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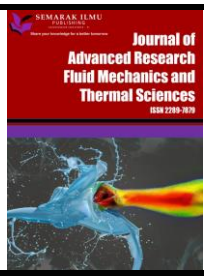

Experimental and Theoretical Study of Propulsion Mechanism with Neutrophil Rotation by using Concentration Marangoni Effect

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ABSTRACT

In the immunological systems, neutrophil propulsion is considered to be driven by the concentration gradient of cytokines. However, the mechanism of propulsion has not been elucidated yet in previous research. In our investigations, assuming that neutrophil moves by the concentration Marangoni effect, the concentration gradient of cytokine on the neutrophil membrane was experimentally examined. The result showed that the cytokine concentration gradient on neutrophil membranes periodically changed. It indicates the possibility that neutrophils move while rotating under the cytokine concentration gradient. However, the neutrophil's rotation is difficult to be observed because experimental verification is limited due to the accuracy and sensitivity of the present measurement system. Therefore, no evidence has been found yet for a relationship between neutrophil velocity and rotation. In this study, assuming that the neutrophil is rotating, this relationship is investigated by theoretical and experimental approaches. As for the theoretical approach, the neutrophil velocity by the concentration Marangoni effect is calculated by using a special mathematical model. From this result, it was found that the velocity is high when the period is short. As for the experimental approach, to obtain the velocity and the concentration gradient on the membrane, neutrophil velocity and distribution of cytokine concentrations are observed with microscopy. In the experiment, the period of rotation was obtained from the time history of the concentration gradient by FFT (Fast Fourie Transform). From the results, the following three findings were obtained. At first, neutrophil velocity is not constant and vibrates. Second, when the rotation period is short, the velocity is fast. Third, when the distance between the neutrophil and the wall is close, the rotation period is longer and the velocity is lower. From these findings, it was concluded that neutrophil rotation is an important role in neutrophil's propulsion mechanism.

1. Introduction

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In recent years, micro-robotic devices based on the driving mechanism of cells have been rapidly developed in the biomedical and nanotechnology fields. In particular, microdevices that mimic immune cells are highly biocompatible and are used in vivo without harmful effects. For example, the contractile behavior of cardiomyocytes and the neutrophil chemotaxis have been used as the power source for various microdevices [1-3]. Drug delivery system (DDS) is often referred to as an application of chemotaxis. There is an example of the treatment of brain blood vessels. Delivery of high-molecular-weight drugs such as peptides and proteins to brain blood vessels had been considered difficult because of the tight binding of nerve cells called glial cells to brain blood vessels [4]. To address this issue, the efficiency of drug delivery to inflammatory regions in the brain has been significantly improved by utilizing neutrophils as drug carriers [5].

Neutrophils are known to move toward higher concentrations of cytokines, but the quantitative relationship between concentration gradients and propulsion has not been clarified yet [6,7]. Some microscale particles like a neutrophil in a liquid move by the Marangoni effect. The Marangoni effect expresses the interfacial tension created by the concentration gradient working on the microparticles. In our previous studies, assuming that neutrophil propulsion by the Marangoni effect, the concentration gradient of cytokine on the surrounding fluid and neutrophil membrane was experimentally examined [8]. The result showed that the cytokine concentration gradient on neutrophil membranes periodically changed. The fact indicated the possibility that neutrophils move while rotating under the cytokine concentration gradient. Numerical analysis showed that the rotation is caused by the adsorption/desorption function and fluidity of the neutrophil membrane [9]. It was also found that neutrophils generate pseudopods near the inner vessel wall for infiltration into organs and decrease the velocity. However, the effect of the distance between the neutrophil and the wall on rotational motion is not understood.

In this paper, assuming that the neutrophil rotates, two issues that have not been clarified in previous studies are investigated. The first one is the elucidation of the quantitative relationship between velocity and rotation. The Second one is the effect of distance from wall on velocity and rotation. To address these issues, it is investigated by theoretical and experimental approaches. As for the theoretical approach, the neutrophil velocity by the concentration Marangoni effect is calculated by using a mathematical model. As for the experimental approach, the distance between the neutrophil and the wall is measured under a microscope, and the neutrophil velocity and rotation period at each distance are obtained.

2. Neutrophil Propulsion Mechanism

In this study, the relationship of the neutrophil velocity and rotation period is investigated. Here, neutrophils are assumed as spherical microparticles because their size range is from 6 μm to 10 μm.

It is generally known that some microscale particles in a liquid move by the Marangoni effect. The Marangoni effect is a passive motion caused by the non-uniformity of the interfacial tension working on microparticles in a liquid [10]. Figure 1 shows the image of the interfacial tension working on the microparticles.

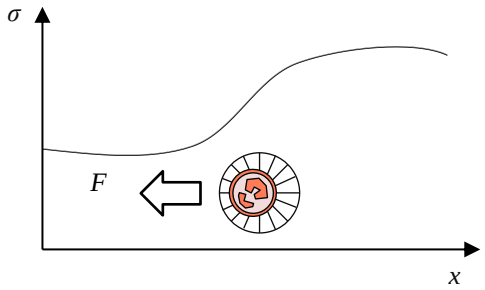


Fig. 1. Image of the interfacial tension working on the microparticle

As shown in the figure, the force *F* is generated in the direction of low interfacial tension. The force *F* working on the neutrophils is expressed by the velocity *V* from Stokes' resistance law [11]. Considering the temperature is constant in vivo, the velocity *V* is expressed as follows referring to the paper by Obana *et al.,* [8]:

$$V = -\frac{2r}{3\eta}\left(\frac{\mathrm{d}\sigma}{\mathrm{d}C}\right)_{\mathrm{T}}\left(\frac{\mathrm{d}C}{\mathrm{d}x}\right) \tag{1}$$

The relationship between interfacial tension σ and concentration C is expressed by Gibbs' adsorption isotherm [12].

$$\sigma = \sigma_0 + RT\ln\left(1-\frac{C}{C_0}\right) \tag{2}$$

where R is the gas constant, T is the temperature, and C_0 is the initial concentration. Differentiating Eq. (2) by C:

$$\left(\frac{\partial\sigma}{\partial C}\right)_{\mathrm{T}} = -\frac{RT}{1-\frac{C}{C_0}} \tag{3}$$

Because the concentration gradient changes periodically, the concentration gradient dC/dx is assumed by sine function.

$$\frac{\mathrm{d}C}{\mathrm{d}x} = A(1+\alpha\sin\omega t) \tag{4}$$

where t is the time, α is the constant, A, ω are the amplitude and frequency of the concentration gradient. Integrating over x at an arbitrary distance d

$$C = Ad(1+\alpha\sin\omega t) \tag{5}$$

From Eq. (1), Eq. (3), Eq. (4), and Eq. (5):

$$V = -\frac{2rRT}{3\eta\left(1-\frac{Ad(1+\alpha\sin\omega t)}{C_0}\right)}A(1+\alpha\sin\omega t) \tag{6}$$

To simplify Eq. (6), β is defined as follows:

$$\beta = \frac{\alpha\frac{Ad}{C_0}}{1-\frac{Ad}{C_0}} \tag{7}$$

Thus, Eq. (6) is expressed as follows:

$$V = \frac{2rRT}{3\eta(1-\beta\sin\omega t)\left(1-\frac{Ad}{C_0}\right)}A(1+\alpha\sin\omega t) \tag{8}$$

Integrating Eq. (8) with time t and averaging, the time-averaged velocity is obtained as follows:

$$\overline{V} = -\frac{\alpha^2+2\alpha-1}{\beta}\cdot\frac{\pi}{\tau}+(2\alpha\beta^2-2\beta^3-2\beta^2-2\beta+1)\tan^{-1}\left(\frac{\tan\frac{5}{4}\tau-\beta}{1-\beta^2}\right) \tag{9}$$

where gas coefficient R = 287 J/kg・K, radius r = 6 μm, temperature T = 293 K, initial concentration C_0 = 2×10-5 kg/m^3, Amplitude A = 7.46 kg/m^4, and distance between location of cytokine generation and neutrophil d = 8 mm.

The theoretical neutrophil velocity is calculated using Eq. (8) and Eq. (9). The theoretical values are compared with experimental values.

Next, to investigate the relationship between velocity and rotation period, Eq. (8) is differentiated by τ (=2π/ω).

$$\frac{dV}{d\tau} = -\frac{2\pi t}{\tau^2}\left(\frac{\alpha\cos\frac{2\pi}{\tau}t}{1+\alpha\sin\frac{2\pi}{\tau}t}+\frac{\beta\cos\frac{2\pi}{\tau}t}{1-\beta\sin\frac{2\pi}{\tau}t}\right)\times V \tag{10}$$

Similarly, to Eq. (9), applicating a numerical integration on the one-cycle range τ, ("dV/dτ"‾ is obtained.

3. Experimental Method

3.1 Isolation of Neutrophils

Neutrophils are isolated from the fresh porcine blood for the experiment. Isolation methods have been referred the literature of Goldsmith *et al.,* [13]. For precipitating red blood cells, the blood with heparin is left for about 60 minutes at room temperature. After this process, the supernatant containing leukocyte components is collected and centrifuged at 2000 rpm for 10 minutes at 4 °C using a cooling centrifuge (KUBOTA, personal cooling centrifuge 2700). To maintain their properties, percoll is used in centrifugation. To get sediments containing neutrophils, the supernatant rich in mononuclear cells is extracted after centrifugation. To adjust the temperature of the dispersion to room temperature (20 °C), the experiment is started 1 hour after isolation.

3.2 Observation Method

Figure 2 shows the schematic diagram of the experiment. The neutrophils are observed under the microscope. In this experiment, a CCD camera (Watec, WAT-910HX) attached to a phase-contrast microscope (Nikon, ECLIPSE TS100-F) is used to capture images of neutrophil motion toward the

direction of cytokine insertion. The frame rate is 30 fps. An observation area is 72 µm in the *x* direction and 48 µm in the *y* direction as shown in Figure 3. The measurement is done with a minimum resolution of 0.1 µm. The temperature during the experiment is set to 20 °C. As shown in Figure 2, 500 µL of the neutrophil dispersion is dropped onto the cover glass of the microscope, and 5 µL of the cytokine solution is inserted into the drop using a micropipette to form a cytokine concentration gradient that works on the neutrophils.

As a chemotactic factor, cytokine IL-8 is used, which is produced by monocytes and lymphocytes. It works on leukocyte cells such as neutrophils. The concentration of cytokine is adjusted by dilution with saline to 20 ng/mL. By antibody-labeling the cytokine solution with a fluorescent substance, the solution emits fluorescence when irradiated with excitation light. Fluorescein-4-isothiocyanate (FITC) is used as a substance for antibody labeling. FITC is a fluorescent dye with a maximum absorption wavelength of 490 nm and a molecular weight of 389.38. It produces yellow-green fluorescence by excitation light. Irradiating the cytokines with the light from a super-high-pressure mercury lamp cut to blue visible light (wavelength λ = 480 ~ 490 nm) by a filter, the antibody-labeled cytokines emit light. The luminance value at that time is measured as the concentration of the cytokines. The cytokine solution labeled with the FITC antibody is inserted from the left side of the observation area, as shown in the schematic diagram in Figure 2. The luminance value in the observation area increases from the left side. In other words, after the insertion of the cytokine solution, it is difficult to observe the right side of the neutrophils clearly, so the coordinates of the neutrophils (x_0, y_0) should be the left side coordinates.

Furthermore, the distance z between the neutrophil and the wall (cover glass) is measured from the amount of revolution of the microscope handle during focus adjustment as shown in Figure 4. Neutrophils are observed at z = 2, 4, 6, 8, and 10 µm.

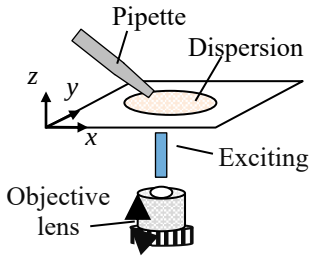


Fig. 2. Schematic diagram of the experiment

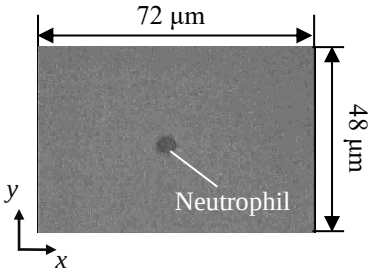


Fig. 3. Image of the observation area

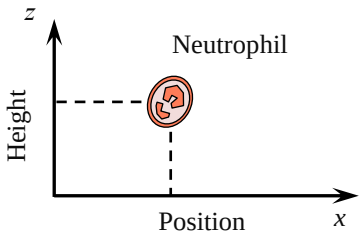
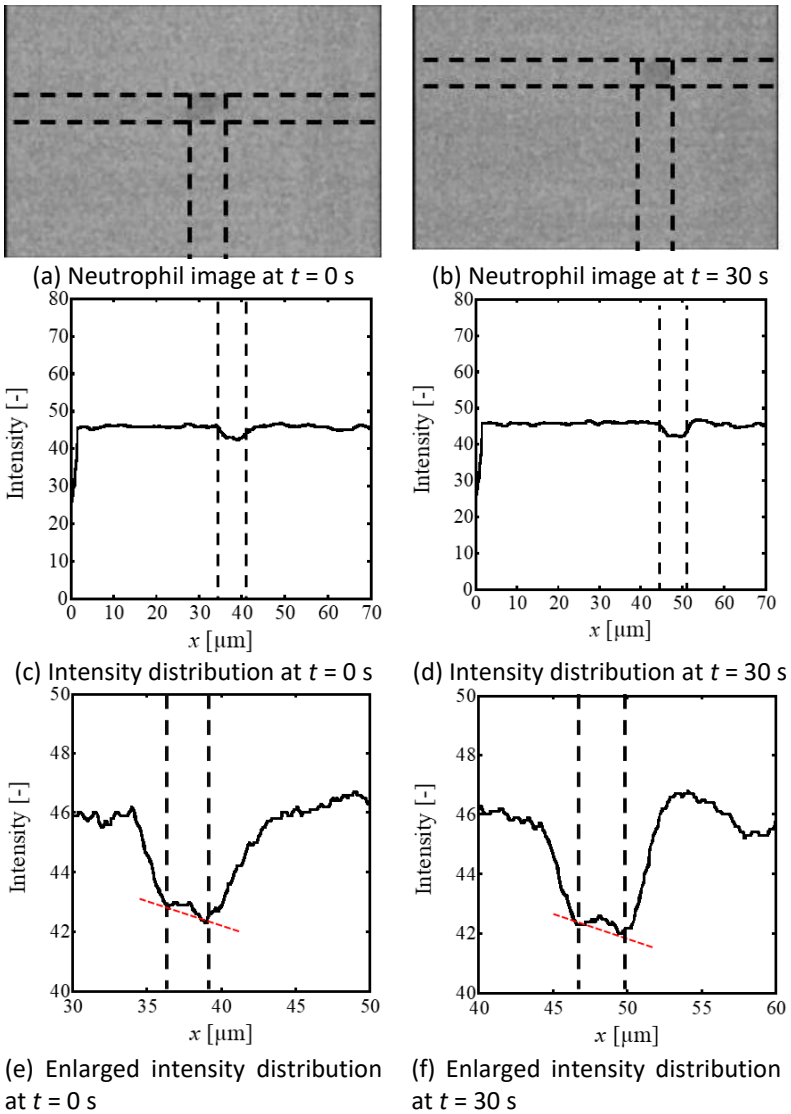


Fig. 4. Distance between neutrophils and the wall

3.3 Method of Obtaining Experimental Values

Figure 5(a) and Figure 5(b) show the images of neutrophil. Figure 5(c) and Figure 5(d) show their luminance distributions at 30-second intervals. The time neutrophils begin to move is defined as t = 0 s. Cytokines are inserted from the left side of the image. This figure shows that the neutrophil moves in the direction of cytokine insertion. From this coordinate history, the velocity is calculated from the distance travelled in 70 seconds. The luminescence intensity is regarded as the cytokine concentration. The y-axis range of the intensity where neutrophils exist is averaged, and the one-dimensional concentration distribution is obtained. The minimum resolution is 0.1 µm while neutrophils range in size from a few µm to 10 µm. To calculate the concentration gradient on the neutrophil membrane, the one-dimensional concentration distribution of the enlarged neutrophil part is shown in Figure 5(e) and Figure 5(f). Averaging in the y-axis direction, the accuracy of the analysis is decreased at the boundary between the surrounding fluid and neutrophils. For this reason,

the concentration gradient on the neutrophil membrane is obtained by linear approximation of 10 points (2 μm) before and after the neutrophil center coordinates in this region shown as a black dotted line in Figure 5(e) and Figure 5(f). The concentration gradient on the neutrophil membrane is calculated every 1/30 second, and the time history is also obtained as shown in Figure 6. The concentration gradient on the membrane of neutrophils vibrates with time. The repeated vibration is considered that the neutrophils are repeatedly rotating in the direction of propulsion. With rotation, neutrophils are prevented from depositing cytokines on the cell membrane and the concentration transport is steady when they move to the concentration gradient. This rotation is caused by the adsorption/desorption function and fluidity of the neutrophil membrane [9].



(a) Neutrophil image at t = 0 s
(b) Neutrophil image at t = 30 s
(c) Intensity distribution at t = 0 s
(d) Intensity distribution at t = 30 s
(e) Enlarged intensity distribution at t = 0 s
(f) Enlarged intensity distribution at t = 30 s

Fig. 5. Calculation method of concentration gradient

The rotation period is measured from the time history of the concentration gradient on the membrane shown in Figure 6. To calculate the rotation period, FFT (Fast Fourier Transform) analysis is applied to the time history of the concentration gradient on the neutrophil membrane. The output points of FFT analysis are 1024 points. Figure 7 shows an example of the results of FFT analysis in Figure 6. The frequency of 0.29 Hz is considered to be the major frequency of neutrophils. As a result, a rotation period of 3.45 s is obtained.

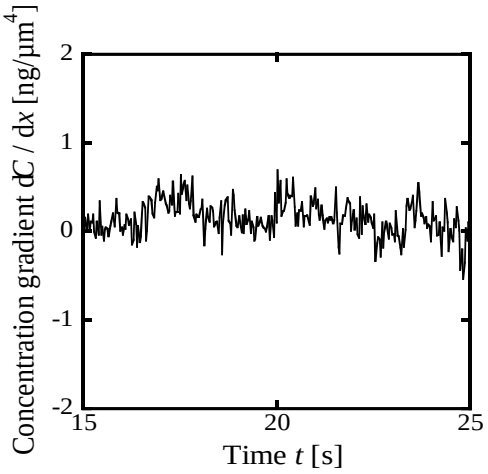


Fig. 6. Time history of concentration gradient

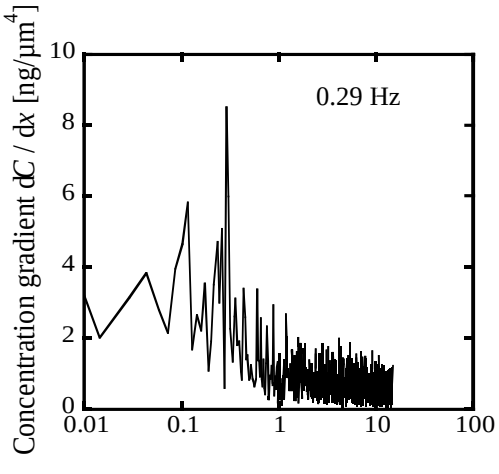


Fig. 7. Typical computational result of FFT analysis

4. Results and Discussion

4.1 Results of Theoretical Calculation

Using Eq. (8), the theoretical value is compared at each condition. Figure 8 shows the theoretical calculation result of velocity time history. Velocity shows periodical waveform with peak values. It indicates that neutrophil velocity is not constant and vibrates. Furthermore, time history contains two different frequencies. It is known that the rotation is caused by the adsorption/desorption function and fluidity of the neutrophil membrane [9]. Considering the properties of cellular membranes, such complex waveforms are predicted to generate by the interaction of cytokine and cell membrane of cytokine. There are substances called ligands on the surface of neutrophils. They bind to specific proteins and contribute to the biological features of the cell. That is why there is the possibility that the two frequencies mean biological features.

Figure 9 shows the integral result of velocity time history. Neutrophil position indicates a monotonically increasing tendency. This result indicates that neutrophils move with periodical motion.

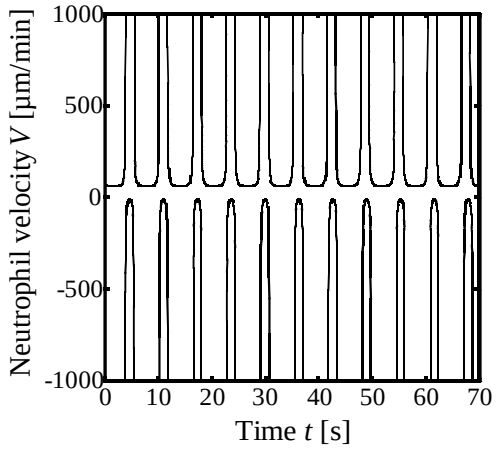


Fig. 8. Calculation result of velocity history

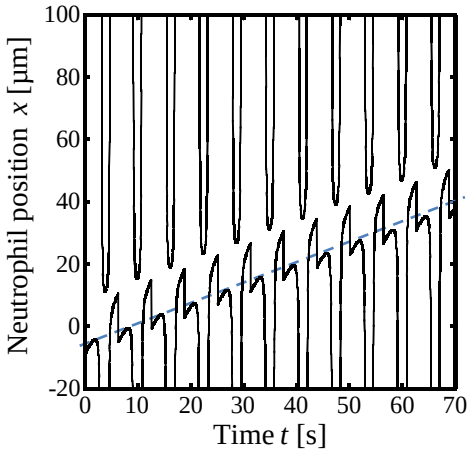


Fig. 9. Calculation result of position history

4.2 Results of Observation Experiments

Figure 10 shows the neutrophil position history. The position history shows that the neutrophils move in the direction of high cytokine concentration by the Marangoni effect. Theoretical calculations show that neutrophils move with periodical motion.

Focusing on t = 0 to 20 s, a step behavior is observed. In other words, neutrophils repeat move and stop in this phase. It indicates the possibility that neutrophil rotation is related to this step behavior. Basically, as a periodical vibrational behavior of cells, Brownian motion is well known. However, the Brownian motion of the cell is an extremely sensitive timescale of approximately 1 μs [14]. Therefore, the vibration examined in this study is different from Brownian motion. For general substances that gain driving force from the Marangoni effect such as gas bubbles, step behaviour isn't observed. It is presumed to be related to biological functions such as interactions between neutrophils and cytokines.

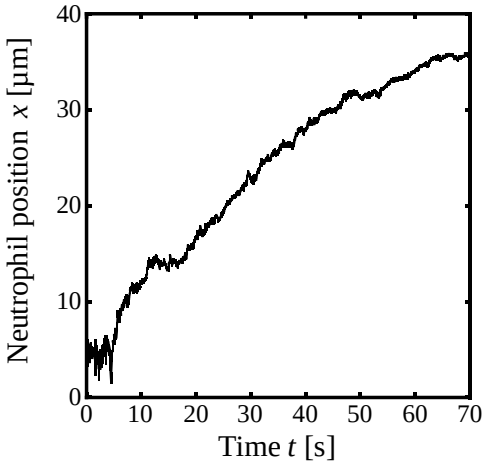


Fig. 10. Neutrophil position history

Figure 11 shows the experimental result of the relationship between distance from the wall and time-averaged velocity. Error bars represent a 95% confidence interval. This graph shows that the neutrophil velocity is slower when the distance is close. Figure 12 shows the relationship between distance from the wall and rotation period. This graph shows that the rotation period is longer when the distance is close. When neutrophils approach the wall, the pseudopods generate and the shape or surface area is changed. Because of this, the move and rotation of neutrophils are reduced. Figure 13 shows the theoretical and experimental value of the averaged velocity at each distance z between the neutrophil and the wall. Theoretical values are calculated by applying physical properties obtained from the observation experiment into Eq. (9). Theoretical values with the same period as the experimental values are obtained. The theoretical values are larger than the experimental values. It is possible that there are factors other than rotation that influence the propulsion mechanism of neutrophils. For example, the work of the pseudopods and the concentration conditions of surrounding fluid are not considered in the present calculations. In both cases theory and experiment, neutrophil velocity is faster when the rotation period is short. the theoretical value is larger than the experimental value. The point is the velocity of neutrophils depends on the period of the rotation. This result suggests the possibility that the neutrophils obtain propulsion force from the rotation.

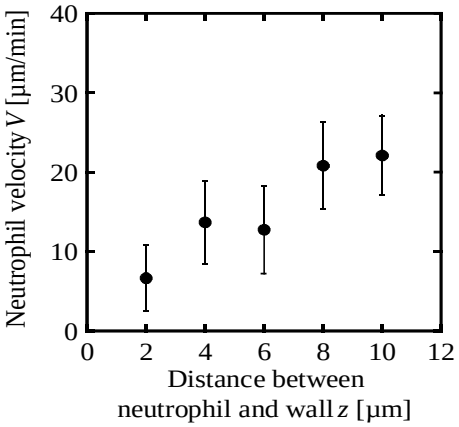


Fig. 11. Neutrophil position history

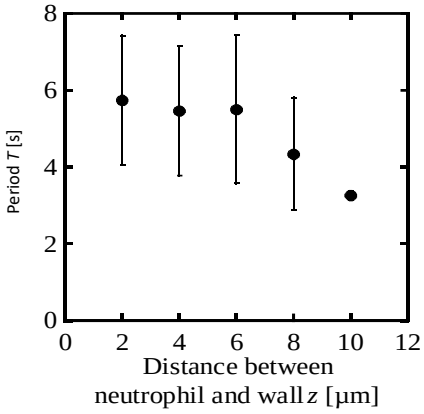


Fig. 12. Relationship between distance from wall and rotation period

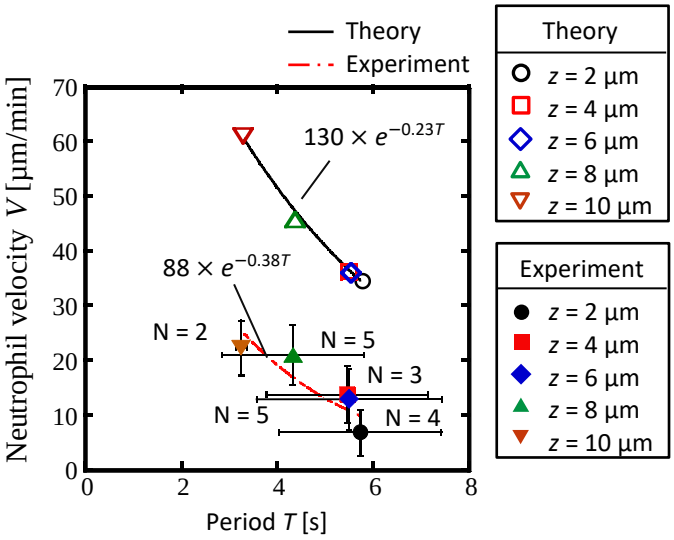


Fig. 13. Relationship between exponentially approximated theoretical values and experimental values

To examine the relationship between velocity and rotation period, dV/dτ is calculated by Eq. (10). dV/dτ means the gradient of the tangent line at each point in Figure 13. Applicating a numerical integration about time on the one cycle range on Eq. (10), dV/dτ is obtained. As in the experiment, this value is calculated for z = 2, 4, 6, 8, and 10 μm. From the results obtained in Figure 13, velocity V is likely to be expressed as a function of rotation period τ. Therefore, the relationship between dV/dτ and τ is obtained by an exponential approximation as follows:

$$\frac{\mathrm{d}\overline{V}}{\mathrm{d}\tau} = 29.8 \times e^{-0.23\tau} \tag{11}$$

Integrating both sides with τ, the following equation is obtained:

$$\overline{V_{\mathrm{th}}} = -130 \times e^{-0.23\tau}$$

To consider velocity as a positive value, $\overline{V_{th}}$ is expressed as follows:

$$\overline{V_{\mathrm{th}}} = 130 \times e^{-0.23\tau} \tag{12}$$

Similarly, from the experimental values shown in Figure 11, the following equation is obtained:

$$\overline{V_{\mathrm{ex}}} = 88 \times e^{-0.38\tau} \tag{13}$$

The ratio of the theoretical value to the experimental value is as follows:

$$\frac{\overline{V_{\mathrm{th}}}}{\overline{V_{\mathrm{ex}}}} = 1.48 \times e^{0.15\tau} \tag{14}$$

Eq. (14) shows the approximated curves are also shown in Figure 13. Regarding the validity of the theory, the tendency that the velocity slows down as the rotation period increase is agreed with the theory and experimental value. But the experimental value is smaller than the theoretical value. From this fact, there are likely factors other than rotation that influence the propulsion mechanism of neutrophils. For example, the work of the pseudopods and the concentration conditions of surrounding fluid are not considered in the present calculations.

Because neutrophil diameter is about 7 μm, measuring below z = 2 μm is difficult. Therefore, the upper limit of period τ is considered to be 6 s. In the future, to consider the lower limit of τ, to measure neutrophils above 10 μm is needed.

There was a hypothesis that neutrophils gain propulsion from rotation. From the periodical waveform of the position history, the neutrophil rotation is considered to be involved in propulsion mechanism. As an example of cells that obtain their driving force by rotation, flagellum is known widely. General flagellar frequencies are 300 ~ 1700 Hz [15]. But the rotation period of neutrophils in this study is under 10 Hz. Therefore, the force by neutrophil rotation is predicted to be very small. It is estimated that neutrophil rotation is not for the drive. Regarding the Marangoni effect, it is known that the velocity of a particle in a dilute surfactant solution decrease with increasing concentration of surfactant. At some point, it reaches a limit to the velocity increase and is no longer affected by further increases in concentration [16]. However, if the particles rotate by themselves, surfactant deposition is prevented. This is one possible reason for neutrophils to rotate. Such biological function of the cell depends on the cell membrane. Cell membranes such as neutrophils have an unbroken asymmetric double-layered structure with a thickness of 5 nm. The outer surface and the inner surface are composed of different lipids and proteins, so the functions of the two surfaces are different. This lipid bilayer is the basic structure of the membrane, and it is quite an impassable barrier for most water-soluble molecules [17,18]. It has been reported that the two layers have respective adsorption and desorption functions [19]. There is the possibility that the two frequency types of waves in the calculation results of velocity and position suggest lipid bilayers work. In future studies, it is needed to investigate the effect of rotation on the membrane surface of neutrophils at a more microscopic scale.

5. Conclusion

In this study, the relationship between velocity and rotation or the effect of distance from the wall on velocity and rotation was investigated by theoretical and experimental approaches. As a result, the following issues are concluded

i. Neutrophil velocity is not constant and vibrates.
ii. When the rotation period is short, the velocity is fast.
iii. When the distance between the neutrophil and the wall is close, the rotation period is longer and the velocity is lower.

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