Periodic and simultaneous quantification of blood viscosity and red blood cell aggregation using a microfluidic platform under *in-vitro* closed-loop circulation

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To evaluate variations of blood circulating in closed loops, hemorheological properties including blood viscosity and red blood cells (RBCs) are quantitatively measured with independent *in-vitro* instruments after collecting blood from a closed loop. But, most previous methods require periodic blood collections which induce several problems such as geometric differences between the fluidic channel and the *in-vitro* method, hemodilution, storage time, and unspecific blood flow rates. To resolve these issues, in this study, blood viscosity and RBC aggregation of blood circulating within a closed loop are measured with a microfluidic platform periodically and simultaneously. To demonstrate the proposed method, *in-vitro* closed-loop circulation is established by connecting several components (peristaltic pump, air compliance unit, fluid divider, and reservoir) in series. In addition, to measure blood viscosity and RBC aggregation, a microfluidic platform composed of a microfluidic device, pinch valve, and syringe pump is created. During each period, blood viscosity and RBC aggregation are measured by monitoring blood flow at constant blood flow, and image intensity at stationary blood flow. The proposed method is first employed to evaluate the effect of hematocrits and dextran concentrations on the RBC aggregation and blood viscosity by using a syringe pump (i.e., specific blood flow-rate). The method is then applied to detect the blood viscosity and RBC aggregation under *in-vitro* closed-loop circulation (i.e., unspecific blood flow-rate). From these experimental demonstrations, it is found that the suggested method can be effectively used to monitor the RBC aggregation and blood viscosity under *in-vitro* closed-loop circulation. Since this method does not require periodic collection from closed-loop circulation or an additional procedure for estimating blood flow-rate with a syringe pump, it will be effectively used to monitor variations of blood circulating in extracorporeal bypass loops. *Published by AIP Publishing.*
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I. INTRODUCTION

Cardiovascular diseases including coronary artery disease, myocardial infarction, and stroke are major causes of death, and are expected to increase gradually.¹ They tend to occur suddenly without any symptoms or warnings and cause unexpected death or serious complications. However, conventional biochemical approaches do not offer significant clues for early detection of cardiovascular diseases.² Since the relationship between cardiovascular diseases and blood rheology was reported,^{3–7} various biophysical properties including blood viscosity,^{8–13} red blood cell (RBC) deformability,^{14–18} RBC aggregation,^{19,20} and the erythrocyte sedimentation rate (ESR)^{21–24} have been suggested to monitor variations in blood. Here, several factors including hematocrit,^{25,26} the cell-free layer,^{27,28} the blood vessel diameter,^{29,30} and white blood cells contribute to varying the biophysical properties of blood.

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To evaluate the biophysical properties of blood circulating in closed loops including hemodialysis,³¹ the cardiopulmonary-bypass loop procedure,^{15,32} and the extracorporeal rat bypass loop,³³ blood is periodically collected from the closed loops. Using the blood collected from the closed loop, stand-alone instruments are employed to evaluate variations in the hemorheological properties of blood. However, the periodic blood collection raises several issues including geometric differences, hemodilution, storage time, and unspecific blood flow rates. First, the hemorheological properties including viscosity or hematocrit can vary owing to geometric differences between the fluidic channel and the in-vitro method.³⁰ Second, since periodic blood collection reduces the blood volume in the closed loop, lactate ring solution or PBS solution is supplied to maintain blood volume constantly.^{15,32} This hemodilution varies hemorheological properties. Third, storage time alter hemorheological properties. Finally, since the blood flow-rate is not unknown under closed-loop circulation, the blood viscosity is measured at wide shear rates, rather than the single shear rate. To resolve these issues, the biophysical properties of blood should be measured under closed-loop circulation and consistent fluidic channels.

A microfluidic platform which provides several advantages including small sample consumption, fast response, and disposability is adopted to measure hemorheological properties, including plasma viscosity,³⁴ blood viscosity,^{35,36} hematocrit,^{37,38} and RBC aggregation.^{19,23,39} However, these techniques still show technical limitations on direct measurement of biophysical properties of blood circulating in a closed loop. On the other hand, a central venous catheter equipped with an impedance measuring device is suggested to measure blood viscosity in the human right atrium.⁹ But, the blood viscosity–shear rate relationship obtained from a standard viscosity meter is required in advance.⁹ Recently, the blood viscosity measurement technique is suggested by using reversal fluid flow switching phenomena in a microfluidic channel.⁴⁰ Afterwards, this method is employed to measure the biophysical properties of blood circulating under in-vitro condition⁴¹ or an ex-vivo circulation loop.^{8,42} Here, a closed-loop circulation is constructed by connecting a Wheatstone-bridge analogue channel with a rat model.⁸ The biophysical properties of blood, including viscosity, flow rate, and pressure, are then periodically evaluated by using reversal fluid switching phenomena in the bridge channel.⁸ However, this method requires a periodically collecting large blood volume (~1 ml) from the closed loop.⁸ After a disposable syringe is filled with blood, it is installed at the syringe pump. Then, the blood flow rate and viscosity are simultaneously measured by using two sequential flow controls for inducing reversal fluid flow in the bridge channel.^{8,41} Because the blood volume in the closed circulation decreases with time, the proposed method shows a limitation in terms of monitoring time. In other words, periodic blood collection and sequential flow-control hinder monitoring variations of biophysical blood properties over a sufficiently long time. Furthermore, since blood viscosity is varied by several factors including plasma proteins, mechanical properties of RBCs, and hematocrit, it does not provide sufficient clues on the variations of blood. Thus, multiple biophysical properties of blood should be measured to effectively monitor blood. For this reason, to effectively monitor variations in blood circulating in a closed loop, multiple hemorheological properties including the blood viscosity and RBC aggregation should be measured to effectively monitor blood, periodically and simultaneously.

In this study, the periodic and simultaneous quantification of blood viscosity and RBC aggregation using a microfluidic platform under *in-vitro* closed-loop circulation is suggested. The proposed method has several distinct advantages when compared to previous methods. First, the proposed method can measure blood viscosity and RBC aggregation under in-vitro closed-loop circulation at time intervals. Second, this method does not require collecting large volumes of blood or sequential flow controls, which hinder long-term and continuous measurements. Here, the blood flow-rate and blood viscosity are quantified with a micro-particle image velocimetry (PIV) technique and a modified parallel flow method (MPFM),⁴³ respectively. As a performance demonstration, the proposed method is first employed to evaluate the effect of hematocrits and dextran concentrations on the RBC aggregation and blood viscosity with a syringe pump (i.e., specific blood flow rate). The method is then applied to detect the blood viscosity and RBC aggregation under in-vitro closed-loop circulation (i.e., unspecific blood flow-rate).

II. MATERIALS AND METHODS

A. Fabrication of microfluidic devices and experimental procedure

A microfluidic device [VA (viscosity-aggregation)] for measuring blood viscosity and RBC aggregation was composed of two inlets (A and B), one outlet (A), and a straight channel (width = 2 mm, length = 12 mm, and depth = 100 μm). Inlet (A) is connected to the middle position of the straight channel. In addition, the other microfluidic device [FD (fluid divider)] was designed to have four ports, and two identical side channels (width = 3 mm, length = 12 mm, and depth = 100 μm) connected with a bridge channel. A silicon-master mold was fabricated using conventional micro-electromechanical-system fabrication techniques, including photolithography and deep-reactive-ion etching. To conduct soft lithography with a silicon-master mold, polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning, Midland, MI, USA) was mixed with a curing agent at a ratio of 10:1. The PDMS mixture was then poured into the silicon-master mold placed in a Petri dish. Air bubbles dissolved in PDMS were completely removed with a vacuum pump. After curing the PDMS in a convection oven at 70 °C for 1 h, the PDMS block was peeled off from the silicon-master mold. The PDMS block was cut with a razor blade. Two inlets (A, B) and one outlet (A) of the VA were punched with a biopsy punch (outer diameter = 0.75 mm). The right-upper port of the FD was punched with a biopsy punch (outer diameter = 0.75 mm). The three other ports of the FD were punched with a biopsy punch (outer diameter = 1 mm). After treating the PDMS block and a glass substrate with oxygen plasma (CUTE-MPR, Femto Science Co., Korea), the VA and FD were prepared by bonding the PDMS block to the glass substrate. The microfluidic device was then placed on an optical microscope (BX51, Olympus, Japan) equipped with a 4 $\times$ objective lens (NA = 0.1). After 1% bovine serum albumin (BSA) solution (~ 5 ml) was dropped into a reservoir, the peristaltic pump was operated at rotation speed of $\omega = 15$ rpm for 10 min. After the BSA solution was completely removed from the in-vitro closed-loop, blood (~ 10 ml) was dropped into the reservoir.

Figure 1 shows the schematics for the periodic and simultaneous measurement of blood viscosity and RBC aggregation using a microfluidic platform under in-vitro closed-loop circulation. As shown in Fig. 1(A), the experimental setup was composed of in-vitro closed-loop circulation and a microfluidic platform, which was intended to quantify the blood viscosity and RBC aggregation. As depicted in Fig. 1(A-a), in-vitro closed-loop circulation was established by connecting several components [peristaltic pump (Techpre pump, Jeniwell, Seoul, Korea), air compliance unit (ACU), fluid divider (FD), and reservoir] with polyethylene tubes (L_5 , L_6 , L_7 , L_8 , and L_9) in series. Here, the dimensions for each tube were as shown in Table S1 (supplementary material). The blood was then circulated by operating the peristaltic pump under an in-vitro closed-loop. To remove the alternating component of the blood flow-rate generated by operating the peristaltic pump, the air cavity in the ACU is adjusted to 2.5 ml with a disposable syringe (3 ml, Kovax-Syringe, Korea Vaccine, Korea).^{44,45} As shown in Fig. S1 (supplementary material), the blood flow-rate remains constant with time (i.e., $Q_{\text{Blood}} = 40.85 \pm 0.59$ ml/h) under in-vitro closed-loop circulation. To stimulate RBC aggregation in blood, a specific concentration of dextran solution (i.e., $C_{\text{dextran}} = 100$ mg/ml) was periodically delivered into the reservoir by operating a syringe pump. Here, a polyethylene tube (L_4) is connected from the driving syringe to the reservoir. As shown in Fig. 1(A-b), a microfluidic platform consists of a microfluidic device (VA), pinch valve (PV) and a syringe pump (neMESYS, Centoni GmbH, Germany) for supplying phosphate-buffered saline (PBS) solution (1 $\times$, pH 7.4, Gibco, Life Technologies, USA). For convenience, the upper and lower areas of the straight channel are named the upper channel and lower channel, respectively. The outlet of the FD was connected to inlet (B) of the VA with a polyethylene tube (L_2). To control the blood flow from the FD to the microfluidic device (i.e., open or close), the PV was installed in front of inlet (B) of the VA. 1 $\times$ PBS (Phosphate-buffered saline) was precisely supplied into inlet (A) of the VA with the syringe pump.

Figure 1(B) represents the flow-rate profile of PBS and dextran solutions (i.e., Q_{PBS} and Q_{Dextran}), and operation of PV (i.e., open, and close) for every period. To measure the RBC aggregation and blood viscosity sequentially, the blood flow-rate was synchronized with the

FIG. 1. Schematics for the periodic and simultaneous measurement of blood viscosity RBC aggregation using a microfluidic platform under in-vitro closed-loop circulation. (A) Schematics of the experimental setup including an in-vitro closed-loop circulation and a microfluidic platform. (a) In-vitro closed-loop circulation established by connecting several components (peristaltic pump, ACU, FD, and reservoir) with polyethylene tubes (L₅, L₆, L₇, L₈, and L₉) in series. After the reservoir was filled with blood (10 ml), a peristaltic pump was set to $\omega = 15$ rpm. The air cavity in the ACU was adjusted to 2.5 ml. A specific concentration of dextran solution (i.e., $C_{\text{dextran}} = 100$ mg/ml) was delivered into the reservoir by operating the syringe pump. (b) A microfluidic platform including a microfluidic device (VA), PV, and syringe pump. The outlet of the FD was connected to the inlet (B) of the VA with a polyethylene tube (L₂). To control the blood flow from the FD to the VA, a PV was installed in front of the inlet (B). 1x PBS solution was supplied into inlet (A) with a syringe pump. (B) Flow-rate profile of two fluids with a syringe pump (i.e., Q_{PBS} and Q_{Dextran}) and PV operation (i.e., open and close) for each period. (C) Quantification of image intensity (I) and blood flow-rate (Q_{Blood}). (a) Quantification of image intensity (I) within the ROI (484×150 pixels) in the upper channel. (b) Quantification of blood velocity fields with a time-resolved micro-PIV technique. The blood flow-rate (Q_{Blood}) was quantified by multiplying averaged blood velocity ($\langle U \rangle$) by the cross-sectional area (A_c) (i.e., $Q_{\text{Blood}} = \langle U \rangle \cdot A_c$). (c) A simple fluidic circuit with discrete elements including fluidic resistances (R_P , R_B), and flow rates (Q_{PBS} , Q_{Blood}). The interface between two fluids was evaluated by converting the gray image into a binary image. (D) For a preliminary demonstration, blood (Hct = 50%) was prepared by adding normal RBCs to autologous plasma. Here, the period was set to $T = 400$ s. The inset shows microscopic images at a specific time (t) [(a) $t = 10$ s, (b) $t = 100$ s, (c) $t = 150$ s, (d) $t = 200$ s, (e) $t = 300$ s, and (f) $t = 400$ s]. During each period, at constant blood flow (i.e., $0 < t < 100$ s), the blood viscosity was quantified with the MPFM. Afterwards, at stationary blood flow (i.e., $100 < t < 400$ s), the RBC aggregation was quantified by evaluating variations in image intensity with respect to time.

PBS flow-rate. PBS solution was supplied as a reference fluid at a flow rate of $Q_{\text{PBS}} = Q_0$ from $t = 0$ to $t = 0.25T$. Here, T denoted the period. The blood flow-rate was stopped or flowed by operating the PV (Supa clip, Pankyo, Korea). In other words, the PV was only opened from $t = 0$ to $t = 0.25T$ for every period. The PV was closed for the remaining duration of each period. To stimulate the RBC aggregation of blood circulating under *in-vitro* closed loop, a specific concentration of dextran solution was delivered at the flow rate of $Q_{\text{Dextran}} = Q_0$ from $t = 0.5T$ to $t = 0.75T$. All the experiments were conducted at a constant temperature (25°C).

B. Quantification of blood velocity, image intensity, and blood-filled width

As shown in Fig. 1(C-a), microscopic images were captured by using an image acquisition system equipped with a microscope and a high-speed camera (FASTCAM MINI, Photron, USA). At intervals of 2 s, two microscopic images were consecutively captured with a high-speed

camera at a frame rate of 1 kHz. The upper channel was only filled with blood. However, the lower channel was partially filled with blood and PBS. The position of the interface between the PBS and blood in the lower channel depended on the viscosity ratio of blood in relation to PBS. To quantify variations in image intensity ($\langle I \rangle$) and blood velocity ($\langle U \rangle$), a region of interest (ROI, 484×150 pixels) was selected within the upper channel. As shown in Fig. 1(C-b), the velocity fields of the blood flow in upper channel are calculated by performing a time-resolved micro-PIV (particle image velocimetry) technique. The size of the interrogation window was 32×32 pixels. The window overlap was 50%. The obtained velocity fields were validated with a median filter. The average velocity of blood flow ($\langle U \rangle$) was calculated as an arithmetic average over the ROI. Then, the blood flow rate in the upper channel ($Q_{\mu\text{PIV}}$) was estimated by multiplying the averaged velocity ($\langle U \rangle$) obtained with the micro-PIV technique by a cross-sectional area with the rectangular channel (A_c) (i.e., $Q_{\mu\text{PIV}} = \langle U \rangle \cdot A_c$). To evaluate the variations in image intensity of blood flow, a specific ROI (484×150 pixels) was selected within the upper channel. The average value of the image intensity over the ROI ($\langle I \rangle$) was calculated by conducting digital image processing with a commercial software package (Matlab, Mathworks, USA). In the lower channel, the blood viscosity is calculated by quantifying the interfacial location between the blood and PBS. As shown in Fig. 1(C-c), to obtain the variations in the blood-filled width (α_{Blood}), the gray image was converted into a binary image by adopting Otsu's method.⁴⁶ By calculating the arithmetic average over the ROI, the blood-filled width (α_{Blood}) was obtained over time.

C. Blood sample preparation

In accordance with the ethics committee of Chosun University Hospital (CUH), all the experiments were performed ensuring that the procedures were appropriate and humane. To stimulate RBC aggregation, various blood samples were prepared by adding human RBCs to autologous plasma, or to specific concentrations of dextran solution. The human concentrated RBCs by removing autologous plasma from whole blood were provided by the Gwangju–Chonnam Blood Bank (Gwangju, Korea). First, to evaluate the effect of the hematocrit on RBC aggregation, the hematocrit of normal RBCs (Hct) (Hct = 30%, 40%, and 50%) was prepared by adding normal RBCs into autologous plasma. Second, to stimulate RBC aggregation, three concentrations of dextran solution (C_{Dextran}) ($C_{\text{Dextran}} = 5, 10, \text{ and } 15 \text{ mg/ml}$) were prepared by diluting dextran (*Leuconostoc* spp., MW = 450–650 kDa, Sigma–Aldrich, USA) with a $1 \times$ PBS solution (pH 7.4, GIBCO, Life Technologies, Korea). Here, the hematocrit of blood was adjusted to 30% by adding normal RBCs to specific concentrations of dextran solution. Finally, a higher concentration of dextran solution ($C_{\text{Dextran}} = 100 \text{ mg/ml}$) was prepared to evaluate variations in RBC aggregation and blood viscosity under in-vitro closed-loop circulation. Here, the hematocrit of blood was adjusted to 30% or 50% by adding normal RBCs into autologous plasma. During this study, all blood tests were completed within 3 h after preparing blood samples.

D. Simultaneous measurement of blood viscosity and RBC aggregation under closed-loop circulation

In this study, the simultaneous measurement of blood viscosity and RBC aggregation using a microfluidic platform under in-vitro closed-loop circulation is demonstrated at an interval of specific duration. Under in-vitro closed-loop circulation, a peristaltic pump induces pulsatile blood flow. With an air compliance unit (ACU),^{44,45} the pulsatile blood flow-rate is stabilized as a constant flow-rate. Thus, the blood viscosity is quantified by monitoring the interfacial location in a single microfluidic channel, especially at consistent blood flow-rates. By clamping the tube with a PV, the blood flow is stopped. Then, the RBC aggregation is measured by monitoring variations in image intensity of the blood flow in the microfluidic channel.

Since a rectangular-shaped microfluidic channel with an extremely low aspect ratio (AR) [i.e., $\text{AR} = \text{depth (h)}/\text{width (w)} = 100/2000$] has a uniform distribution along the channel width, the interface between the two fluids is simply assumed to be a virtual wall. Then, the lower channel is divided into two separated channels connected in parallel. The channel is partially filled with PBS and blood, respectively. For a rectangular-shaped microfluidic channel, the

fluidic resistance (R) is approximately given by $R = 12\mu L/(wh^3)$,⁸ where μ and L denote the fluid viscosity and channel length, respectively. A simple fluidic circuit with discrete elements including fluidic resistances (R_P , R_B) and flow rates (Q_{PBS} , Q_{Blood}) is employed to derive an analytical formula for the blood viscosity. Here, R_P and R_B represent the fluidic resistances of the channels filled with PBS and blood, respectively. In addition, Q_{PBS} and Q_{Blood} denote the flow rates of PBS and blood, respectively. Each fluid channel has the same pressure drop (i.e., $\Delta P = R_P \cdot Q_{PBS} = R_B \cdot Q_{Blood}$). Because each fluid channel has identical dimensions of channel depth (h) and length (L), an analytical formula for the blood viscosity (μ_{Blood}) is simply derived as the following equation:^{43,47}

$$\mu_{Blood} = \mu_{PBS} \cdot \left(\frac{\alpha_{Blood}}{1 - \alpha_{Blood}} \right) \cdot \left(\frac{Q_{PBS}}{Q_{Blood}} \right). \quad (1)$$

In Eq. (1), μ_{PBS} denotes the PBS viscosity. This approach is named the parallel flow method (PFM). However, when the blood-filled width is located far away from the center of the lower channel, the measurement error tends to increase owing to differences in boundary conditions between the virtual wall and the actual interface. For this reason, a correction factor (C_μ) is newly required to compensate this error that inevitably occurs when using a simple analytical model (i.e., PFM). For a given viscosity of a test fluid (μ_{given}), various interfaces are obtained by conducting computational fluid dynamics (CFD) simulations and changing the test fluid flow rate. Then, the fluid viscosity of the test fluid is calculated by inserting the interface (α) into Eq. (1) (i.e., μ_{PFM}). The normalized viscosity (i.e., $\mu_{norm} = \mu_{PFM}/\mu_{given}$) is obtained with respect to the interfacial location. Because the correction factor is calculated by dividing 1 by the normalized viscosity (i.e., $C_\mu = 1/\mu_{norm}$), the correction factors along various interfacial locations are expressed as a function of the interfacial location [i.e., $C_\mu = F(\alpha)$]. Thus, the analytical formula of the blood viscosity is newly derived as the following equation:⁴³

$$\mu_{Blood} = \mu_{PBS} \cdot C_\mu(\alpha_{Blood}) \cdot \left(\frac{\alpha_{Blood}}{1 - \alpha_{Blood}} \right) \cdot \left(\frac{Q_{PBS}}{Q_{Blood}} \right). \quad (2)$$

This approach is named the MPFM.

For a preliminary demonstration, the hematocrit of blood was adjusted to Hct = 50% by adding normal RBCs to autologous plasma. By conducting a time-resolved micro-PIV technique and digital image processing, the image intensity ($\langle I \rangle$) within the upper channel, averaged blood velocity ($\langle U \rangle$) within the upper channel, and blood-filled width (α_{Blood}) within the lower channel were obtained as functions of time, as shown in Fig. 1(D). Here, the period (T) is set to $T = 400$ s. The inset shows microscopic images captured by a high-speed camera at a specific time (t) [(a) $t = 10$ s, (b) $t = 100$ s, (c) $t = 150$ s, (d) $t = 200$ s, (e) $t = 300$ s, and (f) $t = 400$ s]. At a constant blood flow-rate (i.e., $0 < t < 100$ s), the variation in blood viscosity was quantified by monitoring the blood-filled width (α_{Blood}) within the lower channel. Thereafter (i.e., $100 < t < 400$ s), by clamping the tube (L_2) with the pinch valve (i.e., close), the blood flow and PBS flow stopped immediately. The image intensity ($\langle I \rangle$) within the upper channel increased over time. In other words, in the stationary blood-flow condition, the RBC aggregation was quantified by evaluating variations in image intensity with respect to time (i.e., a syllectogram).^{19,48} From this preliminary study, it is certain that the image intensity ($\langle I \rangle$) and blood-filled width (α_{Blood}) can be employed to detect variations in blood viscosity and RBC aggregation.

III. RESULTS AND DISCUSSION

A. Quantitative evaluations of the effect of pinch valve on RBC aggregation measurement

In this study, a pinch valve (PV) was used to stop blood flow in the upper channel immediately. With and without installation of the PV, the transient blood flow contributes to varying the RBC aggregation in the upper channel. Thus, the effect of the PV on the RBC aggregation

measurement was evaluated by varying the hematocrit. The hematocrit of blood was adjusted to 30%, 40%, and 50% by adding normal RBCs into autologous plasma. Instead of a peristaltic pump, blood was supplied into the inlet port (B) of the microfluidic device (VA) with a syringe pump. Thus, the blood and PBS were simultaneously supplied at the same flow-rate (i.e., $Q_{\text{Blood}} = Q_{\text{PBS}} = 1 \text{ ml/h}$) for $0 < t < 0.25 T$. During the remaining portion of each period, the flow rates of blood and PBS were set to zero (i.e., $Q_{\text{Blood}} = Q_{\text{PBS}} = 0$, $0.25 T < t < T$). T denotes the period, where $T = 400 \text{ s}$. When the blood flow-rate was set to zero, the blood flow was stopped after a certain time owing to the compliance effect of the microfluidic device and polyethylene tube.^{43,45} To stop blood flow immediately, the PV was installed between the inlet port (B) and the driving syringe. Here, to determine the effect of transient blood flow on the RBC aggregation measurement, the image intensity ($\langle I \rangle$) of blood flowing in the upper channel was evaluated with and without the PV. As shown in Fig. 2(a), temporal variations in the image intensity ($\langle I \rangle$) were obtained with and without the PV. Blood with the lower hematocrit (Hct = 30%) had a much higher value of image intensity, compared with blood with the higher hematocrit (Hct = 50%). Because blood was supplied into the microfluidic device from the button region of the driving syringe, the hematocrit of blood increased over time owing to continuous RBC sedimentation in the driving syringe.^{19,49} Thus, blood with Hct = 30% shows a distinctive decrease in image intensity over time, compared with blood of Hct = 40% and 50%. When the blood flow rate was set to zero, the blood flow immediately stopped upon the operation of the PV. Image intensity ($\langle I \rangle$) was significantly decreased, compared with the case of no PV. From this result, it is estimated that the transient blood flow has an influence on variations in the image intensity owing to RBC aggregation. Temporal variations in the blood flow-rate obtained with a time-resolved micro-PIV technique ($Q_{\mu\text{PIV}}$) and image intensity ($\langle I \rangle$) of the blood flow in the upper channel were obtained with and without the PV. Here, the hematocrit of blood was fixed at 30%. As shown in Fig. 2(b), when the blood flow rate was set to zero for each period, the blood flow was stopped after a certain time. By conducting a regression analysis of the blood flow rate as $Q_{\mu\text{PIV}} = Q_0 \exp(-t/\lambda_{\text{off}})$,⁴³ the time constant (λ_{off}) was estimated as $\lambda_{\text{off}} = 11.98 \pm 0.72 \text{ s}$. However, the blood flow was periodically stopped immediately ($\sim 0.2 \text{ s}$)

FIG. 2. Quantitative evaluation of the effect of two factors including PV and hematocrit (Hct) on the RBC aggregation measurement. The hematocrit of blood was adjusted to 30%, 40%, and 50% by adding normal RBCs into autologous plasma. With two syringe pumps, blood and PBS were simultaneously supplied at the same flow-rate of $Q_{\text{Blood}} = Q_{\text{PBS}} = 1 \text{ ml/h}$ for $0 < t < 0.25 T$. During each period, the flow rates of blood and PBS were set to zero (i.e., $Q_{\text{Blood}} = Q_{\text{PBS}} = 0$, $0.25 T < t < T$). T denotes the period (i.e., $T = 400 \text{ s}$). (A) Temporal variations in the image intensity ($\langle I \rangle$) with respect to the hematocrit (Hct) and PV installation. (B) Temporal variations in the blood flow-rate ($Q_{\mu\text{PIV}}$) and image intensity ($\langle I \rangle$) with without PV installation. The hematocrit of blood was fixed at 30%. (C) Temporal variations in the shear rate and image intensity for five consecutive cycles. Here, the blood flow was stopped after a certain time without PV installation. (D) Variations in the conventional RBC aggregation index (AI_{conv}) and new RBC aggregation index (AI_{new}) with respect to the hematocrit and PV installation. The inset represented temporal variations for a specific duration of $t_s = 250 \text{ s}$.

by clamping the polyethylene tube with the PV. Without the PV, temporal variations in the shear rate and image intensity ($\langle I \rangle$) for five consecutive cycles were obtained, as shown in Fig. 2(c). The hematocrit of blood was fixed at 30%. For a rectangular-shaped microfluidic channel (VA) with a lower aspect ratio AR (i.e., $AR = h/w = 100/2000$), the shear rate ($\dot{\gamma}$) is approximately given by $\dot{\gamma} = \frac{6Q_{\mu PV}}{wh^2}$. When the shear rate is decreased to less than 25 (i.e., $\dot{\gamma} < 25 \text{ s}^{-1}$), the image intensity tends to increase with time. In other words, the RBCs tend to aggregate after a time constant has elapsed (i.e., $\lambda_{\text{off}} = 11.98 \pm 0.72 \text{ s}$). However, when the PV is employed to stop the blood flow, the RBCs tend to aggregate immediately. Finally, as shown in Fig. 2(d), variations in the conventional RBC aggregation index (AI_{conv}) and new RBC aggregation index (AI_{new}) were obtained with respect to hematocrit and PV installation. Here, owing to the hematocrit variation in blood samples, the minimum value of the image intensity ($\langle I \rangle_{\text{min}}$) decreased with time. Thus, the new RBC aggregation index (AI_{new}) was suggested to consider the effect of the minimum image intensity ($\langle I \rangle_{\text{min}}$) on the RBC aggregation with time.¹⁹ The inset represents temporal variations in $\langle I \rangle$ for a specific duration of $t_s = 250 \text{ s}$. With three factors (S_A , S_B , and S_C), AI_{conv} and AI_{new} were calculated as $AI_{\text{conv}} = S_A/(S_A + S_B)$ ⁴⁸ and $AI_{\text{new}} = S_A/S_C$,¹⁹ respectively. With installation of the PV, the conventional aggregation index (AI_{conv}) increases gradually with increasing hematocrit. Above 40% hematocrit, AI_{conv} remains constant. However, without installation of the PV, AI_{conv} fluctuates with respect to the hematocrit. On the contrary, without respect to PV, the new RBC aggregation index (AI_{new}) is noticeably decreased with increasing hematocrit. From this result, the installation of the PV contributes to consistently measuring RBC aggregation with AI_{conv} and AI_{new} , because the blood flow is immediately stopped in the upper channel.

B. Quantitative evaluations of blood viscosity with the modified parallel flow method (MPFM)

In this study, under periodic on-off blood flows, the blood viscosity was quantified with the MPFM.⁴³ Compared with the PFM,^{47,50,51} the MPFM included correction factors to compensate the inevitable errors due to differences in the boundary conditions of the simple mathematical model. According to the previous work,⁴³ a correction factor inserted in the MPFM was varied depending on channel dimensions (width and depth). Thus, as shown in Fig. S3 (supplementary material), a correction factor was newly obtained by conducting several procedures including CFD analysis, and normalized viscosity with the parallel flow method. As shown in Fig. 3(A), to estimate the correction factors for this microfluidic channel (VA), numerical simulations with CFD software (CFD ACE+, ESI Group, Paris, France) were conducted by varying the flow rate of a test fluid (Q_x). Here, the flow rate and viscosity of the reference fluid were set to $Q_0 = 1 \text{ ml/h}$ and $\mu_0 = 1 \text{ cP}$, respectively. The flow rate of the test fluid (Q_x) was set to range from $Q_x = 1 \text{ ml/h}$ to $Q_x = 18 \text{ ml/h}$ to simulate various interfacial locations (α). Here, the viscosity of the test fluid was fixed as $\mu_{\text{given}} = 1 \text{ cP}$. By conducting numerical simulations, binary images were obtained by increasing the flow-rate ratio of the test fluid in relation to the reference fluid (Q_x/Q_0). As shown in right panels of Fig. 3(A), binary images were obtained with respect to the flow-rate ratio (Q_x/Q_0) [(a) $Q_x/Q_0 = 1$, (b) $Q_x/Q_0 = 3$, (c) $Q_x/Q_0 = 5$, and (d) $Q_x/Q_0 = 8$]. By analyzing several binary images, the interfacial locations were calculated with respect to the flow-rate ratio. As shown in Fig. 3(B), using the PFM, the viscosity of the test fluid (μ_{PFM}) was obtained by inserting the interfacial location (α) and flow-rate ratio (Q_x/Q_0) into Eq. (1). The normalized viscosity of the test fluid was calculated by dividing μ_{PFM} by μ_{given} along various interfacial locations (i.e., $\mu_n = \mu_{\text{PFM}}/\mu_{\text{given}}$). Then, the correction factor (C_μ) was calculated by dividing 1 by the normalized viscosity (i.e., $C = 1/\mu_n$). As shown in Fig. 3(C), variations in the normalized viscosity (μ_n) and correction factor (C_μ) were obtained with respect to the interfacial location (α). In addition, Table S2 (supplementary material) summarized normalized viscosity (μ_n) and correction factor (C_μ) with respect to interfacial location (α). At the middle position of the channel width ($\alpha = 0.5$), the correction factor was evaluated as $C_\mu = 1$. In other words, at $\alpha = 0.5$, the PFM is able to measure the fluid viscosity with sufficient accuracy. However, when the interfacial location was relocated to the wall, the normalized

FIG. 3. Procedure for evaluating the correction factor (C_μ) in the modified parallel flow method (MPFM) and quantitative evaluation of the proposed method. (A) CFD simulation for determining the correction factor by varying the flow-rate ratios. The flow rate and viscosity of a reference fluid were set to $Q_0 = 1$ ml/h and $\mu_0 = 1$ cP, respectively. The test fluid flow rates (Q_x) were set to range from $Q_x = 1$ ml/h to 18 ml/h to simulate various interfacial locations. Four figures represented the variations in the interfacial locations depending on the flow-rate ratio (Q_x/Q_0) [(a) $Q_x/Q_0 = 1$, (b) $Q_x/Q_0 = 3$, (c) $Q_x/Q_0 = 5$, and (d) $Q_x/Q_0 = 8$]. (B) Viscosity measurement of the test fluid with the parallel flow method (PFM) and normalized viscosity (μ_n). (C) Variations in the normalized viscosity (μ_n) and correction factor (C_μ) with respect to the interfacial location (α). (D) Quantitative evaluations of viscosity measurement accuracy of the MPFM for four different concentrations of glycerin solution (C_{glycerin}) ($C_{\text{glycerin}} = 10\%$, 20% , 30% , and 40%) as Newtonian fluid. (a) Variations of viscosity obtained by three methods including the modified parallel flow method (MPFM), flow switching method (FSM), and cone-and-plate viscometer (Cone-and-Plate). (b) Linear relationship between viscosity obtained by the MPFM and the one obtained by the Cone-and-Plate. (c) Linear relationship between viscosity obtained by the MPFM and the one obtained by the FSM.

viscosity decreased. The inevitable errors caused by using the PFM should be compensated by including correction factors in the PFM. In other words, the correction factor was increased when the interfacial location was moved to the wall. Using the MPFM [Eq. (2)], the viscosity of the test fluid remains constant (i.e., $\mu_{\text{MPFM}} = \mu_{\text{given}}$) without respect to the interfacial location. By conducting a regression analysis with a fourth-order polynomial expression, the correction factor (C_μ) was obtained as $C_\mu = 34.041\alpha^4 - 91.75\alpha^3 + 92.393\alpha^2 - 40.977\alpha + 7.7319$. Because the coefficient of linear regression (R^2) has a sufficiently high value of $R^2 = 0.9999$, the polynomial expression of C_μ accurately represents the trends in the correction factor along interfacial locations.

Measurement accuracy of the proposed method (i.e., modified parallel flow method, MPFM) was quantitatively evaluated with two previous methods such as flow switching method (i.e., FSM) and cone-and-plate viscometer (i.e., Cone-and-Plate, HAAKE MARS, Thermo Electron GmbH, and Germany). As shown in Fig. 3(D), using four different concentrations of glycerin solution ($C_{\text{glycerine}}$) ($C_{\text{glycerine}} = 10\%$, 20% , 30% , and 40%) as Newtonian fluid, viscosity of each solution was quantitatively compared by using three representative methods including the cone-and-plate viscometer, flow-switching method, and modified parallel flow method. As shown in Fig. 3(D-a), viscosity evaluated by the MPFM was in good agreement with viscosity obtained by the Cone-and-Plate and the FSM. Furthermore, the linear relationship between the

proposed method and the previous methods was evaluated by conducting linear regression analysis. As shown in Figs. 3(A-b) and 3(A-c), the regression coefficient between the MPFM and the Cone-and-Plate shows a sufficiently high value of $R^2=0.9992$. In addition, the regression coefficient between the MPFM and the FSM gives a sufficiently high value of $R^2=0.9969$. Furthermore, the linear slope of the viscosity obtained by the proposed method was evaluated to be 0.9819 for the cone-and-plate viscometer and 0.9839 for the flow switching method. From these experimental demonstrations, it could be found that the proposed method (MPFM) could be used to measure fluid viscosity with sufficient accuracy, compared with the previous methods including the FSM and Cone-and-Plate.

Under constant blood flow, the blood viscosity obtained with the MPFM was quantitatively compared with the blood viscosity measured with the flow-switching method (FSM).⁴⁰ Here, the hematocrit of blood was adjusted to $Hct=30\%$, 40% , and 50% by adding normal RBCs into autologous plasma. As shown in Fig. 4(A-a), variations in the blood viscosity (μ_{Blood}) were obtained by varying the hematocrit (Hct) and blood flow-rate (Q). The blood viscosity was decreased by increasing the flow rate in the range from $Q=0.5$ to 1 ml/h . The blood viscosity obtained by the proposed method (μ_{MPFM}) was quantitatively compared with the blood viscosity measured with the previous method (μ_{FSM}). As shown in Fig. 4(A-b), the linear relationship between μ_{MPFM} and μ_{FSM} was evaluated by conducting a linear regression analysis. As evidenced by the linear slope less than 1, the MPFM underestimates the blood

FIG. 4. Quantitative evaluation of blood viscosity using the MPFM under periodic on-off blood flow-rate. (A) Quantitative evaluation of the blood viscosity with the proposed method (MPFM) and the previous method (FSM) under steady blood flow. (a) Variations in the blood viscosity (μ_{Blood}) with respect to the hematocrit (Hct) and blood flow rate (Q). (b) Quantitative comparison of the blood viscosity obtained by the MPFM (μ_{MPFM}) and the FSM (μ_{FSM}). (c) Variations in the blood viscosity with respect to the shear rate and hematocrit. (B) The effect of dextran solution on blood viscosity measurement by using the MPFM and Cone-and-Plate. (a) Variation of blood viscosity of normal blood ($Hct=50\%$) with respect to the shear rate. (b) Variation of blood viscosity of dextran blood ($Hct=50\%$) with respect to the shear rate. (C) Periodical quantification of the blood viscosity under pulsatile on-off blood flow. With two syringe pumps, the blood and PBS were supplied at the same flow rate of $Q_{\text{Blood}}=Q_{\text{PBS}}=1\text{ ml/h}$ for 100 s of each period. The blood and PBS were stopped for the remaining time ($100\text{ s} < t < 400\text{ s}$) of each period. (a) Temporal variations in the blood-filled width (α_{Blood}) with respect to the hematocrit. (b) Variations in α_{Blood} for 50 s with respect to the hematocrit. (c) Periodic quantification of the blood viscosity with respect to the hematocrit.

viscosity, compared with the FSM. In addition, the coefficient of linear regression has a sufficiently high value of $R^2 = 0.9696$. From this result, the suggested method (i.e., MPFM) is able to measure blood viscosity with consistency, compared with the previous method (i.e., FSM). For this reason, in this study, the blood viscosity was measured with the MPFM. The MPFM was employed to measure the blood viscosity with respect to the shear rate and hematocrit. As shown in Fig. 4(A-c), the blood viscosity decreased with increasing shear rate. In other words, the blood exhibited a shear-thinning behavior. In addition, increases in the hematocrit increase blood viscosity.

To analyse the effect of dextran solution on blood viscosity measurement, two blood samples were prepared by adding normal RBCs into $1 \times$ PBS solution and a specific dextran solution (5 mg/ml), respectively. Here, hematocrit was fixed to 50% for two blood samples. Then, two methods (MPFM and Cone-and-Plate) were employed to measure the blood viscosity of two blood samples through wide shear rates ranging from 10 s^{-1} to 1000 s^{-1} . Figures 4(B-a) and 4(B-b) show blood viscosity of normal blood (i.e., normal RBCs in PBS solution) and dextran blood (i.e., normal RBCs in dextran solution) with respect to the shear rate. As a result, the MPFM underestimated blood viscosity, compared with the Cone-and-Plate. In addition, dextran solution contributed to increasing blood viscosity. Since blood flow behaves as non-Newtonian fluid, variation of blood viscosity could be best fitted with the power-law model (i.e., $\mu = \mu_0 \dot{\gamma}^n$). A higher value of regression coefficient (i.e., $R^2 > 0.96$) indicated that the power-law model could give a best approximation of blood viscosity with respect to the shear rate. First, for normal blood as shown in Fig. 4(B-a), μ_0 and n were estimated as (1): ($\mu_0 = 5.999$ and $n = -0.134$) for the MPFM and (2): ($\mu_0 = 8.456$ and $n = -0.143$) for the Cone-and-Plate, respectively. μ_0 obtained by the MPFM was underestimated about 19%, compared with that obtained by the Cone-and-Plate. Normalized deviation of the power index (n) obtained by both methods was less than 7%. Second, for dextran blood as shown in Fig. 4(B-b), μ_0 and n were estimated as (1): ($\mu_0 = 10.631$ and $n = -0.209$) for the MPFM and (2): ($\mu_0 = 11.904$ and $n = -0.178$) for the Cone-and-Plate, respectively. μ_0 obtained by the MPFM was estimated to be about 11%, compared with that obtained by the Cone-and-Plate. Both methods provided a very similar value of power index. From this demonstration, it was found that the blood viscosity obtained by the MPFM was underestimated within 20%, compared with that obtained by the Cone-and-Plate. In addition, dextran solution contributed to increasing blood viscosity through wide shear rates.

Under periodic on-off blood flow, the blood viscosity was periodically quantified with the MPFM. Here, the hematocrit of blood was adjusted to 30%, 40%, and 50% by adding normal RBCs into autologous plasma. As shown in Fig. 1(b), the flow rate of blood was precisely controlled with a syringe pump. Simultaneously, as a reference fluid, PBS was controlled with a syringe pump. For every period of 400 s, the blood and PBS were supplied at the same flow rate of $Q_{\text{Blood}} = Q_{\text{PBS}} = 1 \text{ ml/h}$ for 100 s. The flow rate of blood and PBS were set to zero value for the remaining duration of 300 s. As shown in Fig. 4(C-a), temporal variations in the blood-filled width (α_{Blood}) were obtained by varying the hematocrit (Hct) (Hct = 30%, 40%, and 50%). When the flow rates of both fluids were set to $Q = 1 \text{ ml/h}$, the blood-filled width (α_{Blood}) increased transiently with time. In addition, the blood-filled width increased with increasing values of the hematocrit. Here, a consistent blood-filled width was employed to calculate the blood viscosity under a steady flow condition. As shown in Fig. 4(C-b), under a constant flow condition, variations in α_{Blood} were obtained for 50 s for each period. Using the variations in the blood-filled width, the blood viscosity was calculated with Eq. (2) as the MPFM. Then, temporal variations in the blood viscosity were obtained with increasing hematocrit. As shown in Fig. 4(C-c), the blood viscosity increases gradually for blood with 30% hematocrit. As shown in Fig. 2(a), continuous sedimentation in the driving syringe increases the hematocrit. Thus, the image intensity of the blood flow decreases with time, and the blood-filled width increases with time. However, for the blood with higher hematocrit (Hct = 40% and 50%), the blood viscosity remained constant with time. In other words, higher values of the hematocrit (i.e., Hct > 40%) hinder RBC sedimentation in the driving syringe.

C. Measurement of RBC aggregation and blood viscosity under periodic on-off fluid delivery with the driving syringe

When a driving syringe filled with blood was aligned in the gravitational direction, the RBCs migrated toward the button area of the syringe. Because blood was supplied into the microfluidic device from the button area of the syringe, the hematocrit of blood in the microfluidic channel increased with time. According to our previous work,¹⁹ a continuous ESR in a conical pipette-tip causes the hematocrit to vary with time. Thus, the RBC aggregation varied depending on the continuous variations in the hematocrit in the conical pipette-tip. In this demonstration, the proposed method was employed to quantify the RBC aggregation and blood viscosity under periodic on-off fluid delivery with a driving syringe. Three concentrations of dextran solution (C_{dextran}) ($C_{\text{dextran}} = 5, 10, \text{ and } 15 \text{ mg/ml}$) were prepared to stimulate RBC aggregation. To effectively induce RBC sedimentation in the driving syringe, the hematocrit of blood was adjusted to 30% by adding normal RBCs into each concentration of dextran solution. For every period of 400 s, the blood and PBS were supplied at the same flow rate of $Q_{\text{Blood}} = Q_{\text{PBS}} = 1 \text{ ml/h}$ for 100 s. After that, the flow rates of blood and PBS were set to zero for the remaining period of 300 s.

Figure 5(A-a) shows variations in RBC-depleted layers in the driving syringe under periodic on-off delivery of blood with an elapse of time (t) ($t = 0, 100, 500, 900, 1300, 1700, \text{ and } 2100 \text{ s}$). Here, the hematocrit of blood was adjusted to 30% by adding normal RBCs into a specific concentration of dextran solution (i.e., $C_{\text{dextran}} = 15 \text{ mg/ml}$). After 500 s, dextran solution

FIG. 5. Simultaneous measurement of the RBC aggregation and blood viscosity under periodic on-off fluid delivery with driving syringes. The hematocrit of blood was fixed at 30%. (A) Temporal variation in the image intensity ($\langle I \rangle$), and blood-filled width (α_{Blood}) with respect to the concentration of dextran solution. (a) Temporal variations in the RBC-depleted layers in the driving syringe under periodic on-off delivery of blood. (b) Temporal variations with respect to the specific concentration of dextran solution (C_{dextran}) ($C_{\text{dextran}} = 0, 5, 10, \text{ and } 15 \text{ mg/ml}$). (c) Temporal variations in α_{Blood} with respect to the concentration of dextran solution. (B) Quantitative evaluation of the RBC aggregation and blood viscosity with respect to C_{dextran} . (a) Temporal variations in the conventional RBC aggregation index (AI_{conv}) with respect to C_{dextran} . (b) Temporal variations in the new RBC aggregation index (AI_{new}) with respect to C_{dextran} . (c) Temporal variations in the blood viscosity (μ_{Blood}) with respect to C_{dextran} .

(i.e., RBC-depleted layer) and RBCs (i.e., RBC-rich layer) were distinctively separated. From snapshot images of blood in the driving syringe, it is found that the ESR occurs significantly before 500 s in the driving syringe. To monitor the variation in the blood flow in the microfluidic channel owing to RBC sedimentation in the driving syringe, temporal variations in image intensity ($\langle I \rangle$) were obtained by varying the concentrations of dextran solution. As shown in Fig. 5(A-b), blood with dextran solution of 15 mg/ml has a higher value of image intensity, compared with the control blood. Here, the hematocrit of the control blood was adjusted to 30% by adding normal RBCs into $1 \times$ PBS. In addition, for every period of the stationary blood-flow condition, variations in the image intensity ($\Delta \langle I \rangle = \langle I \rangle_{\max} - \langle I \rangle_{\min}$) increased at higher concentrations of dextran solution. The minimum value of the image intensity ($\langle I \rangle_{\min}$) decreased up to 500 s. After that, $\langle I \rangle_{\min}$ remained constant with time.

To monitor the effect of the hematocrit variations on the blood viscosity, temporal variation in the blood-filled width (α_{Blood}) were obtained by varying the concentration of dextran solution, as shown in Fig. 5(A-c). For each period of fluid delivery, the blood-filled width increases at higher concentrations of dextran solution. The control blood has a consistent value of the blood-filled width with time. However, blood prepared with dextran solution increases the blood-filled width gradually with time.

To quantify the variation in the RBC aggregation of blood flowing in the microfluidic channel, the conventional RBC aggregation index (AI_{conv}) was calculated as $AI_{\text{conv}} = S_A / (S_A + S_B)$ by temporal variations in the image intensity, as shown in Fig. 5(A-b). Temporal variations in AI_{conv} were obtained by varying the concentrations of dextran solution, as shown in Fig. 5(B-a). As a result, AI_{conv} remained constant with time. The inset shows variations in AI_{conv} with respect to concentration of dextran solution. In other words, the conventional RBC aggregation index (AI_{conv}) tends to increase gradually at higher concentrations of dextran solution. To consider the effect of the minimum value of the image intensity ($\langle I \rangle_{\min}$) on the RBC aggregation due to continuous RBC sedimentation in the driving syringe, the new RBC aggregation index (AI_{new}) was calculated as $AI_{\text{new}} = S_A / S_C$ with temporal variations in the image intensity.¹⁹ As represented in Fig. 5(B-b), the blood with a higher concentration of dextran solution has a higher value of AI_{new} , compared with the control blood. For the blood prepared with dextran solution, AI_{new} decreases gradually with time. However, the AI_{new} value of the control blood remains consistent, and is a much lower value than that of the blood with dextran solution.

Using Eq. (2) as the MPFM, variations in the blood viscosity were obtained by varying the concentration of dextran solution, as shown in Fig. 5(B-c). As a result, the dextran solution contributes to increasing the blood viscosity with time. In other words, initially, the variation in the blood viscosity was not significant depending on the concentration of dextran solution. However, after a certain time elapsed, RBC sedimentation in the driving syringe increases the blood viscosity, whereas that of the control blood remains constant with time.

From this result, the new RBC aggregation index (AI_{new}) is more effective to monitor variations in the RBC aggregation due to continuous RBC sedimentation the driving syringe. Simultaneously, the blood viscosity can be effectively used to monitor variations in the hematocrit of blood flowing in a microfluidic channel.

D. Measurement of blood viscosity and RBC aggregation under in-vitro closed-loop circulation

As a final demonstration, the proposed method was applied to measure the blood viscosity and RBC aggregation under in-vitro closed-loop circulation, as shown in Fig. 1. To remove the alternating blood flows generated by operating a peristaltic pump, an ACU^{44,45} was installed between the peristaltic pump and a FD. Here, the air cavity was adjusted to 2.5 ml inside the ACU. To quantify the effect of the ACU on flow stabilization, sequential microscopic images were captured with a high-speed camera at a frame rate of 1 kHz and an interval of 1 s. As shown in Fig. S1(A) (supplementary material), after selecting a specific ROI (730×310 pixels) within the left side channel of the FD (width = 3 mm, and depth = 100 μm), the blood velocity fields were obtained by conducting a time-resolved micro-PIV technique.

As shown in Figs. S1(B) and S1(C) (supplementary materials), using the velocity fields along the channel width, the flow-rate of blood flowing in the channel ($Q_{\mu\text{PIV}}$) was calculated by integrating the blood flow-rate along the channel width. Figure S1(D) (supplementary materials) shows temporal variations in $Q_{\mu\text{PIV}}$ with and without the ACU. By installing the ACU between the peristaltic pump and FD, the flow rate of blood flowing in the FD remains constant with time (i.e., $Q_{\text{Blood}} = 40.85 \pm 0.59 \text{ ml/h}$). However, without ACU, the blood flow-rate largely fluctuated with time. Thus, by installing the ACU between the peristaltic pump and FD, the blood viscosity can be consistently measured under steady blood flow. Here, the hematocrit of blood was adjusted to 30% or 50% by adding normal RBCs into autologous plasma. Thereafter, a blood sample ($\sim 10 \text{ ml}$) was dropped into the reservoir. In addition, to stimulate RBC aggregation of the blood circulating under in-vitro closed loop, a specific concentration of dextran solution ($C_{\text{dextran}} = 100 \text{ mg/ml}$) was supplied with a syringe pump from $t = 200 \text{ s}$ and $t = 300 \text{ s}$ for every period of 400 s (i.e., $T = 400 \text{ s}$), as shown in Fig. 1(B).

Temporal variations in the image intensity ($\langle I \rangle$) for normal blood ($\text{Hct} = 50\%$) were obtained with and without addition of dextran solution, as shown in Fig. 6(a). As a result, when supplying dextran solution into blood circulating under an in-vitro closed loop, the image intensity significantly increases with time. However, the image intensity for normal blood (i.e., normal RBCs in autologous plasma) remains constant with time. To quantify the variations in the blood viscosity under in-vitro closed-loop circulation, temporal variations in the blood-filled width (α_{Blood}) were obtained with and without addition of the dextran solution, as shown in Fig. 6(b). With the addition of the dextran solution, the blood-filled width (α_{Blood}) tends to decrease, compared with normal blood. A time-resolved micro-PIV technique was employed to quantify the flow rate of blood circulating under an in-vitro closed loop. Using an analytical formula for the depth of correlation (DOC),^{52,53} the DOC was estimated to be $364.6 \mu\text{m}$. Because the DOC is sufficiently larger than the channel depth (i.e., $\text{DOC} > h = 100 \mu\text{m}$), it is reasonably estimated that all RBCs can contribute to calculating the velocity fields in the ROI selected within the upper channel. In other words, our micro-PIV technique measures the

FIG. 6. Periodic and simultaneous measurement of the blood viscosity and RBC aggregation under in-vitro closed-loop circulation. (a) Temporal variations in the image intensity ($\langle I \rangle$) with and without the addition of dextran solution ($C_{\text{dextran}} = 100 \text{ mg/ml}$). The hematocrit was fixed at 50%. (b) Temporal variations in the blood-filled width (α_{Blood}) with and without the addition of dextran solution. The hematocrit was fixed at 50%. (c) Temporal variation in the blood flow-rate ($Q_{\mu\text{PIV}}$) with and without the addition of dextran solution. (d) Temporal variations in AI_{conv} and AI_{new} with respect to the hematocrit (Hct) (Hct = 30% and 50%) and addition of the specific concentration of dextran solution. (e) Temporal variations in the blood viscosity with respect to the hematocrit and addition of the specific concentration of dextran solution.

velocity fields averaged through the depth directions. According to Eq. (2), the measurement accuracy of the blood viscosity is influenced by the flow-rate ratio of PBS in relation to blood (i.e., $Q_{\text{PBS}}/Q_{\text{Blood}}$). Here, the flow rate of PBS was supplied with a syringe pump. However, the blood flow-rate was quantified by performing a time-resolved micro-PIV technique. Thus, it is required to verify the relationship between the flow rate of blood obtained with the micro-PIV technique ($Q_{\mu\text{PIV}}$) and the flow rate of blood supplied with the syringe pump ($Q_{\text{syringe pump}}$). As shown in Fig. S2 (supplementary material), variations in $Q_{\mu\text{PIV}}$ were obtained by varying $Q_{\text{syringe pump}}$ and the hematocrit. Here, the hematocrit of blood (Hct) was adjusted to Hct = 30%, 40%, and 50% by adding normal RBCs into autologous plasma. According to a linear regression analysis performed with EXCEL (Microsoft, USA), all coefficients of linear regression have sufficiently higher values of $R^2 > 0.99$. In addition, the slope of linear regression (i.e., slope = $\Delta Q_{\mu\text{PIV}}/\Delta Q_{\text{syringe pump}}$) varied from 0.978 to 1.094 with respect to the hematocrit [i.e., (a) slope = 0.978 for Hct = 30%, (b) slope = 1.036 for Hct = 40%, and (c) slope = 1.094 for Hct = 50%]. From this result, it is found that the micro-PIV technique can measure the flow rate of blood (Hct = 30%–50%) within measurement errors ranging from -2.2% to 9.4% , compared with the blood flow-rate delivered with a syringe pump. Since the blood flow-rate is accurately estimated with the micro-PIV technique, an additional calibration procedure is not employed to measure the blood flow-rate with the micro-PIV technique. Thus, using the time-resolved micro-PIV technique, as shown in Fig. 6(c), temporal variations in the blood flow-rate ($Q_{\mu\text{PIV}}$) were obtained with and without addition of the dextran solution. As a result, dextran solution contributes to decreasing the flow rate of blood in a microfluidic device, compared with normal blood.

To quantify the RBC aggregation of blood, temporal variations in AI_{conv} and AI_{new} were obtained by changing blood samples or adding dextran solution. As shown in Fig. 6(d), the conventional RBC aggregation index (AI_{conv}) remains constant with time. AI_{conv} increases at higher hematocrit values. The dextran solution contributes to elevating the conventional RBC aggregation index (AI_{conv}). For normal blood, the new RBC aggregation index (AI_{new}) increases at lower hematocrit values. In addition, AI_{new} remains constant without respect to time. However, with the addition of dextran solution, the new RBC aggregation index (AI_{new}) increases significantly with time. Temporal variations in blood viscosity were obtained by changing blood samples, as shown in Fig. 6(e). As a result, the blood viscosity is consistently measured over time. The blood viscosity increases at higher hematocrit values. The dextran solution contributes to significantly increasing the blood viscosity, compared with normal blood.

From these experimental demonstrations, it is found that the suggested method can be effectively used to monitor the RBC aggregation and blood viscosity under in-vitro closed-loop circulation. Compared with previous works,^{8,41} this method does not require periodic collection from closed-loop circulation, or additional procedures for estimating the blood flow-rate with a syringe pump. However, this method was suggested for measuring two properties of several biophysical properties (i.e., blood viscosity, blood viscoelasticity, RBC aggregation, RBC deformability, hematocrit, and so on). In other words, additional research will be required to measure most biophysical properties of blood under in-vitro conditions. In the near future, the suggested method will be effectively used to monitor variations in the biophysical properties of blood circulating in extracorporeal loops.

IV. CONCLUSION

In this study, the blood viscosity and RBC aggregation under in-vitro closed-loop circulation were simultaneously measured with a microfluidic platform at intervals of 400 s. For each period, the blood viscosity and RBC aggregation were periodically quantified at constant blood flow and stationary blood flow, respectively.

First, the quantitative evaluation of RBC aggregation was quantitatively measured by varying hematocrit and the installation of a PV. By clamping the tube with the PV, the blood flow was stopped within 0.2 s. The PV was effective to consistently measure RBC aggregation. Second, under steady blood flows, blood viscosity was continuously measured by the MPFM.

Blood viscosity obtained by the MPFM was underestimated within 20%, compared with that obtained by the conventional cone-and-plate viscometer. The blood viscosity increased gradually for blood with 30% hematocrit owing to continuous ESR in a driving syringe. However, blood with higher values of the hematocrit ($Hct = 40\%$, and 50%) had consistent blood viscosity over time. Third, for blood with various concentrations of dextran solution, RBC aggregation and blood viscosity were simultaneously measured under periodic on-off delivery of the driving syringe. Due to the continuous ESR in the driving syringe, hematocrit of blood supplied into the microfluidic device (VA) from the driving syringe was gradually increased. RBC aggregation decreased over time. Blood viscosity increased with an elapse of time. At last, as an ultimate goal of this study, the proposed method was applied to detect the blood viscosity and RBC aggregation of blood circulating in a closed-loop. To stimulate the RBC aggregation, a specific concentration of dextran solution was periodically supplied with a syringe pump. Blood viscosity and RBC aggregation were gradually increased over 4000 s.

From these experimental demonstrations, it was found that the suggested method could be effectively used to monitor RBC aggregation and blood viscosity of blood circulating under an in-vitro closed-loop. This method does not require periodic collection from closed-loop circulation, or additional procedures for estimating the blood flow-rate with a syringe pump. In the near future, this suggested method will be effectively used to monitor variations in the biophysical properties of blood circulating in extracorporeal bypass loops.

SUPPLEMENTARY MATERIAL

See [supplementary material](#) for the summary of tube specifications (Table S1), quantitative evaluation of the effect of the ACU on the blood flow stabilization (Fig. S1), data of correction factors with respect to the interfacial location (Table S2), and the relationship between the blood flow-rate obtained with the micro-PIV and the blood flow-rate delivered with a syringe pump (Fig. S2).

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