Dielectrophoretic force measurement of red blood cells exposed to oxidative stress using optical tweezers and a microfluidic chip

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Received: 7 April 2017 / Revised: 21 June 2017 / Accepted: 30 June 2017 / Published online: 10 July 2017
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Abstract Red blood cell (RBC) dysfunction is often associated with a pathological intervention, and it has been proposed as a critical risk factor for certain lethal diseases. Examining the cell viability of RBCs under various physiological conditions is essential and of importance for precise diagnosis and drug discovery in the field of medicine and pharmacy. In this paper, we report a new analytical method that employs dielectrophoretic (DEP) force measurements in absolute units to assess the viability, and potentially the functionality of RBCs. We precisely quantify the frequency-dependent DEP forces of the RBCs by using a micro-electrode embedded chip combined with optical tweezers. DEP characteristics are known to be well-correlated with the viability of biological cells, and DEP forces are measured in both fresh and long-term stored RBCs to investigate the effect that the storage period has on the cell viability. Moreover, we investigate the DEP behavior of RBCs when exposed to oxidative stress and verify whether EDTA protects the RBCs from an oxidant. From the experiments, it is found that the fresh RBCs without oxidative stress display very high DEP forces over the entire frequency range, exhibiting two cutoff

frequencies. However, both the RBCs stored for the long-term period and exposed to oxidative stress reveals that there exist no significant DEP forces over the frequency range. The results indicate that the DEP forces can serve as a useful parameter to verify whether the RBCs in certain blood are fresh and not exposed to oxidative stress. Therefore, it is believed that our system can be applied to a diagnostic system to monitor the cell viability of the RBCs or other types of cells.

Keywords DEP measurement · Red blood cell · Oxidative stress · EDTA · Protective effect

1 Introduction

Under normal conditions, the physiological concentration of reactive oxygen species (ROS) plays a critical role in cell signaling pathways, which in turn regulate normal cell function. For example, ROS accumulation in hematopoietic stem cells (HSCs) at an early developmental stage forces the HSCs to leave the stem cell niche, enter the cell cycle and proliferate into erythrocytes [1–3]. ROS-mediated oxidative stress activates Ca^{2+} -permeable ion channels, promotes cytosolic Ca^{2+} activity, and triggers erythrocyte apoptosis after their physiological life span, which corresponds to an essential cellular process to remove the defective or aged erythrocytes [4]. Ca^{2+} and ROS are related to important intracellular signaling molecules, and Ca^{2+} has been known to accelerate ROS generation by enhancing the metabolism [5]. On the other hand, pathogenesis via ROS disrupts the homeostasis of the redox balance and deteriorates erythrocytes in blood, leading to pathological cell dysfunction and death. In a clinical setting, excessive ROS generation is known to be a

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major contributor of several congenital disorders of erythrocytes, including sickle cell anemia and thalassemia [6–9]. Furthermore, membrane lipid peroxidation caused by oxidative stress alters the dielectric properties of the erythrocyte membrane, induces membrane instability, and eventually reduces the erythrocyte's deformability. Such membrane peroxidation is closely linked to acquired erythrocyte disorders, such as hemolysis [10–14].

Previous studies [15, 16] revealed that in the case of sickle erythrocytes, in vitro antioxidant supplementation effectively suppressed the pathological consequence of ROS-mediated erythrocyte dysfunction. Accordingly, it seems that antioxidant supplementation holds great potential to deliver reliable therapy against ROS-mediated erythrocyte disorders. However, there is still a shortage of efficient methods to precisely and easily measure the severity of the damage to cells from oxidative stress and whether the cells can be protected from oxidants through the addition of some reagents. Therefore, it seems necessary to develop a simple, efficient analytical tool for single-cell analysis.

In this paper, we report on an efficient method based on DEP that can be used to monitor whether a single biological cell has deteriorated under constant oxidative stress and the level of protection from the oxidant resulting from the addition of a reagent. The experimental set-up adopted for this method is comprised of optical tweezers combined with a microelectrode-embedded microfluidic chip that is used to generate the DEP force. The measured DEP force is considered to be a measure that estimates the biological state of a cell, such as the viability.

Specifically, we observe the deterioration of RBCs exposed to oxidative stress and investigate the protective effect of the added reagent on the RBCs. Hydroperoxide is used as an oxidant to provide the oxidative stress, and we adopt EDTA as a test reagent since it is known to be a calcium ion chelator that forms chelates with metal ions across the cell membrane. Based on the experimental results, the protective effect of EDTA on RBCs is discussed in terms of drug biochemistry.

2 Materials and methods

2.1 Sample preparation

A whole blood sample from a healthy volunteer who provided informed consent was collected in BD Vacutainer tubes (3.0 ml, BD-Plymouth, PL6 7BP, UK) containing 0.184M K₂-EDTA. The RBCs were extracted using a centrifuge at a speed of 1200 rpm for 12 min after removing buffy coat and plasma. A hematocrit level of ~45% was confirmed via centrifugation [17, 18]. A

0.83 µg/µl solution of Dulbecco's modified eagle medium (DMEM; no glucose, L-glutamine (0.584 g/L), sodium bicarbonate (3.8 g/L)) was prepared prior to the test. Various reagents were added to the solution containing RBCs, and their DEP behaviors were monitored. Of these, *tert*-butyl hydroperoxide is known to act as an oxidant while EDTA is expected to provide protective effects [19, 20]. The reagents used in the solution were adjusted to achieve the following fixed concentrations: 0.83 µg/µl DMEM, 0.1 µl of 70% *tert*-butyl hydroperoxide, 600 µM EDTA. All reagents used in this study were purchased from Sigma–Aldrich (St. Louis, MO, USA).

We prepared three solutions to analyze the DEP behavior of the RBCs. The first solution used in the control experiment was fabricated by adding the RBCs in DMEM solution. The second solution was made by adding EDTA in the control solution to test whether and how EDTA affects the normal RBCs. The third solution was made by adding 0.1 µl of *tert*-butyl hydroperoxide (70% aqueous solution) in the control solution to expose the RBCs to a harsh oxidative environment. All tubes with different solutions were incubated simultaneously at 37 °C for 30 min in a shaking water bath [21]. The sample preparation described above was completed within 3 h after blood collection.

2.2 Experimental setup

We recently introduced a new DEP force measurement system comprised of optical tweezers with a microelectrode-embedded microfluidic device that can generate the DEP force [22, 23]. For this measurement, the DEP force acting on each cell was monitored as a function of the AC frequency. The system shown in Fig. 1 consists of a diode laser with the wavelength of 1064 nm, a quadrant photodiode (QPD) sensor, a CCD camera, a microfluidic DEP chip mounted on a motion controller and the accompanying optics including dichroic mirrors. The entire data acquisition and analysis procedures were fully automatized using Labview software for the overall measurement time to be effectively shortened (<5 min).

The microelectrode-embedded microfluidic chip was fabricated using thermal evaporation and lift-off techniques on a thin slide glass (24 × 50 mm and 300 µm thick), on which the RBC was then placed to take the DEP force measurements [22, 23]. Both arms (~100 µm wide) of a Cr electrode pair pattern was separated by ~100 µm each other, as shown in Fig. 2. This Cr electrode pattern is ~150 nm thick. The electrode-patterned slide glass was washed with acetone and methanol, and then repeatedly rinsed in deionized water.

A polyester film (~500 µm thick) punched with a ~1 cm diameter hole was placed onto the patterned slide

Fig. 1 **a** Schematic of a DEP force measuring system consisting of an optical tweezer set-up and a microfluidic device. **b** Schematic illustrating the main dysfunction by ROS generation in red blood cells

glass using a high-vacuum-sealing compound (HiVAC-G, Shin-Etsu Chemical Co.). The hole in the chip acted as a liquid chamber or reservoir over the electrode pattern. Also, a thin slide glass (24 × 50 mm, ~0.15 mm thick, Marienfeld-Superior) was used as a cover to prevent solution evaporation from the liquid chamber.

2.3 DEP measurement of RBCs

The RBCs were incubated in each tube before the DEP measurement. 1 μ l of each solution containing the RBCs was introduced into the reservoir of the micro-electrode chip using a micro-pipette. A single RBC was trapped using a highly focused laser beam, and its relative displacement from the center of the beam focus was monitored using the QPD sensor. The DEP force acting on each RBC was estimated from the relative displacement based on the same calibration procedure described in Refs. [24] and [25]. The frequency-dependent DEP forces of the RBCs were measured with various reagents while the AC frequency applied to the electrode pair varied from 10 to 10 MHz.

3 Results and discussion

3.1 Double shell DEP model of a trapped cell

When an alternating voltage is applied, the DEP behavior of a single cell corresponds to an electro-dynamic phenomenon in which a spatially non-uniform electric field induces a net force on the field-induced dipole of the dielectric particle, namely, a single cell. The DEP force (F_D) acting on a homogeneous dielectric particle can be represented using the following equation [26, 27].

$$F_{DEP} = 2\pi r^3 \epsilon_m \text{Re}[K(\omega)] \nabla |E|^2$$

where ϵ_m is the complex permittivity of the medium solution, r represents the radius of the homogeneous particle (the trapped cell), and $|E|^2$ is the root mean square value of the electric field strength. The real part of the CM factor, $\text{Re}[K(x)]$ indicates that the DEP force acting on the particle varies with the applied frequency (x) and particle polarizability. Since the sign of the FD is dependent on the polarity of the $\text{Re}[K(x)]$, the trapped cell may exhibit

Fig. 2 Characterization of the DEP behavior of RBCs with respect to the AC frequency. **a** Schematic of the measurements of the DEP force movement of the RBCs in a non-uniform electric field (the electrode pattern is placed in the x - y plane and the z -axis is perpendicular to the electrode pattern). Here, an RBC is positioned at the origin ($x = 0 \mu\text{m}$, $y = 0 \mu\text{m}$, $z = 10 \mu\text{m}$) for measurement, which is represented by the “+” mark, **b** the frequency-dependent DEP behavior of both the fresh RBCs and the RBCs stored for 22 and 40 days. **c** Double-shell DEP model covered by two concentric layers (membrane and cell wall) of a trapped cell

positive or negative DEP behavior and will consequently move toward or away from the electrodes [26, 27].

A proper DEP model explaining how each dielectric component within it is arranged, is necessary to calculate $K(x)$ and thus understand the frequency-dependent DEP behavior of a single cell. In general, a double-shell DEP model has been widely accepted as a representative model for a single biological cell [28–31]. In the model, biological cells are thought to be composed of three concentric layers (cell wall, membrane, and cytoplasm), each of which possesses its permittivity and electrical conductivity. A typical schematic depicting the double shell model is shown in Fig. 1c. In the model, the frequency-dependent CM factor $K(x)$ is given as shown below [28, 29].

$$K(\omega) = \frac{\epsilon_c^* - \epsilon_m^*}{\epsilon_c^* + 2\epsilon_m^*}$$

where ϵ_c^* and ϵ_m^* respectively represent the complex

permittivity of the single cell and the solution medium. The complex permittivity can be given as $\epsilon^* = \epsilon - i\sigma/\omega$, where ϵ , σ and ω represent the relative permittivity, the electric conductivity, and the angular frequency of the applied electric field, respectively, and the double-shell model is formulated as a dielectric sphere (cytoplasm) covered by two concentric layers (membrane and cell wall) [30, 31].

$$\epsilon_{cell}^* = \epsilon_1^* \frac{\left(\frac{r_1}{r_1}\right)^3 + 2\left(\frac{\epsilon_{23}^* - \epsilon_1^*}{\epsilon_{23}^* + 2\epsilon_1^*}\right)}{\left(\frac{r_1}{r_1}\right)^3 - \left(\frac{\epsilon_{23}^* - \epsilon_1^*}{\epsilon_{23}^* + 2\epsilon_1^*}\right)}, \text{ and}$$

$$\epsilon_{23}^* = \epsilon_2^* \frac{\left(\frac{r_2}{r_3}\right)^3 + 2\left(\frac{\epsilon_3^* - \epsilon_2^*}{\epsilon_3^* + 2\epsilon_2^*}\right)}{\left(\frac{r_2}{r_3}\right)^3 - \left(\frac{\epsilon_3^* - \epsilon_2^*}{\epsilon_3^* + 2\epsilon_2^*}\right)}$$

where ϵ_1^* , ϵ_2^* , and ϵ_3^* correspond to the complex permittivities of the cell wall, membrane, and cytoplasm, respectively. Once the alternating voltage is applied, the DEP force is exerted onto a trapped cell and makes it move either in the positive (toward a higher electric field) or in the negative (toward a lower electric field) direction. It is believed that this phenomenon can be applied to the development of an analytical tool to characterize the cell viability because the direction and magnitude of the DEP force are determined by several factors, including the polarity of the CM factor, the geometrical and electrical properties of the cells, and the medium.

Prior to this report, some studies have investigated how closely cell viability is related to the DEP behaviors of biological cells, such as the cell viability of yeast cells, and consequently it strongly affected their DEP behavior, including DEP force and cutoff frequency [22, 32, 33]. Kim and coworkers reported that a live yeast cell has two cutoff frequencies and exhibits very strong DEP forces over the entire frequency range [22]. However, the DEP spectrum of a dead yeast cell has a single cutoff frequency, and the cell exhibits low DEP forces over the entire frequency range. This difference may be attributed to the cell membrane of a live cell that is less conductive than that of a dead cell, which has been confirmed by conducting a computer simulation using the double shell model [22].

The results given above suggest the intuitive idea that the cell viability of RBCs is similarly related to the DEP behavior. Accordingly, we speculate that the cell viability of RBCs and the protective effect of some reagents on RBCs can also be monitored using a similar set-up with optical tweezers.

The following sections present the systematic study of the DEP behavior of RBCs with variations in the storage period as well as the addition of oxidant or protective reagents.

3.2 DEP analysis of the RBCs with different storage period

In general, RBCs in blood extracted from a human are known to deteriorate over time. To examine the severity of the deterioration of RBCs during long-term storage, we investigated the frequency-dependent DEP behavior of both the fresh RBCs and those stored for 22 and 40 days. Figure 2a depicts a schematic of the DEP forces acting on the RBCs placed at the origin of the microfluidic chip. Each RBC is trapped with a focused laser beam and is positioned at the origin ($x = 0 \mu\text{m}$, $y = 0 \mu\text{m}$) with a height of $10 \mu\text{m}$ from the chip surface ($z = 10 \mu\text{m}$).

Figure 2b shows the frequency-dependent DEP behavior of the fresh RBCs and those stored for 22 and 40 days, and the DEP forces can be seen to be highly dependent on the storage time. The fresh RBCs exhibit a very strong, positive DEP over a wide frequency range from 7 kHz to 10 MHz with two clear cutoff frequencies, which is similar to that of viable yeast cells [34–36]. At 100 kHz, the maximum DEP force is $\sim 0.429 \text{ pN}$ for fresh RBCs and $\sim 0.198 \text{ pN}$ for RBCs stored for 22 days. In contrast, the RBCs stored for 40 days exhibit a weak DEP pattern over the same frequency range. The majority of DEP forces are measured within the $\pm 0.1 \text{ pN}$ range, which is much smaller than that of the fresh RBC. These results imply that the storage period affects the viability of the RBCs and metabolic systems in the stored RBCs do not operate in a proper way. Consequently, the cell viability modulates the dielectric characteristics around the cell membrane.

This variation in the DEP forces of RBCs according to the storage period may be explained as follows. In fresh RBCs, active metabolism takes place, and ion channels in the cell membrane operate properly, maintaining a constant intracellular ion environment. This induces a large difference in the ion environment inside and outside the cell, so a large DEP force acts on the RBC due to the difference in the dielectric properties. On the other hand, the RBCs stored for 40 days seem not to be viable. Their ion-channels may not operate properly, and thus the cell membrane becomes more conductive with passive ion penetration across the membrane, resulting in a concentration equilibrium [37–39]. Therefore, the ion concentration in the cytosol and outside the cell would be nearly the same, resulting in weaker DEP forces because the polar structure of the membrane, normally formed by functioning ion-channels, has been significantly destroyed.

The cell viability is known to be affected when some chemicals are added as well as when the storage period increases [40]. In our experiment, the addition of some chemicals is believed to change the dielectric characteristics of the RBCs and then their DEP behaviors. Accordingly, we consider that an investigation into whether the

addition of chemicals mitigates or preserves the DEP force of the RBCs is an interesting, practical topic in the field of single-cell drug screening.

3.3 Protection of the RBCs protective reagent of EDTA

The DEP force is considered to be a good measure of whether RBCs have been exposed to oxidative stress and how effectively they have been protected by the addition of a reagent. In this section, the oxidative effect of hydroperoxide and the protective effect of EDTA are demonstrated and discussed in terms of the DEP phenomenon. The same fresh blood is used for all further experiments to precisely investigate how these reagents affect the RBCs.

We used DMEM (D), hydroperoxide (H), and EDTA (E) as test reagents. Hydroperoxide was adopted as an oxidant to apply the oxidative stress to the RBCs. EDTA was selected as a protective reagent to prevent the RBCs from being oxidized. The test solution (FDH) to apply the oxidative stress to the RBCs was manufactured by adding hydroperoxide in the control solution (FD) with RBCs and DMEM ($0.83 \mu\text{g}/\mu\text{l}$). The test solution (FDHE) for the protection of the RBCs was prepared by adding EDTA in the FDH solution, and its EDTA concentration was adjusted to $600 \mu\text{M}$. For better understanding, we emphasize that the FDHE solution is a mixed solution of fresh blood, DMEM, hydroperoxide, and EDTA.

Figure 3 shows the oxidative effect of hydroperoxide and the protective effect of EDTA on the DEP characteristics of the RBCs exposed to oxidative stress. As expected, the fresh RBCs in the solution (FD) with no reagents exhibit strong positive DEP forces (e.g. $\sim 0.809 \text{ pN}$ at 300 kHz) over a wide frequency range (10 kHz–7 MHz). In contrast, the fresh RBCs in the FDH solution containing hydroperoxide display no significant DEP forces over the entire frequency range, which is similar to the experimental result for the RBCs stored for 40 days (Fig. 2b). This implies that the RBCs in the FDH solution are severely oxidized by hydroperoxide and are seemingly unviable. On the other hand, the RBCs in the FDHE solution exhibit considerable DEP forces (e.g. $\sim 0.202 \text{ pN}$ at 300 kHz) in the frequency range from 20 kHz to 10 MHz, indicating that the RBCs are in part protected from oxidative stress with the assistance of EDTA.

Why this phenomenon happens can be explained through the following description. In general, EDTA is known to be an intracellular calcium ion chelator [41]. Since EDTA is a small molecule that can penetrate a plasma cell membrane, it passes through the membrane and chelates Ca^{2+} in the cytosol. Ca^{2+} in the cell is known to accelerate ROS generation and activation during signal

Fig. 3 **a–c** Microscopic image of a RBC treated with FD, FDEH, and FDH, **d** optical trapping of the RBCs in the microfluidic device by using objective lens, and **e** frequency-dependent DEP forces of the RBCs exposed to various solutions with different reagents. Here, F = fresh blood, D = DMEM, H = hydroperoxide, and E = EDTA. For instance, FDHE indicates a mixed solution of fresh blood, DMEM, hydroperoxide, and EDTA

transduction [42], and the decrease in the Ca^{2+} concentration through chelation is thought to prevent the RBCs from being additionally damaged by ROS generation, which improves the viability relative to the RBCs in the FDH solution with considerable DEP forces over a wide frequency range.

The above results indicate that when the RBCs are stored for an extended period or are exposed to oxidative stress, they display very weak DEP forces over the entire frequency range. The cells exposed to oxidative stress were partially protected by the addition of EDTA, exhibiting considerable DEP forces. This means that it is quite reasonable to consider the DEP force as an estimate of cell viability. Furthermore, cell viability or drug efficacy can be monitored by taking DEP measurements of a single cell.

4 Conclusion

We demonstrated the DEP force measurements of RBCs in various solutions using a set-up comprised of optical tweezers with a micro-electrode embedded microfluidic chip. The experimental results for both the fresh RBCs and the RBCs stored for 40 days indicate that the DEP force of the RBCs would be a good measure to verify whether the cells are viable and how fresh they are. Interestingly, we

confirmed that the RBCs became non-viable in the presence of hydroperoxide due to oxidative stress.

We investigated the means to estimate the protection of RBCs by measuring the DEP forces of RBCs in mixed solutions of hydroperoxide and EDTA. The results indicate that EDTA effectively mitigates the oxidative stress and prevents the RBCs from being oxidized, which results in considerable DEP forces. From the results, it is plausible that the DEP force can be considered as a measure of cell viability. Accordingly, we suggest that our measuring system comprised of optical tweezers and a microfluidic chip is applicable for the single-cell diagnosis of cell viability and drug efficacy.

Acknowledgements This study was supported in part by the National Research Foundation of Korea (NRF) with a grant funded by the Korean Government (NRF-2013R1A2A2A03005767, NRF-2013R1A1A2053613 and NRF-2015R1A5A7037674).

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