Optical Detection of Red Blood Cell Aggregation in a Disposable Microfluidic Channel

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The aggregability of red blood cells (RBCs) was determined by laser backscattering light analysis in a microfluidic channel. Available techniques for RBC aggregation often adopt a rotational Couette-flow using a 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 the application of vibration-induced shear. With the present apparatus, the aggregation indexes of RBCs can be measured easily with small quantities of a blood sample. The measurements with the present aggregometer were compared with those of LORCA and the results 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 can incorporate the disposable element that holds the blood sample.

1. Introduction

Red blood cells (RBCs) in normal human blood tend to form linear and branched aggregates. Such aggregation is frequently referred to as the 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 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 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.

Increased RBC aggregability 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-

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proteins, especially fibrinogen (Rampling, 1999).

Various techniques for measuring RBC aggregation have been developed and are described elsewhere (Zhao et al., 1999). A photometric method to record light intensity has been employed widely to quantify aggregation due to its simplicity. Recording the light intensity either backscattered (Schmid-Schönbein et al., 1982 ; Hardeman et al., 2001) from or transmitted (Baskurt et al., 1998) 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."

In addition, aggregometers using photometric analysis after a sudden cessation of shear stress have been developed. Commercially available models include the Erythroaggregometer (Regulest, France), Myrenne aggregometer (Myrenne, Germany), and LORCA (R&R Mechatornics, Netherlands). These commercial instruments employ different geometries for the rotational shearing system such as a 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 ($t_{1/2}$), and M-index.

In order to measure aggregation indexes, it is often required to initialize the blood sample by disaggregating RBC aggregates. In general, RBC aggregation is a reversible dynamic phenomenon, so that the increase in the shear rate breaks up large RBC aggregates into smaller ones. Between shear rates of 5.8 and 46 s^{-1}, each doubling of shear rate resulted in the reduction of an aggregate size by 50% and vice versa (Schmid-Schönbein and Wells, 1971). The decrease in aggregate size is manifested by the shear-thinning behavior of blood leading to a decrease in blood viscosity with an increasing shear rate (Schmid-Schönbein, 1976). In a shear flow, however, the disaggregation of the rouleaux presents a complex problem involving the deformation of cells (Sakalak and Chien, 1983 ; Hardeman et al., 2001) and tank-treading (Schmid-Schönbein and Wells, 1969). Chien et al. (1990) 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 horse blood, however, a much higher shear rate is needed for complete disaggregation. It has been known that incomplete disaggregation could cause serious problem in the commercial aggregometer (Hardeman et al., 2001). Thus, the rotational shearing systems should be able to generate a high shear rate by increasing the rotational speed. Thus, these rotational shearing systems complicate the construction of these instruments and as a result, they are 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 RBC aggregation indexes with a minimal blood sample.

The current study describes an innovative approach to a photometric aggregometer adopting a vibration-induced disaggregation mechanism, which replaces the complex rotational Couette-flow apparatus with a disposable microfluidic-channel. The essential feature of the proposed aggregometer is the vibration-induced disaggregating mechanism, which enables to replace the conventional, rotational Couette-flow type apparatus with a disposable microfluidic channel. The disposability after use makes it possible for the present aggregometer to be used in RBC aggregation measurement in a clinical setting, which is another objective of the present study. The advantages of this design are its simplicity (i.e., no rotating parts and easy to operate), low cost, and it is disposable after each 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 six 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 six 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, RBCs were washed three times with an isotonic phosphate buffered saline (PBS, pH = 7.4, 290 mOsmol/kg) and resuspended in serum, which was prepared using a Gel & Clot Activator containing Vacutainers (BD, Franklin Lakes, NJ).

2.2 Apparatus and operational procedure

Figure 1 is a schematic diagram of the light-backscattering 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 (0.1 ml) is placed into the microfluidic channel. The width and depth of the channel are 4.0 mm and 200 μm, 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, 1.5 mW) and a photo diode are used to obtain backscattered intensity. The vibration mechanism consists of a function generator, amplifier, and speaker. A jig, attached to the speaker diaphragm, is connected to the slit.

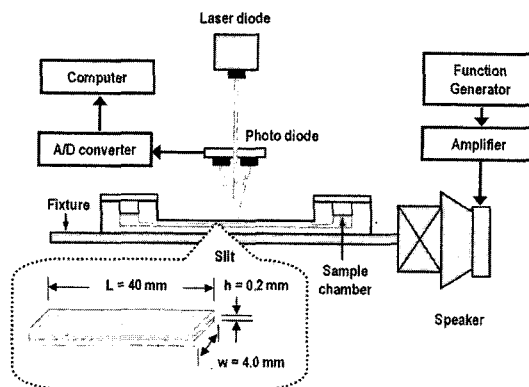Figure 2 shows the effect of vibrations on RBC

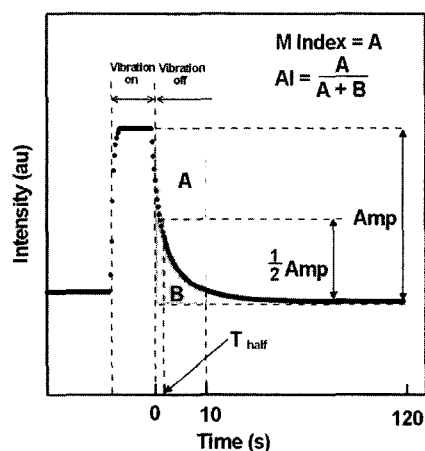**Fig. 1** Schematic diagram of the laser-backscattering aggregometer with vibrating mechanism

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

aggregation. When a vibration is applied to the sample with a preset frequency and amplitude for 20 s, the backscattered intensity increases sharply and reaches a plateau value. However, beyond a critical vibration intensity, the plateau value cannot be increased anymore. The plateau intensity is strongly proportional to the frequency intensity such as vibration frequency and amplitude. Then, this implies that RBC aggregates in the blood sample are completely disaggregated by the vibration-induced shearing mechanism. Further increase of vibration intensity should be avoided since there may occur mechanical hemolysis due to the high shear vibration (Shin et al., 2003a; 2003b).

It is worthy to note that incomplete disaggregation could cause serious error in measuring aggregation parameters (Hardeman et al., 2001). Either vibration frequency or amplitude should be sufficiently high to prevent RBCs from aggregating each other. Thus, the optimal vibrating conditions were carefully chosen for the complete disaggregation of RBCs without hemolysis. In the present study, the vibrating frequency and amplitude are fixed at 100 Hz and 0.5 mm, respectively. At this condition, RBCs are completely disaggregated and the corresponding light intensity reaches an asymptotic maximum, which cannot be increased by further increasing vibration intensity. In fact, the disaggregation mechanism of RBCs is known quite complex and unknown so that there are few studies to theoretically predict the aggregation behavior of RBCs. Moreover, even though the vibration-induced disaggregation mechanism is adopted in the present study, further study is required to understand the working principle comparing with rotational shearing principle.

Typical tests were conducted as follows: The test fluid is poured into the test slit. Then, the test microchannel is mounted on the jig attached to the speaker diaphragm. For disaggregating RBC aggregates, the defined vibration is applied for 20 s and is then stopped. 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 over time, which is called the syllectogram as shown in Fig. 2. It is noteworthy that the more the aggregation occurs, the more the light transmits and the less the backscattered light becomes. In the syllectogram after sudden cessation of the vibration, the conventional mathematical representation of the syllectogram adopts the bi-exponential representation (Hardeman et al., 2001) from the bottom onward as follows

$$I(t) = I_f \cdot e^{-t/T_f} + I_s \cdot e^{-t/T_s} + I_0$$

where T_f and T_s denote the time constants of (fast) rouleaux formation and (slow) three-dimensional aggregate formation, respectively. At time $t=0$, the maximum intensity is obtained, $I(t=0) = I_{max}$. Aggregation parameters as measures of RBC aggregation are determined from the curve-fitted equation. These parameters of the aggregation were well defined in the previous study (Hardeman et al., 2001). They were as follows:

(1) *Amplitude* (*Amp*): The difference between maximum light intensity and the light intensity at 120 s ($I_{max} - I_0$), 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*," indicating a characteristic time constant to reach a half level of aggregation. This may be regarded as the exponent of the single exponential curve-fitted equation.

(3) *M*-index: The area over the syllectogram over a 10 s time period (A), indicating degree of the accumulated aggregation during 10 s.

(4) *Aggregation Index* (*AI*): The ratio of the area over the syllectogram (A) to the total area (A+B) over a 10 s time period, indicating the normalized degree of accumulated aggregation.
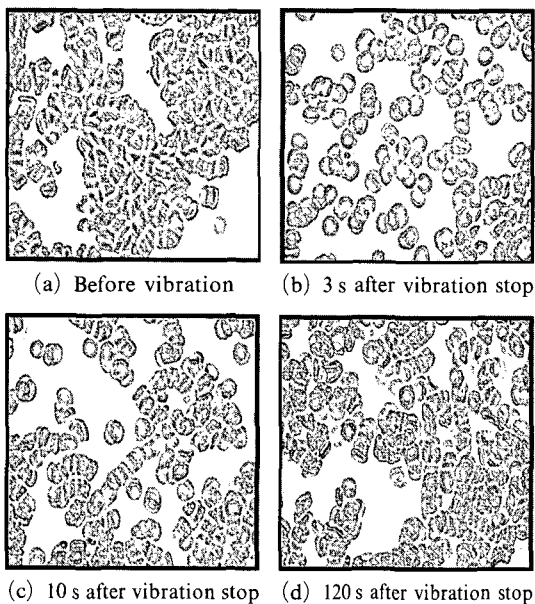
2.3 Assessment of instrument precision

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

3. Results and Discussions

Figure 3 shows a microscopic examination of RBCs in the blood sample (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 Fig. 3(b). This fact implies that the vibration mechanism has been proven to replace the previous rotational shearing system for the disaggregation of RBC aggregates. After the vibration stops, RBCs tend to aggregate immediately as shown in Fig. 3(c). When $t = 120$ s after vibration for Fig. 3(d), RBC aggregation is highly developed and there is a high degree of aggregation including rouleaux and the rouleaux network.
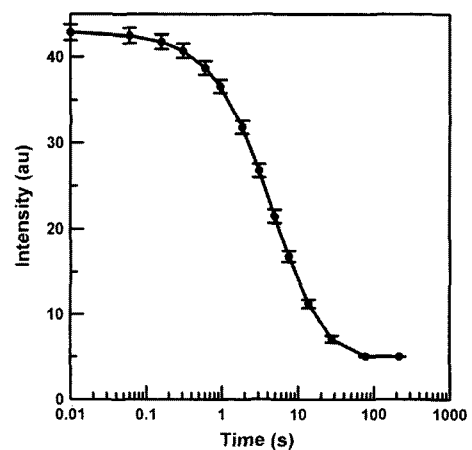
The kinetics of RBC aggregation and disaggregation were studied using the present apparatus. A blood sample (0.2 ml) was placed in the test slit. Figure 4 shows the typical kinetics of aggregation and disaggregation for RBC suspension in autologous plasma (RIP) in a microchannel. The output signal of the photo-detector, which is proportional to the intensity of the backscattered light, is plotted along time. 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 from 30 repeated measurements on the aliquots of the same blood sample, are shown in Table 1. The most repeatable parameter was AI, with a CV of 3.4%. The M index and the half time constants had somewhat higher CVs (4.73%), and the Amp was characterized by high CV value (i.e., less precision). Other repeated measurements on different samples yielded a similar pattern of precision for the different aggregation parameters. In addition, Table 1 compares the values for RBC aggregation indexes measured by the present aggregometer and a commercial aggregometer (LORCA). It is found that the

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

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

Fig. 3 Microscopic examination of RBCs at various time

Fig. 4 Syllectogram for normal, whole blood sample

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

Aggregation Indexes	Present				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	2.9	0.28	8.3	1.7	4.8	0.16	25	1.2
CV (%)	8.1	6.6	4.6	3.4	14	4.0	15	2.4
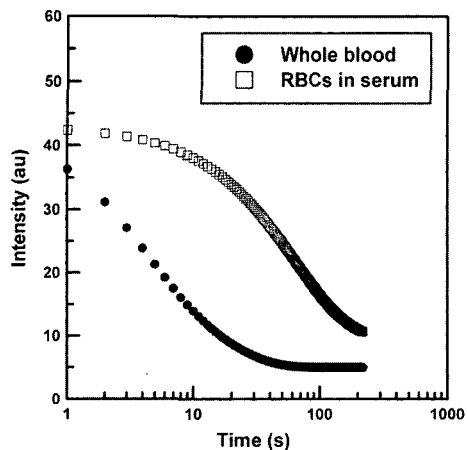
Fig. 5 Comparison of light intensity for whole blood and defibrinogated blood (RBC suspension in serum)

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

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

test results provide a good correlation between the two instruments with less than a 5.0% 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 of 40%. The solid circles indicate the backscattered intensity of whole blood ; and the open rectangles indicate those of RIS. The elimination of fibrinogen results in a slow decrease of the backscattered-intensity and a small amplitude of the I range. Both syllectogram in Fig. 5 can be mathematically represented with exponential function, respectively. Using these mathematical representations, the detailed effect of fibrinogen on the RBC-aggregation indices is summarized in Table 2.

Table 2 shows a significant difference in the aggregation indexes between whole blood and the fibrinogen-free blood (RBC in serum). For example, the M-index and the AI of RIS decreased significantly, 87.2% and 84.0%, from that of whole blood, respectively. The Amp of RIS decreases 19.4% from that of whole blood. These different indexes were exaggerated in the half-time $(t_{1/2})$, whereby the RIS increased eight times that of whole blood. As stated earlier, the half-time $(t_{1/2})$ is similar to the exponent (τ)

when the syllectogram is curve-fitted with single exponential function, $I(t) = Amp\, e^{-t/\tau} + I_0$. The larger the exponent becomes, the slower the intensity decreases and the less the aggregation progresses. These results indicate that the fibrinogen is a key plasma protein, which plays an important role on RBC aggregation. In other words, the fibrinogen-free blood has less aggregability of RBCs than whole blood. These aggregation parameters show quantitatively the effect of the fibrinogen on the RBC aggregation characteristic with the proposed instrument.

4. Conclusions

The present study described a newly developed disposable-aggregometer, which integrated optical detecting method, disposable microfluidic channel, and vibration-induced disaggregating mechanism. Additionally, the present study demonstrated that the results that were taken with the proposed aggregometer showed a correlation with those of a commercial aggregometer (LORCA). As a proof of principle, the proposed instrument measured and compared the aggregation indexes for whole blood and the fibrinogen-free blood. 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 since the kinetics of RBC disaggregation or aggregation may be a key issue to understand the blood rheology in microcirculation. Conclusively, the present study demonstrated the novel features of the present optical aggregometer, which can be used in a clinical setting

due to its disposability.

Acknowledgments

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