Microfluidic-based measurement of erythrocyte sedimentation rate for biophysical assessment of blood in an *in vivo* malaria-infected mouse

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This study suggests a new erythrocyte sedimentation rate (ESR) measurement method for the biophysical assessment of blood by using a microfluidic device. For an effective ESR measurement, a disposable syringe filled with blood is turned upside down and aligned at 180° with respect to gravitational direction. When the blood sample is delivered into the microfluidic device from the top position of the syringe, the hematocrit of blood flowing in the microfluidic channel decreases because the red blood cell-depleted region is increased from the top region of the syringe. The variation of hematocrit is evaluated by consecutively capturing images and conducting digital image processing technique for 10 min. The dynamic variation of ESR is quantitatively evaluated using two representative parameters, namely, time constant (λ) and ESR-area (A_{ESR}). To check the performance of the proposed method, blood samples with various ESR values are prepared by adding different concentrations of dextran solution. λ and A_{ESR} are quantitatively evaluated by using the proposed method and a conventional method, respectively. The proposed method can be used to measure ESR with superior reliability, compared with the conventional method. The proposed method can also be used to quantify ESR of blood collected from malaria-infected mouse under *in vivo* condition. To indirectly compare with the results obtained by the proposed method, the viscosity and velocity of the blood are measured using the microfluidic device. As a result, the biophysical properties, including ESR and viscosity of blood, are significantly influenced by the parasitemia level. These experimental demonstrations support the notion that the proposed method is capable of effectively monitoring the biophysical properties of blood.

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I. INTRODUCTION

According to previous studies on blood biophysical properties including viscosity,^{1–6} viscoelasticity,^{7–12} deformability,^{13–18} aggregation,^{19–22} and erythrocyte sedimentation rate (ESR),^{23–27} various cardiovascular diseases cause changes in the biophysical properties of the blood. The biophysical properties of the blood have been measured to effectively monitor disease states or their progresses. Among the biophysical properties, the ESR suggested by Westergren²⁸ in 1921 has been clinically used to detect infection²⁴ or inflammatory diseases, including anemia,²⁹ kidney disease,³⁰ thyroid disease,³¹ rheumatoid arthritis,³² atherosclerosis,²³ and even cancers.^{33,34} ESR represents a setting distance where anti-coagulated red blood cells (RBCs) fall down in a vertical tube after 1 h. The variation of ESR depends on physiological

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conditions, such as plasma protein level and hematocrit. In addition, ESR is an indicator of RBC aggregation and blood viscosity at low shear rate conditions. However, the conventional ESR measurement method has several disadvantages, such as long measurement time (~ 1 h), large volume consumption (~ 2 mL), high cost due to bulk-sized instruments and specimen tubes, tedious cleaning procedure, and difficulty in quality control.^{24,35} The accuracy of ESR measurement is influenced by several factors, including vertical installation angle and contamination of the specimen tubes. Furthermore, the conventional ESR measurement method provides only single value for each blood sample after 1 h. Therefore, the conventional approach is insufficient for obtaining the dynamic behaviour of RBCs during experiments because the interface between the RBC-depleted region and the RBC-rich region is not clearly shown in the specimen tube.

To monitor the dynamic variation of RBCs in a vertical tube, several methods, including electrical impedance method^{36,37} and light-emission diode method,³⁸ were introduced. Among these methods, the electrical impedance method was demonstrated to monitor the similar trend of ESR through measuring the conductivity using two electrodes installed in the tube. However, this method requires bulk-sized instruments and complex signal processing routine. In addition, this approach cannot resolve the intrinsic issues of the conventional ESR measurement method. By considering the fact that the ESR value is dominantly determined by RBC aggregation, several methods, including capillary electrophoresis,³⁹ Doppler optical coherence tomography,⁴⁰ and optical aggregometry,⁴¹ were suggested to measure the aggregation index (AI) as an indirect measurement method for ESR. However, these indirect methods have not been sufficiently used in extensive clinical applications, compared with the conventional ESR measurement method. For this reason, the development of a new method that is based on the conventional ESR measurement method is required to resolve the intrinsic problems of the conventional ESR measurement method.

Thus, in this study, a simple but effective ESR measurement method is proposed to quantify the biophysical properties of blood by using a microfluidic device. After being filled with blood, a disposable syringe is turned upside down, and aligned at 180° with respect to gravitational direction. When delivering blood sample into the microfluidic device, hematocrit is decreased due to RBC sedimentation in the syringe. That is, when the blood flow in the syringe is sufficiently slow, RBCs tend to be aggregated and formed as rouleaux, due to the presence of acute phase protein, especially fibrinogen. The aggregated RBCs fall downward at a constant velocity, under the gravity effect. Therefore, the hematocrit level is smaller at the top position of the syringe, compared with that at the bottom part. The dynamic variation of ESR in the syringe is evaluated by monitoring the temporal variation of image intensity within a specific region of interest (ROI). The value of ESR is quantified using two representative parameters, namely, time constant (λ) and ESR-area (A_{ESR}). Compared with previous ESR measurement methods, the proposed method has several distinctive advantages. First, the proposed method can conduct ESR measurement for test sample with small volume (~ 0.2 mL), for a short period of time (~ 10 min), and still under similar configuration of the conventional ESR method. Second, the proposed approach can provide quantitative information on the dynamic variation of ESR by evaluating temporal intensity variation in the image of RBCs, which flow in a microfluidic channel. Third, the measurement errors caused by installation and contamination of the test tube can be minimized because the disposable syringe is always inversely installed by the syringe pump. In addition, the combination of the disposable syringe and microfluidic device eliminates tedious cleaning procedures of the test tube. Finally, the proposed method does not require bulk-sized expensive ESR instruments and specimen tubes.

The performance of the proposed method is optimized by quantitatively evaluating several parameters, including the installation angle of a disposable syringe (upright, horizontal, and vertical), base solution [plasma, $1 \times$ phosphate-buffered saline (PBS)], hematocrit ($Hct = 20\% - 50\%$), and delivery flow rate of blood sample. To simulate various ESR values, blood samples are prepared by the addition of different concentrations of dextran solution. At first, the ESR value is measured using a disposable syringe, which is similar to the conventional ESR measurement method. To quantify ESR in the disposable syringe, the temporal variation of liquid volume in blood sample is obtained as a function of time. After then, the dynamic variation of ESR is evaluated using the

parameters including λ and A_{ESR} . The same blood samples are then used to evaluate the performance of the proposed method. The experimental results obtained from the proposed method are compared with those obtained from the similar configuration of the conventional ESR method. Finally, as clinical application of the proposed method, blood samples are collected directly from an *in vivo* malaria-infected mouse, rather than *in vitro* malaria-infected blood samples. The proposed method is applied to quantify the variation of the ESR with respect to the malaria infection rate. In addition, to compare with the variation of ESR with respect to the malaria infection rate, the viscosity and velocity as biophysical properties of blood samples are quantitatively measured using the microfluidic device, which is demonstrated in this study. Thereafter, the variations of blood biophysical property are intensively discussed with respect to the malaria infection rate.

II. THE PROPOSED ESR MEASUREMENT METHOD

An effective method for evaluating the ESR of blood in a disposable syringe by using a microfluidic device is proposed. After being filled with blood, a disposable syringe is inversely installed. By supplying a blood sample into the microfluidic device, the RBC-depleted region is expanded from the top of the syringe because RBCs migrate through plasma toward the downward direction. Hematocrit level tends to decrease at the top position in the syringe continuously. To evaluate the temporal variations of hematocrit level in the syringe, blood samples are intentionally supplied from the top position of the syringe into a microfluidic channel. Then, the hematocrit level of blood sample flowing in the microfluidic channel is decreased continuously, under certain experimental condition. Taking into account the fact that image intensity is reciprocally increased depending on the hematocrit level, the variation of the hematocrit level in the syringe was indirectly measured by monitoring temporal variations of image intensities in the microfluidic channel. Therefore, the dynamic variation of ESR can be evaluated by detecting the temporal variation of image intensity in a specific ROI in the microfluidic channel. To demonstrate the feasibility and usefulness of the proposed method, the experimental setup is composed of the following components: two syringe pumps for delivering blood sample and PBS solution, and a microfluidic analogue of Wheatstone-bridge device for measuring the temporal variations of average blood velocity ($\langle U \rangle$), blood viscosity (μ_{Blood}), and image intensity (I) in a specific ROI.

As shown in Fig. 1(A)–(a), the effect of delivery direction on ESR measurement is investigated for three different installation angles of the syringe (θ) ($\theta = 0^\circ$, 90° , and 180°). The installation angle is defined with respect to the gravitational direction. In other words, when the syringe is aligned at zero degree ($\theta = 0^\circ$), the RBC-depleted region starts to form from the top position of the syringe. The blood sample is supplied from the bottom position of the syringe into the microfluidic device; thus, the blood sample is consistently supplied with minimal RBC sedimentation for a certain period. However, when the syringe is aligned at 90° or 180° , the hematocrit of blood streaming in the microfluidic channel is decreased continuously due to RBC sedimentation in the disposable syringe.

As depicted in Fig. 1(A)–(b), the microfluidic analogue of Wheatstone-bridge device is composed of two inlets, two outlets, and two identical side channels connected with one bridge channel.¹ In addition, two pinch valves for the inlet (B) and outlet (B) are employed to control fluidic flows as on or off. To quantify the change in blood hematocrit passing through the microfluidic channel due to RBC sedimentation in the syringe, two biophysical properties, namely, blood viscosity and average blood velocity, are quantitatively measured using flow-switching manipulation in the bridge channel⁴² and a micro-PIV (particle image velocimetry) velocity field measurement technique, respectively. In addition, the average hematocrit of the blood sample, which is collected from the outlet (A) at 15 min intervals for 75 min, is measured using a centrifugal-based hemocytometer, after closing the inlet (B) and outlet (B) with pinch valves.

As shown in Fig. 1(B), during the delivery of blood from the disposable syringe aligned at $\theta = 180^\circ$ into a microfluidic device, the RBC-depleted region starts to form from the top position of the syringe due to RBC sedimentation. Thus, the hematocrit of the blood sample flowing

FIG. 1. Schematic diagram of the ESR measurement method in which a blood sample is delivered into a microfluidic device from a disposable syringe. (A) Experimental setup including syringe pumps to deliver blood and $1 \times$ PBS solution, and the microfluidic device for measuring average blood velocity ($\langle U \rangle$), blood viscosity (μ_{Blood}), and image intensity (I) in a ROI. (a) Installation angle of a syringe (θ) ($\theta = 0^\circ, 90^\circ$, and 180°) with respect to the gravitational direction. (b) The microfluidic device is composed of two inlets (A and B), two outlets (A and B), and two identical side channels with one bridge channel. In addition, two pinch valves are installed to control the fluidic flows of inlet (B) and outlet (B). Using the microfluidic device, three representative parameters, namely, average blood velocity, blood viscosity, and intensity variations, are monitored as a function of time. (B) When the syringe pump is aligned at inverse gravity direction ($\theta = 180^\circ$), the ESR in the disposable syringe depends on the relative velocity difference between sedimentation velocity ($U_{\text{sedimentation}}$) and delivery velocity (U_{delivery}) [(a) $U_{\text{sedimentation}} < U_{\text{delivery}}$, (b) $U_{\text{sedimentation}} > U_{\text{delivery}}$]. After a certain time ($t = t_0 + \lambda_0$), RBC-depleted region is clearly formed due to RBC sedimentation, only when the sedimentation velocity is greater than the delivery velocity. (C) Temporal variation of image intensity, which depends on relative velocity difference between the sedimentation and delivery velocities. When the sedimentation velocity is less than the delivery velocity ($U_{\text{sedimentation}} < U_{\text{delivery}}$), the temporal variation of image intensity is remained constantly. However, the temporal variation is increased abruptly when the sedimentation velocity is greater than the delivery velocity ($U_{\text{sedimentation}} > U_{\text{delivery}}$).

in the microfluidic channel is gradually decreased because the blood sample located at the top region of the syringe is first delivered into the microfluidic device. The ESR of blood in the disposable syringe is strongly influenced by the relative velocity difference between sedimentation velocity ($U_{\text{sedimentation}}$) and delivery velocity (U_{delivery}). Thus, when the sedimentation velocity is less than the delivery velocity ($U_{\text{sedimentation}} < U_{\text{delivery}}$) [Fig. 1(B)–(a)], the RBC-depleted region is not observed. However, when the sedimentation velocity is greater than the delivery velocity ($U_{\text{sedimentation}} > U_{\text{delivery}}$) [Fig. 1(B)–(b)], the RBC-depleted region is formed after a certain time ($t = t_0 + \lambda_0$). As such, the delivery flow rate of blood should be appropriately selected for an effective measurement of ESR. After closing the two pinch valves for inlet (B) and outlet (B), the blood sample is delivered from the top region of the syringe to inlet (A), and then collected from outlet (A). At this point, the blood sample passes through the left-side channel of the microfluidic device. To monitor temporal variation of the hematocrit of blood passing through the microfluidic channel, microscopic images are consecutively captured using a high-speed camera. The temporal variation of image intensity is obtained using digital image processing for a specific ROI in the left upper-side channel.

Figure 1(C) shows the temporal variation of image intensity with respect to relative velocity difference between the sedimentation and delivery velocities. When the sedimentation velocity is smaller than the delivery velocity ($U_{\text{sedimentation}} < U_{\text{delivery}}$), the temporal variation of

image intensity is remained constantly. Insets (a)-(b) represent grayscale images captured at $t = 0$ s and $t = 400$ s, respectively. However, when the sedimentation velocity is larger than the delivery velocity ($U_{\text{sedimentation}} > U_{\text{delivery}}$), the image intensity tends to increase abruptly. Inset (c) denotes the grayscale image captured at $t = 400$ s.

From this preliminary study, the image intensity (I) is approximately assumed as

$$I(t) = \alpha + \beta \exp\left(-\frac{t}{\lambda}\right). \quad (1)$$

To quantify the ESR value using this mathematical formula, λ can be determined using regression analysis. In addition, A_{ESR} is mathematically defined as

$$A_{\text{ESR}} = \int_0^{t_{\text{total}}} I(t) dt. \quad (2)$$

Here, t_{total} represents the total measurement time. In this study, the total measurement of the proposed method is completed within 10 min for convenience. Therefore, the two representative parameters, λ and A_{ESR} , are used to evaluate the ESR of the blood.

III. MATERIALS AND METHODS

A. Fabrication of a microfluidic device and experimental setup

A microfluidic device was designed to have two inlets, two outlets, and two identical side channels (width = 1000 μm , length = 10 mm) with one bridge channel (width = 200 μm). The depth of the microfluidic channel was fixed at 50 μm . A silicon mold for the microfluidic device was fabricated using the conventional micro-electro-mechanical-system technique. Thereafter, polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning, USA) was poured on the silicon mold positioned in a petri dish. The air bubbles dissolved in the PDMS were removed using a vacuum pump. After curing the PDMS in a convection oven at the temperature of 80 °C for 2 h, the PDMS block was peeled off the silicon mold. After treating oxygen plasma on the PDMS block and on a slide glass, the PDMS block was bonded on the glass substrate to fabricate a microfluidic device.

The microfluidic device was mounted on an optical microscope (4 $\times$ objective lens, Nikon, Tokyo, Japan) combined with a high speed camera (PCO, Germany). Disposable syringes (BD Science, 1 mL, Singapore) of syringe pumps (neMESYS, Centoni GmbH, Germany) were connected to inlets (A)–(B) by using polyethylene tubes (I.D. = 250 μm). To remove or collect blood samples from outlets (A)–(B), polyethylene tubes were connected to the outlets. To control the fluidic flow of a reference fluid prepared for measuring blood viscosity, two pinch valves were installed at inlet (B) and outlet (B).

B. Measurement of biophysical properties of blood

To evaluate the variation of hematocrit of blood flowing in a microfluidic channel, the viscosity and average velocity of the blood were measured using the microfluidic channel. In addition, the average value of hematocrit was evaluated using the blood sample collected from the microfluidic device. Firstly, to measure blood viscosity (μ_{Blood}) by using the microfluidic device, 1 $\times$ PBS solution (pH 7.4, Bio Solution, Korea) was used as a reference fluid. Using a co-axial cylinder viscometer (DV-II, Brookfield, USA), the viscosity of the reference solution (μ_{Ref}) was measured to be 1 ± 0.05 cP. When a test blood and reference fluid are delivered into specific inlets at the same flow rate ($Q_{\text{Blood}} = Q_{\text{Ref}}$), the flow direction of each fluid in the bridge channel is determined by the relative viscosity of test blood in relation to the viscosity of reference fluid ($\mu_{\text{Blood}}/\mu_{\text{Ref}}$). By increasing the flow rate of reference fluid, a hydrodynamic balancing condition occurs. Immediately, flow direction is changed from left to right (or right to left) in the bridge channel. By monitoring the flow rate of reference fluid ($Q_{\text{Ref}}^{\text{SW}}$), where the reverse flow

is induced in the bridge channel, blood viscosity is measured as $\mu_{\text{Blood}} = \mu_{\text{Ref}} Q_{\text{Ref}}^{\text{SW}} / Q_{\text{Blood}}$ with high accuracy and good reliability.^{1,42} Second, temporal variation of velocity field was measured through consecutive flow images at a frame rate of 500 Hz for 2.8 s. After selecting the specific ROI (330 × 260 pixels) in the left-upper side channel, the velocity fields of the blood flow were obtained using a time-resolved digital PIV technique.⁴³ Thereafter, the average velocity of blood flow ($\langle U \rangle$) was estimated for the ROI. Lastly, after closing the pinch valves of inlet (B) and outlet (B), pure blood samples were collected from outlet (A) at 15 min intervals for 75 min. Subsequently, the average hematocrit of the blood sample was measured using a centrifugal-hemocytometer. All experiments were carried out under a consistent room temperature of 25 °C.

C. Image processing technique for ESR measurement

To monitor the ESR of blood in a disposable syringe, pure blood samples were delivered into the microfluidic device after the pinch valves of inlet (B) and outlet (B) were closed. To evaluate the temporal variation of hematocrit of blood passing through the left side channel, blood images were captured consecutively at a frame rate of 2 Hz for 600 s. After selecting a specific ROI (300 × 340 pixels) in the left-upper side channel, the average pixel value for the ROI was estimated using commercial software (Matlab, Mathworks, USA). Thereafter, the temporal variation of the average pixel value was evaluated for each blood sample.

D. Blood sample with malaria parasite and Giemsa staining

According to the POSTECH Ethics Committee, all the procedures used in the experiments were considered as appropriate and humane. Twenty male ICR mice (6–7 weeks old, body weight of 34.2 ± 1.3 grams) were prepared and randomly assigned into two groups: normal group (n = 6) and malaria parasite group (n = 14). All mice had been carefully housed for one week, under the standard living conditions of light and temperature.

The *Plasmodium berghei* ANKA strain as rodent malaria parasites was provided by Kyungpook National University (Dr. Yeongchul Hong, Department of Parasitology, School of Medicine, Daegu, 700–422, South Korea). Mice of the malaria parasite group were infected through intraperitoneal injection of 200 μL parasitized RBCs. The malaria infection rate, which is called as parasitemia, depends on the elapsed time.^{44,45} Thus, to prepare various values of parasitemia, blood samples were collected from the tail vein of the mice from the malaria parasite group during specifically indicated times ranging from 1 day to 12 days. In addition, blood samples were collected from mice of the normal group.

To stain malaria parasite in each RBC with Giemsa solution, as the first step, thin blood smears on the slide glass were fixed with 99% methanol for 5 min. Subsequently, the slide glass was dried for 2 min, and the slide was immersed in a 10% Giemsa solution (Merck, Darmstadt, Germany), which was diluted with deionized water for 25 min. Thereafter, the slide was rinsed with tap water and dried. The malaria parasites, which were stained with the Giemsa solution on the slide glass, were examined under a fluorescent microscope (Carl Zeiss Axiovert 200 M, Oberkochen, Germany) with $\times 100$ oil-immersion objective lens. To monitor parasitemia level in the mice of the malaria parasite group, an area of Giemsa-stained blood film where erythrocytes were distributed was chosen. The slide was then moved to adjacent fields randomly. The number of erythrocytes was continuously counted for four slides (n = 4). The parasitemia level was calculated using the average number of malaria-infected RBCs per 1000 RBCs. After finishing the experiments, all mice of the two groups were sacrificed.

IV. RESULTS AND DISCUSSION

A. Biophysical properties depending on inlet hematocrit and syringe delivery direction

During blood delivery from a disposable syringe to the microfluidic device, the hematocrit of the blood is changed due to RBC sedimentation in the disposable syringe. Thus, the hematocrit of blood flowing in a microfluidic channel is temporally changed depending on the hematocrit of the inlet blood sample and the flow direction in the syringe. To measure the temporal

variation of the hematocrit of blood flowing in the microfluidic device due to RBC sedimentation, two biophysical properties, namely, blood viscosity and average blood velocity, were measured using a microfluidic device. In addition, the blood hematocrit collected from the outlet (A) was measured at 15 min intervals for 75 min. Human blood was provided by a blood bank (Daegu-Kyeongbuk Blood Bank, Korea). RBCs in the plasma suspension were adjusted to have three different hematocrits (30%, 40%, and 50%). Subsequently, the blood sample was delivered into inlet (A) at a flow rate of 2 mL/h by using a syringe pump. The PBS solution was simultaneously supplied into the inlet (B) by using the other syringe pump.

First, the blood viscosity of each blood sample for three different installation angles of a syringe ($\theta = 0^\circ$, 90° , and 180°) was measured at 15 min intervals for 75 min. Figure 2(A) shows the temporal variations of blood viscosity with respect to hematocrit level [(a) $\circ = 30\%$, (b) $\triangle = 40\%$, and (c) $\square = 50\%$] and installation angle of the syringe (θ) [(a) $\theta = 0^\circ$, (b) $\theta = 90^\circ$, and (c) $\theta = 180^\circ$]. When the blood sample is delivered within a syringe in the gravitational direction, blood viscosity increases consistently with respect to hematocrit (Hct). The viscosity of the blood sample with Hct = 50 is remained consistently, even without considering the delivery direction of blood. This result implies that RBC sedimentation in the syringe is nearly negligible for blood samples with high hematocrit (Hct = 50%). However, for blood samples with hematocrit of less than 50%, the viscosity of blood depends on both delivery direction and hematocrit of the blood sample. Therefore, the RBC sedimentation in the syringe occurs dominantly for blood samples with low hematocrit (i.e., $\text{Hct} \ll 50\%$). When a blood sample is delivered into the syringe in the horizontal direction ($\theta = 90^\circ$), the temporal variation of blood viscosity is largely increased compared with the other installation angles.

Second, to quantify the hemodynamic properties due to RBC sedimentation in the syringe, average blood velocity ($\langle U \rangle$) was measured by using a time-resolved PIV technique with respect to inlet hematocrit and delivery direction of blood samples. Figure 2(B) shows the

FIG. 2. Temporal variations of blood viscosity (μ_{Blood}), average blood velocity ($\langle U \rangle$), and average hematocrit (Hct) with respect to inlet hematocrit ranging from 30% to 50% and syringe installation angles ranging from 0° to 180° . (A) Temporal variations of blood viscosity (μ_{Blood}) measured by fluid flow-switching manipulation in the Wheatstone-bridge analogue of the microfluidic device. (B) Temporal variations of average blood velocity ($\langle U \rangle$) estimated over a specific ROI in the left upper-side channel. (C) Temporal variations of average hematocrit (Hct) measured with a centrifugal-based hemocytometer for blood sample collected at the outlet.

temporal variations of average blood velocity with respect to blood hematocrit and installation angle of the syringe (θ) [(a) $\theta = 0^\circ$, (b) $\theta = 90^\circ$, and (c) $\theta = 180^\circ$]. The temporal variations of average blood velocity remained constant during vertical delivery of blood ($\theta = 0^\circ$) in the syringe. This result indicates that blood samples are consistently delivered into the microfluidic device when the syringe is aligned at zero angle (or vertical direction). In other words, when blood sample is delivered from the bottom position of the syringe to the microfluidic device, the temporal variation of hematocrit is considered as negligible. Furthermore, the average blood velocity decreases at high hematocrit conditions. However, when the syringe was aligned at the horizontal or inverse direction, the average blood velocity has higher values due to RBC sedimentation in the syringe, compared with that of the vertical delivery direction. RBC sedimentation in the syringe seems to increase the area of RBC-depleted region, which contributes to the decrease of hematocrit of blood passing through the microfluidic channel. In addition, the temporal variation of average blood velocity is increased, especially in the horizontal delivery direction. This variation trend is similar to that of blood viscosity.

Lastly, after closing inlet (B) and outlet (B) with pinch valves and delivering blood sample into the inlet (A) with a syringe pump, the blood sample was collected only from outlet (A) at 15 min intervals for 75 min. The blood hematocrit was measured using a centrifugal-based hemocytometer. Figure 2(C) shows the temporal variations of hematocrit with varying inlet hematocrit and installation angle of the syringe [(a) $\theta = 0^\circ$, (b) $\theta = 90^\circ$, and (c) $\theta = 180^\circ$]. As hypothesized, the hematocrit is remained constantly, without respect to inlet hematocrit. However, when a blood sample with low hematocrit is horizontally delivered in a syringe, the temporal variation of the average hematocrit is increased largely due to RBC sedimentation in the syringe.

These results show that ESR measurement would be more effective when blood samples are delivered in the syringe at the inverse gravitational direction ($\theta = 180^\circ$), especially in samples with low hematocrit.

B. Quantitative evaluations of the proposed method

For an effective ESR measurement, several parameters including different base solutions, hematocrit (Hct = 20%–50%), and delivery flow rate of blood sample were evaluated. To determine proper hematocrit for effective measurement of ESR, the ESR values of normal blood samples with different hematocrits (20%, 30%, 40%, and 50%) and different base solutions (PBS, plasma) were quantitatively evaluated by using a similar configuration of the conventional ESR method. In this study, to remove the effect of the delivery flow rate of blood, the ESR value was measured in a disposable syringe, without operating a syringe pump. After loading a disposable syringe (1 mL) with blood sample (1 mL), snap shot images were continuously captured with a digital camera (D700, Nikon, Japan) at 30 s intervals for 8.3 h. Subsequently, liquid volume (ΔV_{liquid}) was measured by analyzing the interface between RBC-depleted region and RBCs-rich region.

Figure 3(A) shows temporal variations of liquid volume (ΔV_{liquid}) with respect to the inlet hematocrit of the normal blood sample. Several images in the right side of Fig. 3(A) represent variation of liquid volume due to RBC sedimentation of blood sample (RBCs in plasma suspension, Hct = 20%), at different elapsed times [(a) $t = 0$ h, (b) $t = 2$ h, (c) $t = 4$ h, (d) $t = 6$, and (e) $t = 8$ h]. As expected, the liquid volume increases gradually at a lower hematocrit. In addition, RBCs in plasma suspension has larger variation of liquid volume than RBCs in PBS suspension because plasma proteins induce RBC aggregation. Based on these results, the hematocrit is adjusted to 20% for an effective ESR measurement by using a disposable syringe. Thereafter, to create various ESR values by using a configuration similar to the conventional ESR setup, dextran solution (MW = 70 kDa, Sigma Aldrich, USA) was added into RBCs suspended in PBS solution.^{19,22,46,47} Blood samples (RBCs in PBS suspension, Hct = 20%) mixed with three different concentrations of dextran solution ($C_{dextran}$) [(a) $C_{dextran} = 0.5\%$, (b) $C_{dextran} = 1\%$, and (c) $C_{dextran} = 1.5\%$] were carefully prepared. First, a disposable syringe was loaded with 1 mL of blood sample. To monitor temporal variations of liquid volume, snap shot images were

FIG. 3. ESR results measured by detecting the temporal variations of liquid volume (ΔV_{liquid}) in a disposable syringe, without operating syringe pump. (A) Temporal variations of the liquid volume with respect to hematocrit level (20%–50%), and base solution (plasma, PBS). Typical images show the variations of liquid volume due to RBC sedimentation with respect to time (t) [(a) $t=0$ h, (b) $t=2$ h, (c) $t=4$ h, (d) $t=6$ h, and (e) $t=8$ h]. (B) Temporal variations of ΔV_{liquid} with respect to blood sample (RBCs in PBS suspension, Hct=20%) mixed with different concentrations of dextran (C_{dextran}) ($C_{\text{dextran}}=0.5\%$ – 1.5%) for 1 h. (C) Quantitative evaluations of λ and A_{ESR} with respect to different concentrations of dextran solution.

consecutively captured at 30 s intervals for 1 h, which corresponds to the total measurement time of the conventional ESR technique. As represented in Fig. 3(B), the temporal variations of the liquid volumes were obtained with respect to the concentration of the dextran solution. As a result, the liquid volume of the blood sample ($C_{\text{dextran}}=0.5\%$) linearly increased for 1 h. However, the liquid volumes of the other two concentrations ($C_{\text{dextran}}=1\%$, 1.5%) were saturated in a relatively short time. Given that dextran solution induces RBC aggregation, the liquid volume increased linearly or was saturated in a short period of time. To evaluate the dynamic variation of ESR using a similar configuration of the conventional ESR method, the liquid volume (ΔV_{liquid}) can be approximately modeled as $\Delta V_{\text{liquid}}(t) = \alpha + \beta \exp(-t/\lambda)$, which model is similar to Eq. (1).

Here, λ was estimated by using regression analysis. In addition, A_{ESR} is expressed as

$$A_{\text{ESR}} = \int_0^{t_{\text{total}}} \Delta V(t) dt. \quad (3)$$

Here, the experimental measurement time (t_{total}) was fixed at 1 h. Using the temporal variation information of the liquid volume, two representative parameters (λ and A_{ESR}) were estimated quantitatively, as shown in Fig. 3(C). As a result, λ tends to decrease at a high concentration of dextran solution, which indicates that blood sample mixed with high concentration of dextran solution takes a short time to complete RBC sedimentation in a disposable syringe. In addition, A_{ESR} tends to increase with respect to the concentration of the dextran solution.

These results demonstrate that two representative parameters proposed in this study can be used sufficiently to evaluate ESR values with sufficient accuracy.

The comparative advantage of the proposed method using a microfluidic device was demonstrated by measuring the ESR of the same blood sample, which was tested in advance using a configuration similar to that of the conventional ESR technique. As shown in Fig. 4(A), the experimental setup was composed of a syringe pump with a disposable syringe and a microfluidic device. To evaluate RBC sedimentation in the syringe, the disposable syringe was aligned at 180° with respect to the gravitational direction. After closing inlet (B) and outlet (B) with pinch valves, blood sample was delivered into inlet (A), and then collected from outlet (A). To monitor the temporal variation of the hematocrit of blood flowing in the microfluidic channel due to RBC sedimentation in the syringe, microscopic images were consecutively captured with a high-speed camera at 0.5 s interval for 10 min. Using the captured microscopic images, temporal variation of image intensity (I) was obtained by conducting digital image processing over a specific ROI in the left-upper side of the microfluidic channel. Figure 4(B) shows a typical temporal variation of image intensity, especially at a blood flow rate of 1 mL/h ($Q_{\text{Blood}} = 1 \text{ mL/h}$). Insets (a)–(c) show microscopic images captured with respect to time (t) [(a) $t = 0 \text{ s}$, (b) $t = 200 \text{ s}$, and (c) $t = 600 \text{ s}$]. Attributing to RBC sedimentation in the disposable syringe, the hematocrit of blood streaming in the microfluidic channel tends to decrease with respect to time, which increased image intensity. The result implies that ESR can be quantitatively evaluated by analyzing the temporal variation of image intensity.

To quantify the value of ESR by using the proposed method, a mathematical formula for image intensity (I) was approximately assumed as Eq. (1). This equation is similar to the formula for the liquid volume, as previously discussed. From the temporal variations of image intensities, λ was then estimated by conducting regression analysis using the analytical formula. In addition, A_{ESR} was evaluated by integrating the temporal variations of image intensity captured for 10 min. As represented in Fig. 4(C), to quantitatively evaluate the effect of blood flow rate on the two characteristic parameters, temporal variations of image intensity were obtained with varying blood flow rate ranging from 0.6 mL/h to 2 mL/h. In other words, λ and A_{ESR} were evaluated with respect to blood flow rate (Q_{Blood}). Results show that the time constant increases

FIG. 4. Quantitative evaluations of ESR value by using the proposed method with respect to the flow rate of blood (Q_{Blood}) and performance evaluations of the proposed method using blood samples mixed with different concentrations of dextran solution. (A) A schematic diagram of the experimental setup used for the performance evaluation of the proposed method by delivering blood sample in the inverse gravitational direction ($\theta = 180^\circ$) into the microfluidic device. Blood samples are delivered into the inlet (A), and collected from the outlet (A), especially by closing the inlet (B) and outlet (B) with pinch valves simultaneously. Temporal variations of ESR are monitored in a specific ROI of the left upper-side channel. (B) The temporal variation of image intensity is estimated by conducting digital image processing technique for gray-scale images captured with a high-speed camera at 500 ms intervals for 10 min. Insets (a)–(c) are typical images captured at a certain time [(a) $t = 0 \text{ s}$, (b) $t = 200 \text{ s}$, and (c) $t = 600 \text{ s}$]. (C) Quantitative evaluation of λ and A_{ESR} with respect to the flow rate of blood, which ranges from 0.6 mL/h to 2 mL/h. (D) Performance evaluation of the proposed method for blood samples with different concentrations of dextran solution. (a) Temporal variations of image intensity (I) with respect to different concentration of dextran solution (C_{dextran}) ($C_{\text{dextran}} = 0.5\%$, 1% , and 1.5%). (b) Quantitative evaluations of λ and A_{ESR} with respect to different concentrations of dextran solution.

FIG. 5. Quantitative comparisons of the proposed method and the conventional method. (A) Variation of time constant (λ) for three different blood samples. (B) Variation of ESR-area (A_{ESR}) for three different blood samples.

at a high blood flow rate condition. This finding indicates that the RBC sedimentation rate is decreased at a high blood flow rate. The ESR-area is decreased at a high flow rate. The two representative parameters obtained by the proposed method are strongly influenced by the blood flow rate. Therefore, it is important to select an appropriate flow rate of blood delivery for effective ESR measurement by using the proposed method. In this demonstration, the blood flow rate was conveniently fixed at $Q_{\text{Blood}} = 1 \text{ mL/h}$. Using a blood sample (RBCs in PBS suspension, $\text{Hct} = 20\%$) mixed with different concentrations of dextran (C_{dextran}) ($C_{\text{dextran}} = 0.5\%$, 1% , and 1.5%), microscopic images were captured with a high-speed camera. Quantitative image intensity distribution was obtained by conducting digital image processing for each image. As shown in Fig. 4(D)–(a), image intensity (I) was obtained with respect to concentration of the dextran (C_{dextran}). For the blood sample with low concentration of dextran solution ($C_{\text{dextran}} = 0.5\%$), the image intensity increased gradually with respect to time. However, for the blood sample with high concentration of dextran solution ($C_{\text{dextran}} = 1\%–1.5\%$), the image intensity was saturated in a relatively short time. In addition, the lapse of time for searching peak is decreased at a high concentration of dextran solution. As shown in Fig. 4(D)–(b), λ tends to decrease with respect to the concentration of dextran solution. However, the other parameter, A_{ESR} , tends to increase with respect to the concentration of dextran solution. These experimental results demonstrate that the proposed method can be effectively used to monitor variations of ESR quantitatively. In addition, λ and A_{ESR} are effective for quantifying ESR values of blood samples.

Lastly, to compare the performance of the proposed method with that of the conventional ESR technique, the relationship between the proposed method and the conventional method was evaluated by conducting a linear regression analysis with respect to λ and A_{ESR} . As shown in Fig. 5(A), taking into account the fact that R^2 estimated by the linear regression analysis is greater than 90%, the λ estimated by the proposed method has a strong linear relationship with that obtained by the conventional method. In addition, the A_{ESR} obtained by the proposed method is proportional to that evaluated by using the conventional method, as shown in Fig. 5(B).

In this comparison study, the proposed method has the ability to measure ESR with good reliability, compared with the conventional method. Furthermore, the two parameters, namely, λ and A_{ESR} , are considered as promising factors for evaluating temporal variations of ESR quantitatively.

C. Biophysical comparison study using *in vivo* malaria-infected mice

Malaria-infected RBCs induce to morphological and rheological alternations. RBCs lose their deformability, which leads to block microcirculation of vital organs.^{48,49} To determine the biomechanical properties of malaria-infected RBCs, several methods including mechanical-based approaches^{48–53} and electrical impedance-based approaches^{54,55} have been employed by

using a microfluidic device. As a clinical demonstration using the proposed method, blood sample was collected from an *in vivo* malaria-infected mouse at indicated times. Hematocrit was adjusted to 20% by adding 1x PBS solution into the blood. Thereafter, the proposed method was applied to evaluate the variation of ESR with respect to parasitemia level. To determine the appropriate flow rate of blood for effective ESR measurement, two different flow rates (0.5 and 1 mL/h) were selected. Normal blood and malaria-infected blood with high parasitemia level (30%) were prepared from the normal and malaria-infected mice, respectively. As shown in Fig. 6(A), temporal variation of image intensity (I) was obtained with respect to the flow rate (Q_{Blood}) of normal and malaria-infected blood samples. When blood sample was delivered at a flow rate of 1 mL/h, the image intensity (I) remained constant after initial transient behavior, without respect to blood sample. Given that the sedimentation velocity of RBCs is less than that of blood in the disposable syringe, the image intensity of blood traveling in the microfluidic channel remained consistent, without respect to blood sample. Thus, for an effective ESR measurement by using the proposed method, the flow rate for the blood sample was reduced to 0.5 mL/h. As a result, the blood sample collected from a malaria-infected mouse has a higher intensity value in a relative short time, compared with the blood of a normal mouse. To inspect the morphological variation of RBC appearance as a function of parasitemia level, microscopic images of normal and malaria-infected RBCs were captured with a CCD (charged-coupled device) camera. Figure 6(B)–(a) shows a typical microscopic image of normal RBCs. The morphological appearance of malaria-infected RBCs is distinctively changed depending on the development stage (ring, trophozoite, and schizont), as shown in Fig. 6(B)–(b). Figure 6(B)–(c) shows the microscopic images of blood sample with parasitemia levels ranging from 4.7% to 30%.

Temporal variations of image intensity obtained by the proposed method for various mouse bloods at different parasitemia levels were measured. λ was estimated with respect to parasitemia level, as shown in Fig. 6(C). The time constant decreased with increasing level of parasitemia. By conducting a linear regression analysis, a sufficiently high value of R^2 (i.e., $R^2 = 76\%$) was obtained. Therefore, λ depends on parasitemia distinctively. When a normal mouse is

FIG. 6. Clinical demonstration of the proposed method for blood samples extracted from normal and *in vivo* malaria-infected mice. (A) Temporal variations of image intensity (I) with respect to blood flow rate (Q) and two different blood samples (normal blood, malaria-infected blood). The flow rate of blood delivered by a syringe pump was controlled at 0.5 and 1 mL/h. Normal blood sample was collected from normal mouse. In addition, blood sample with 30% parasitemia level was prepared from a malaria-infected mouse at a prescribed time. (B) Microscopic images of blood samples collected from the normal and malaria-infected mice. (a) A typical image of normal RBCs. (b) Microscopic images of malaria-infected RBCs with respect to development stage, including ring, trophozoite, and schizont. (c) Microscopic images at different parasitemia level ranging from 4.7% to 30%. (C) Quantitative evaluations of λ with respect to the parasitemia level. (D) Variations of A_{ESR} with respect to the parasitemia level. (E) Variation of average blood velocity ($\langle U \rangle$) with respect to the parasitemia level. (F) Variations of viscosity of whole blood (Hct = 50%) and plasma as a function of parasitemia level.

infected by malaria parasite, λ is decreased in proportion to the parasitemia level. As shown in Fig. 6(D), A_{ESR} is increased in proportion to the parasitemia level (i.e., $R^2 = 72\%$). These experimental results demonstrate that the two ESR parameters including λ and A_{ESR} can be effectively used to monitor the parasitemia level of an *in vivo* malaria-infected mouse.

To compare with the results obtained by the proposed method indirectly, the blood biophysical properties, including average velocity and viscosity, were measured with respect to parasitemia level. Unlike the sample preparation for ESR measurement, the hematocrit of mouse blood was adjusted to 50% by removing autologous plasma. After aligning the syringe to have zero degree with respect to the gravitational direction, the blood sample was consistently supplied into the microfluidic device at a flow rate of 0.5 mL/h. As shown in Fig. 6(E), the average blood velocity ($\langle U \rangle$) was obtained with varying parasitemia level. The average blood velocity is consistent at 2.834 mm/s. Results indicate that the blood sample is consistently delivered into the microfluidic channel, without respect to the parasitemia level. In addition, after considering the fact that the average velocity is theoretically estimated at approximately 2.778 mm/s, the difference in velocity obtained by the experimental method and theoretical estimation is less than 2%. This finding implies that the blood sample is supplied into the microfluidic device at a specific flow rate, with reasonable accuracy.

The viscosity of whole blood (Hct = 50%) and plasma were measured by using the microfluidic device, with respect to the parasitemia level. According to the shear rate formula for the rectangular channel,⁴² the shear rate of blood flow is approximately estimated to be 333.3 s^{-1} . As shown in Fig. 6(F), the viscosity of whole blood increases linearly with respect to parasitemia level, which can be considered as one of the promising factors for monitoring parasitemia level of an *in vivo* malaria-infected mouse. This result is comparable with the previous result with respect to parasitemia level.^{56,57} However, the plasma viscosity remained constant, without respect to the parasitemia level. This result implies that the blood viscosity of malaria-infected mouse is dominantly determined by RBCs rather than the plasma. Therefore, the viscosity of whole blood is more effective in the monitoring of parasitemia level, compared with that of plasma.

These experimental results demonstrate that the biophysical properties including ESR and blood viscosity can be used as promising factors to effectively monitor the variation of the parasitemia level.

V. CONCLUSION

In this study, a simple but effective ESR measurement method was demonstrated for the biomechanical assessment of blood sample by using a microfluidic device. Taking into account a conventional ESR measurement method, a disposable syringe was aligned at 180° with respect to the gravitational direction and filled with blood sample. The variation of hematocrit level was quantitatively evaluated using several biophysical properties, including viscosity and velocity of blood flowing in the microfluidic channel. In addition, the hematocrit level was intermediately monitored by using blood sample collected from the microfluidic device. To quantify the dynamic variation of ESR of blood sample, two representative parameters, namely, λ and A_{ESR} , were evaluated using the temporal variation information of the image intensity of blood in a specific ROI of the microfluidic channel. To select a proper hematocrit level for an effective ESR measurement, the blood sample was loaded into a disposable syringe (1 mL), without operating syringe pump. The variations of liquid volume were measured by analyzing the RBC-depleted layer with respect to the base solution (plasma, PBS) and hematocrit level (Hct = 20%–50%). The two parameters have a distinctive influence on the variation of liquid volume. From this result, the blood hematocrit was adjusted to 20% during the ESR measurement. To check the performance of the proposed method, blood samples with different ESR values were prepared by adding different concentrations of dextran solution. By using a similar configuration of the conventional ESR method, the temporal variations of the liquid volume were quantitatively evaluated for 1 h. The same blood samples were simultaneously applied to evaluate the performance of the proposed method. The measurement results were compared

with those obtained by using a similar configuration of the conventional method. As a result, the proposed method could be used to measure ESR with high consistency. Furthermore, λ and A_{ESR} were considered as primary factors for evaluating the variation of ESR quantitatively. The proposed method was finally applied to quantify the ESR of the blood sample collected from an *in vivo* malaria-infected mouse. As a result, the blood velocity remained consistent, without respect to the parasitemia level. The ESR and blood viscosity linearly varied with respect to the parasitemia level. In the near future, the proposed method will be used to evaluate variations of the biophysical properties of blood samples collected from patients with various cardiovascular diseases.

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