Engineering developments in hemorheology

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Red blood-cell analyzer (LORCA)*

Abstract

The capability of red blood cells to deform is of crucial importance for both macro and microcirculation. A satisfying technique for the measurement of this deformation is lacking so far. We developed and tested in various studies an instrument for automatic measurement of red cell deformability by laser diffractometry. Recently, it appeared that the applicability of the instrument could be extended for measuring another structural hemorheological parameter, red blood cell aggregation. In this communication, a description of the Laser-assisted Optical Rotational Cell Analyzer (LORCA) is given, followed by the general methodology for the measurement of both red blood-cell deformability and aggregation. It demonstrates the practical versatility of this instrument in the field of hemorheology.

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2.1 Introduction

The extreme deformability of red blood cells (RBCs) enables them to pass capillaries with a diameter approximately twice as small as the diameter of the RBC at rest. This property is also responsible for the surprisingly low viscosity at high shear rates in the large arteries, although these cells take up almost 50 volume % of the whole blood. It can therefore be expected that even a slight increase in RBC rigidity may cause important disturbances in both micro and macrocirculation. A large variety of diseases were described in association with less deformable RBCs (for a review, see ref. 64). However, except in some hematological disturbances, it is less clear whether this phenomenon is the cause or the result of the underlying disease. Clinical interventions, like injection with radio contrast media, plasma expanders, drugs, etc. can also affect RBC deformability. Furthermore, hemorheologists are interested in the effect of disturbed RBC deformability on whole blood viscosity under various experimental conditions. Therefore, there is a need for a sensitive and reproducible technique evaluating the capability of RBCs to deform under stress. A survey of various existing techniques can be found elsewhere. Due to its simplicity, filtration of RBC suspensions through filters with 3-5 μm pores has become very popular. Besides the original whole blood filtration technique, the more laborious filtration of washed RBCs (more or less completely depleted of rigid, filter clogging, white blood cells) is performed most frequently. Other variations, regarding the performance of the filtration technique are used, e.g., positive or negative pressure, filters having different numbers of pores varying from many thousands to ca. 30 or to even one single pore and fabricated from various materials. Filterability has also been analyzed by measuring the pressure built up across the membrane during a short or longer period, by the transit time related to a certain volume of blood (detected by light sensors) or by the number of RBCs (detected by the disturbance of an electric field across the membrane). The latter technique, measuring the mean cell transit time, has recently been extended with pulse-shape analysis. The mere fact that so many variations of RBC filtration were and still are described illustrates that this technique is rather cumbersome and full of pitfalls. Especially the variability between the individual filters should worry each investigator. Unfortunately, but this applies to all existing techniques, a calibration standard is lacking.

An instrument, the Ektacytometer, using laser diffraction analysis of RBCs under varying shear stress was commercially available up until a few years ago. Although this instrument had a poor technique for detection of the laser pattern and was not thermostated, it gave the impetus for much excellent
Based on the ektacytometric principle, we have developed a new instrument with a video camera for detection of the laser-diffraction pattern, thermostation unit and an ellipse fit algorithm for calculating the Elongation Index ($EI$). The instrument was initially called LORD (Laser-assisted Optical Rotational Deformability meter). Later it appeared to have a few other applicabilities beyond the measurement of RBC deformability, e.g., quantitative analysis of RBC aggregation indices, RBC relaxation rate, etc. and was therefore renamed LORCA (Laser-assisted Optical Rotational Cell Analyzer). In this communication we describe some characteristics of this instrument in relation to the measurement of both RBC deformability and RBC aggregation indices.

### 2.2 Methods and instrument description

#### 2.2.1 Sample preparation

Human blood was anticoagulated with K$_3$-EDTA (f.c. 4.7 mM), Li-heparin (f.c. 7.5 USP.U/ml) or Na$_2$H-citrate (f.c. 10.5 mM). For measurement of RBC deformability, either 5, 10, 15, 20, 25 or 50 µl of blood was diluted in 5 ml of a solution of 0.14 mM polyvinylpyrrolidone (PVP, M=360 000, Sigma) in phosphate buffered saline (pH 7.4). A large quantity of this PVP - saline solution was prepared and stored in 5-ml portions at −70 °C. The viscosity at 37 °C of the PVP medium was 31 mPa·s.

RBC deformability was also measured in non-anticoagulated blood directly diluted in the PVP medium. PVP - blood mixtures were always prepared immediately prior to the $EI$ measurement. RBC aggregation indices were analyzed in duplicate in whole blood. Prior to the measurements, a volume of blood was oxygenated during 0, 5, 10 or 15 min with at least 3 volumes of air or a 95% O$_2$ - 5% CO$_2$ gas mixture at room temperature on a rollerbank.

#### 2.2.2 Instrument description

The basic instrument, containing the laser, thermostated measuring system, stepper motor and video camera, is shown in Fig. 2.1. The laboratory setup consisted further of an IBM-compatible PC plus printer. Details of the measuring system are depicted in Fig. 2.2. Both concentric cylinders of the Couette system are made of glass.
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Fig. 2.1. Laser-assisted Optical Rotational Cell Analyzer (LORCA). Basic instrument (right), computer monitor (left) showing diffraction pattern of elongated RBC, Elongation Index - stress curve, etc.

The inner cylinder (the bob) can be moved vertically with a lever, after which the outer cylinder (the cup) can be removed for cleaning or replacing. The gap between the cylinders is 0.3 mm and can be filled with ca. 1.5 ml of test suspension, either manually or with a pump, connected to a flush channel. The light source, a diode laser (670 nm, 4 mW), is integrated in the bob, with a prism, scatter intensity sensors and a temperature control unit (set to 37 °C). The measured sample temperature is displayed at the front of the instrument. The cup is driven by a stepper motor (0 - 10 rps, 10 000 steps per revolution), controlled by the computer.

A commercial version of the LORCA is manufactured by R&R Mechatronics, P.O. Box 225, 1620 AE Hoorn, the Netherlands.

2.2.3 Determination of RBC deformability

In the application mode for RBC-deformability measurements (Fig. 2.2), the laser beam traverses the diluted blood suspension and is diffracted by RBCs in the volume. The diffraction pattern is projected on a screen monitored by a CCD-video camera, linked to a frame grabber integrated with the computer. The diffraction pattern is shown on-line, on part of the computer screen.
Fig. 2.2. Schematic drawing of LORCA measuring and detection system for RBC deformability determination.

The gap is filled with 1-2 ml of PVP diluted blood. Each subsequent step of the measuring procedure, viz., filling the cup with PVP diluted blood, adjustment of camera diaphragm, measurement of RBC deformability, printing the results and emptying the cup, is guided by interactive software having a graphical user interface.

The measurement program can be influenced by parameters displayed in an options menu. It is possible to set the shear-stress range at which RBC deformability is measured. The program generates a user-specified number of shear stresses uniformly spaced on a logarithmic scale. The shear stress applied to the cells is calculated by multiplication of the suspending medium viscosity and the mean geometric shear rate ($\gamma_r$) in the gap. The latter, controlled by the rotational speed, is calculated as:

$$\gamma_r = \frac{2\omega (r_c^2 - r_b^2)}{r^2 (r_c^2 - r_b^2)}$$  \hspace{1cm} (2.1)

where $\omega$ = rotational speed, equal to $2\pi N/60$ where $N$ = number of revolutions/min

$r_b, r_c$ = radius of the inner (bob) and inside outer (cup) cylinder, respectively

$r$ = the geometric mean radius of the gap ($\sqrt{r_b r_c}$).

For such small gaps $r^2$ may be equalled to $r_b r_c$ and the equation becomes:

$$\gamma_r = \frac{4\pi (r_c^2 - r_b^2)}{60 (r_c^2 - r_b^2)} N$$  \hspace{1cm} (2.2)

When deforming under increasing shear stress, RBCs change gradually from the biconcave towards a prolate ellipsoid morphology and orient themselves along the flow vector in the gap, i.e., tangential to the axis. This is accompanied
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by a transition from a circular into an elliptic diffraction pattern (Fig. 2.3) which is oriented perpendicular to the orientation of the elongated cells.

\[
\text{Elongation index (EI) } = \frac{A - B}{A + B}
\]

*Fig. 2.3. Change in diffraction pattern during RBC elongation; calculation of elongation index (EI).*

The Elongation Index (EI) is used as a measure of RBC deformability. It is calculated from an isointensity curve in the diffraction pattern using ellipse fitting and is defined as \((A-B)/(A+B)\), where \(A\) and \(B\) are the vertical and horizontal axes of the ellipse, respectively. The EI is determined a user-specified number of times at each shear stress and the mean value is plotted versus the corresponding shear stress (Pa) on the computer screen. Increase of shear stress in logarithmic steps from 0.3 to 53 Pa results initially in a rapid increase of EI until \(\sim 10\) Pa, followed by a slower increment (see Fig. 2.3). For comparison of various blood samples, the near-maximum value of EI at 30 Pa was chosen. The EI for normal cells at rest is zero and may increase to 0.6 at 30 Pa.

The instrument is calibrated with 5 \(\mu\)m diameter polymer microspheres (EI = 0), while an additional calibration can be done with a standard ellipse-pattern put on the screen, having a major \((A)\) and minor \((B)\) axis such that \((A-B)/(A+B)\) is 0.6 \((EI = 0.6)\).
2.2.4 Determination of RBC-aggregation parameters

RBC-aggregation indices are measured with the setup shown in Fig. 2.4. The laser beam is now backscattered by undiluted blood and the intensity of the backscattered light is detected by the photodiodes. The signal is further processed by the computer.

![Schematic drawing of LORCA measuring system for the determination of RBC-aggregation parameters.](image)

This mode of the LORCA has also an interactive graphical user interface. The measuring program includes, aside from the complete aggregation measurement, the following options: RBC disaggregation, syllectogram display and analysis, reiteration procedure to find minimal shear rate necessary to prevent aggregation, aggregation at low shear. Furthermore, there is another option menu for the desired measuring time, sample rate, shear rate, saving and presentation of results, etc.

For measurement of RBC aggregation, the gap is filled with 1-2 ml of oxygenated EDTA-blood. The optional disaggregation shear rate and its duration before the motor abruptly stops are normally set to 500 s\(^{-1}\) and 5 s. Backscattered light is measured by the photo-diode sensors located in the bob. The analog signal is digitized by the computer and expressed in arbitrary units (au).

The Syllectogram (light backscatter versus time, Fig. 2.5), obtained after abruptly stopping the motor, consists of an initial increase in backscatter intensity (upstroke), caused by the loss of elongation and orientation of the RBC, immediately followed by a decrease in the backscatter intensity, due to RBC aggregation. The program also has an option to display the syllectogram with a logarithmic time scale. Figure 2.5 indicates most aggregation indices. The part of the syllectogram between the start of the aggregation \((I_{\text{rop}})\) and the final level corresponding to full aggregation \((I_0)\) are approximated by a bi-exponential curve with a fast and a slow component:
$I(t) = I_0 + I_f \cdot e^{-t/T_f} + I_s \cdot e^{-t/T_s}$, \hspace{1cm} (2.3)

where $I(t)$ and $I_0$ are the intensity of the backscatter intensity at time $t$ and infinity, respectively. $I_f$ and $I_s$ are the initial backscatter intensities of the fast and slow component, and $T_f$ and $T_s$ are the involved time constants. Measuring time is set at 2 minutes since $I(t)$ is usually stable after this period. The following indices are calculated from the syllectogram (Fig. 2.5): $I_{dis}$, $I_{top}$, $t_{top}$, Aggregation Index ($AI$) defined as $[area/A(area(A+B))] \times 100 \%$, Amplitude ($Amp$), $t_{1/2}$ and the upstroke=$I_{top}$-$I_{dis}$.

![Figure 2.5](image-url)

**Fig. 2.5.** RBC aggregation characterized by the syllectogram: lightscatter before and various times after abruptly stopping the motor. RBC morphology corresponding to various parts of the curve is indicated. From left to right: elongated flow orientated and disaggregated RBC, undeformed randomly orientated and disaggregated RBC, RBC aggregated in rouleaux. $t_{AI}$ is normally set at $t_{top}+10$ s. Peak duration is exaggerated.

Furthermore the mean square error (Fit Error), describing the similarity of the measured syllectogram and the syllectogram reconstructed according to the function above, is determined. To get an indication whether there is any additional (secondary) aggregation at low flow, the ratio of $I$ at an optional low shear rate (e.g., $I_{10}$ at 10 s$^{-1}$) and $I$ at stasis ($I_0$) can be calculated. In case of additional flow-induced aggregation, a Flow-to-Stasis Aggregation Ratio (FSAR) value of $<1$ can be expected. To find the threshold shear rate needed to prevent aggregation, $\eta_{max}$, Bauersachs et al.\textsuperscript{6} described a reiteration procedure for the Myrenne aggregometer. We modified this procedure somewhat and
introduced an option to perform a reiteration procedure starting with seven user-defined shear rates, with or without an alternating disaggregation shear rate. A choice can be made between a plot of $I - I_{dis}$ versus the shear rate for determination of $\gamma_{dmin}$ (Fig. 2.6) or $I$ directly versus the shear rate for determination of $\gamma_{max}$ (according to Bauersachs). Since both parameters indicate the same threshold, they will be referred to by us in the future as threshold shear rate or $\gamma_{thr}$. The threshold shear stress can be calculated as $\gamma_{thr}$ times the viscosity at that shear rate.

![Graph](image)

**Fig. 2.6.** RBC aggregation; $\gamma_{thr}$ determination. Result of reiteration procedure (see text); difference of $I$ at various shear rates with $I_{dis}$ plotted versus shear rate.

It may not always be suitable to use the full aggregation program. For routine purposes it is recommended to use the following parameters:

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Extend of aggregation (amplitude)</td>
<td><em>Amp</em> (au)</td>
</tr>
<tr>
<td>2</td>
<td>Kinetics of aggregation</td>
<td>$t_j$ (s)</td>
</tr>
<tr>
<td>3</td>
<td>Aggregation index (extend + kinetics)</td>
<td><em>AI</em> (%)</td>
</tr>
<tr>
<td>4</td>
<td>Tendency for aggregation or strength of formed aggregations (threshold shear stress; $\eta =$ suspension viscosity)</td>
<td>$\gamma_{thr} \times \eta$ (Pa)</td>
</tr>
<tr>
<td>5</td>
<td>Deviation of normal aggregation process</td>
<td><em>Fit Error</em> (au$^2$)</td>
</tr>
</tbody>
</table>

The instrument is calibrated for aggregation measurements with a suspension of polymer microspheres yielding a fixed light scatter.
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2.3 Results

2.3.1 RBC deformability

Effect of anticoagulant
For each of 3 donors, identical EI-stress curves were found in both non-anticoagulated blood and blood anticoagulated with EDTA, heparin or citrate (data not shown). Measuring the EI of the same samples stored for 6 hours at room temperature, but in freshly made dilutions in PVP did not influence the results.

Effect of sample size
As can be seen from table 2.1 there is, numerically, a slight trend for increasing $EI$ at 30 Pa ($EI_{30}$) with increasing sample size up to 20 µl of blood (in 5 ml of PVP) followed by a plateau. Presumably this is caused by a less precise ellipse fit when the diffraction pattern intensity decreases with a decreasing number of RBCs.

<table>
<thead>
<tr>
<th>µl blood</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
<th>30</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>$EI_{30}$</td>
<td>0.570</td>
<td>0.578</td>
<td>0.583</td>
<td>0.584</td>
<td>0.585</td>
<td>0.585</td>
<td>0.585</td>
</tr>
<tr>
<td>$SD$</td>
<td>0.011</td>
<td>0.008</td>
<td>0.005</td>
<td>0.008</td>
<td>0.006</td>
<td>0.006</td>
<td>0.007</td>
</tr>
</tbody>
</table>

Table 2.1. Effect of sample size (µl blood) on measured Elongation Index. Mean value and $SD$ for 6 different healthy donors.

Based on these results it is recommended to use EDTA-anticoagulated blood within 6 hours after venapuncture and to perform the RBC deformability measurement in a freshly prepared dilution of 25 µl of blood in 5 ml of the PVP solution.

Relative error in determination of EI
The relative error ($SD/EI \times 100\%$) of 15 subsequent measurements with 25 µl of the same sample of EDTA-blood diluted in PVP just before the assay, for each of the applied shear stresses is shown in table 2.2. Further results of $EI$ determination with the LORCA, compared to results obtained by RBC filtration, under normal and experimental conditions are reported elsewhere.
Table 2.2. Relative error of Elongation Index measurement at various shear stresses; 15 subsequent measurements of the same RBC-PVP mixture.

2.3.2 RBC-aggregation indices

Effect of anticoagulant

Although there was a tendency for lower values of heparin anticoagulated blood in comparison to EDTA anticoagulated blood with respect to both extend and kinetics of RBC aggregation, the differences were not significant (table 2.3). Since thrombocyte aggregates may be associated with the use of heparin these could interfere with RBC-aggregate formation, which might explain the lower $\gamma_{hr}$-values, i.e., less firm aggregates, found in heparin anticoagulated blood. The decreased $AI$ (lower $Amp$, higher $t_{\%}$) for citrated blood, when compared to EDTA or heparin anticoagulated blood, is undoubtedly mainly due to the 1:9 dilution factor since equal dilution of EDTA-blood with physiological saline yields similar aggregation indices (data not shown).

Table 2.3. Effect of anticoagulant on RBC aggregation indices. Mean of three experiments ±SD.

Oxygenation

The effect of venous blood oxygenation on RBC-aggregation indices is shown in table 2.4. The presence of HbO$_2$ results in increased backscatter intensities and an extended $I$ range. Therefore, all absolute $I$-values alter with changes in the $O_2$-saturation percentage. This also has consequences for the value assigned to area A (Fig. 2.5), which is usually taken as ‘Aggregation Index’ in similar
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studies reported in the literature. Since the value of area $A$ expressed as percentage of area $(A+B)$ is not influenced by % $O_2$ saturation, we decided to use this ratio as Aggregation Index ($AI$). To enable comparison of other aggregation parameters of various blood samples, however, all samples are routinely oxygenated before testing.

$AI = \frac{\text{area } A}{\text{area } (A+B)} \times 100\%$.

<table>
<thead>
<tr>
<th>O$_2$ sat. (%)</th>
<th>$I_{ds}$ (au)</th>
<th>Amp (au)</th>
<th>$T_f$ (sec)</th>
<th>Area $A$ (au*s)</th>
<th>$AI$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venous blood</td>
<td>64</td>
<td>24</td>
<td>13</td>
<td>2.49</td>
<td>62</td>
</tr>
<tr>
<td>Oxygen. blood</td>
<td>99</td>
<td>47</td>
<td>26</td>
<td>2.56</td>
<td>61</td>
</tr>
</tbody>
</table>

Table 2.4. Effect of oxygenation of venous blood on RBC-aggregation indices.

Table 2.5. Effect of incubation time of venous blood with air or a 95% $O_2$ - 5% CO$_2$ mixture on $O_2$ sat % and pO$_2$.

Table 2.5 shows that rotation of a blood sample in a tube with at least 3 volumes of air or a 95% $O_2$ - 5% CO$_2$ mixture for 10 - 15 min on a roller bank is sufficient to achieve a HbO$_2$ saturation of 99-100 %. Based on these results it is recommended to use fresh EDTA anticoagulated blood, which is oxygenated with at least 3 volumes of air during 15 min for measurement of RBC aggregation with the LORCA.

Relative error in determination of aggregation parameters

Relative errors ($SD/\text{mean} \times 100\%$) were calculated for various RBC aggregation indices after 11 subsequent measurements in fresh, oxygenated EDTA blood (table 2.6). Regarding the absolute $I$-values it was noted that, except $I_{ds}$, at least part of the variation is caused by the slight tendency of these values to increase with time. For the recommended parameters the following relative errors were found: $Amp$ 2.8%, $T_f$ 3.1%, $AI$ 3.0%, $\gamma_{th}$ 8.4%, while the Fit-Error range was 0.033 - 0.068 au$^2$.

Further results of RBC-aggregation indices obtained with the LORCA under normal and experimental conditions will be described in a separate communication.
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<table>
<thead>
<tr>
<th>Parameter</th>
<th>Rel err (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$I_{dis}$</td>
<td>7.4(*)</td>
</tr>
<tr>
<td>$I_{top}$</td>
<td>3.8(*)</td>
</tr>
<tr>
<td>$T_{top}$</td>
<td>4.1</td>
</tr>
<tr>
<td>$I_{10}$</td>
<td>4.3(*)</td>
</tr>
<tr>
<td>$I_{o}$</td>
<td>6.9</td>
</tr>
<tr>
<td>FSAR</td>
<td>3.4</td>
</tr>
<tr>
<td>AI</td>
<td>3.0</td>
</tr>
<tr>
<td>AMP</td>
<td>2.8</td>
</tr>
<tr>
<td>$t_{50}$</td>
<td>3.7</td>
</tr>
<tr>
<td>$T_{fast}$</td>
<td>3.1</td>
</tr>
<tr>
<td>$T_{slow}$</td>
<td>7.1</td>
</tr>
<tr>
<td>$y_{hr}$</td>
<td>8.4</td>
</tr>
</tbody>
</table>

Table 2.6. Relative error of RBC aggregation indices (11 subsequent analysis on the same oxygenated EDTA blood). Fit-Error range: 0.033 – 0.068 au. (*)Tendency to increase with time.

2.4 Discussion

The LORCA offers a very reproducible method for the analysis of red blood cell deformability. The most characteristic difference with a similar instrument, the Ektacytometer, is the real time detection of the diffraction pattern by a video camera followed by computerized analysis. Both the added temperature control and the possibility of measuring RBC aggregation indices as well, warrant a high practical versatility of this instrument in the field of hemorheology. Studies on further applications are in progress.
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