Optical Detection of Red Blood Cell Aggregation under vibration

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진동장에서의 적혈구 응집성을 측정하는 광학적 방법

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Abstract

Aggregability of red blood cells (RBCs) was determined by a laser backscattering light analysis in a microfluidic channel. Available techniques for RBC aggregation often adopt a rotational Couette-flow using bob-and-cup system for disaggregating RBCs, which causes the system to be complex and expensive. A disposable microfluidic channel and vibration generating mechanism were used in the proposed new detection system for RBC aggregation. Prior to measurement, RBC aggregates in a blood sample were completely disaggregated by applying vibration-induced shear. With the present apparatus, the aggregation indexes of RBCs can be easily measured with small quantities of blood sample. The measurements with the present aggregometer were compared with those of LORCA and showed a strong correlation between them. The aggregability of the defibrinogenated blood RBCs is markedly lower than that of the normal RBCs. The noble feature of this design is the vibration-induced disaggregation mechanism, which enables to incorporate disposable element that holds the blood sample.

1. Intorduction

Red blood cells (RBCs) in normal human blood tend to form linear and branched aggregates. Such aggregation is frequently referred to as rouleaux formation, which is similar to a stack of coins. This RBC aggregation, which can be observed both in vitro and in vivo, is found to be responsible for much of the increase in the blood viscosity at low shear rates. Combined with other cellular constituents, white cells and platelets, the plasma constituents contribute significantly to the aggregation characteristics. Thus, aggregation is a result of the interaction of erythrocyte membrane and plasma proteins such as fibrinogen and globulins[1].

In a creased RBC aggregability has been observed in various pathological diseases, such as diabetes, thrombosis, myocardial infarction, vascular diseases, and hematological pathologies.1 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 charateristics of blood. The major cause of aggregation is the presence of large plasma-proteins, especially fibrinogen [2].

Various techniques for measuring RBC aggregation have been developed and are described elsewhere [3]. A photometric method to record light intensity has been widely employed to quantify aggregation due to its simplicity. Recording the light intensity either back-scattered [4, 5] from or transmitted [6,] through RBCs under defined shearing conditions has been used to assess different aspects of RBC aggregation. The time course of light transmission (or back scattering) is known as a “syllectogram.”

Additionally, aggregometers using phtometric analysis after sudden cessation of shear stress have been developed, which are commercially available such as Erythroaggregometer (Regulest, France), Myrenne aggregometer (Myrenne, Germany), and

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LORCA (R&R Mechatornics, Netherlands). These commercial instruments employ different geometries for the rotational shearing system such as cone-plate, parallel plates and concentric bob-cup systems. These instruments analyze the syllectogram using a curve-fitting program and determine the aggregation indices such as ***AI (aggregation index), half-time ($t_{1/2}$), M-index***, etc.

In order to measure aggregation indexes, it is often required to initialize the blood sample by disaggregating RBC aggregates. In general, the RBC aggregation is a reversible dynamic phenomenon, so that the increase in shear rate breaks up large RBC aggregate to smaller ones. Between shear rates of 5.8 and 46 s^{-1}, each doubling of shear rate resulted in the reduction of aggregate size by 50% and vice versa [7]. The decrease in aggregate size is manifested by the shear-thinning behavior of blood leading to a decrease in blood viscosity with increasing shear rate [8]. In a shear flow, however, the disaggregation of rouleaux presents a complex problem involving the deformation of cells [9, 10] and the tank-treading [12]. Chien et al. [13] have investigated the disaggregation mechanism under oscillatory shear stress and showed that erythrocytes in a doublet separate from each other by rolling rather than sliding of the sheared cell.

For RBC disaggregation, most of the current techniques including the commercial aggregometers adopt a rotational shearing system. In order to obtain a complete disaggregation, a high shear rate above 500 s^{-1} should be applied. For hyperaggregated blood samples such as cryoglobulinemia and with horse blood, however, much higher shear rate is needed for complete disaggregation. It has been known that incomplete disaggregation could cause serious problem in the commercial aggregometer [10]. Thus, the rotational shearing systems should be able to generate such a high shear rate by increasing rotational speed. Thus, these rotational shearing systems cause the instruments to be complex to design and expensive.

In addition, they require labor-intensive cleaning after each measurement. Hence, these current techniques, while useful in a research setting, are not optimal for day-to-day clinical use. Furthermore, these systems require about 2 ml of blood samples, which is relatively very large compared to the chemical assay. Therefore, it is necessary to develop a simple and labor-free instrument that can measure the aggregation index of RBCs with minimal volume of blood sample.

The current study describes an innovative approach to a photometric aggregometer with vibration-induced disaggregation mechanism. The rotational shearing system is replaced with a simple vibration-aided disposable microfluidic-channel containing a blood sample. The advantages of this design are its simplicity (i.e., no rotating parts and ease of operation), low cost, and disposability after use..

2. Materials and methods

2.1 Sample preparation

Blood was obtained from six normal, healthy volunteers who were not on any medications and who provided informed consent (age range 25-40 years and male/female participants). The blood samples used in the experiments were not pooled from more than one individual subject and all analyses were completed within 6 hours after blood collection. The samples of venous blood were drawn from the antecubital vein and collected in an EDTA containing Vacutainers (BD, Franklin Lakes, NJ). The blood samples used in the experiments were not pooled from more than one individual subject and all analyses were completed within 6 hours after blood collection. The samples of venous blood were drawn from the antecubital vein and collected in an EDTA containing Vacutainers (BD, Franklin Lakes, NJ). Then, to eliminate fibrinogen of the blood samples, the RBCs were washed three times with an isotonic phosphate buffered saline (PBS, pH=7.4, 290mOsmol/kg) and resuspended in serum, which was prepared using a Gel & Clot Activator containing Vacutainers (BD, Franklin Lakes, NJ).

2.2 Apparatus and operation procedure

Figure 1 is a schematic diagram of the light-reflection aggregometer, which consists of a disposable test slit with an inlet reservoir, vibration mechanism, laser diode, photodiode, and a computer data acquisition system. The blood sample (0.3 ml) is filled into the microfluidic channel. The width and depth of the channel are 4.0 mm and 200 m, respectively. The slit which is integrated with an inlet reservoir is designed to be disposable. The slit is made of transparent polystyrene using micro-injection molding. A laser diode (650 nm, 5mW) and a photo diode are used to obtain a laser-transmit intensity. The vibration mechanism consists of a function generator, amplifier, and speaker. A jig attached to the speaker diaphragm is connected to the slit. A blood sample in the slit is vibrated for 20 s. Then, RBC aggregates in the blood sample are disaggregated by the vibration-induced shear. The effects of frequency and amplitude of vibration are then examined and optimal values were determined for the RBC disaggregation. The optimal vibrating condition for RBC disaggregation should be carefully

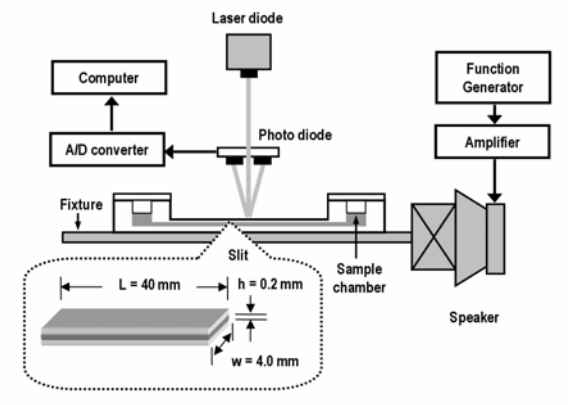


Fig. 1 Schematic diagram of the laser-reflection aggregometer with vibrating mechanism

chosen to ensure that there is no hemolysis due to the vibration [14, 15]. In the present study, the vibrating frequency and amplitude are fixed at 100 Hz and 0.5 mm, respectively.

Typical tests were conducted as follows: The test fluid is filled into the test slit. Then, the test microchannel is mechanically mounted on the jig attached on the speaker diaphragm. For disaggregating RBC aggregates, the defined vibration is applied for 20 s and stops. Then, the laser beam emitting from the laser diode traverses the blood sample and is backscattered from the blood sample. The backscattered light is detected by the photodiode which is linked to the data acquisition system by a computer. When the vibration stops suddenly, the disaggregated RBCs start to aggregate. The light intensity is recorded through time, which is called the syllectogram as shown in Fig. 2. Aggregation parameters as measures of RBC aggregation are determined from the syllectogram using a curve-fitting program. These parameters of the aggregation are well defined in the previous study [Hardeman et al., 2001] as follows:

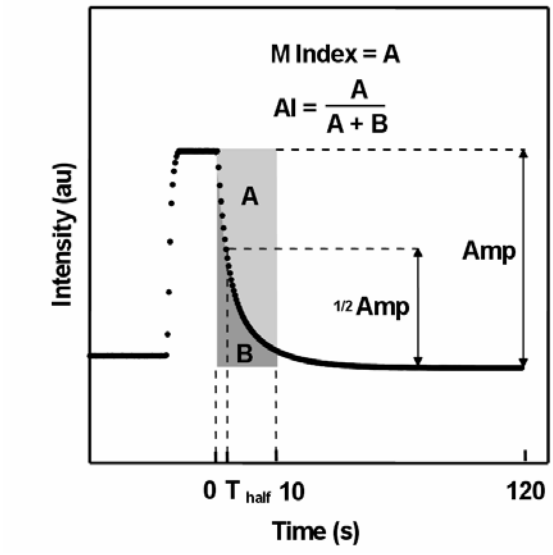


Fig. 2 Light intensity versus time for a blood sample before and after vibration

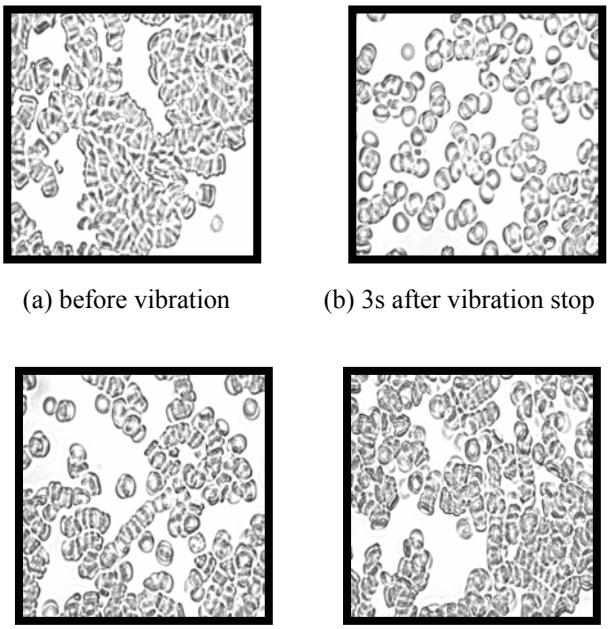
1. ***Amplitude (Amp):*** The difference between the maximum light intensity and the light intensity at 120 s, indicating the extent of RBC aggregation.
2. ***Half time ($t_{1/2}$):*** The time required to reach a light intensity of "***minimum intensity + 1/2 Amp.***"
3. ***M-index***: The area under the syllectogram over a 10 s time period.
4. ***Aggregation Index (AI)****:* The ratio of the area under the syllectogram to the total area over a 10 s time period.

2.3 Assessment of instrument precision

Instrument precision for each parameter was assessed by calculating the coefficient of variation (CV) of 10 measurements on aliquots of healthy donor blood samples; each measurement consisted of triplicate determinations followed by introducing a new aliquot. To determine the sensitivity of the aggregation parameters, two types of blood samples were prepared with and without fibrinogen protein in blood. The standardised difference between these two groups for each measured parameter was calculated

3. Results and Discussions

Figure 3 shows microscopic examination of RBCs in the blood sample for (a) before vibration, (b) right after vibrating for 30 s, (c) 10 s after vibration stops and (d) 120 s after vibration stops. As the vibration-induced shear applies, aggregated RBCs in Fig. 3(a) disaggregate gradually as shown in Figs. 3(b). This fact implies that the vibration mechanism is proven to replace the previous rotational shearing system for disaggregation of RBC aggregates. After the vibration stops, RBCs tend to aggregate, immediately as shown in Fig. 3(c). At t =120 s after vibration for Fig. 3(d), RBC aggregation is highly developed and there shows high degree of aggregation including rouleaux and the rouleaux network. The kinetics of RBC aggregation and disaggregation were studied using the present apparatus. Blood sample (0.3 ml) was placed in the test slit. Figure 4 shows the typical kinetics of aggregation and disaggregation for RBC suspension in autologous plasma (RIP) in a microchannel. The output signal of the photo-detector, which is proportional to the intensity of backscattered light, is plotted along time.



(a) before vibration (b) 3s after vibration stop

(c) 10s after vibration stop (d) 120s after vibration stop

Fig. 3 Microscopic examination of RBCs at various time (a) before vibration, (b) 3 s after vibration, (c) 10 s after vibration and (d) 120 s after vibration.

Aggregation indexes are determined from a syllectogram using a curve-fitting program as indicated in Fig. 4.

The mean, standard deviation (SD) and CV of various aggregation parameters obtained for 30 repeated measurements on aliquots of the same blood sample are

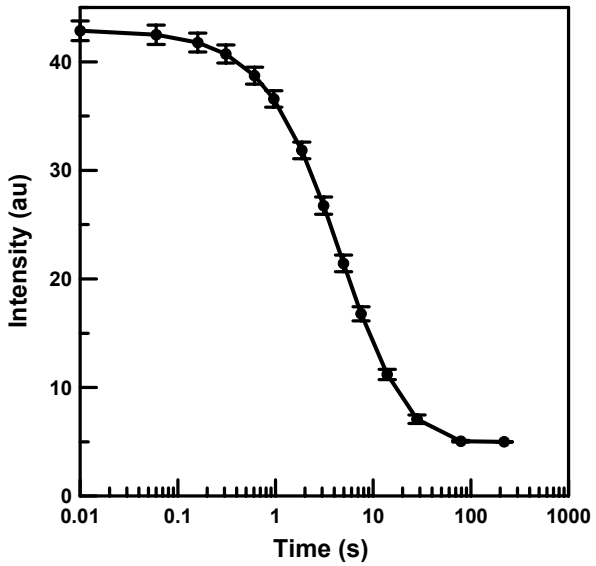


Fig. 4 Syllectogram for normal, whole blood sample

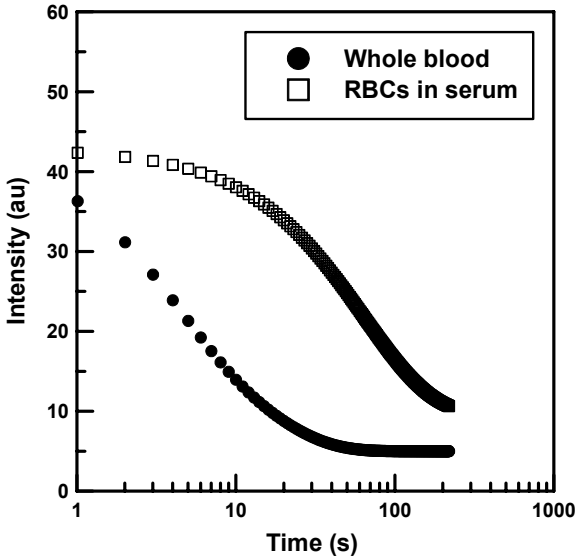


Fig. 5 Comparison of light intensity for whole blood and defibrinogated blood (RBC suspension in serum)

measurements on aliquots of the same blood sample are shown in Table 1. The most repeatable parameter was AI, with a CV of 1.97%. The M index had a somewhat higher CV (4.73%), and the half time constants were characterized by high CV values (i.e., less precision). Other repeated measurements on different samples yielded a similar pattern of precision for the different aggregation parameters. Additionally, Table 1 compares the values for RBC aggregation indexes measured by the present aggregometer and a commercial aggregometer (LORCA). It is found that the test results provide a good correlation between two instruments with less than a 5.6 % error rate.

Figure 5 shows the effect of fibrinogen on RBC-aggregation by comparing two syllectogram curves of normal, whole blood and defibrinogenated blood. The defibrinogenated blood was obtained by suspending RBCs-in-serum (RIS) at the same hematocrit 40%. The solid circles indicate the backscattered intensity of whole blood; and open rectangles indicate those of RIS. The elimination of fibrinogen results in slow decrease of the backscattered-intensity and a small amplitude of I range. The detailed effect of fibrinogen on RBC-aggregation indices is summarized in Table 2.

Table 2 shows significant difference of aggregation indexes between the whole blood and the fibrinogen-free blood (RIS). For example, the M-index and AI of RIS decreases significantly 87.2% and 84.0% from that of the whole blood, respectively. The Amp of RIS from that of the whole blood. These different indexes were exaggerated in the half time, which of the RIS increases 8 times of that of the whole blood. These results show quantitatively the effect of the fibrinogen on the RBC

Table 1 Comparison of RBC aggregation parameters measured by the proposed and LORCA aggregometers.

Aggregation Indices	Rheoscan - A				LORCA			
	Amp (au)	$t_{1/2}$ (s)	***M Index*** (au*s)	***AI***	***Amp*** (au)	$t_{1/2}$ (s)	***M Index*** (au*s)	***AI***
Mean	36	4.2	179	50	35	4.0	175	49
SD	0.91	0.28	8.3	1.7	4.8	0.16	25	1.2
CV (%)	2.5	6.6	4.6	3.4	14	4.0	15	2.4

Table 2 Aggregation parameters and percentage difference for whole blood and defibrinogated blood

Aggregation Indices	Whole blood	RBCs in serum	Percentage difference
Amp (au)	36	29	19.4% ↓
$t_{1/2}$ (s)	4.2	36	757% ↑
M Index	179	23	87.2% ↓
AI	50	8.0	84.0% ↓

aggregation characteristic with the proposed instrument.

4. Conclusions

The aggregation of RBCs was measured with the proposed apparatus. The results which were taken with the proposed aggregometer showed a correlation with those of a commercial aggregometer (LORCA). Using the proposed instrument, fibrinogen was found to be the major determinant of the RBC aggregation. Although the proposed apparatus measured successfully the characteristics of RBC aggregation, further study is needed with regard to the kinetics of the vibration-induced disaggregation. The present study, as a proof of principle, demonstrated the novel feature of the present optical aggregometer, which can be used in clinical setting due to its disposability after use.

Acknowledgments

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