Comparison of light-transmission and -backscattering methods in the measurement of red blood cell aggregation

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Abstract. Light-transmission and light-backscattering methods are commonly used to determine red-blood-cell (RBC) aggregation. Even though the results reveal good correlations between the parameters that are measured by these two methods, the methods themselves yield quite different values. The objective of this research is to investigate and delineate the characteristics of the two optical methods. We measured RBC aggregation by using a newly developed microchip-based aggregometer. An orthogonal polarization technique, wherein multiple scattering causes polarized light to be depolarized and passed through an orthogonal polarizer, was applied to the backscattering method. Our results were also compared to those of conventional aggregometers [laser-assisted optical rotational cell analyzer (LORCA)], and revealed that the backscattering method yielded higher aggregation indices than the transmission method and LORCA. However, the backscattering method with orthogonal polarization yielded the same values of aggregation indices as the transmission method. These agreements between the two methods were also found in measurements of RBC aggregability in various concentrations of dextran solutions. © 2010 Society of Photo-Optical Instrumentation Engineers.  

Keywords: erythrocyte; aggregation; backscattering; transmission; orthogonal polarization.

Paper 09375RR received Aug. 26, 2009; revised manuscript received Jan. 13, 2010; accepted for publication Jan. 15, 2010; published online Mar. 25, 2010.

1 Introduction

Red blood cells (RBCs) in normal human blood tend to form linear and branched aggregates. This RBC aggregation plays an important role in the flow of blood, particularly in the microvascular system. Increased aggregation of RBCs has been observed in various diseases, such as diabetes, HIV infection, myocardial infarction, thrombosis, sepsis, and stroke. Thus, the degree of RBC aggregation is widely accepted as a major determinant of hemorheological characteristics. However, although RBC aggregation certainly affects low-shear blood viscosity, its effects on in vivo flow resistance remain uncertain.¹

Various techniques for measuring RBC aggregation have been developed and are described elsewhere.² Because of their simplicity, optical methods that record the intensity of light have been widely employed to quantify aggregation.³ Different aspects of RBC aggregation have been assessed by recording the intensity of light that is either backscattered from RBCs or transmitted through RBCs.⁴⁻⁵ Commercial aggregometers, namely, laser-assisted optical rotational cell analyzer (LORCA), (R&R Mechatronics, The Netherlands) and the Myrenne aggregometer (Myrenne GmbH, Germany), have been developed to support these two optical techniques, respectively. LORCA analyzes the backscattered light in a concentric bob-cup system, while the Myrenne aggregometer analyzes the transmitted light by using a cone-plate system. Both methods are widely used for examining the behavior of RBC aggregation under zero—or steady-shear conditions. After cells are completely disaggregated through the application of a dynamic high-shear flow, they are suddenly exposed to a zero-flow condition. Then, the intensity of light is recorded over time and the resulting syllectogram is analyzed with a curve-fitting program to determine aggregation indices such as the AI (aggregation index), half-time (t₁/₂), and M index.

Light-transmission and -backscattering methods yield quite different results.² For instance, backscattering analysis yields a higher value of the (aggregation index AI) and a smaller value of t₁/₂ (half-time) than transmission analysis. Because of these different values of RBC AIs, experimental results of transmission method cannot be directly compared to those of backscattering method. In order to compensate the differing values of the AI that are yielded by the two methods, a modified definition of AI was introduced for the transmission method.³ For example, an analysis of the transmission syllectogram for 10 s yields higher values of AI than that for 120 s. These results were reconfirmed in our recent study.⁶ However,
there is still a lack of understanding as to why these two methods yield different values, even though the same light-scattering techniques were adopted.

Recently, an orthogonal polarization (OP) spectral (OPS) imaging technique, which can visualize in vivo blood flow through vessels near the skin, was introduced. The essential feature of OPS imaging is that polarized light can be depolarized by multiple scattering and the depolarized light can pass through an orthogonal polarizing filter. The directly scattered light, which is still polarized, cannot pass through the orthogonal polarizing filter. After eliminating the directly scattered light from the skin and tissues, the multiple-scattered and depolarized light can be detected. These results make it possible to visualize in vivo blood flow near the skin. It was also proposed that this OP technique could be used to understand the optical measurement of RBC aggregation.

To understand the differences between the backscattering and transmission methods, we need a new method for delineating the mechanisms of the optical measurement of RBC aggregation. Therefore, in our study, the OP technique was adopted to analyze the detected light and investigate the characteristics of the two methods for determining RBC aggregation. We measured RBC aggregation with a newly developed microchip-based aggregometer that was reported in our previous study.

2 Materials and Methods
2.1 Sample Preparation
Venous blood samples were obtained from 10 healthy male volunteers (between 25 and 32 years of age), who were not on any medication and who provided informed consent. Thirty milliliters of blood was obtained from each donor using vacuum tubes (Vacutainers, 6 mL, BD, Franklin Lakes, New Jersey) that contained (K2)EDTA as the anticoagulant. We note that a complicated, multistage process is not required for preparing blood samples, because whole blood can be directly used, as is the case with other aggregometers. However, in this study, a series of processes was conducted to vary the concentration of dextran and/or to adjust the hematocrit.

The whole blood was centrifuged at 800 g for 5 min. The plasma anduffy coat were then removed. To adjust the hematocrit of the blood samples, the RBCs were washed three times with an isotonic phosphate buffered saline (PBS) solution (pH 7.4, 290 mOsmol/kg). Then, the washed RBCs were resuspended in autologous plasma with the hematocrit being fixed at 45%. For comparing the sensitivities of the various methods, the washed RBCs were resuspended in isotonic PBS-polymer solutions that were prepared by using various concentrations of the nonionic, water-soluble, polymer dextran, molecular weight (MW) 70 K D (Dex70). There was no hemolytic presence during the entire test, and all analyses were completed within 4 h following the collection of blood.

Intra-assay variation was calculated as the coefficient of variation (CV) of 10 repeated measurements on the same sample. Biological variation was expressed as the CV of the data obtained using the control blood samples from the 10 donors.

2.2 Apparatus and Operational Procedure
Figure 1 illustrates a schematic of the experimental apparatus for backscattering and transmission methods with a polarizer and orthogonal polarizers.

![Fig. 1 Schematic diagram of the experimental apparatus for backscattering and transmission methods with a polarizer and orthogonal polarizers.](image)

Typical test procedures were conducted as follows. The test fluid was placed in the chamber of a microchip. The microchip was then mechanically mounted onto the jig, which was 10 mm apart from the magnet-rotating mechanism. For disaggregating the RBC aggregates, the stirrer in the microchip rotated at a user-selectable speed for disaggregation of preexisting RBC aggregates (900 rpm in the present study),
following which the rotating shear is stopped abruptly. Either
light reflection or transmission from the suspension is...mission methods.

The use of the LORCA (RR Mechatronics, Hoon, The
Netherlands) as a reference aggregometer has been previously
described. In brief, the system consists of a Couette geometry
that is composed of a glass cup and a precisely fitting bob
with a 0.3-mm gap between the cylinders; the RBC suspen-
sion is contained in the gap. The beam from a laser built into
the bob is directed onto the sheared sample. The reflected
light is recorded by two photodiodes located in the bob and
analyzed by a microcomputer. The sample is first sheared at a
user-selectable shear rate for the disaggregation of preexisting
RBC aggregates (600 s−1 in the present study), following
which the shearing is stopped abruptly. Light reflection from
the suspension is recorded for 120 s, and the resulting syllec-
gram is analyzed to calculate several indices that reflect
both the magnitude and duration of aggregation.

3 Results
Prior to the main experiments, the present study examined the
biological variation of RBC aggregation using two different
methods. The CV values for measurements on the control
samples from the 10 donors were somewhat different for each
aggregation parameter, but no significant difference was
found between the two methods. For both methods, the lowest
biological variations were observed for AI, whereas the high-
est CVs were for the kinetic parameter, t1/2. In addition, the
CV values of the 10 repeated measurements on the same
sample were <5% for all the aggregation parameters that
were measured with the two methods. Similar results can be
found elsewhere.9,10

Figure 2 shows typical syllectograms for the backscatter-
ing and transmission methods. While the stirrer rotated,
aggregated cells were dispersed and the surface area of the scat-
tering attained a peak. Thus, the intensity of backscattering
plateaued at a maximum level, whereas the transmission in-
tensity was minimal. After the stirring suddenly stopped, the
intensity of the backscattered light rapidly decreased and the
transmission intensity rapidly increased with the lapse of
time. These two syllectograms appear to be symmetric. How-
ever, there are a surprising number of asymmetries that cause
the two methods to yield different values of aggregation indi-
ces.

The adoption of the OP technique for the backscatter-
ing method caused an apparent change in the syllectogram,
as shown in Fig. 2. The backscattering method with the OP tech-
nique revealed a new syllectogram (dotted line in Fig. 2),
which showed an apparent deviation from the syllectogram of
backscattering without OP. In Fig. 2, the syllectogram for
backscattering yields steeper decrease rather than that for

Fig. 2 Syllectograms for backscattering with/without OP and for trans-
mision methods.

method: (i) Amplitude (AMP), the total change in the inten-
sity of reflected light during the period of 120 s; (ii) aggrega-
tion half-time (t1/2), the time for AMP to halve; (iii) surface
area (SA), the area above the syllectogram over the first 10 s;
(iv) AI, the ratio of the area above the syllectogram to the sum
of the areas above and below the curve over the first 10 s; and
5) T1 and T2, the time constants of the fast rouleaux formation
and the slow 3-D aggregate formation, respectively. However,
due to similar trends across the listed aggregation parameters,
two main parameters (AI, t1/2) were selected and presented for
comparing the difference between the light transmittance and
light backscattering signals.

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In Eq. (1), Tf and Ts, respectively represent the time con-
stants of the fast rouleaux formation and the slow 3-D ag-
gregate formation.

At time t=0, the maximum intensity was obtained [i.e.,
I(0)=Imax]. As measures of RBC aggregation, the parameters
of aggregation were determined from the syllectogram by us-
ing a curve-fitting program, as shown in Fig. 2.5–6 The fol-
lowing parameters were defined based on the backscattering

I(t) = Ie−αTf + Ie−αTs + I0

In Eq. (1), Tf and Ts denote the time constants of the aggre-
gation process. Aggregation can be considered a multistage
process (i.e., a double-, rouleaux-, or 3-D-aggregate forma-
tion), which takes only minutes. In the biexponential equa-
tion [viz., Eq. (1)], Tf and Ts, respectively represent the time con-
stants of the fast rouleaux formation and the slow 3-D ag-
gregate formation.

Journal of Biomedical Optics 027003-3
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backscattering with OP and these two curves result in significant changes in the analyzed AIs, which are described in Fig. 3.

Figure 3 shows the AIs, AI and $t_{1/2}$, which are measured by varying the height, $H$, of the sample chamber. In our measurements, the three following different methods were compared: backscattering without OP ($B$), backscattering with OP ($B_{op}$); and transmission without OP ($T$). These results were also compared to those of the commercial LORCA aggregometer. As shown in Fig. 3(a), at the intermediate range of the height of the sample chamber (0.75 $< H < 1.7$ mm), the backscattering measurements of AI showed good agreement with the LORCA results. We recall that LORCA, which adopts the backscattering method, has a flow gap of 0.3 mm in the concentric cylindrical geometry. The transmission measurements ($T$) show quite smaller values of AI than those of backscattering, even though they are independent of the chamber height. However, the backscattering measurements with OP ($B_{op}$) show good agreement with the transmission measurements ($T$). The AI values for $B_{op}$ become smaller than those for $B$ and are almost the same as those for $T$. In other words, the OP technique eliminates the difference between the backscattering and transmission methods in terms of AI measurements.

Furthermore, the backscattering measurements with OP yield independence of the chamber height.

Similar results were also found in the results for the halftime ($t_{1/2}$). The measurements of the half-time for the four cases ($B$, $T$, $B_{op}$, and LORCA), are shown in Fig. 3(b). The backscattering measurements ($B$) show good agreement with the LORCA results ($t_{1/2} = 1.65 \pm 0.15$ s) at the intermediate range of the height of the sample chamber (0.75 $< H < 1.7$ mm). The transmission measurements ($T$) yield a considerably larger value ($t_{1/2} = 3.17 \pm 0.23$ s) than the backscattering measurements. However, the backscattering measurements with OP ($B_{op}$) show good agreement with the transmission measurements. Even though a somewhat unstable region exists at the low range of the chamber height, the half-time for $B_{op}$ shows good agreement with that for $T$.

4 Discussion

The backscattering analysis with adopting OP technique yielded good agreement with the transmission analysis. These results might give a clue for understanding the differences between backscattering and transmission methods. Because of the characteristics of the OP technique, only the depolarized light was detected, and the polarized light was excluded from the $B_{op}$ analysis. The light that penetrated deeply into the cell suspensions and that underwent multiple scattering became depolarized, whereas the light that reflected off the surface-layer cells and directly returned to the orthogonal polarizer remained polarized. The surface-layer cells are the cells located near the surface of blood samples wherein the incident light begins to penetrate. Recall that the polarized light cannot pass through the orthogonal polarizer. Thus, the difference between $B$ and $B_{op}$ was caused by the elimination of the polarized light that was directly backscattered by the cells of the surface layer. Note that $B_{op}$ indicated lower AIs than $B$.

Therefore, it is necessary to explain why the backscattered light from the surface-layer cells causes the AI to be overestimated in the analysis of $B_{op}$. When red cells are disaggregated, the surface area of the cells is maximized. Most of the incident light may be backscattered from the surface-layer cells, and some from the deep, suspended cells. Thus, any small change of RBC aggregation in the surface-layer cells may affect significantly on the backscattered intensity, relatively. In fact, as the cells start to aggregate, the corresponding surface area decreases and gates of incident light to penetrate open up. Then, the extent of the light that is backscattered from the surface cells may decrease proportional to the surface area, whereas the extent of the light that penetrates through the surface-layer cells may increase and the possibility of backscattering in bulk cells may increase. However, the backscattered light from bulk cells may be blocked by the surface-layer cells. Therefore, the total backscattered light from cells can rapidly decrease when cells start to aggregate. Thus, the effect of surface-layer cells on backscattering may be significant in the initial stages of aggregation, which cause the values of AI and $t_{1/2}$ to be relatively overestimated. Thus, backscattering analysis ($B$) yields a higher aggregation-index than transmission ($T$) and backscattering with OP ($B_{op}$).

The adoption of the OP technique in backscattering analysis may minimize the effect of the behavior of surface-layer cells. In that sense, $B_{op}$ analysis may be very similar to trans-
mission analysis, because both are based on light that is scattered by bulk cells. This may be the reason why the AIs of backscattering with orthogonal polarization ($B_{\Theta}$) and transmission ($T$) decreased with the concentration of dextran, the difference became negligibly small.

The AIs did not significantly vary with the chamber height for $T$ and $B_{\Theta}$. In other words, both $T$ and $B_{\Theta}$ were independent of the chamber height. However, backscattering showed strong dependencies with regard to the chamber height. Because of the nonlinear characteristics of the $B_{\Theta}$ syllectogram, it is difficult to interpret the dependence on the chamber height of backscattering analysis. We note that when the chamber height was small, the AIs of backscattering became the same as those of $B_{\Theta}$ and $T$.

For comparing the sensitivities of the various methods, RBC aggregation was measured by four different methods ($B$, $B_{\Theta}$, $T$, and LORCA) with varying concentrations of dextran in PBS, as shown in Fig. 4. For AI and $t_{1/2}$, the measured results showed a slight difference between $B_{\Theta}$ and $T$, especially at low concentrations (1.5–2%) of the dextran solution. As the dextran concentration increased, the RBC aggregation increased and the difference between $B_{\Theta}$ and $T$ decreased. In addition, for all the methods, there were significant correlations between the dextran concentration and the aggregation parameters. These results showed the same patterns as those measured by LORCA.

5 Conclusion

This paper has described an important methodological study related to the measurement of RBC aggregation. With respect to the light-transmission and light-backscattering methods, this study clearly demonstrated the differences between the data obtained by the two methods, which lead to significantly different calculations of the parameters. Furthermore, the difference was eliminated or minimized if the light reflected from the cells close to the surface was filtered by sending polarized light to the RBC suspension and using an orthogonal polarizer in the pathway of the reflected-scattered light. This is an important finding that may impact on the development of better instruments for measuring RBC aggregation. However, the findings of the present study do not imply that one methodology is more acceptable and valid than the other for testing RBC aggregation in clinical practice. Instead, because the measurements obtained by the two methods yielded comparable results, we reconfirmed that both methods are still valid and acceptable.

Acknowledgments

This work was supported by the Korea Research Foundation (KRF) Grant No. 2009-0080636 funded by the Korea government (MOST).

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