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Measurement of red blood cell aggregation by analysis of light transmission in a pressure-driven slit flow system

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Abstract

The aggregation characteristics of red blood cells (RBCs) were measured using a newly developed light-transmission slit rheometer. Conventional methods of RBC disaggregation such as the rotational Couette system were replaced with a pressure-driven slit flow system with a vibrational mechanism. Using a vibration generator, one can disaggregate the RBC aggregates stored in the slit. While shear stress decreases exponentially, instantaneous pressure and the transmitted light intensity were measured over time. Applying an abrupt shearing flow after disaggregation caused a rapid elongation of the RBCs followed by loss of elongation with the decreasing shear stress. While the shear stress is further decreasing, the RBCs start to re-aggregate and the corresponding transmitted intensity increases with time, from which the aggregation indices can be obtained using a curve-fitting program.

Keywords : aggregation, red blood cell, light, transmitted intensity

1. Introduction

Red blood cells (RBCs) in normal human blood tend to form linear and branched aggregates. Under normal circumstances, RBC aggregate resembles stacks of coins and are therefore called rouleaux. These rouleaux can grow end-to-end or side-to-end, and upon completion, form 3D-networks. Obviously the side to end format has a considerable effect on the viscosity of blood, which rises in an exponential way from flow to stasis. The RBC aggregation plays an important role in blood flow, particularly in the microvascular system. Increased RBC aggregability has been observed in various pathological diseases, such as diabetes, thrombosis, myocardial infarction, vascular diseases, and hematological pathology (Chien and Sung, 1987). 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 (Chen *et al.*, 1970; Rampling, 1999).

Thus, the characteristics of RBC aggregation have been investigated and various techniques for quantification of this aggregation process have been proposed (Zhao *et al.*, 1999). A measurement of the erythrocyte sedimentation rate (ESR) was commonly used due to its simplicity even though it was an indirect measurement of RBC aggregation (Houbouyan *et al.*, 1998). In addition, there have been other methods for measuring RBC aggregation such as image analysis method in a flow chamber (Chen *et al.*, 1994), and ultrasound back-scattering technique (Boynard *et al.*, 1987).

Recently, a photometric method to record light intensity under defined shearing conditions has been widely employed to quantify aggregation characteristics (Schmid-Schönbein *et al.*, 1982). The time course of either light transmission (Baskurt *et al.*, 1998) or back scattering (Hardeman *et al.*, 2001), after a sudden decrease in the shear rate following a period of high shear, is known as a "syllectogram." The syllectometric principle for measuring RBC aggregation has been implemented in commercial aggregometers including LORCA (Mechatronics, Netherlands), Erythro-aggregometer (Regulest, France) and Myrenne-aggregometer (Myrenne, GmbH, Germany). These commercial instruments employ different geometries and shearing systems for disaggregation such as cone-plate, parallel plates and concentric bob-cup systems.

Although there are many methods and instruments for measuring RBC aggregation, as described above, most of the current techniques including the commercial aggregometers adopt a rotational shearing system. These rotational shearing systems cause the instruments to be complex and expensive. In addition, they require labor-intensive clean-

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ing after each measurement. Hence, the current techniques, while useful in a research setting, may not be optimal for day-to-day clinical use. Therefore, it is necessary to develop a simple, labor-free and low-cost instrument that can measure the aggregation index of RBCs with a minimal blood sample volume.

The current study describes an innovative approach to a light-transmission slit rheometer (LTSR) with the adoption of a vibration-induced disaggregation mechanism. The rotational shearing system is replaced successfully with the proposed vibration mechanism with auxiliary high shear flow in the slit. In addition, this article discusses the operating principles and various phenomena observed throughout the procedures.

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). Then, to eliminate fibrinogen of the blood samples, the 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 operation procedure

Fig. 1 is a schematic diagram of the laser-transmission slit-rheometer (LTSR), which consists of a vacuum generator, disposable test slit with two reservoirs, vibration mechanism, pressure transducer and photometric system. The blood sample is sheared in the slit channel with a gap of 0.46 mm, width of 4.6 mm and length of 135.0 mm. The slit which is integrated with two chambers is designed to be disposable. The slit is made of transparent polystyrene using micro-injection molding. The length and gap of the slit were chosen to ensure that the friction loss in the slit was the dominant loss in the system (Shin *et al.*, 2004b). The photometric system consists of a diode laser (650 nm, 5 mW) and a photodiode.

A blood sample in the disposable element is vibrated for 20 s for disaggregation of the RBCs. Then, the vibration-induced shear disaggregates the RBC aggregates in the blood sample. The effect of the frequency and amplitude of vibrations was widely examined and optimal values were determined for the RBC disaggregation. The optimal vibrating conditions for RBC disaggregation should be chosen carefully to ensure that there is no hemolysis due to the vibrations (Shin *et al.*, 2003). In the present study, the vibrating frequency and amplitude for RBC disaggregation are defined as 100 Hz and 0.5 mm, respectively.

After the vibration, typical tests were conducted as follows: At time $t = 0$, the valve between the vacuum generator and the waste chamber is opened, allowing the fluid to flow through the slit and to be collected in the waste chamber as driven by the differential pressure. When the differential pressure reaches an equilibrium, the test fluid stops flowing. While the fluid is flowing through the slit, the transmitted light and the pressure differential are mea-

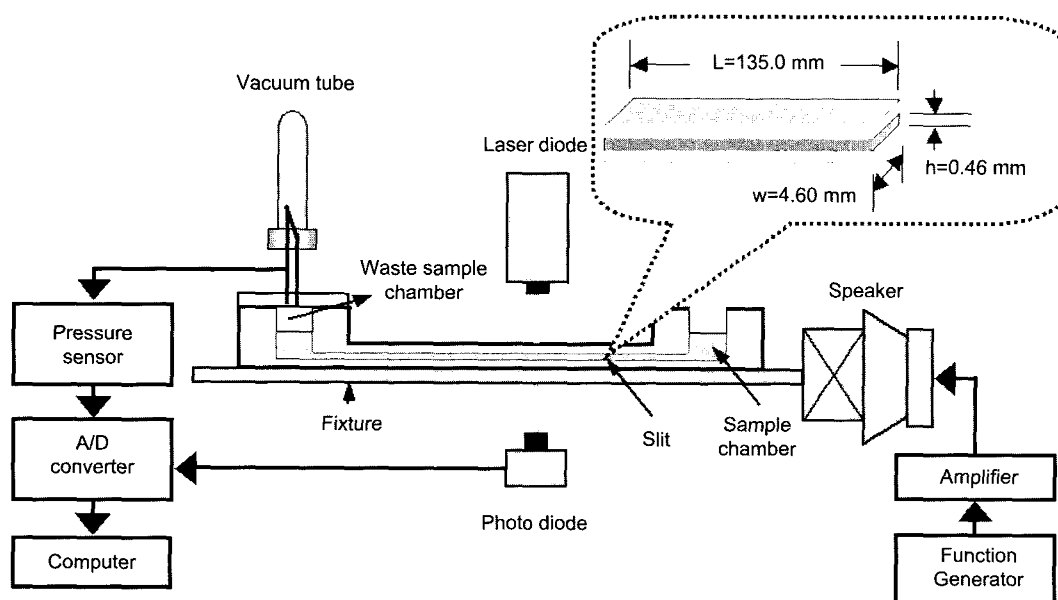

Fig. 1. Schematic diagram of the laser-transmission slit-rheometer (LTSR).

sured every 0.01 s. The transmitted light-time curve is called the syllectogram, from which the aggregation indices are determined using a curve-fitting program. The pressure-time data can be used to determine the shear stress and shear rate at an arbitrary time.

A detailed description of the stress-shear rate relation can be found in a previous study (Shin *et al.*, 2004b). A brief description is as follows: In deriving the stress-shear rate relation in the slit rheometer, the important assumptions are 1) a fully developed, isothermal, laminar flow; 2) no slip at the walls; and 3) air in the vacuum chamber as an ideal gas. On the assumption that the product of pressure $P(t)$ and volume $V(t)$ in the vacuum chamber at time t is constant, $P_iV_i = P(t)V(t)$, where the subscript i represents the initial state of the experiment. The instantaneous pressure $P(t)$ is recorded in the computer file. The flow rate at time t can be determined as $Q(t) = \frac{d}{dt}\left(\frac{P_iV_i}{P(t)}\right)$.

In addition, the pressure difference through the slit can be expressed as $\Delta P = \{P_A - P(t)\}$ and the corresponding wall shear stress as $\tau_w(t) = \Delta P(t)H/\{(1+2H/w)L\}$. The shear rate at the slit wall is obtained from the classical Weissenberg-Rabinowitsch equation

$$\dot{\gamma}_w(t) = -\frac{dV_z}{dz}\bigg|_w = \frac{1}{3}\dot{\gamma}_{aw}\left[2 + \frac{d\ln Q}{d\ln \tau_w}\right] \tag{1}$$

where $\dot{\gamma}_{aw}$ is $6Q/wH^2$. It is worth noting that the optical measurement of anisotropy in the laser-transmission reflects the RBCs aggregated at all depths in the pressure-driven slit flow. Hence the RBC aggregates experience shear levels from zero up to the wall shear rates. Additionally, detailed results including calibration can be found in our previous studies (Shin *et al.*, 2004a; 2004b).

3. Results and discussion

Fig. 2 shows the effect of vibrations on RBC aggregation. When vibrations are applied to the sample with a preset frequency and amplitude, the transmitted intensity decreases exponentially and reaches a minimum. When the vibrations are turned off, the transmit intensity increases exponentially. The longer the duration of the non-vibration is, the higher transmitted intensity is observed. In addition, the minimum value remains constant if the same vibration applies to the blood sample. It is worthy to note that incomplete disaggregation could cause serious error in measuring aggregation parameters (Hardeman *et al.*, 2001). Thus, either vibration frequency or amplitude should be sufficiently high to prevent RBCs from aggregating each other. However, the vibrating conditions for RBC disaggregation should be carefully chosen since there may occur mechanical hemolysis due to the high shear vibration (Shin *et al.*, 2003a; 2003b). Thus, the optimal vibrating conditions were carefully chosen for the

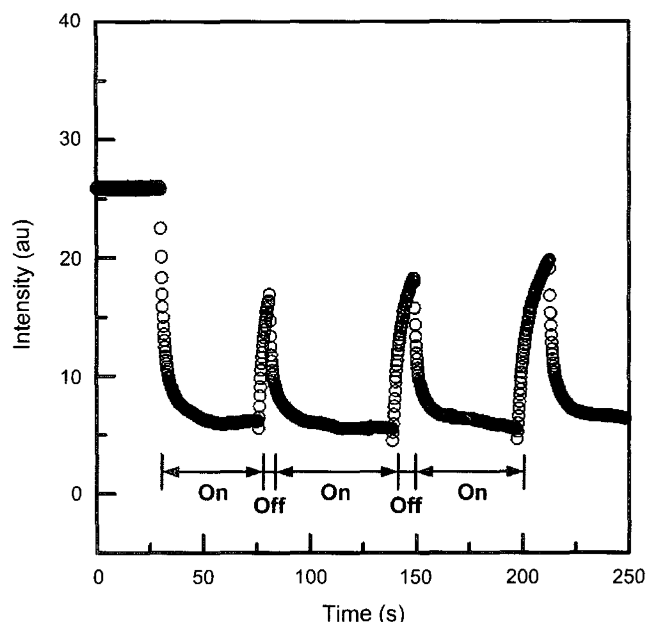

Fig. 2. Effect of vibration on the disaggregation of RBC.

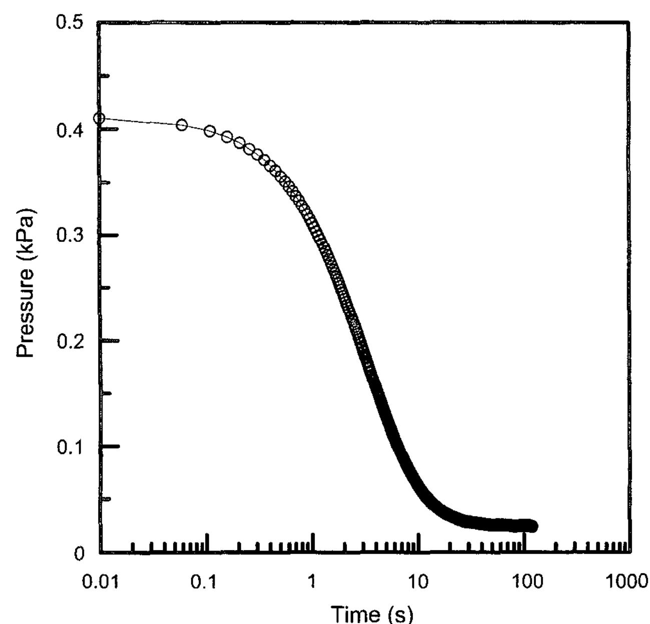

Fig. 3. Pressure differential versus time for a blood sample.

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 reach an asymptotic minimum, which cannot be lowered by further increasing vibration intensity.

The above vibration procedure is followed by the application of a decreasing pressure differential, which causes the pressure-driven shear flow though the slit. Fig. 3 shows the differential pressure variations of a blood sample over

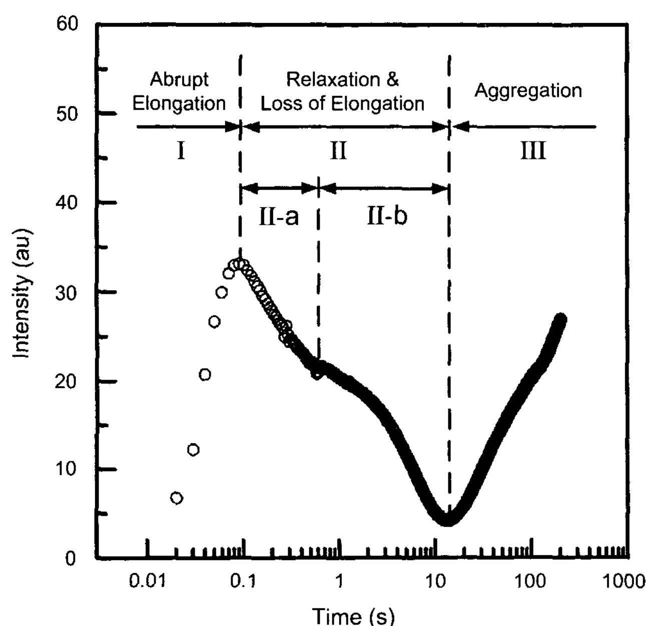

Fig. 4. Time courses of the transmitted intensity with applying abrupt pressure differential.

time. As time passed, the differential pressure between the vacuum chamber and atmosphere decreased exponentially. Typically, it took approximately one minute to reach an asymptote for blood samples. In our previous study (Shin *et al.*, 2004b), the initial high differential pressure resulted in a large deformation of the RBCs. Thus, the high differential pressure in the present study would result in the deformation of the RBCs.

Fig. 4 shows the syllectogram (transmitted intensity versus time) for a normal blood sample. With the abrupt application of the pressure differential, a rapid elongation and subsequent slow elongation loss of the RBCs are observed. They are followed by the RBC aggregation process. The syllectogram in Fig. 4 consists of three regions, which involve distinct, different mechanisms and processes related with RBC aggregation and elongation. The transmitted light intensity is expressed in arbitrary units (au).

Region-I in Fig. 4 indicates a rapid elongation when disaggregated RBCs are exposed to abrupt shearing conditions. As the RBCs are elongated, the transmitted intensity increases. The rapid elongation process ends within 100 *ms*, which includes a response time of the solenoid valve (approximately 10 ms). One may attempt to calculate the characteristic time of RBCs for abrupt elongation. The calculated time, however, may not be accurate since the pressure keep decreasing with time. It was recently reported that the relaxation time constant of RBCs in whole blood is 271 ms (Bronkhorst *et al.*, 1995), whereas that of RBCs in a PBS solution is 120 ms (Baskurt *et al.*, 1995). These are interesting issues, however, which are beyond the scope of the present study. Thus, further investigation is strongly required in order to understand cell membrane rheology.

Meanwhile, *Region-II* in Fig. 4 indicates the gradual decrease of transmitted intensity over time. The decrease of the transmitted intensity is mainly caused by the loss of elongation corresponding to the decreasing pressure as shown in Fig. 3. Since the RBCs were completely disaggregated before the measurement, the decrease of intensity in *Region-II* should not be considered as a result of RBC disaggregation. Similar phenomenon was observed also in the commercial aggregometer, LORCA (Hardeman *et al.*, 2001). Baskurt and Meiselman (1996) investigated the initial increase of backscattering intensity right after sudden cessation of shear and found that it is due to the loss of the elongation and orientation of RBCs. Under most physiological conditions, RBC shape change in response to deforming forces is a reversible process, with the cell recovering its original biconcave shape when the forces are removed; for normal adult human RBC the shape recovery process is complete within 100 – 200 msec (Hochmuth *et al.*, 1979; Linderkamp and Meiselman, 1982). Evans (1989) has clearly indicated the in vivo importance of the cells dynamic viscoelastic rigidity, and that the characteristic time for shape recovery is a significant factor in the distribution and flow of red cells through the small microvessels. Thus, the cell relaxation characteristics can be further investigated using the present apparatus.

It is noteworthy that there is an inflection point in *Region-II*. It seems that the early part of the *Region-II* may be related more with the rapid relaxation of RBCs, and the latter part of the *Region-II* is related with the gradual decrease of elongation. In addition, the final value in the *Region-II* shows the same or slightly lower value than the initial intensity in *Region-I*, which indicates the same degree of disaggregation at $t = 0$.

Next, *Region-III* in Fig. 4 shows the aggregation behaviour of the whole blood sample. As the RBCs aggregate, the transmitted intensity increases. As the shear flow decreases with time, mainly the 1-D rouleaux are formed followed by the slower formation of the 3-D networks. In fact, various indices, reflecting the total extent, kinetics, avidity and pattern of aggregation, are defined and calculated in the syllectogram of *Region-III* in Fig. 4. These indices were well defined in a previous study (Hardeman *et al.*, 2001): *Amplitude* (*Amp*) is the difference between the minimum light intensity and the light intensity at 120 s, indicating the extent of RBC aggregation. The term, *half time* ($t_{1/2}$), is defined as the time required to reach a light intensity of "*minimum intensity* + 1/2 *Amp*." *M*-index is the area under the syllectogram over a 10 s time period and the *Aggregation Index* (*AI*) is the ratio of the area under the syllectogram to the total area over a 10 s time period.

The dependence of RBC aggregation on the plasma protein, fibrinogen, is depicted in Fig. 5, which compares the

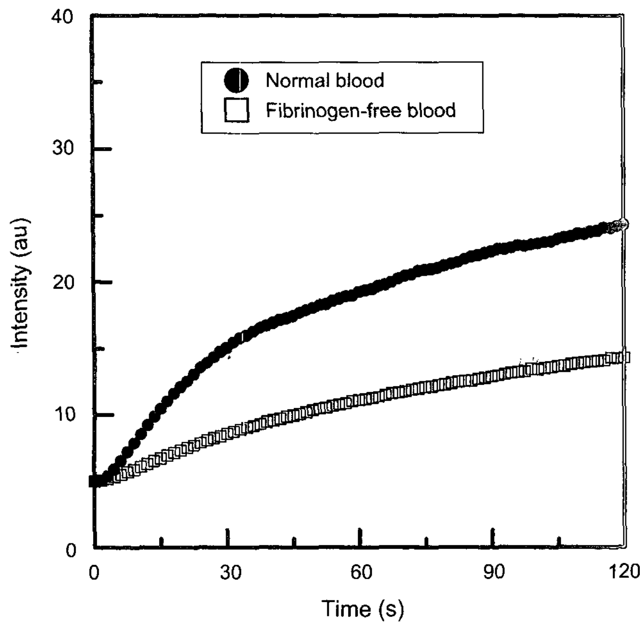

Fig. 5. Comparison of syllectogram for whole blood and RBC suspension in serum.

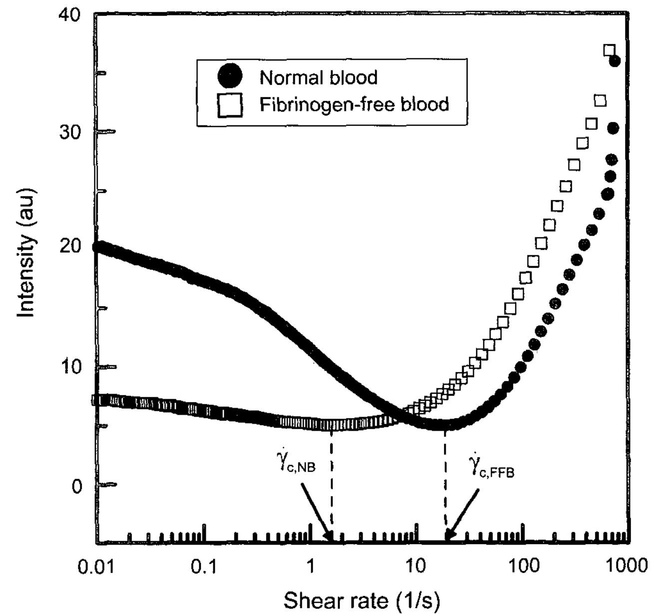

Fig. 6. Transmit intensity versus shear rate for whole blood and RBC suspension in serum.

two syllectograms of normal and fibrinogen-free blood for *Region-III*. The solid circles indicate the transmitted intensity of whole blood; and open rectangles indicate those of RBC suspension in serum. The elimination of fibrinogen results in the overall decrease of intensity and a smaller amplitude of I range. Various aggregation indices are obtained using a curve-fitting program and are listed in Table 1. Although fibrinogen is generally known as a major factor for RBC aggregation, fibrinogen-free blood showed a slow, but only a 40% reduction of aggregation. Thus, plasma proteins other than fibrinogen also play an important role in aggregate formation.

Fig. 6 shows the transmitted intensity against the shear rate for normal and fibrinogen-free blood samples in *Region-II and III*. Due to the decrease in pressure, the experiments were conducted from high to low shear rates over time. While the shear rate decreased, the normal blood tended to aggregate at a higher shear rate than the fibrinogen-free blood. For these results, a critical shear rate is determined as the shear rate at which the RBCs start to aggregate. In other words, the critical shear rate indicates a minimal shear rate needed to prevent aggregation, which is the same definition of the threshold shear rate of the LORCA measurements (Hardeman *et al.*, 2001). As shown in Fig. 6, the critical shear rate for normal blood is higher than that for the fibrinogen-free blood. This indicates that normal blood has a higher aggregability than the fibrinogen-free blood.

Table 1 summarizes the indices of aggregation for the normal and the fibrinogen-free blood samples ($n = 6$). Normal blood shows much larger values of *Amp* and *M-index* than the fibrinogen-free blood. The *half time* of the fibrinogen-free blood is longer than that of the whole blood. The above three indices are dimensional such as arbitrary unit (*au*) or time, whereas the Aggregation Index, *AI*, is a non-dimensional parameter. The *AI* of normal blood is 38% larger than that of the fibrinogen-free blood. As discussed earlier, the critical shear rate of the normal blood is larger than that of the defibrinogenated blood. With these indices, one can determine the aggregability of blood samples.

Table 1. Effect of fibrinogen on RBC aggregation indices ($n = 6$)

Aggregation indices	Present		LORCA	
	Normal blood	Fibrinogen-free blood	Normal blood	Fibrinogen-free blood
Amp (au)	18.1 ± 3.2	6.6 ± 2.9	23.7 ± 2.6	19.7 ± 1.3
$T_{1/2}$ (s)	27.8 ± 0.8	36.9 ± 0.7	2.8 ± 0.0	11.4 ± 0.4
M Index	20.0 ± 2.1	5.6 ± 1.2	N/A	N/A
AI	0.12 ± 0.01	0.08 ± 0.01	0.58 ± 0.0	0.25 ± 0.0
$\dot{\gamma}_c(1/s)$	16.3 ± 2.9	1.5 ± 0.5	85 ± 0.0	47.5 ± 3.5

In addition, Table 1 compares the values measured by the present aggregometer with those of the commercial aggregometer (LORCA). In general, the results show fair agreement, but the data cannot be compared quantitatively. This is due to the intrinsic differences of the photometric method between the two instruments: The present instrument measures light transmittance through the RBCs, whereas LORCA examines the light reflectance from them. Due to the inherent difference in optical measurement, Baskurt *et al.* (1998), who had the same problem as ours, introduced new aggregation indices for the light transmission analysis. Since both analyses of transmittance and reflectance, however, measure the same phenomenon of RBC aggregation, it might be interesting to consider further how the results of the two instruments may be compared quantitatively.

4. Conclusions

Although the present slit aggregometer was described as an instrument capable of measuring the characteristics of RBC aggregation, further development is needed with regard to the reproducibility and sensitivity of the measured aggregation characteristics. Besides an evaluation of the total aggregation extent, the present method allows for a judgement on the kinetics of the aggregation process, which also requires further investigation. In addition, the vibration-induced disaggregation mechanism should be examined further for hyperaggregated blood samples, which can cause a serious problem due to incomplete disaggregation in the commercial aggregometer. The novel features of the laser-transmission slit rheometer are the vibration-induced disaggregation system and the critical shear rate as a measure of aggregation tendency, which are successfully demonstrated in the present study.

Acknowledgments

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References

Baskurt, O.K. and H.J. Meiselman, 1996, Determination of red blood cell shape recovery time constant in a Couette system by the analysis of light reflectance and ektacytometry, *Biorheology*, **33**, 487-501.

Baskurt, O.K., H.J. Meiselman and E. Kayar, 1998, Measurement of red blood cell aggregation in a "plate- plate" shearing system by analysis of light transmission, *Clin. Hemorheol. Microcirc.* **19**, 307.

Boynard, M., J.C Lelievre and R. Guillet, 1987, Aggregation of red blood cells studied by ulstrasound backscattering, *Biorheology* **24**, 451.

Bronkhorst, P.J.H., G.J. Streekstra, J. Grimbergen, E.J. Nijhof, J.J. Sixma and G.J. Brakenhoff, 1995, A new method to study shape recovery of red blood cells using multiple optical trapping, *Biophysical J.* **69**, 1666.

Chen, S., G. Barshtein, B. Gavish, Y. Mahler and S. Yedgar, 1994, Monitoring of red blood cell aggregability in a flow-chamber by computerized image analysis, *Clin. Hemorheol.* **14**, 497.

Chien, S. and L.A. Sung, 1987, Physiochemical basis and clinical implications of red cell aggregation, *Clinical Hemorheology* **7**, 71.

Chien, S., S. Usami, R.J. Dellenback, M.I. Gregersen and L.E. Nanninga, 1970, Shear-dependent interaction of plasma proteins with erythrocytes in blood rheology, *Am. J. Physiol.* **219**, 143.

Evans, E.A., 1989, Structure and deformation properties of red blood cells: concepts and quantitative methods. *Methods in Enzymology* **173**, 3.

Hardeman, M.R., J.G.G. Dobbe and C. Ince, 2001, The Laser-assisted Optical Rotational Cell Analyzer (LORCA) as red blood cell Aggregometer, *Clin. Hemorheol. Microcirc.* **25**, 1.

Hochmuth, R.M., P.R. Worthy and E.A. Evans, 1979, Red cell extensional recovery and the determination of membrane viscosity. *Biophys. J.* **26**, 101.

Houbouyan, L.L., M. Delamaire, A. Beauchet, M. Gentil, G. Cauchois, A. Taccoen, J.P. Yvert, N. Montredon, M.F. Roudaut, S. Zhao, A. Goguel, G. Potron, M. Boisseau and J.-F. Stoltz, 1998, Multicenter study of an erythro-aggregometer : quality control and standardization, *Clin. Hemorheol. Microcirc.* **17**, 299.

Linderkamp, O. and H.J. Meiselman, 1982, Geometric, osmotic, and membrane mechanical properties of density-separated human red cells. *Blood* **59**, 1121.

Rampling, M.W., 1999, Rouleax formation; its cause and consequences, *In: Cardiovascular flow modeling and measurement with application to clinical mdecine.* S.G. Sajidai, G.B. Nash and M.W. Rampling, Ed., Clarendon Press, Oxford, pp. 183-193.

Schmid-Schönbein, H., E. Volger, P. Teitel, H. Kiesewetter, U. Dauer and L. Heilmann, 1982, New hemorheological techniques for the routine laboratory, *Clin. Hemorheol.* **2**, 93.

Shin, S., S.W. Lee and Y.H. Ku, 2004a, Measurements of blood viscosity using a pressure-scanning slit rheometer, *KSME Int. J.* **18**, 1036.

Shin, S., Y.H. Ku, M.S. Park, J.H. Jang and J.S. Suh, 2004b, Measurement of Red Cell Deformability and Whole Blood Viscosity using Laser-Diffraction Slit Rheometer, *Korea-Australia Rheology J.* **16**, 85.

Shin, S., Y.H. Ku, M.S. Park, S.Y. Moon, J. H. Jang and J.S. Suh, 2004c, Laser-diffraction slit rheometer to measure red blood cell deformability, *Rev. Sci. Instr.* **75**, 559.

Shin, S., Y.H. Ku, J.S. Suh, S. Y. Moon and J.Y. Jang, 2003, Characteristics of Blood Flow Resistance Under Transverse Vibration: Red Blood Cell Suspension in Dextran-40, *Annals of Biomedical Eng.* **31**, 1077.

Zhao, H., X. Wang and J.F. Stolz, 1999, Comparison of three optical methods to study erythrocyte aggregation, *Clin. Hemorheol. Microcirc.* **21**, 297.