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A noble RBC aggregometer with vibration-induced disaggregation mechanism

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Abstract

The aggregation of red blood cells (RBCs) is a major determinant of blood flow resistance passing through various veins. Available techniques for measuring RBC aggregation often require pretreating and washing after each measurement, which is not optimal for day-to-day clinical use. A laser reflection technique has been combined with a vibration-aided disaggregation mechanism, which shows significant advances in aggregometer design, operation and data analysis. The essential features of this design are in its simplicity and a disposable element that is in contact with the blood sample. Using extremely small quantities of blood, the RBCs subjected to vibrations can be quickly and completely disaggregated. This is followed by measuring the backscattered light intensity. The measurements with the present sensor were compared with those of a commercial aggregometer and a strong correlation was found between them. The newly-developed optical aggregometer can measure the RBC aggregability difference between young and old cell suspension with ease and accuracy.

Keywords : aggregation, red blood cell, vibration, light, reflection

1. Introduction

Red blood cells (RBCs) in normal human blood tend to form linear and branched aggregates. Such aggregation forms face-to-face morphology similar to a stack of coins, which is called rouleaux. Furthermore, the RBC aggregation is reversible, whereby RBCs disperse in a high shear environment. The reversible aggregation has a strong correlation with shear-thinning blood viscosity (i.e., a decrease in blood viscosity with an increase in the shear rate due to the dispersion of the aggregates). Since the RBC aggregation has been known to be a major determinant of the in vitro rheological properties of blood, it continues to be of interest in the field of hemorheology (Rampling *et al.*, 2004; Meiselman *et al.*, 1999; Stoltz *et al.*, 1999). In sepsis (Baskurt *et al.*, 1997), diabetic mellitus (Bauersachs *et al.*, 1987), myocardial ischaemia (Dormandy *et al.*, 1982), and renal failure (Hein *et al.*, 1987), increased RBC aggregation is observed but the causes of the diseases are different.

Even though the RBC aggregation is of great importance, there is still a lack of additional information. For example, a mechanism that governs this form of cell–cell interaction is not sufficiently known. There are two current hypotheses for the aggregation mechanism: the cross-bridging hypothesis and the depletion layer hypothesis (Neu *et al.*, 2002). Neither of these hypotheses has been generally accepted. The major cause of aggregation, however, has been known to be the presence of large plasma-proteins, especially fibrinogen. Recently, there is an increasing amount of experimental evidence indicating that RBC cellular properties can markedly affect aggregation with the term "RBC aggregability", which describes the cell's intrinsic tendency to aggregate (Rampling *et al.*, 2004).

Various techniques for measuring RBC aggregation have been developed and are described elsewhere (Rampling, 1988). Typical techniques can be briefly summarized as follows: (i) *Direct microscopic technique*: The most obvious approach to quantify RBC aggregation is to take photographs of the diluted blood sample which is placed between a slide and a coverslip and to analyze them with counting numbers of cellular units being either a mono-dispersed cell or cellular aggregates (Chien and Jan, 1973). This technique is labor-intensive and time-consuming; (ii) *Electrical impedance technique*: Capacitance and resistance were measured in a rectangular channel over time (Pribush *et al.*, 1999). This technique has been widely used in measuring platelet aggregation and there are commercial instruments available such as the Chrono-log aggregometer (Havertown, PA, USA); (iii) *Light intensity method:* The

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principle of this technique is to record light intensity over time (which is known as a "syllectogram") and analyze the transient characteristics of the recorded intensity, as a measure of RBC aggregation. Recording the light intensity either back-scattered (Zhao *et al.*, 1999; Hardeman *et al.*, 1994) from or transmitted (Shvartsman *et al.*, 2001; Shin *et al.*, 2004) through the RBCs under defined shearing conditions, has been used to assess different aspects of the RBC aggregation. Recently, other techniques including ultrasound backscattering method (Boynard *et al.*, 1987) have been reported.

The light transmission (or back scattering) technique has been further developed and is commercially available in models such as LORCA®(R&R Mechatronics, Amsterdam, Netherlands), Erythroaggregometer (Regulest, France), and Myrenne aggregometer (Myrenne, Roetgen, Germany). One of these instruments, LORCA, consists of a laser diode, a thermostated bob-cup measuring system, a step motor and photodiodes attached to a microcomputer. To disperse the RBC aggregates in the sample prior to testing, the sample is highly sheared in a Couette system made of glass, with a gap of 0.3 mm between the cylinders. Then, light intensity is recorded over time and the syllectogram is analyzed with a curve-fitting program to determine the aggregation indexes such as the *AI* (aggregation index), half-time ($t_{1/2}$) and *M*-index (Hardeman *et al.*, 1994).

For the 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} has to be applied. For hyperaggregated blood samples such as cryoglobulinemia and those with horse blood, however, a much higher shear rate is needed for complete disaggregation. It has been known that an incomplete disaggregation could cause serious problems in the commercial aggregometer. Thus, the rotational shearing systems should be able to generate a high shear rate by increasing the rotational speed. Thus, these rotational shearing systems cause the instruments to be expensive and difficult to design. 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.

Therefore, it is necessary to develop a simple and labor-free instrument that can measure the aggregation index of RBCs with minimal blood sample. The current study describes an innovative approach to a vibration-aided optical biosensor to detect RBC aggregation. The rotational shearing system is replaced with a simple vibration-aided disposable element containing a blood sample. The advantages of this design are its simplicity, low cost, and easy to use.

2. Materials and methods

A blood sample in the slit is vibrated for 40 s. Then, the 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 the optimal values are determined for the RBC disaggregation. The optimal vibrating condition for the RBC disaggregation should be carefully chosen to ensure that there is no hemolysis due to the vibrations (Shin *et al.*, 2003). In the present study, the vibrating frequency and amplitude are fixed at 150 Hz and 0.5 mm, respectively.

The present study developed a backscattered-light sensor and system for detecting RBC aggregation as shown in Fig. 1. The system consists of a disposable test slit with an inlet reservoir, vibration mechanism, laser diode, photo-diode, and a computer data acquisition system. The blood sample (9.6 μl) is placed on the micro-slit with a gap of 0.2 mm, a width of 1.2 mm and a length of 40.0 mm. The slit, which is made of glass, is designed to be disposable. A laser diode (650 nm, 1.5 mW) and a photo-diode are used to obtain laser-reflection intensity. The vibration mechanism consists of a function generator, amplifier, and a speaker. A jig, attached to the speaker diaphragm, is connected to the slit. The detail description can be found elsewhere (Shin *et al.*, 2005).

Typical tests were conducted as follows: The test fluid (9.6 μl) is placed on a test slit and sealed with a sealant. Then, the test slit is mechanically mounted onto the jig, which is attached to the speaker diaphragm. For disaggregating the RBC aggregates, the defined vibration is applied for 40 *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. Indexes of the aggregation as a measure of the RBC aggregation are determined from the syllectogram using a curve-fitting program.

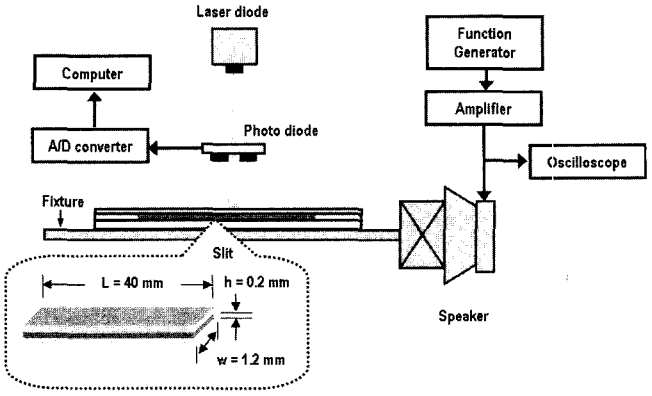

Fig. 1. Schematic diagram of the light-reflection aggregometer with vibrating mechanism.

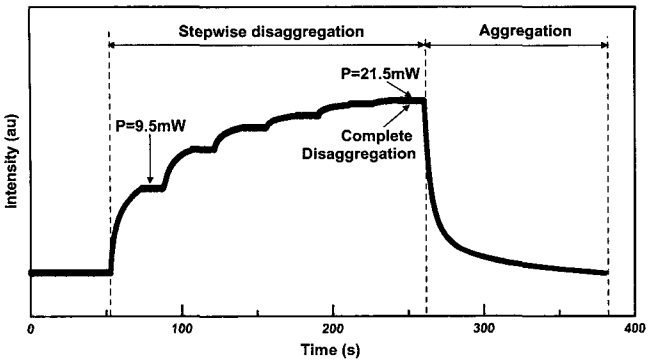

Fig. 2. Light intensity versus time for a blood sample with stepwise increase and sudden cessation of vibration.

The essential feature of the present aggregometer is the use of a vibration mechanism to disaggregate the red blood cells and aggregates. The test slit attached to the vibrator is electromagnetically vibrated, in which the blood cells are dispersed within a short period of time. Another essential feature of the present aggregometer is the use of a disposable slit, which is designed to plug-in and -out of the test instrument. The disposable slit can be made of various materials including glass, silicon, and PMMA.

3. Results and discussion

The kinetics of RBC aggregation and disaggregation were studied using the present apparatus. Fig. 2 shows the typical kinetics of aggregation and disaggregation in a sample of whole blood from a healthy donor. The output signal of the photo-detector, which is proportional to the intensity of the backscattered light, is plotted along time. In the present study, the vibration frequency was fixed at 150 Hz. The vibration amplitude was varied by manipulating the input power of the speaker, which was measured. As shown in Fig. 2, a stepwise increase of input power causes the increase of the backscattering light intensity, which reaches a plateau value. Then, the light intensity cannot be further increased by vibrations. This fact implies that there are no longer RBC aggregates that can be dispersed in the blood sample, which was demonstrated through a microscopic examination of the RBCs in the blood sample before and after the vibrations in a previous study (Shin *et al.*, 2004). These results imply that the present vibration-mechanism is sufficient enough to disaggregate the RBC aggregates and the present vibrating mechanism can successfully replace the conventional rotating shear mechanism.

Aggregation indexes are determined from a syllectogram using a curve-fitting program as indicated in Fig. 3. The measured parameters of the aggregation kinetics are well defined in a previous study (Hardeman *et al.*, 1994): *Amplitude* (*Amp*) is the difference between the maximum

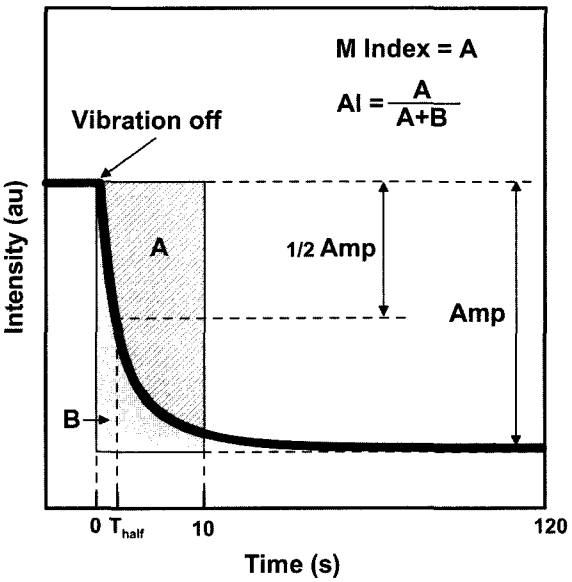

Fig. 3. Schematic diagram for defining aggregation indexes using a syllectogram.

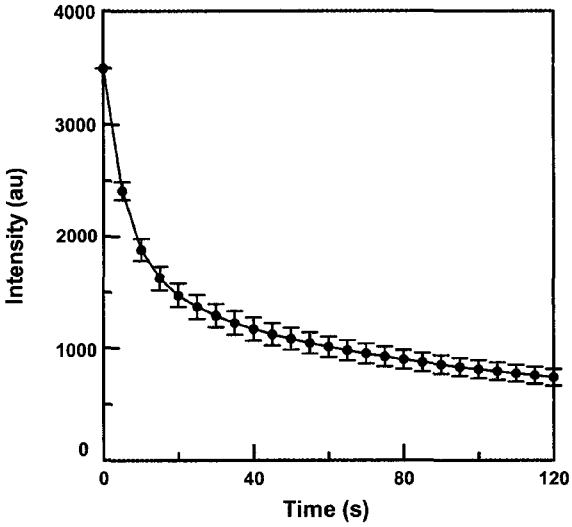

Fig. 4. Syllectogram for normal, whole blood sample with reproducibility.

light intensity and the light intensity at 120 s, indicating the extent of RBC aggregation. The *Half time* ($t_{1/2}$) is defined as the time required to reach a light intensity of "*minimum intensity + 1/2 Amp.*" The *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.

Fig. 4 shows a typical syllectogram of aggregation for normal blood after the cessation of vibrations. Aggregation indexes are determined from the syllectogram using a curve-fitting program. In addition, Fig. 4 shows the reproducibility of the present apparatus from 10 repeated measurements on the aliquots of the same blood sample. The

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

Aggregation Indices	Present				LORCA			
	Amp (au)	$t_{1/2}$ (s)	M Index (au)	AI (%)	*Amp* (au)	$t_{1/2}$ (s)	M Index (au)	AI (%)
Mean	40.0	4.55	188	47.0	41.9	4.50	198	47.1
SD	0.16	0.11	3.0	0.61	0.85	0.12	5.62	0.65
CV (%)	0.40	2.56	1.55	1.30	2.02	2.65	2.84	1.37
Percentage difference (%)	4.8	1.1	5.3	0.2	–	–	–	–

mean, standard deviation (SD) and CV of various aggregation parameters are shown in Table 1. The most reproducibility was found in the *Amp* index, with a CV of 0.4%. The *M* index, half time and *AI* showed high reproducibility with low CV values (less than 2.56%). 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 the RBC aggregation indexes measured by the present aggregometer and a commercial aggregometer (LORCA). It was found that the test results provided a good correlation between the two instruments with less than a 5.0% error rate.

Fig. 5 shows the effect of cell density on RBC-aggregation by comparing the two aggregation indexes of young and old cells suspended in an autologous plasma. In fact, cell density is directly related with cell age. In other words, the older the cells are, the higher the density red cells become. Thus, through high speed centrifugation (15,000 rpm, 20 min), a density-based separation can be achieved. As shown in Fig. 5, the young cells have a longer half time constant and a smaller *AI* than the old cells. The detailed

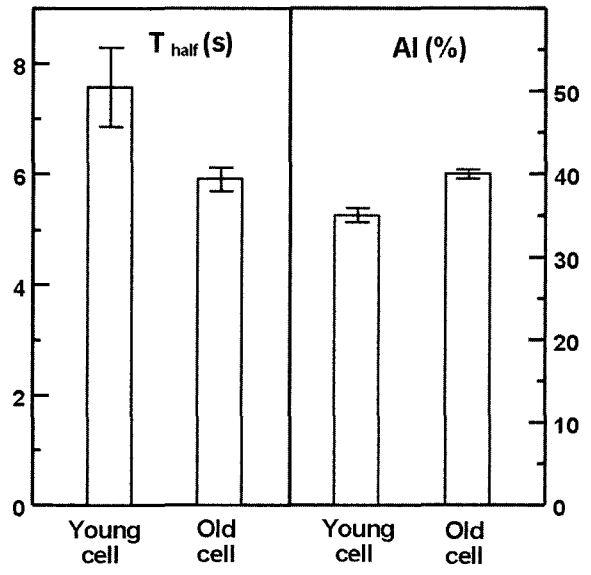

Fig. 5. Comparison of aggregation indexes for young and old cells suspended in autologous plasma.

Table 2. Aggregation indexes and percentage difference for young and old cells suspended in autologous plasma

Aggregation Indexes	Young cell	Old cell	Percentage Difference
Amp (au)	55.6	56.2	1.80 (%) ↑
$t_{1/2}$ (s)	7.57	5.91	21.9 (%) ↓
M Index	194.2	224	15.3 (%) ↑
AI (%)	35	40	14.3 (%) ↑

RBC-aggregation indexes are summarized in Table 2. The *M-index* and the *AI* of the old cell suspension increased significantly, 15.32% and 14.3%, from that of young cell suspension, respectively even though the *Amp* of both suspensions showed a slight difference (1.8%). These results indicate that the old cells have a higher aggregability than the young cells.

The objective of the present aggregometer is to measure the aggregability of red blood cells in a simple, disposable device without adding any reagents. Current aggregometers, which are frequently used to measure the RBC aggregation, are a useful and powerful tool in this area. While various aggregometers have been employed to diagnose deformability-related cell diseases, they usually require a long preparation time (2 min) for the RBC disaggregation and cleaning after each measurement. Such requirements lead to a cumbersome and time-consuming process, which prevents previous techniques from being used in clinical settings. Here, we describe a vibration-aided aggregometer with a disposable micro-slit, which eliminates the above hindrances of the current technology.

The measuring principle of the present and LORCA aggregometers are almost the same except for the shearing system for disaggregation. LORCA adopts a rotational Couette flow between the two concentric cylinders, in which the shear stress causes the dispersion of the RBC aggregates. The proposed aggregometer utilizes a vibration-driven disaggregation mechanism in a slit, whereby the alternating accelerating force causes the disaggregation of the RBC aggregates. The vibration-aided disaggregation mechanism is simple and efficient in dispersing red blood cells within a short time. In addition, the required time can

be minimized by increasing the vibration intensity such as vibration frequency and amplitude. Highly increased vibrations, however, may result in hemolysis of the cells. To prevent from happening, it is important that an optimal vibration intensity level be determined.

4. Conclusions

The present aggregometer is a new tool that can probe RBC aggregability with high resolution and reproducibility. The aggregation indexes measured with the present aggregometer were in close agreement to those obtained with the LORCA aggregometer. The present instrument could also differentiate between high (old)- and low-density (young) cells in one blood sample. With proper technological improvements including automation, measuring the aggregability of several hundred samples per day now seems possible.

Acknowledgments

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