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Photometric measurements of red blood cell aggregation: light transmission versus light reflectance

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Abstract. Red blood cell (RBC) aggregation is the reversible and regular clumping in the presence of certain macromolecules. This is a clinically important phenomenon, being significantly enhanced in the presence of acute phase reactants (e.g., fibrinogen). Both light reflection (LR) and light transmission (LT) from or through thin layers of RBC suspensions during the process of aggregation are accepted to reflect the time course of aggregation. It has been recognized that the time courses of LR and LT might be different from each other. We aim to compare the RBC aggregation measurements based on simultaneous recordings of LR and LT. The results indicate that LR during RBC aggregation is characterized by a faster time course compared to simultaneously recorded LT. This difference in time course of LR and LT is reflected in the calculated parameters reflecting the overall extent and kinetics of RBC aggregation. Additionally, the power of parameters calculated using LR and LT time courses in detecting a given difference in aggregation are significantly different from each other. These differences should be taken into account in selecting the appropriate calculated parameters for analyzing LR or LT time courses for the assessment of RBC aggregation. © 2009 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.3251050]

Keywords: erythrocyte rouleaux formation; syllectometry; acute phase reactions.

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1 Introduction

The reversible aggregation of red blood cells (RBCs) is a physiological phenomena influenced by both plasma and cellular properties.¹⁻³ This process is significantly affected in various pathophysiological processes (e.g., acute phase reactions),^{4,5} it influences *in vivo* blood flow,⁶⁻⁸ and thus has clinical significance. Most methods used to quantitate aggregation are based on monitoring the optical properties of RBC suspensions. The level of light transmission (LT) through or light reflectance (LR) from RBC suspensions during aggregation reflects the time course of this process. The time course of LT or LR recorded following the dispersion of existing aggregates in RBC suspensions can be analyzed and various indices reflecting both the kinetics and the overall intensity of aggregation can be calculated.⁹ Such measurements of RBC aggregation using LR data from RBC suspensions have been termed "syllectometry"¹⁰ and have been developed into a commercial instrument.¹¹ LT data have also been successfully used in various instruments developed to quantitate RBC aggregation,¹²⁻¹⁴ Both LT and LR methods utilize similar approaches to calculate parameters reflecting the time course and the intensity of aggregation.^{11,12,14}

In general, both LT and LR of RBC suspensions during aggregation are expected to be influenced by the same physi-

cal alterations of the suspension properties. The average particle size grows while the number of particle, including both individual cells and aggregates, become smaller during the course of aggregation, resulting in decreased LR and increased LT. Although these two properties of RBC suspensions (LR and LT) reflect the same process, it has been demonstrated^{15,16} that their time courses are not identical (i.e., they are not mirror images of each other). Therefore, it might be expected that at least the parameters reflecting the kinetics of aggregation may differ when measured using LR or LT data. However, it is not clear if differences of such parameters can influence the power of the methods or instruments to detect alterations in aggregation. This study was designed to compare (1) the parameters calculated using LR and LT data recorded during the aggregation process following an abrupt cessation of shear and (2) the power of these parameters to detect the experimental alterations in the aggregation behavior of RBC suspensions.

2 Materials and Methods

2.1 Preparation of RBC Suspensions

Venous blood samples (~25 ml) were obtained from 10 healthy male volunteers, aged between 25 to 52 yr, following the guidelines for hemorheological laboratory methods.¹⁷ A tourniquet was applied to locate the antecubital vein prior to venipuncture and kept in place during the blood sampling.

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Blood samples were obtained using vacuum tubes (Vacutainer; Becton-Dickinson, New Jersey) containing dipotassium ethylenediamine-tetraacetic acid (K_2EDTA , 1.8 mg/ml) as the anticoagulant. All blood sampling was completed within 2 min after the application of the tourniquet.

A 5-ml aliquot of each sample was saved for control measurements and the remaining 20 ml of each sample was centrifuged at $1400 \times g$ for 6 min, the buffy coat removed, and the plasma aspirated and saved. RBCs were washed twice with isotonic phosphate-buffered saline (PBS, pH=7.4), then resuspended as described in the following.

Native plasma was diluted with PBS at ratios of 1/3 and 1/2 to yield altered plasma containing 66 and 50% of the components in undiluted plasma. Washed RBCs from each donor were resuspended in the diluted plasmas at 0.4 l/l hematocrit. Diluting plasma with PBS results in reduced RBC aggregation due to lower concentrations of proaggregating plasma proteins.¹⁸ Additionally, washed RBCs from each donor were suspended in PBS containing 1% dextran 500 (500 kDa, Sigma Chemical Company, St. Louis, Missouri) at 0.4 l/l hematocrit, resulting in increased RBC aggregation compared to suspensions in undiluted native plasma.¹⁹

The hematocrit of all samples was adjusted to 0.4 l/l by adding or removing calculated amounts of suspending medium after determining hematocrit by the microcapillary method.¹⁷ Since the degree of hemoglobin oxygenation has been demonstrated to influence RBC aggregation parameters, especially those calculated²⁰ based on the time course of LR, all samples were carefully oxygenated prior to measurement by rolling 3 ml of suspension in a horizontal 15-ml polypropylene tube for 5 min at 37°C. All measurements of LT and LR were performed at 37°C.

2.2 Recordings of LT and LR during RBC Aggregation

LT and LR were recorded simultaneously during RBC aggregation using the laser-assisted optical rotational cell analyzer (LORCA; RR Mechatronics, Hoorn, The Netherlands) which was modified to record LT in addition to the usual LR. The system has been previously described elsewhere.¹¹ In brief, it consists of a Couette shearing system consisting of a glass cup and a precisely fitting bob with a 0.3-mm gap between the cylinders; the RBC suspension is contained in the gap. The outer cylinder is rotated by a stepper motor controlled by a digital computer. The beam from a laser diode (670 nm, 4 mW) mounted in the bob is directed onto the sheared sample, and the reflected light is recorded by two photodiodes in the bob. The LR signal from these two photodiodes was obtained from the LORCA and fed to an analog-to-digital converter (USB-6009; National Instruments, Texas) via a signal conditioning circuit to adjust signal amplitude and offset. Additionally, a phototransistor (BP101, Siemens, Germany) was positioned immediately adjacent to the outside of the cup in the pathway of the laser light to monitor the transmitted light through the blood sample, and connected to a second channel of the analog-to-digital converter. LR and LT signals were digitized at a sampling rate of 10 Hz and recorded on a separate digital computer using LabView 8.2 (National Instruments, Texas).

LT and LR were recorded during the usual aggregation protocol of the LORCA. Briefly, the sample is first sheared at

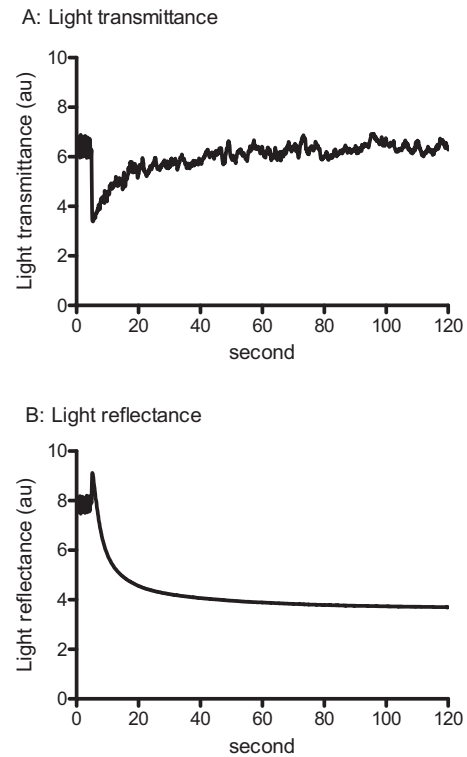


Fig. 1 Time course of (a) LT through and (b) LR from blood between the bob and cup of the LORCA during RBC aggregation.

a shear rate of 500 s^{-1} to disperse preexisting RBC aggregates, following which the shear is abruptly stopped. LT and LR data are recorded for 120 s using software developed using LabView 8.2, and the resulting LR or LT versus time profiles analyzed to determine several indices reflecting both the magnitude and time course of aggregation. The following parameters were calculated: (1) amplitude (AMP), the total change in intensity of transmitted or reflected light during the 120-s period; (2) aggregation half time ($T_{1/2}$), the time required to reach a LT or LR level corresponding to 50% of AMP; (3) surface area (SA), the area below (for LT) or above (for LR) the time course curve during the first 10 s; and (4) aggregation index (AI), the ratio of the area below (for LT) or above (for LR) the time course curve to the sum of the areas above and below the curve during the first 10 s. Additionally, time constants for the fast (T_{fast}) and slow (T_{slow}) components of RBC aggregation were calculated by fitting LR or LT versus time data to a double-exponential equation:

$$I_t = a + b \exp(-t/T_{\text{fast}}) + c \exp(-t/T_{\text{slow}}). \quad (1)$$

In this equation T_{fast} reflects the time course of 2-D rouleaux formation, while T_{slow} represents the formation of 3-D structures resulting from secondary aggregation. Accordingly, the terms b and c correspond to the changes of reflected or transmitted light intensity due to these processes, whereas the a term is the light intensity at the end of the measurement period.⁹

Table 1 Aggregation parameters calculated using LT and LR data for whole blood, RBC suspensions in 1/3 and 1/2 diluted plasma, and 1% dextran 500.

	LT			
	Whole Blood	RBCs in 1/3 Diluted Plasma	RBCs in 1/2 Diluted Plasma	1% dextran 500
AMP (au)	4.09±0.32	1.69±0.28**	0.74±0.13**	3.75±0.27
SA (au)	179.30±17.45	48.58±8.49**	19.96±2.95**	182.80±9.90
AI	43.11±1.95	31.70±6.42	33.26±8.19	45.97±1.73
$T_{1/2}$ (s)	5.75±0.56	14.30±2.81*	20.22±4.63**	4.87±0.50
T_{fast} (s)	3.32±0.35	7.35±2.12	10.25±5.32	3.07±0.47
T_{slow} (s)	48.38±7.39	102.4±37.9	68.25±22.01	61.61±19.29
	LR			
	Whole Blood	RBCs in 1/3 Diluted Plasma	RBCs in 1/2 Diluted Plasma	1% dextran 500
AMP (au)	4.58±0.26	5.38±0.20*	4.77±0.24	8.55±0.45**
SA (au)	305.41±17.75 ^{†††}	207.40±21.13	136.33±21.59**	621.60±57.47**
AI	66.29±2.31 ^{†††}	38.29±3.96**	27.54±3.71**	76.81±0.47*
$T_{1/2}$ (s)	2.27±0.22 ^{†††}	7.89±1.09*	13.66±2.50**	1.16±0.03
T_{fast} (s)	2.35±0.21 [†]	5.89±0.73	12.46±3.17*	1.26±0.03*
T_{slow} (s)	19.47±1.92 ^{††}	38.62±4.6*	38.34±7.27**	26.04±5.87

All hematocrits adjusted to 0.4 l/l. Data are mean±SE; $n=10$ for each type of suspension. Difference from whole blood: *, $p<0.05$; **, $p<0.01$. Difference of LR results from LT results are shown *only* for the whole blood samples: †, $p<0.05$; ††, $p<0.01$; †††, $p<0.001$.

2.3 Calculations and Statistics

The results of the measurements on RBC suspensions with normal and modified aggregation are expressed as mean±standard error (SE). Statistical comparisons were done using one-way analysis of variance (ANOVA) followed by appropriate posttests. Additionally, standardized differences from control values were calculated by dividing the mean difference between control and modified RBC suspensions by the “pooled standard deviation” of the control plus modified data. That is,

$$\text{St.Diff.} = \frac{M_c - M_m}{SD_p}, \quad (2)$$

where M_c and M_m are the means of the selected parameter for control and modified samples prepared using RBC from 10 donors. SD_p was calculated as the square root of the average of the squared standard deviations (SD_c and SD_m) of the data from the control and modified samples being compared:²¹

$$SD_p = \left(\frac{SD_c^2 + SD_m^2}{2} \right)^{1/2}. \quad (3)$$

The standardized difference has been accepted as a measure of the power of a specific measurement condition to detect a difference between two groups.²²

3 Results

Typical time courses of LT and LR signals for normal blood at 0.4 l/l hematocrit are presented in Fig. 1. LT exhibits a very sudden decrement following the abrupt cessation of shear, reaching a minimum in about 0.1 s. This minimum level of LT is followed by an increase that is initially rapid during the first several seconds and then occurs at a slower rate. Although this time course was clearly visible, there were rapid, random fluctuations of LT with amplitudes sometimes about one-third of the initial decline [Fig. 1(a)]. The LR time course [Fig. 1(b)] is characterized by an initial peak after the shearing is stopped and then an initial fast decrement of LR. The change in LR then slows over several seconds and continues to change slowly throughout the recording up to 120 s. There were no random fluctuations in LR similar to those seen for LT.

A comparison of LT and LR curves for whole blood during aggregation indicates a faster time course for LR and the time constants $T_{1/2}$, T_{fast} , and T_{slow} , with all three constants being significantly smaller than those calculated using LT data (Table 1). Additionally, the SA and AI parameters were higher if calculated using LR data while AMP values calculated by LR and LT for whole blood were not significantly different (Table 1).

Parameters calculated using LT and LR curves of RBC suspensions in diluted plasma and in 1% dextran 500 are also shown in Table 1. AMP and SA values calculated using LT were very significantly reduced by plasma dilution and $T_{1/2}$ was significantly increased, whereas AI, T_{fast} , and T_{slow} were not sensitive to dilution. In contrast, the time course parameters calculated using LR were more sensitive to plasma dilutions while AMP and SA were not; there was even an increase of AMP above whole blood for the 1/3 diluted plasma.

RBC suspended in 1% dextran 500 were characterized by very significantly higher AMP and SA when calculated using LR ($p < 0.01$) data, while time course parameters were less affected (Table 1). Interestingly, none of the parameters calculated using LT were sensitive to the enhanced RBC aggregation in these dextran 500 suspensions.

Standardized differences from normal blood values for RBC suspensions with modified aggregation were calculated using the data presented in Table 1. The calculated values indicate an interesting contrast between results using LT or LR [Figs 2(a)–2(c)]: (1) parameters reflecting the overall extent of aggregation (i.e., SA) and thus those less affected by the kinetics of aggregation had a higher power to detect altered aggregation if calculated using LT curves; and (2) kinetic parameters reflecting the time course of aggregation generally had higher power if calculated using LR curves. Note that the low standardized differences for RBC in 1% dextran 500 [Fig. 2(c)] confirm the insensitivity of LT-based parameters for alterations in these highly aggregating suspensions (Table 1).

Since the differences between the calculated values of AMP using LT or LR data were most obvious for this parameter [Fig. 2(b)], the AMP term was further evaluated to determine its dependence on the duration of time after the start of aggregation process (Table 2). AMP values calculated using LT data for whole blood exhibited a significant dependence on the duration (e.g., at 10 s it was 62% of the value reached at 120 s), while this dependence was less pronounced for LR data (e.g., at 10 s, 86% of the value at 120 s). This dependence of AMP on the duration after stopping shear became more pronounced for RBC suspended in diluted plasma: (1) for 1/3 diluted plasma, AMP calculated at 10 s was 43% of the 120 s value for LT and 56% for LR and (2) for 1/2 diluted plasma, AMP calculated at 10 s using LT data was 38% of the 120 s value and 42% for LR. Thus, both LT and LR curves had slower time courses for RBCs suspended in diluted plasma. Standardized differences for AMP calculated using LT or LR at various time points after the start of aggregation offer additional insight into the two measurement methods: in 1/2 diluted plasma suspensions, standardized differences for AMP using LR decreased with increasing time period after the start of aggregation whereas AMP using LT data was insensitive to the elapsed time (Fig. 3).

4 Discussion

Our results indicate that the time course of LT and LR recorded simultaneously during RBC aggregation are significantly different: (1) LR changes faster during aggregation and (2) LT data are characterized by random fluctuations of the signal superimposed on the characteristic time course, reflecting the aggregation process. These fluctuations are similar to

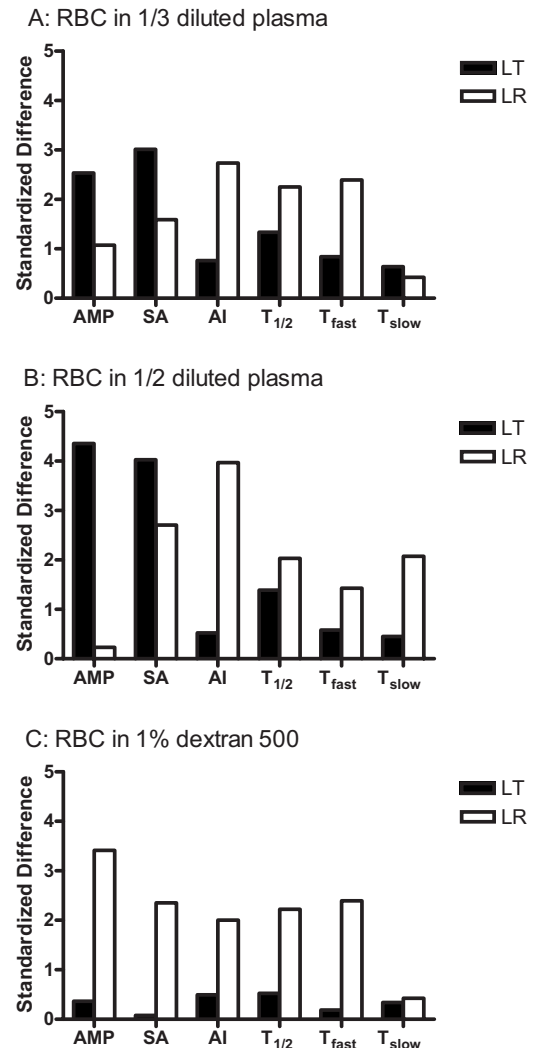


Fig. 2 Standardized differences from whole blood of RBC aggregation parameters for RBC suspended in (a) 1/3 and (b) 1/2 diluted plasma and in (c) 1% dextran 500. The parameters were calculated using LT or LR data in Table 1.

those reported by Singh and Kumavarel who suggest that they represent the instantaneous changes in aggregate size and number of cells per aggregate.²³

The difference in the time course of LT and LR signals was also reflected by the calculated parameters that are widely utilized by instruments developed to quantitate RBC aggregation^{11–14} (Table 1). These findings confirm the previous report by Gaspar-Rosas and Thurston,¹⁵ who also reported faster time courses for reflected light during RBC aggregation when using a chamber consisting of two parallel glass plates. Their calculations of time constants yielded values consistent with those obtained via LT and LR in this study. It is notable that the time constants calculated via LT data reported by Gaspar-Rosas and Thurston were strongly influenced by the thickness of the blood layer, with data reported for 0.125- and 0.508-mm layers. However, since the thickness of the blood layer between the bob and cup in the LORCA is 0.3 mm, their time constants are not directly comparable with our values.¹⁵

Table 2 AMP parameters calculated at various time points after the sudden stop of shearing for whole blood and RBCs suspended in 1/3 and 1/2 diluted plasma.

		LT				
AMP		10 s	20 s	40 s	60 s	120 s
Whole blood		2.52±0.22	2.98±0.24	3.46±0.27	3.75±0.29	4.09±0.32
RBC in 1/3 diluted plasma		0.72±0.10	0.96±0.12	1.24±0.17	1.42±0.22	1.69±0.28
RBC in 1/2 diluted plasma		0.28±0.04	0.38±0.05	0.50±0.07	0.59±0.09	0.74±0.13
		LR				
AMP		10 s	20 s	40 s	60 s	120 s
Whole blood		3.94±0.22	4.23±0.23	4.45±0.25	4.55±0.25	4.58±0.26
RBC in 1/3 diluted plasma		3.02±0.23	3.92±0.19	4.65±0.17	5.00±0.17	5.38±0.20
RBC in 1/2 diluted plasma		2.00±0.20	2.88±0.23	3.70±0.22	4.12±0.22	4.77±0.24

Data are mean±SE; $n=10$ for each sample.

The difference between the LT and the LR time relations during RBC aggregation can be explained based on the relative contribution of RBCs located at various depths of the blood film under investigation. It can be assumed that RBCs close to the surface facing the incident light are mostly responsible for the reflected light, while transmitted light intensity is affected by the entire thickness of the RBC suspension.¹⁵ RBC aggregation kinetics might well be influenced by geometrical constraints due to the presence of solid surfaces (e.g., parallel plates or cylindrical bob used in this study), thereby affecting the time course of aggregation. Supporting this explanation, Gaspar-Rosas and Thurston report that the values of aggregation time constants calculated using LR data are less affected by blood layer thickness than those using LT data.¹⁵

The different time courses and time constants for LT and LR also influenced the sensitivity of the calculated parameters to modified RBC aggregation. There is an interesting contrast

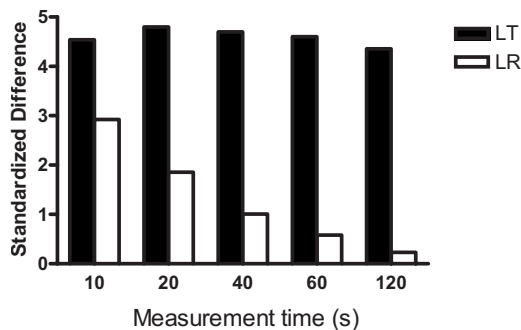


Fig. 3 Standardized differences of AMP for RBC suspended in 1/2 diluted plasma compared to whole blood; values were calculated using LT and LR data at various time points after the start of aggregation (Table 2).

between the results based on LT or LR data (Figs. 2 and 3): (1) the AMP parameter reflecting the overall extent of aggregation was very sensitive to changes due to plasma dilution, but only if this parameter was calculated using LT data. AMP values calculated using LR data showed no change for 1/2 dilution and an unexpected increase of AMP even though the concentration of pro-aggregating proteins was reduced; (2) parameters reflecting the time course of aggregation ($T_{1/2}$, T_{fast} , and T_{slow}) were more sensitive to alterations in RBC aggregation due to plasma dilutions if they were calculated using LR data; and (3) all parameters reflecting both the extent and time course of aggregation were sensitive to the enhanced aggregation in 1% dextran 500 solution.²⁴ only if they were calculated using LR data. In particular, AMP and SA parameters using LR data were doubled in the dextran suspensions compared to whole blood, while there were no significant changes of these parameters using LT data. Such striking differences also suggest different dynamics of aggregate formation at the locations within the suspension that are sensed by LT and LR monitoring. Gaspar-Rosas and Thurston also reported contrasting features for LT and LR such as different dependences of aggregation time constants on blood layer thickness: aggregation time constants based on LT decreased with increasing blood layer thickness, while they were slightly increased if based on LR data.¹⁵

A major aim of this study was to compare the power of various parameters to detect given alterations in RBC aggregation when calculated using LT or LR data. Standardized differences (see Sec. 2.3) between RBC suspensions with normal and experimentally altered aggregation (i.e., plasma dilution or 1% dextran 500) were used as an indicator of the power for a parameter.²² A contrast similar to that already discussed was also obvious when considering standardized difference data (Fig. 2), with the results for 1/2 plasma dilution being typical. First, the AMP parameter, which reflects

the extent of RBC aggregation over the 120-s recording period, was the most powerful parameter (i.e., parameter with highest standardized difference) to reduced aggregation due to plasma dilution when using LT data, while the same parameter based on LR had the least power and was almost equal to zero [Fig. 2(b)]. Second, the SA parameter based on LT data had a high standardized difference while the same parameter calculated based on LR data has only a modest power. Note that the SA parameter is affected by both the magnitude of the signal change due to aggregation and its time course, and therefore reflects a mixture of both properties. Third, AI, $T_{1/2}$, T_{fast} , and T_{slow} exhibited higher power if they were calculated using LR data. Finally, standardized differences for RBC suspended in PBS containing 1% dextran 500 confirmed the insensitivity of parameters calculated using LT data.

The sensitivity of the AMP parameter calculated using LR data was significantly affected by the length of the period selected for analysis, with the power for RBC suspended in diluted plasma being highest if the analyzed period following the abrupt cessation of shear was shorter. This dependence on analysis period can be interpreted as being related to the fast time course of LR. It can be assumed that LR from the layers of a RBC suspension changes between a maximum corresponding to the totally disaggregated status and a minimum level corresponding to extensive aggregation. Since LR has a faster time course, the signal could approach the minimum level for both normal and reduced RBC aggregation suspensions if the analysis period is sufficiently long, thereby preventing detection of the given alteration. Alternatively, if calculations were limited to shorter time periods, differences in aggregation tendency would more likely to be detected, and thus reducing the analysis time to shorter periods is strongly recommended for measurements based on LR. Note that due to the slower time course of the LT signal, the duration of analysis period did not affect the AMP parameter to the same extent.

In overview, our results indicate that RBC aggregation parameters calculated using LR and LT versus time data may not be directly comparable when using the same mathematical approaches for the two modalities. Furthermore, the power of the parameters calculated using LR and LT data for detecting alterations of RBC aggregation also differ from each other. These characteristics of the parameters therefore should be considered when selecting appropriate indices to quantitate RBC aggregation. In general, when using measurements based on LR, either parameters sensitive to the time course of RBC aggregation should be used or the analysis period should be short. Alternatively, when using measurements based on LT, parameters sensitive to the overall extent of RBC aggregation (e.g., AMP, SA) are appropriate.

Acknowledgments

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