this area is now focused on the observation of the lysis or interaction of cell under different reaction mechanisms. By using the improved miniaturization apparatus and protocol, the further cell reaction mechanism is potential to be examined.

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