

Orientation and elongation of RBC in searle flow in relation to forward scattering

Rainer Bayer^a, Markus Greweling^a, Thomas Wimmer^a, Alexander V. Priezzhev^b

^a Heinrich-Heine-University, Department of Laser Medicine, P.O.Box 1007, 40001 Düsseldorf, FRG

^b Physics Department, Moscow State University, 119899, Moscow, Russia

ABSTRACT

It is well accepted, that in whole blood as well as in blood suspensions light transmission increases, when shear stress is applied. Up to now it is not clear to what extent the changes in forward scattering are related to the orientation of the RBC in flow or to their elongation. If the latter would be true, forward scattering could be used as a simple parameter for RBC deformability. For our present investigation we used the method of laser diffraction in combination with image analysis to determine RBC elongation. Simultaneously forward scattering was measured by a photo detector, placed in the center of the non-diffracted laser beam. When slowly increasing the shear stress from 0-500 dyn/cm² the light intensity measured by the photo detector first increased steeply, reaching a maximum of transmission at about 25 dyn/cm², followed by a mono-exponential (elongation related) decay, reaching a "steady state" at shear stresses producing maximum elongation (450-500 dyn/cm²). But, the decrease of transmission was only present, if the hematocrit (HCT) of the sample was > 0.5%. At a HCT <=0.5%, only an exponential increase of transmission was detected, reaching a „steady state“ at about 25 dyn/cm². In the apparatus used, the orientation of RBC is complete, if a shear stress of 15 to 25 dyn/cm² is applied. Hence, at low shear stresses the increase of transmission is a consequence of RBC orientation. At low HCT, RBC-elongation (due to shear stresses between 25- 500 dyn/cm²) does not influence forward scattering. The elongation related decrease of light transmission observed at high HCT (1%- 6%) may be explained by an increase of the area of deformed RBC, promoting the formation of additional cell layers. As a consequence multiple scattering will reduce transmission. Alternatively, during RBC-elongation the cross section of interaction, comprising absorption and scattering, may be altered.

Keywords: laser diffraction, forward scattering, searle viscometer, erythrocyte deformability, erythrocyte orientation

1. INTRODUCTION

Since the pioneering work of Schmidt-Schönbein^{11, 12} it is well known, that in whole blood as well as in blood suspensions light transmission increases, when shear stress is applied. This property was assumed to be the consequence of desaggregation, orientation and elongation of the RBC. The results presented by the group of Schmidt-Schönbein based on experiments with whole blood or blood, diluted in low viscosity media. Though applying high shear rates, rather low shear stress could be generated (about 20 dyn/cm²).

Previous results² of our group have demonstrated, that the orientation of erythrocytes in a laminar flow is completed if a shear stress >25dyn/cm² is applied and, the elongation coefficient (E) - determined at shear stresses >25 - can be regarded as a parameter of RBC elongation.

When investigating the kinetics of spontaneous aggregation by means of backward scattering, Priezzhev et al.¹⁰ detected a short lasting increase of the backward scattering signal, when suddenly removing a high shear rate from whole blood. Since this signal seemingly was smaller in blood samples drawn from diseased donors, a reduced flexibility of RBC was supposed to underlie this transient. In regard to forward scattering a reversed effect was postulated, i.e. a decrease of light transmission during elongation of RBC. If this holds true - instead of the time consuming determination of the viscoelastic properties of RBC - transmission of sheared blood could be used as a parameter for erythrocyte elongation.

On order to test the hypothesis of A.V. Priezzhev, the viscoelastic properties of human RBC were investigated using laser diffractoscopy (ektacytometry with image analysis) in combination with the detection of forward scattering.

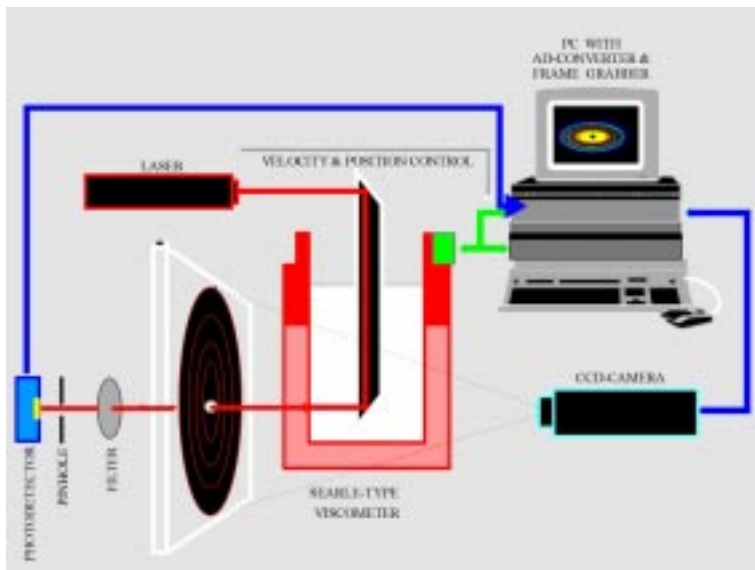


Figure 1: Experimental set-up of the laser diffractoscope (for further explanation see text)

2. MATERIAL AND METHODS

2.1. Blood preparation and solutions

Blood was drawn from the cubital vein of healthy volunteers in heparinized tubes (Vacutainer, Becton & Dickinson). The suspending medium contained 210 g/l dextran (MW 60000, Schiwa) in a MOPS buffered saline (140 mM/l NaCl, 5 mM/l KCl, 5mM/l glucose, 3 mM/l MOPS buffer). The pH was adjusted to 7.4, the viscosity of the suspending medium (22-24 cpoise) controlled with a Hoeppler viscometer (Haake), the osmolarity (305 mOsm) by micro osmometry (Knauer). For measurement of RBC deformability 4 ml of the dextran containing solution and 0.2 ml of blood (hematocrit approx. 2%) were gently mixed by hand or on a roller mixer and filled in the laser diffractoscope. All measurements were carried out at room temperature.

3.2. The laser diffractoscope

The method to measure elongation of RBC by means of laser diffraction has already been described previously¹⁻⁴. Figure 1 shows schematically the experimental set-up. A viscometer is used to produce well defined shear stress within a gap of 0.5 mm between two transparent cylinders. The rotating inner cylinder ($R_i = 24.5$ mm) offers the advantage of effortless gap filling and emptying and, most importantly, the absence of lens effects of the outer ($R_o = 25$ mm), non-rotating cylinder (Searle-system) due to its plane front face. The inner cylinder can be driven with velocities in the range of 0 to 500 rpm, corresponding to shear rates between 0 and 2620 /s. The velocity and the position of the inner cylinder is controlled by a photo detector, in order to gain the current shear rate allowing to shoot the diffraction pattern at a defined position. The laser beam (He-Ne-Laser, 20 mW) is passed into the rotating cylinder via an aluminized dove prism. Undisturbed transmission from the prism to the gap is achieved by phase-matching. The gap is filled with RBC, suspended in an isotonic solution of high viscosity. In this manner, the RBC are exposed to variable shear stress, depending on solution viscosity and shear rate. The diffracted laser beam is projected on a reflection screen and photographed with a CCD camera. The BAS video signal is digitized ("Fast screen machine II", 8 bit, real-time, maximum resolution 736 x 560 pixels) and transferred to a 486 PC for display and further analysis.

As in ektacytometry⁵⁻⁷ the image analysis is based on light- intensity measurement. After A/D conversion discrete intensity values are attached to each pixel, which over a range of 256 intensity steps are linearly related to the intensity of incoming light. The main issue of laser diffractoscopy is to extract intensity information from up to 412.160 points. This offers the opportunity to compare light intensity at different loci and to evaluate areas of selected intensity forming circles or ellipsoids of equal light intensity. These iso-intensity lines represent the geometric form of RBC. Elongated RBC diffract collimated light according to their shape. Circular RBC yield a circular diffraction pattern, elliptical RBC yield an elliptical pattern with the same eccentricity, but rotated by 90°. The diffraction intensity distribution becomes wider as particles get

smaller, the minor axis of an ellipsoid isointensity line corresponding to the major axis of the sheared RBC and vice versa. Since the distribution of points of equal intensity form circles or ellipses a linear correlation can be applied (using the square of loci of each pixel) to determine the parameters of ellipses.

From each diffraction pattern a series of isointensity lines and the corresponding E values are calculated. Circular RBC yield a circular diffraction pattern, elliptical RBC yield an elliptical pattern with the same eccentricity, but rotated by 90°^{8,9}. The diffraction intensity distribution becomes wider as particles get smaller, the minor axis of an ellipsoid isointensity line corresponding to the mean major axis of the sheared RBC and vice versa.

For an elliptical pattern, the extent of elongation is given by

$$\text{elongation [E]} = \frac{\text{major axis} - \text{minor axis}}{\text{major axis} + \text{minor axis}}$$

Due to the noise of CCD chips, the elongation coefficient [E] calculated for the low intensity range includes rather high errors (correlation coefficients for isointensity lines < 0.9000). Close to the center of the diffraction image (high intensity range) the light of the non-diffracted laser beam adds a circular intensity distribution to the elliptical one. Consequently, the inner and outer isointensity lines are not included to determine the average E (with standard deviation SD) for each diffraction picture. The method error for this determination of RBC elongation has been previously shown to be about 0.5-1% (variation coefficient)^{1,3}.

In laminar Searle- (or Couette-) flow the shear stress applied is a function of the viscosity of the suspending fluid, the rotation speed of the outer cylinder and the geometry of the cylinders. From the analysis of error of measurement it emerged that at low shear rates, which correspond to the steep part of the elongation curve, small changes of the gap width or unsteady cylinder rotation are one of the main causes of error¹. Therefore a computer assisted control of motor speed and, most importantly, a position control device were introduced to avoid small fluctuations of gap width and to monitor the rotation of the cylinder continuously. Such, the variation coefficient could be reduced to less than 0.5%.

3.3. Application of tangential mechanical stress

The force produced within the gap of the viscometer can be given in terms of shear stress f [dyne/cm²] which is determined by the product of shear rate [1/s] and viscosity of the suspending medium [cpoise]^{6,7}

In the following experiments the apparatus described here is also used for continuous application of mechanical stress in order to cause a graded damage of RBC^{4,13}.

3.4. Detection of forward scattering

In order to detect forward scattering a hole with a diameter of 4 mm was drilled in the reflection screen, just in the center of the diffraction pattern. 45 cm behind the reflection screen and 1 cm in front of the photo detector (Hamamatsu S 1722-02-1A) a filter holder (with gray filters) and a 2 mm pinhole was placed. An amplifier allowed to control gain and offset of the photo detector output. After AD-conversion the data were transferred to the computer.

4. RESULTS AND DISCUSSION

The gap width within the viscometer is only 0.5 mm. However, due to not perfectly polished surfaces of the cylinders and/or eccentricity of the cylinder axes, small deviations of the gap width may exist¹. In consequence, the thickness of the blood layer and the amount of light transmitted may vary. As shown in the polar plot of figure 2a a maximum of light was detected at a cylinder position of 90°, a minimum with nearly half of maximum at 0°. To avoid erroneous results, the data on forward

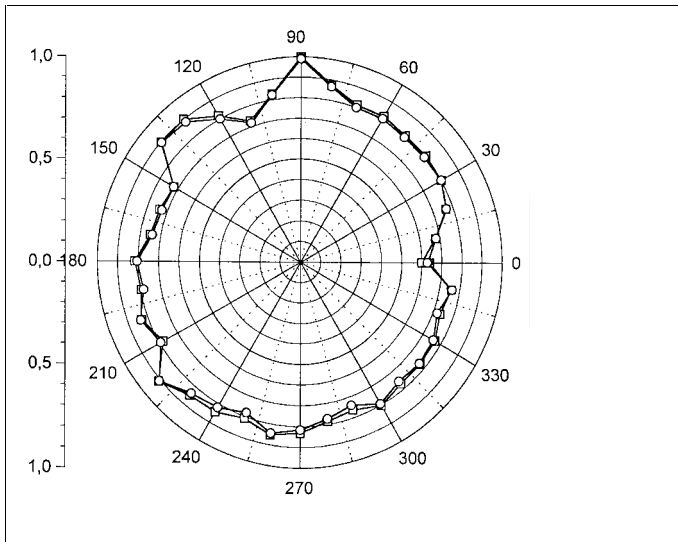
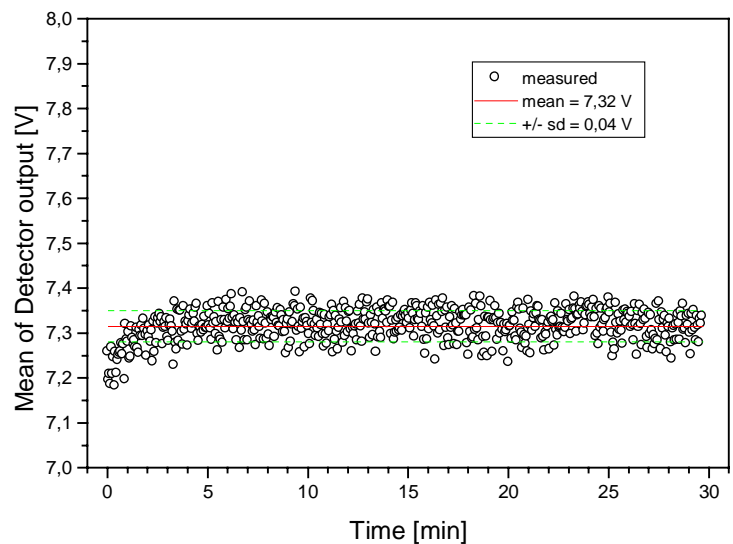


Figure 2a: Polar plot of relative photo detector output as depending on the position of the inner cylinder of the viscometer. The maximum at 90° was set to 1. HCT : 2%, shear stress 54 dyn/cm². Two consecutive measurements

Figure 2b: Stability of the laser. Approximately 5 to 10 minutes after switching on the He-Ne-laser (0 s) the detector output is stable (+/- 0.5%).



scattering shown below each represent mean values of the photo detector outputs, collected during one round of the inner cylinder.

The He-Ne-Laser used was very stable unless it was switched on 5 to 10 minutes prior to the measurements (see figure 2b). Using venous blood, diluted in an aqueous saline, the oxygen saturation of the RBC may vary within the samples used or may be altered during the experimental procedure. Figure 3 shows an example of the photo detector output as depending on the shear stress applied. It is obvious that over the whole range of shear stress the transmission is higher in oxygenated than in partly deoxygenated blood. This is in accordance with the well known light absorbing properties of blood at the wavelength of 633 nm. To exclude effects of O₂-saturation on the light transmitted, all blood samples were oxygenated with pure oxygen for 5 minutes.

In order to measure the elongation of RBC 0.2 ml of whole blood were added to 4 ml of the dextran containing saline. After gently mixing the suspension – usually by shaking the vials manually – it was filled in the viscometer. Repetitive measurements with suspension from the same donor gave an excellent reproducibility (variation coefficient 0.5-1%). However this did not hold true in respect to the data on forward scattering. The filled symbols in figure 4 show the results of two consecutive measurements of the photo detector output as depending on shear stress. Obviously there is a parallel shift of the data. Normalizing the data by setting the maximum photo detector output to “1”, completely removed the difference within the curves. When the samples – instead of manually mixing – were rolled for 10 minutes on a roller mixer (see open symbols in figure 4), no major differences could be detected within repetitive measurements.

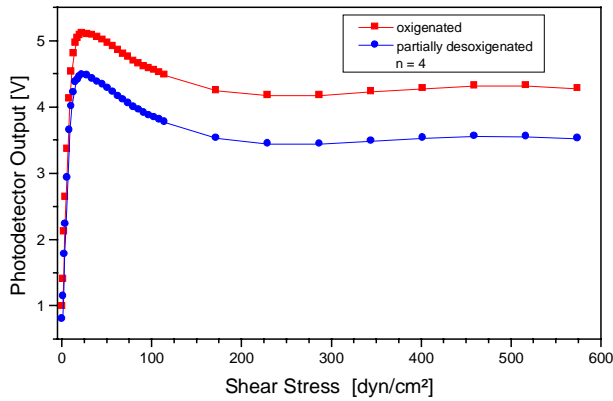


Figure 3: Forward scattering as depending on O₂-saturation. HCT 2%, mean values of n = 4.

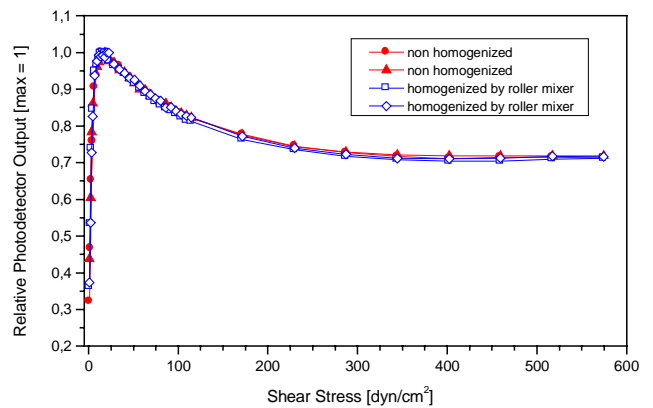
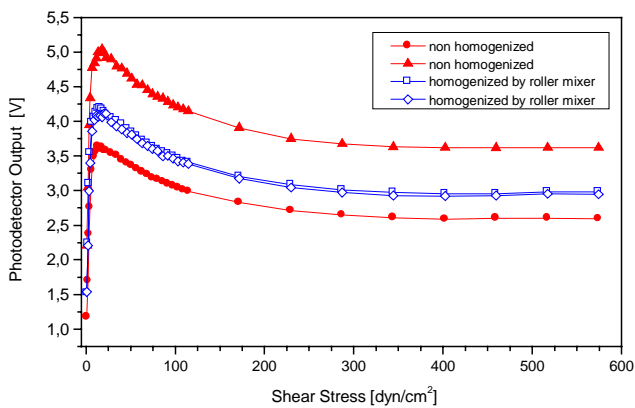


Figure 4: Forward scattering at shear stresses between 1 to 572 dyn/cm². Example for the effect of the mode of mixing the samples before measuring (for further explanation see text).

We assume, that in the shift of the photo detector signal is a result of non homogeneous dispersion of the erythrocytes in the dextran saline. In consequence, to avoid that, all samples were carefully mixed on a roller mixer before exposing them to defined shear stress.

As already described by our group ^{4, 13} RBC can be exposed to defined mechanical stress by exposing the cells within the viscometer to shear stresses exceeding 220-240 dyn/cm². The loss of flexibility depends on the extent (dyn/cm²) and the duration of exposure. It is irreversible and not accompanied by hemolysis. Figure 5 (upper plot) shows the elongation as a function of shear stress applied. Prior to the measurement, the stock RBC suspension of a donor was divided in 7 samples and each of them exposed to a shear stress of different duration but equal extent (512 dyn/cm²).

As plot demonstrates, the longer the erythrocytes were mechanically stressed, the stronger the loss of elongation observed. The forward scattered light, detected by the photo detector is shown in the middle of figure 5. The control curve (0 s mechanical stress) is biphasic (see also figures 3 and 4). Up to a shear stress of 20-25 dyn/cm² there is an evident increase of the photo detector voltage, followed by a comparably small exponential decay. Since results presented earlier ² gave evidence that in the shear stress range up to 25 dyn/cm² the cells become oriented in the laminar flow, whereas the elongated is rather poor, the first part of the function may easily explained by an increase of transmission associated with the orientation. The decay coincides with and increasing elongation of the cells. This finding supports the hypothesis of Priezzhev ¹⁰, predicted from investigations on backward scattering. When damaging the cells two phenomena can be detected: 1) a - more or less parallel - shift to higher (duration of stress 4 to 64 s) or to lower detector outputs (>64 s); 2) a partial to total loss of the decay. The latter becomes more prominent if the photo detector output is normalized by setting the maximum voltage detected within each set of data to "1" (lower plot). The analysis of relative photo detector output reveals, that with the increase of stress duration the amplitude of the decay decreases. Since in parallel the elongation decreases (upper plot of figure 4) the decrease of forward scattering may be taken as a parameter for flexibility. However to quantify this dependence, more experiments are needed.

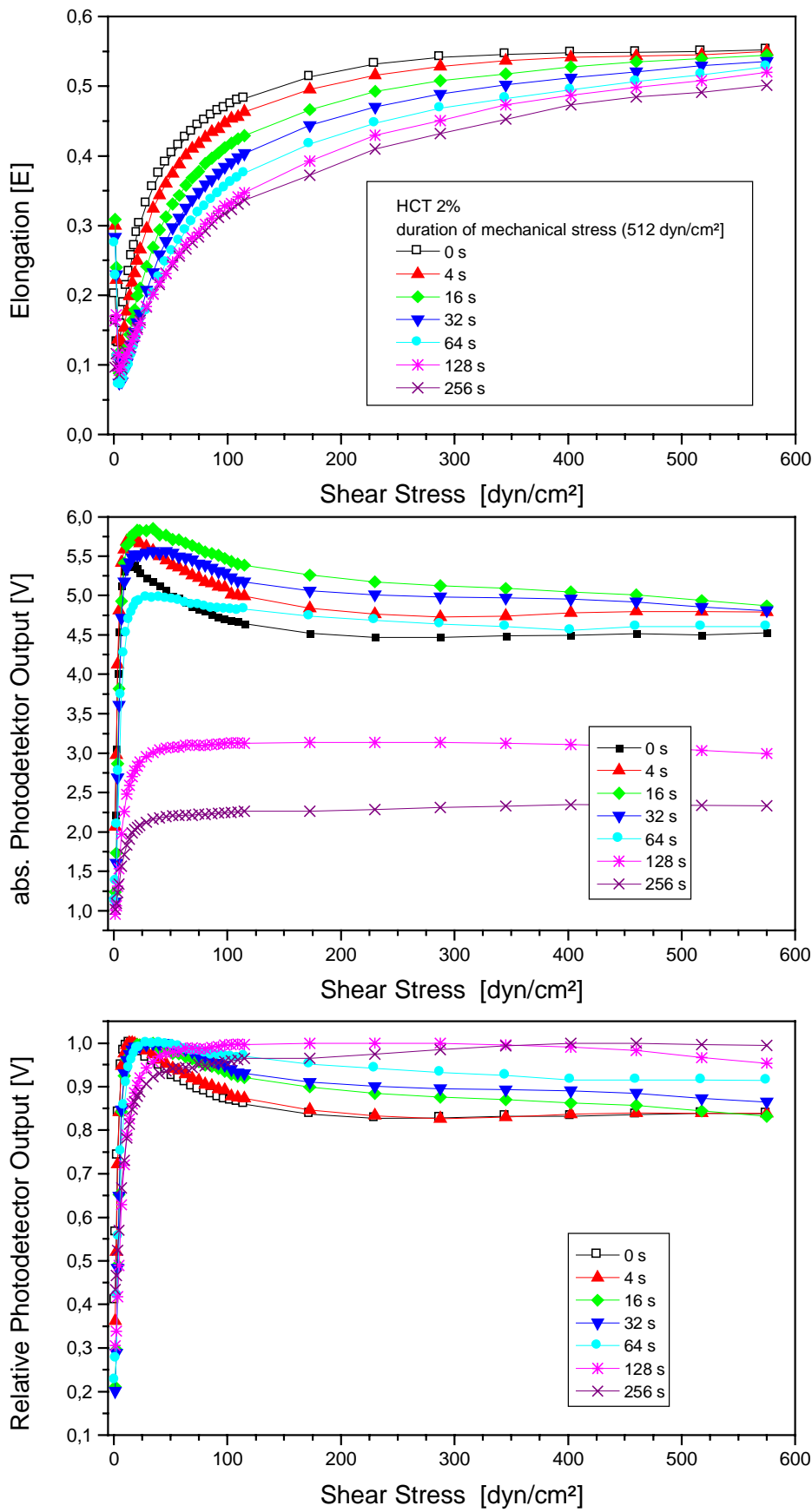


Figure 5. Elongation and forward scattering (absolute and relative photo detector output) after defined mechanical damage of the RBC.

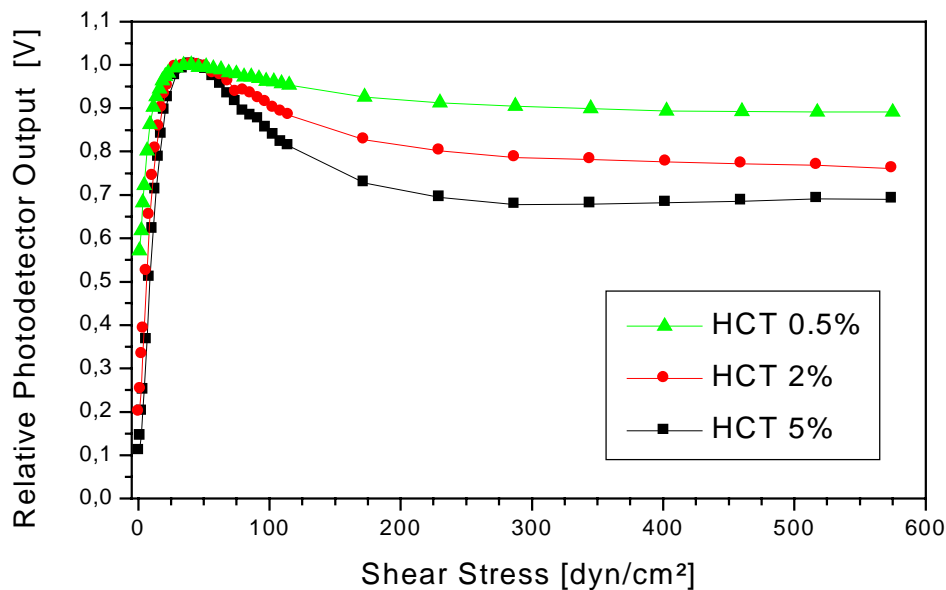


Figure 6: Forward scattering (relative photo detector output) as depending on the hematocrit (HCT) of the suspension

The results presented so far, were done with diluted blood (0.2 ml to 4ml dextran saline) giving a hematocrit of approximately 2%. When the hematocrit is varied, forward scattering as a function of shear stress changes. Since the absolute values of transmission strongly depend on the cell concentration, in figure 6 the relative photo detector output voltage are plotted against shear stress. It is obvious, that the elongation associated decay in the shear stress range of 20 to 572 dyn/cm² is less pronounced, if the hematocrit is lowered. At a hematocrit equal or smaller than 0.5% the decay of the curve disappears. In consequence, when using this low hematocrit, the above described (see figure 5) effect of reduced elongation on the decay is not detectable. On the other hand the data shown in figure 6 may give reference for the mechanism underlying the decay of forward transmission during the elongation of RBC.

We suppose that RBC elongation is accompanied by an increase of the area of the cells. This in turn may promote the formation of additional cell layers. As a consequence multiple scattering will reduce transmission. Alternatively, during RBC-elongation the cross section of interaction, comprising absorption and scattering, may be altered.

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