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Measurement of cell aggregation characteristics by analysis of laser-backscattering in a microfluidic rheometry

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Abstract

The aggregation characteristics of red blood cells (RBCs) are known as important factors in the microvascular flow system, and increased RBC aggregation has been observed in various pathological diseases, such as thrombosis and myocardial infarction. This paper describes a simple microfluidic device for measuring the RBC aggregation by integrating a microfluidic slit rheometry and laser-backscattering technique. While a decreasing-pressure mechanism was applied to the microfluidic rheometry, a syllectogram (the light intensity versus time) showed an initial increase and a peak caused by the high shear stress-induced disaggregation, immediately followed by a decrease in the light intensity due to RBC aggregation. The critical shear stress (CST) corresponding to the peak intensity was examined as a new index of the RBC aggregation characteristics. The CST of RBCs increased with increasing aggregation-dominating protein (fibrinogen) in the blood plasma. The essential feature of this design was the combination of the rheometric-optic characterization of RBC aggregation with a microfluidic chip, which may potentially allow cell aggregation measurements to be easily carried out in a clinical setting.

Keywords : cells, aggregation, shear stress, microfluidics, rheometry

1. Introduction

Analyzing dynamic particle aggregation in fluidic samples is of immense importance in industrial and clinical analyses. Applications include measuring nano-particle aggregating characteristics in nano-fluids and red blood cell (RBC) aggregation in blood. In fact, RBCs in normal human blood tend to form linear and branched aggregates under low shear-flowing conditions, which form a face-to-face morphology similar to a stack of coins (so called "rouleaux"). This RBC aggregation can be observed both in vitro and in vivo and is found to be responsible for much of the increase in blood viscosity at low shear rates. Combined with other cellular and plasma constituents contribute significantly to the aggregation characteristics. Thus, aggregation is a result of the interaction of the erythrocyte membrane and plasma proteins such as fibrinogen and globulins (Stoltz *et al.*, 1999). Furthermore, this aggregation tendency is known to be one of the major determinants of the blood viscosity with hematocrits, plasma proteins, and RBC deformability so that it continues to be of interest in the field of clinical hemorheology (Rampling *et al.*, 2004).

Increased RBC aggregation has been observed in various pathological diseases, such as diabetes, thrombosis, myocardial infarction, vascular diseases, and hematological pathology. In addition, RBC aggregation is known to be one of the major determinants of blood viscosity. Thus, the degree of RBC aggregation is widely accepted as a very important determinant for the hemorheological characteristics of blood. The major cause of aggregation is the presence of large plasma-proteins, especially fibrinogen (Rampling *et al.*, 1999).

A large number of experimental techniques and methods have been developed to analyze RBC aggregation, including the electrical impedance method (Pribush *et al.*, 1999) and ultrasound technique (Boynard *et al.*, 1987). Recently, optical aggregometry has become the method of choice for many clinical diagnostic applications (Shin *et al.*, 2005; Hardeman *et al.*, 2001), which is based on the time-varying detection of laser-light scattering for RBC aggregation at stasis. In optical aggregometry, the cells should be completely disaggregated, by applying either a high rotational shear flow or vibrational flow, where they are suddenly exposed to zero-flow conditions, prior to testing. Light-scattering signals, with respect to time, provide information about the cell aggregation characteristics.

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Each of these methods allows for the examination of RBC behavior in response to a static mode of aggregation at stasis (i.e., zero flow condition). Even though these methods define meaningful parameters representing RBC aggregation, these measurements are quite different from the actual *in-vivo* flow environment (Yalchin *et al.*, 2004). Furthermore, most current methods, while useful in a research setting, are not suitable for day-to-day clinical use due to their time-consuming and labor-intensive processes. Our recent study (Shin *et al.*, 2006), which proposed a new vibrational disaggregation mechanism, has encountered difficulty in reducing the vibration noise for use in a clinical application as well as in analyzing the complex results due to combined effects of vibration and pressure-decreasing mechanisms.

The present study proposes a simple design for an aggregometry and a new aggregation index, which adopted pressure-decreasing microfluidics and a laser-backscattering technique. While the applied pressure decreased with time, a syllectogram (the light intensity versus time) showed an initial increase, and a peak caused by the high shear stress-induced disaggregation, immediately followed by a decrease in the light intensity due to RBC aggregation. The critical shear stress (CST) corresponding to the peak intensity was examined as a new index of RBC aggregation characteristics.

Thus, the present study adopted the pressure-decreasing mechanism, but with excluding the vibration-induced disaggregation mechanism and adjusting range of shear stresses (0~20 Pa) suitable in microfluidics. By adopting this decreasing shear flow mechanism without vibration mechanism, it was possible to detect critical shear stress, whereby the RBC aggregation characteristics showed transitional phenomena. The measurements of the critical shear stress were compared by varying the fibrinogen concentrations in blood plasma.

2. Materials and method

2.1. Sample preparation

Blood was obtained from healthy volunteers who provided informed consent. Venous blood samples were drawn from the antecubital vein and collected in Vacutainers (6 ml, BD, Franklin Lakes, NJ, USA) that contained the anticoagulant, (K_2) EDTA. It is noteworthy that this newly proposed method does not require complex multi-step processes to prepare blood samples since whole blood it required. In this study, however, a series of processes were conducted in order to vary the amount of fibrinogens in blood plasma.

Thus, whole blood was centrifuged at 800×g for 5 min. Plasma and buffy-coat were then removed. Then, to eliminate fibrinogen of the blood samples, RBCs were washed three times with an isotonic phosphate buffered saline (PBS, pH = 7.4, 290 mOsmol/kg). Using a Gel & Clot Activator containing Vacutainers (BD, Franklin Lakes, NJ), serum was obtained. Then, fibrinogen was added into the serum and mixed well using a vortex stirrer. Then, the washed RBCs were resuspended in a fibrinogen-added serum with fixed hematocrits at 45%. There was no any hemolytic presence during the entire test and all analyses were completed within two hours after the collection of blood.

2.2. Apparatus and operation

Fig. 1 shows a schematic of the light backscattering microfluidic aggregometer, consisting of a slit rheometry, a vacuum generating mechanism, a light source and detection system, a pressure transducer, a computer data acquisition system and a microscopic imaging system. The vacuum generating mechanism contained a syringe, a step motor, a solenoid valve and a control processor. Using a precision differential pressure transducer (Druck, PMP-4170), variations in the pressure differential were measured between the vacuum and sample chambers with respect to time, $\Delta P(t)$, from which the shear stress was mathematically calculated.

The laser-light scattering detection system consisted of a 1.5 mW diode laser (637 nm, Lanics, Korea) and a photodiode (FDS1010, ThorLabs, NJ, USA). The laser beam was directed to the center of the microchannel. The beam diameter was narrower than the 4 mm channel width so as to avoid scattering from channel walls. Two photodiodes were mounted along the microchannel, which was centered at the laser beam. The setup was interfaced with a PC and a data acquisition card (PCI-MIO-16E-4, NI, USA). Data were processed with LabView software (National Instruments). Data were collected over a period of 250 s. The microfluidic chip consisted of a microchannel, a sample reservoir and a waste sample reservoir. The channel had a depth, width and length of 200 μm, 4 mm and 40 mm, respectively. This single channel chip required a sample volume of 200 μl. In addition, the microscopic imaging

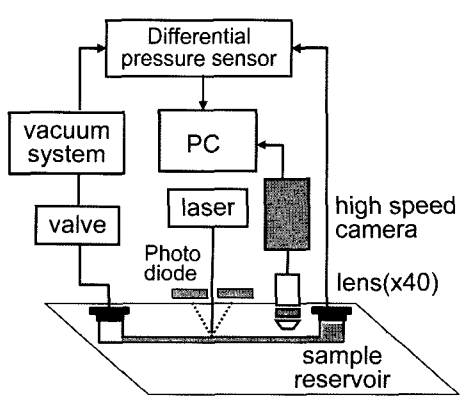

Fig. 1. Schematics of the optical aggregometer using a microfluidic rheometry.

system consisted of a lens (40X) assembly and a high speed camera (FastCam, USA).

Plastic microfluidic chips were fabricated by Sewon Meditech. Inc. (Daegu, Korea), using a precision injection molding technique. Since the microfluidic chip consists of a microchannel, a sample reservoir, and a waste sample reservoir, there is no need for an external sample chamber or waste chamber. For flow-induced disaggregation, the optimal design was a straight channel with two inlets. The channel having a depth of 200 μm, was 4 mm wide and 40 mm long. This single channel chip requires a sample volume of 200 μl. Flows were generated by applying an initial pressure differential of 12 kPa, between the waste and sample chambers. This was achieved using a vacuum generation system equipped with a 5-ml syringe. At this initial pressure differential, the averaged shear stress is beyond 10 Pa. The gap and length of the microchannel were chosen to ensure that the friction loss in the microchannel was significantly greater than the loss in other parts of the system.

2.3. Experimental operation

Typical tests were conducted as follows: When test fluid is loaded into a sample chamber, it is penetrated into the microchannel due to surface tension. At time $t = 0$, the valve between the vacuum generating system and waste chamber was opened, allowing the fluid to flow through the microchannel, due to the differential pressure, and to be collected in the waste chamber. When the differential pressure reached equilibrium, the test fluid stopped flowing. While the fluid was flowing through the microchannel, the backscattered light and pressure differential were measured every 0.01 s.

A detailed description of stress-pressure relation can be found in our previous study (Shin *et al.*, 2006). Briefly, the pressure difference through the slit can be expressed as $\Delta P(t) = \{P_{sample}(t) - P_{waste}(t)\}$ and the corresponding wall shear stress can be written as $\tau_w(t) = \Delta P(t)H/\{(1+2H/W)L\}$. The optical measurement of anisotropy, in laser-backscattering, reflects RBCs at all depths of the pressure-driven slit flow and RBC aggregates experience shear levels from zero to wall shear stress. Thus, the average shear stress was adopted throughout the present study to represent shear-flow characteristics in the microchannel. It is worthy to note that shear stress can be determined by pressure measurements, whereas shear rate determination requires measuring pressure and flow rate.

3. Results and discussion

While fluid flows through the microchannel, the differential pressure decreases and the corresponding RBC aggregation characteristics change. Fig. 2 shows differential pressure variations, over time, for a blood sample. As time passed, the differential pressure between the vacuum and

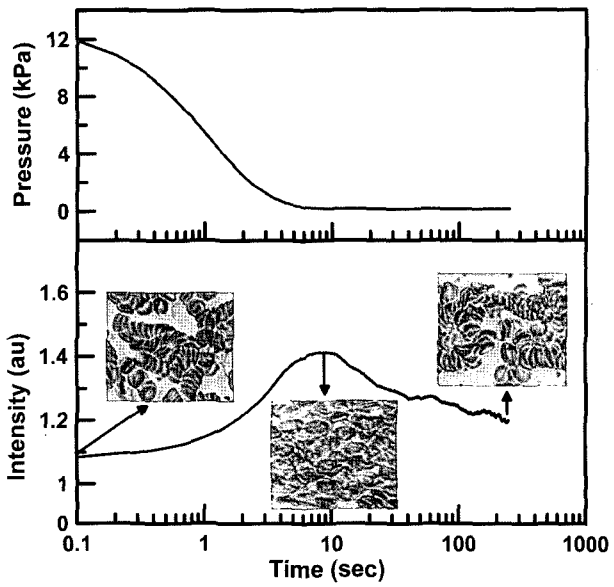

Fig. 2. Pressure and light intensity versus time for a blood sample.

sample chambers exponentially decreased since the waste sample chamber was filled with the fluid flowing from the sample chamber. Typically, it took approximately 30 seconds to reach an asymptote.

Fig. 2 shows the essential features of the present simultaneous pressure and light intensity measurements, and illustrates the typical syllectogram of RBC aggregation and disaggregation for a blood sample in the microchannel flow, while the differential pressure decreases with time. The time-course curves of the backscattered light, shown in Fig. 2, called *syllectogram*, show initial increases due to the disaggregation of RBCs at a high-shear flow, followed by a decrease of the intensity. The decreasing intensity was due to the re-aggregation of RBCs at a low-shear flow. In the initial process, a strong shear-flow condition was applied to the blood cells, which caused the disaggregation of RBCs. As the shear-flow decreased, the RBCs started to re-aggregate.

These experimental interventions with the syllectogram were verified by microscopic images obtained at different times, as shown in Fig. 2. The photographs in Fig. 2 were taken in-situ during an additional experiment in a 50 μm $\times$50 μm microchannel using a high speed camera. The photograph at center was not clear due to its high speed of flow. Though the center photograph is slightly suffered from afterimage, it may help to understand the RBC aggregation-related light intensity. In other words, since the center image showing disaggregated RBCs shows larger surface area for backscattering than the photographs before and after experiment, the light intensity corresponding to the center image was higher than initial and final intensities. Thus, the point of inflexion in the syllectogram indicates the critical point for the start RBC re-aggregation, by over-

coming the shear-flow environment. The critical point in Fig. 2 can be described as the critical shear stress (CST), where a blood sample would re-aggregate during decreasing shear-flow.

In the present syllectogram, aggregation intensity was scanned under a wide range of shear stresses. Such flows are quite similar to physiological blood flow. Pulsatile blood flow in arteries exhibits high-to-low shear conditions corresponding to systolic and diastolic points. In fact, RBCs experience quite a wide range of shear stress depending upon the temporal and spatial conditions of the blood vessels, which can result in the dis-aggregation and re-aggregation of RBCs. RBC aggregation characteristics under dynamic conditions, however, have neither been studied nor measured. Conventional aggregation indexes are defined at stasis (zero-flow condition), which may or may not represent the actual characteristics of RBC aggregation. Thus, RBC aggregation measurements, under dynamic conditions, may be more realistic in representing the *in-vivo* characteristics of RBC aggregation than conventional static ones.

Fig. 3 shows the typical kinetics of RBC aggregation against the shear stress for four different blood samples with various fibrinogen concentrations. Due to the characteristics of the decreasing shear flow, the test proceeded from high-to-low shear stress over the course of the experiment. During the high shear process, the backscattered light intensity increased, and exhibited a peak value due to the progressive break-up of the cellular aggregates. The light intensity then tended to rapidly decrease below the shear stress corresponding to this peak point, which is defined as *critical shear stress* (CST). Thus, the CST was easily determined as the point where maximum light intensity occurred with a range of shear stress levels.

Fig. 4 shows the CST measurements for four different fibrinogen concentrations. As the fibrinogen concentration increased, the CST measurements increased proportionally. The CST ranged from 0.2~1.2 Pa for fibrinogen concentrations of 0~400 mg/dL, while the present CST measurement precision was 0.01 Pa. At low concentrations (i.e., 0 and 50 mg/dL), the CST measurements did not show any significant differences. As the fibrinogen concentration was increased (200 and 400 mg/dL); however, the CST measurements showed distinct differences compared to that for 50 mg/dL (203 and 353% increases, respectively). These significant differences for higher fibrinogen concentrations provide potential for detecting the RBC aggregability of hyper-aggregated blood samples, which may be frequently observed in cardiovascular or thrombotic diseases. The CST could be a good index to represent the RBC aggregation characteristic in order to quantitatively distinguish such effects of plasma proteins on RBC aggregation.

In the present measurements, the aggregation intensity was scanned over a wide range of shear stresses. Thus, the CST indicates an average shear stress whereby the RBCs start to re-aggregate by overcoming the shear flow environment. The formation of RBC aggregates depends on two counteracting forces; shear force due to bulk fluid flow and the aggregating force of cells and plasma. The latter is difficult to measure directly, but the present system was able to measure this force in terms of the CST with ease and simplicity. This implies that the CST can be considered as an aggregating force between RBCs or RBC aggregates in overcoming the environmental shear force.

Since the present study adopted a shear-decreasing

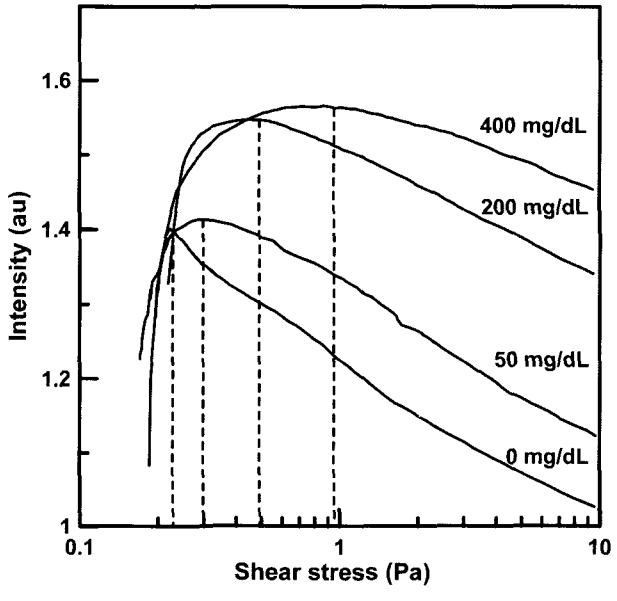

Fig. 3. Light intensity versus shear stress for RBC suspensions in serum, with various fibrinogen concentrations.

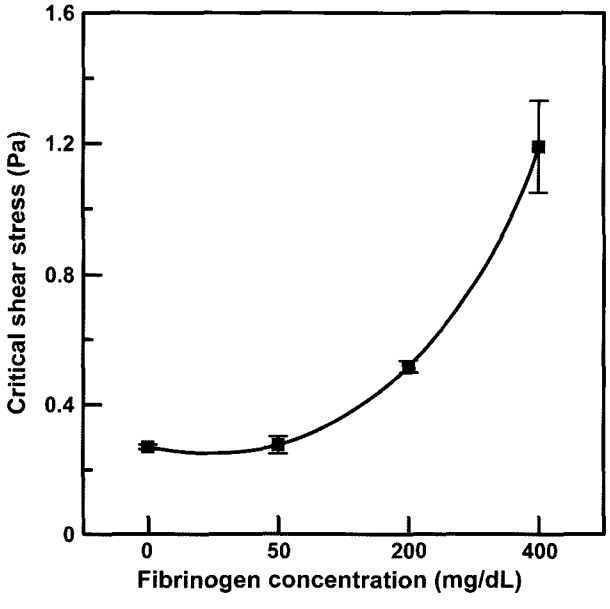

Fig. 4. Effect of fibrinogen concentration on critical shear stress as a measure of RBC aggregability.

unsteady flow, there was an important but difficult issue on the characteristic time of RBC aggregation. In fact, the aggregation of red blood cells is a dynamic process, with a finite time constant. The objective of the present study is to find an aggregating force between cells which may be in *quasi-equilibrium* with external shear force while shear stress is varying. The question is how fast RBCs respond to flow and dis-aggregate and re-aggregate when shear flow condition changes rapidly. Based on our understanding, aggregated cells can be immediately disaggregated when vibration is applied (Shin *et al.*, 2005), whereas aggregation process needs time to progressively form 1-D rouleaux and 3-D aggregation of red cells.

In fact, the conventional aggregation indices such as t_{fast} and t_{slow} determined represent the time constants for 1-D rouleaux and 3-D aggregation formation and typical values are 1.5~3.0 s and 10~20.0 s for normal healthy control, respectively. Considering that there are 10~20 number of RBCs in a 1-D rouleaux formation, the time constant for bi-cellular aggregation may be estimated as 1/20~1/10 of t_{fast} and the short time constant of bi-cullular aggregation may not significantly affect the present critical shear stress measured in a shear-decreasing unsteady flow. However, it may require a systematic and microscopic investigation for the time constant of bi-cellular aggregation.

In stead of the time constant of RBC aggregation, the proposed critical shear stress may be validated with by comparing a threshold shear rate which has a similar physical definition. The threshold shear rate, defined as a minimal shear rate needed to prevent RBC aggregation, can be determined with iteration procedure of applying steady shear rates (Hardeman *et al.*, 2001). The critical shear stress in the present study also represents the value for minimal shear stress that is needed to break down existing aggregates. The similarity between two parameters can be directly compared in a quantitative manner if viscosity data is available. Thus, the present study conducted an additional experiment to measure critical shear stress, threshold shear rate and viscosity of a normal whole blood sample obtained from the antecubital vein. The critical shear stress and threshold shear stress show 1.2 Pa and 1.8 Pa, respectively. These values reveal fairly same even though the measuring methods are different. These results imply indirectly that even though the time constant of bi-cellular aggregation is yet unknown, the critical shear stress may represent the value for minimal shear stress that is needed to break down existing aggregates. Of course, there require further works to understand the aggregation dynamics examined in a shear-varying microchannel flow.

The novel features of this new RBC aggregation measurement include: 1) the use of a disposable microchannel; 2) the combination of microfluidic rheometry and optical detection. Due to the adoption of the disposable microchannel, cleaning after each measurement has been eliminated and; thus, RBC aggregability tests can be conducted within any clinical environment. Also, the decreasing shear flow also provided information matching the *in-vivo* blood flow, including pulsation in an arterial flow. Another feature of this technology is that it does not, in principle, require either a large amount of blood or additional manipulations.

4. Conclusion

The present microfluidic aggregometry is a new method that can probe RBC aggregating force with a high resolution capable of detecting the effects of fibrinogen concentration on cell aggregation. Furthermore, the present aggregometer may provide an assessment of cell's aggregation property under dynamic conditions, which are similar to *in-vivo* blood flow dynamics. In the present study, a new method of evaluating dynamic RBC aggregability was presented by directly measuring aggregating forces in terms of critical shear stress in a decreasing shear flow. The advantages of this design are its simplicity (i.e., no moving parts), ease of operation, and disposability after each use. Although the present microfluidic aggregometer was described as an instrument capable of measuring RBC aggregation, further investigation is strongly needed with regard to the effect of time constant of aggregation on critical shear stress, clear evidence of correlation between light intensity and RBC aggregation, and reproducibility of the measured aggregation characteristics.

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