*Journal of Biophotonics*

JOURNAL OF BIOPHOTONICS

RESEARCH ARTICLE OPEN ACCESS

# Laser Aggregometry Assessment of Blood Microrheology in a Slit Fluidic Channel Covered With Endothelial Cells

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**Received:** 19 August 2024 | **Revised:** 16 September 2024 | **Accepted:** 19 September 2024

**Funding:** This work was supported by Russian Science Foundation Grant No. 22-15-00120.

**Keywords:** blood microrheology | critical shear stress | endothelial cells cultivation | laser aggregometry | microfluidics | red blood cells aggregation

## ABSTRACT

The blood rheology in vitro in glass or plastic microfluidic chips is different from that in vivo in blood vessels with similar geometry. Absence of vascular endothelium is suggested to cause these discrepancies. This work aims to perform in vitro measurements of blood microrheologic parameters in a slit microfluidic channel covered with endothelial cells (HUVEC). The laser aggregometry was employed to measure the intensity of laser light, backscattered from the blood flow, as a function of shear stress to evaluate the hydrodynamic strength of red blood cells (RBC) aggregates in terms of critical shear stress (CSS). The results demonstrated a decrease in CSS accompanied by an increase in the accuracy of its measurement at similar shear stresses when endothelial cells were present in the channel. The findings hold valuable implications for advanced approaches for endothelization of microfluidic devices, facilitating the study of blood flow dynamics in physiologically more relevant environment.

## 1 | Introduction

Blood circulates throughout the body via a complex network of tube-like vessels, which permeate the tissues and organs, facilitating the transport of respiratory gases and products of cellular metabolism. The rheological behavior of blood significantly influences the efficiency of its transport functions [1]. In addition to the geometric characteristics of the blood vessels that affect the flow velocity profiles, blood rheology on the microscale level (microrheology) is influenced by the aggregation and deformation properties of red blood cells (erythrocytes, RBC). They constitute the most abundant cellular fraction of blood and determine its non-Newtonian character [2].

The ability of RBC to form aggregates upon membrane contact (RBC aggregation) is a significant aspect of blood microrheology modulation at low shear rates [3]. When shear rates are insufficient to disperse aggregates, RBC aggregation promotes the axial migration of RBC clusters into the central area of large vessels decreasing the frictional flow resistance [4]. However, significant enhancement of RBC aggregation observed in diabetes mellitus [5, 6], arterial hypertension [7], inflammation [8, 9], hereditary hematological pathologies [10] leads to a pathological increase in blood apparent viscosity, potentially resulting in vaso-occlusions. Therefore, the assessment of RBC aggregation within microcirculation is essential for preventing irreversible tissue necrotic damage caused by oxygen supply deficiencies due to microrheological abnormalities, described above.

The laser aggregometry technique is a photometric approach used to quantitatively assess RBC aggregation [11]. This technique monitors changes in the intensity of laser light scattered

**Abbreviations:** CSS, critical shear stress; HUVEC, human umbilical vein endothelial cells; RBC, red blood cells; TNFα, tissue necrosis factor alpha.

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from whole blood samples, depending on the size of RBC aggregates in the sample. Several aggregometers were commercially proposed [11], each with its own experimental chamber geometry. The Laser-assisted Optical Rotational Cell Analyzer (LORRCA, RR Mechatronics, Hoorn, the Netherlands) [12] utilizes a Couette system for shearing RBC suspensions. The chamber consists of a rotating glass cup and a precisely fitting coaxial bob, through which a laser beam is directed, and the intensity of backscattered light is detected. Myrenne aggregometer (MA-1, Myrenne GmbH, Roetgen, Germany) [13] measures forward scattering during light transmission through a blood suspension, placed inside a cone-plate shearing system composed of two transparent plastic disks, one of which is static while the other one rotates. The RheoScan AnD-300 aggregometer (Rheomeditech, Seoul, Korea) offers an alternative approach, utilizing an experimental chamber composed of a disposable transparent plastic microfluidic chip connected to a pumping device serving as a shearing system for assessing RBC aggregation under flow conditions [14].

The advantages of employing plastic microfluidic chips for diagnosing blood rheology have been convincingly demonstrated [15, 16]. Microchannels within these chips can mimic the dimensions and structure of blood vessels. This allows for more relevant modeling of tissue perfusion. Microchannels also provide precise control over flow conditions, enabling the investigation of blood microrheology under various shear rates and flow patterns. The miniaturization of microfluidic chips makes them suitable for compact, portable medical devices. Additionally, microfluidic chips operate with smaller volumes of liquids, leading to a significant reduction in reagent and biomaterial consumption. This reduction in volume also lowers the risk of contamination and minimizes sample loss during research. However, the rheological behavior of blood in such chips in vitro is different from that in vivo. One of the main challenges still not explained is that flow resistance in microcapillaries in vivo is higher than in vitro in similar vessels made of plastic or glass [17]. Vascular endothelium, which is, the thin layer of cells forming the interior surface of blood vessels, is suggested to cause these discrepancies [18].

The aim of the present study was to evaluate the rheological impact of endothelial cells on the blood flow in the slit microfluidic channel of the K-01 chip, utilized in the RheoScan AnD-300, by assessing changes in the hydrodynamic strength of RBC aggregates under shearing flow in norm and in pathology (arterial hypertension).

## 2 | Materials and Methods

### 2.1 | Blood Sampling

The experiment involved the blood samples of six healthy male donors (aged 20–30 years) and five donors with arterial hypertension (AH). The AH group included donors of both genders aged over 54 years. Anthropometric data on the AH donors, as well as clinical information about their comorbidities and corresponding medicine that constitutes their conservative therapy, are provided in the supporting information (Table S1).

The blood from cubital vein was sampled into Vacuette EDTA K3E tubes. Scientific research using donors blood samples was performed according to the Permission 11/22 dated 05.12.2022 issued by Ethical Committee of International Educational Medical Center of M.V. Lomonosov Moscow State University. Informed consent was obtained from all subjects involved in the study. All manipulations were carried out in accordance with Declaration of Helsinki.

### 2.2 | Cultivation of Human Umbilical Vein Endothelial Cells (HUVEC)

Human umbilical vein endothelial cells (HUVEC) were provided by unique scientific installation "Collection of cell cultures" of the Center for Collective Use of the Koltzov Institute of Developmental Biology of Russian Academy of Sciences (Moscow, Russia). The HUVEC cells were cultured under standard cultivation conditions (incubation at 37°C and 5%-$CO_2$) in M199 medium supplemented with Earle's salts (PanEco, Russia), containing 10% FBS (fetal bovine serum, HyClone, Cytiva, USA), 1× Glutamax (Gibco, Thermo Fischer, USA), 1× ITS (insulin-transferrin-selen, Gibco), 10 ng/mL EGF (Invitrogen, USA), 10 ng/mL FGF (PeproTech, Thermo Fischer, USA), and 8000 U/mL heparin (Sigma Aldrich, USA). The cells were cultured in collagen-coated culture flasks. Upon reaching confluence, the cells were passaged using Versene solution (PanEco) and 0.05% trypsin-EDTA (Gibco). Cells from passages 2–6 were used for experiments.

For laser aggregometry measurements, the suspension of the HUVEC was injected into the microchannel of the K-01 chip pre-coated with type I collagen. Prior to the cells injection, the chips were sterilized with UV-irradiation. After the injection, the chips were held in the incubator for several hours to let all the cells in the suspension attach to the surface of the channel. During the first 30 min of the incubation period, the chip has been periodically rotated to maintain the homogeneous coverage the channel surfaces. To initiate the activation of endothelium (i.e., simulating inflammatory conditions), HUVEC were cultured inside the channel of the chip in the medium containing 20 ng/mL TNFα for at least 16 h.

### 2.3 | Laser Aggregometry Measurements

Laser aggregometry measurements were performed in microfluidic chips K-01 utilized in aggregometer RheoScan AnD-300. The schematic lay-out of the chip and the geometric characteristics of the slit microchannel are provided in Figure 1A. The plastic microchip is comprised of two reservoirs connected with a slit microchannel. Figure 1B provides an explanation of the measurement procedure. The chip was filled with 500 μL of a donor's blood and plugged to the pump device which can apply pressure difference between the two reservoirs. The central part of the slit microchannel was illuminated with a laser diode ($\lambda = 635$ nm, power 1.5 mW). When the measurement starts, the pressure impulse is applied and the blood suspension begins to flow through the microchannel. During the measurements, time dependencies of the shear stress in the microchannel and the intensity of the light, backscattered from the blood stream inside the microchannel, are monitored. The curve of

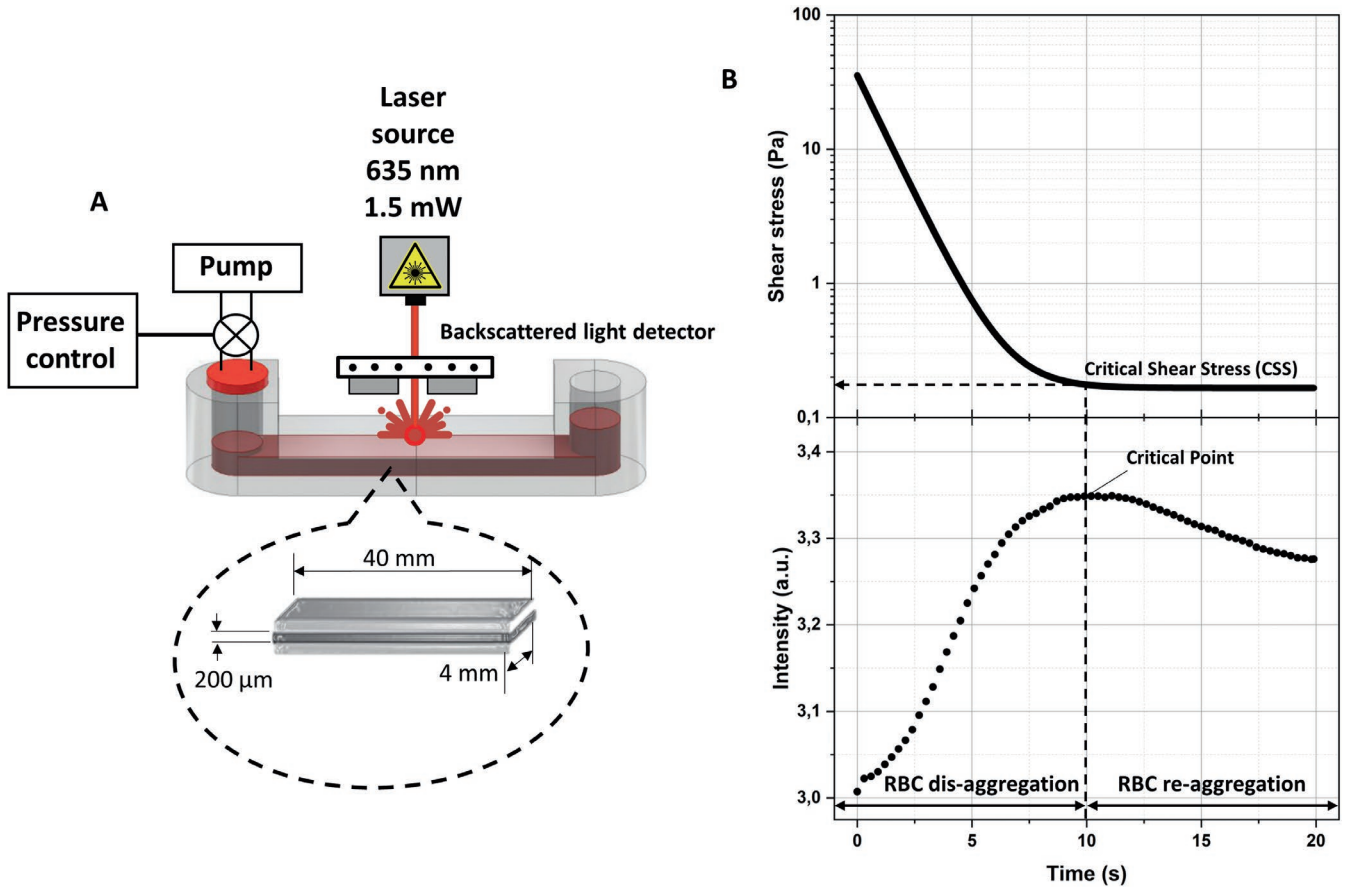


**FIGURE 1** | Laser aggregometry using the microfluidic chip K-01 utilized in RheoScan AnD-300 (RheoMeditech, Korea). (A) Schematic layout and the geometrical characteristics of the experimental chip with the microfluidic channel involving measuring the backscattered light intensity; (B) the time dependencies of shear stress inside the channel (upper graph) and backscattered light intensity (lower graph) with indicated areas, related to the processes of "RBC disaggregation" and "RBC re-aggregation." A critical point between these two processes corresponds to hydrodynamic strength of aggregates in term of critical shear stress (CSS).

back-scattered light intensity time dependence referred to as syllectogram has a specific profile: it has the area where the intensity grows and the area where the intensity decreases. The first area corresponds to the time period when the shearing forces inside the channel are high enough to disperse the RBC aggregates in the blood flow. Thus, the number of aggregates decreases and the sizes of the prevailing scattering objects, single cells, become smaller than those of the aggregates. In this case, the intensity of light backscattered from the microchannel becomes higher, so the output signal of the detector increases. As the pressure difference comes to zero, the shearing forces inside the channel are not sufficient enough to suppress RBC aggregation. RBC starts to aggregate, and therefore the size of the scattering objects increases. That means that during this period of the measurement the intensity of light scattered in forward direction increases and the output signal of the detector decreases. The extremum of this profile corresponds to the critical point when two opposing processes—disaggregation and aggregation—are in equilibrium. Evaluating the value of the critical time, one can assess critical shear stress (CSS)—the minimal shear stress required to prevent RBC from aggregation [19]. Thus, CSS characterizes the hydrodynamic strength of RBC in the blood suspension. More details are available in the publications of the RheoScan developers group [14, 20].

### 2.4 | Statistical Data Analysis

For the processing and plotting of all data, developed applications in python language were used. In the results section, the box plots in the figures show the first quartile (Q1) to the third quartile (Q3) of the data, with a median line inside. The whiskers represent the standard deviations with the mean values (white diamond-shaped dots). For independent data samples, Mann–Whitney *U* test was performed to assess the statistical significance of differences between samples. For dependent data samples, Wilcoxon signed-rank test was carried out to assess the statistical significance of differences between samples. Two samples were significantly different if the *p* value was less than 0.05.

## 3 | Results

### 3.1 | Viability of the Endothelial Cells, Cultured in the Slit Microfluidic Channel

Figure 2A demonstrates the transmittance microscopy images of HUVEC, covering the surfaces of the microchannel of the K-01 fluidic chip. Figure 2B demonstrates the results of the viability test of HUVEC inside the microchannel. The assay was performed using cell-staining with Calcein-AM

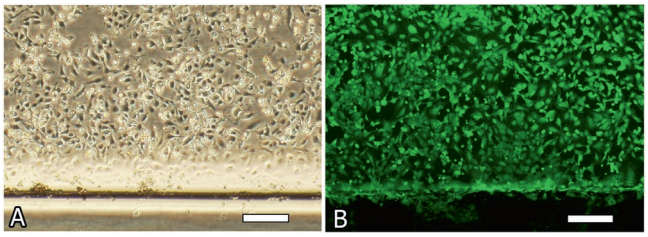


**FIGURE 2** | Results of the viability test of endothelium cells (HUVEC), cultivated in the slit microchannel of the fluidic chip K-01, utilized in the commercial medical device RheoScan AnD-300 (RheoMeditech, Korea). (A) Transmittance microscopy image of the endothelium coverage of the microchannel; (B) fluorescence microscopy image of the endothelium cells, stained with Calcein AM, tracking live cells in the microchannel. Measure bar corresponds to 200 μm.

(calcein-acetoxymethyl ester), generally used as a live/dead assay [21]. Calcein-AM is a non-fluorescent compound that can penetrate the cell membrane. In live cells it is hydrolyzed by endogenous esterases, forming calcein, a highly fluorescent green product.

One can see that HUVEC, which cover the surfaces of the microchannel, exhibit intense fluorescence (Figure 2B) due to intracellular accumulation of calcein, indicating that they are in the physiologically normal state without apoptotic alterations. More microscopic images of the endothelial cells, cultivated inside the fluidic channel, are available in supporting information (Figures S1 and S2).

### 3.2 | Impact of Endothelial Cells (HUVEC) Cultivation on the Blood Flow in the Slit Microfluidic Channel

Figure 3 demonstrates the results of the impact of the modification of the microchannel by HUVEC cultivation on the CSS values. Prior to the insertion of blood into the fluidic chip, its microchannel was pipetted thoroughly to get rid of the growth media and endothelial cells, which did not attach to the channel surface.

It is seen that CSS values of the blood of donors, suffering from arterial hypertension, were statistically significantly higher than CSS, measured in blood samples of healthy donors: 209 ± 52 mPa in healthy blood versus 306 ± 69 mPa in AH (w/o HUVEC coverage) and 142 ± 44 mPa in healthy blood versus 234 ± 47 mPa in AH (with HUVEC coverage). While the increase was observed with and without HUVEC cultivation, in case of the presence of endothelium the statistical significance of this effect was two times higher according to the Mann–Whitney *U* test. It indicates that the cultivation of endothelium cells in the fluidic channel allows to significantly improve the sensitivity of laser aggregometry to microrheological disturbances of the blood flow, which accompany arterial hypertension. The observed results correspond with the data on changes in apparent viscosity of blood of patients, suffering from AH. We measured the viscosity of blood in the control group and group of patients suffering from AH by rotational viscometer RM100 CP1000 (Lamy Rheology, France; acquired in the frame of M.V. Lomonosov Moscow State University Development Program). It was obtained that the whole blood apparent viscosity

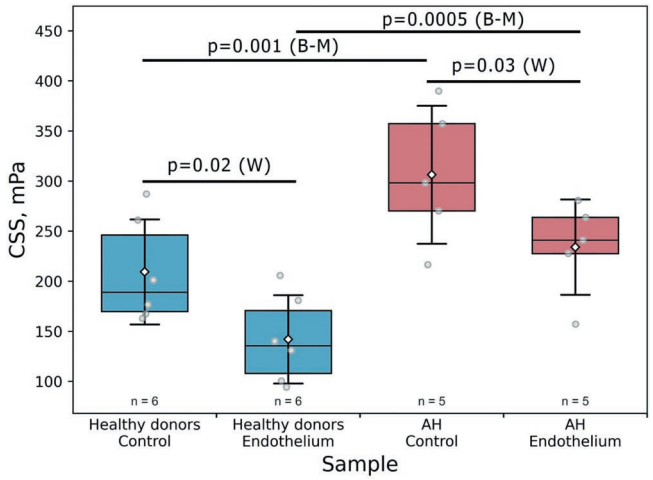


**FIGURE 3** | Impact of endothelial cells (HUVEC) cultivation inside microfluidic channel on the critical shear stress (CSS) of the whole blood of healthy individuals and patients suffering from arterial hypertension (AH). Each point represents the average value of CSS of one individual. M.W., Mann–Whitney *U* test; W, Wilcoxon signed-rank test.

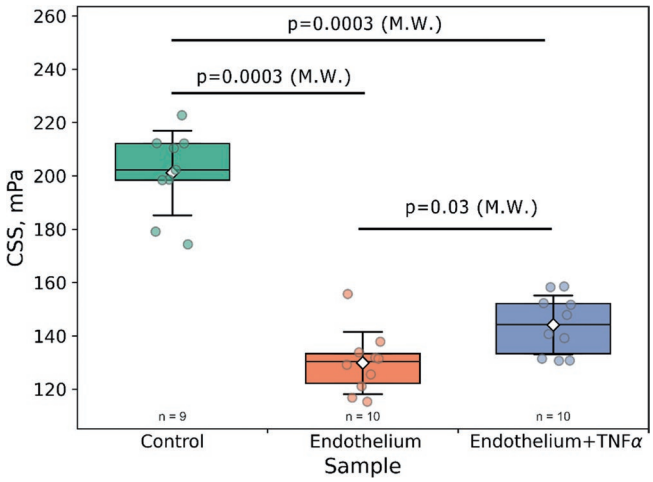


**FIGURE 4** | Impact of the TNFα activation of endothelial cells (HUVEC), cultivated inside microfluidic channel of chip K-01, on the critical shear stress (CSS) of the whole blood of a healthy individual. Each point represents one CSS measurement of the individual. M.W., Mann–Whitney *U* test.

in case of AH was elevated to the value 6.8 ± 1.9 mPa·s in comparison with the control group value of 4.7 ± 0.7 mPa·s under measured rotational speed 1000 1/s. The increase in the viscosity may be associated with an increase in the hydrodynamical strength of RBC aggregates in the case of AH, as was observed in this work in laser aggregometry measurements.

The coverage of microchannel with endothelium surprisingly led to the statistically significant decrease in CSS in both norm and arterial hypertension blood samples. We examined this phenomenon in more details using only blood of a healthy donor. We also made additional CSS measurements in microchannels, modified with TNFα activated endothelial cells. The results are demonstrated in Figure 4. Presence of HUVEC in the fluidic channel again resulted in significant decrease in CSS in comparison with bare chips (from 219 ± 57 to 131 ± 11 mPa),

and the activation of HUVEC with TNFα did not change the trend whereas the average CSS increased to a smaller extent (144 ± 10 mPa) in comparison with intact endothelium.

## 4 | Discussion

Despite the numerous advantages of microfluidic chips, their application in hematology remains limited. One of the most significant limiting factors is the absence of a physiologically relevant environment for RBC when studying blood flow in the channels of plastic microchips. The blood flow within microchip channels deviates significantly from that in physiological conditions due to the inherent interactions between blood and the polymer material composing the chip. In particular, the commonly used polymer polydimethylsiloxane (PDMS) has a high capacity for adsorbing hydrophobic molecules onto its surface [22]. This phenomenon can influence blood rheology by affecting interactions between blood cells in close proximity to the channel surface. The use of less hydrophobic materials such as polymethyl methacrylate, polystyrene, cyclic olefin copolymer, or glass only partially mitigates these undesired effects. Cultivating vascular endothelial cells in microchannels is a valuable approach to overcome this obstacle, as it allows for a physiologically more relevant environment for the blood flow [18, 23]. In our experiments, HUVEC effectively attached to the surface of the channel with the preliminary exposition of the channel to collagen, although usage of other adhesive compounds such as fibronectin or gelatin is possible [24]. Application of external shearing stress when measuring RBC aggregation/disaggregation properties under the flow conditions did not affect physiological state of endothelial cells, cultured in the microfluidic channel. Finally, the cultivation of endothelium in the microchannel of the fluidic chip K-01 for RheoScan AnD-300 aggregometer significantly increased the sensitivity and precision of the measurements of CSS as a parameter of hydrodynamic strength of RBC aggregates in the blood of donors with arterial hypertension. We suggest that the observed improvement in measurement quality, expressed by the reduction in the deviations of experimental data, was a result of creating more optimal hydrodynamic conditions for blood flow in the microchip with slit rectangular geometry. However, the absence of complete understanding of the mechanisms, underlying the impact of endothelium on blood rheology, still stands as an important limitation factor.

Considering results of the laser aggregometry and rheometric measurements, the blood of arterial hypertension patients is characterized with increased apparent viscosity and increased CSS. Meanwhile, coverage of the surfaces of the slit microchannel with HUVEC decreased CSS in the flow of blood of both healthy and arterial hypertension donors (Figure 3). The similar effects were observed upon activation of endothelium with TNFα (Figure 4). Such effects cannot be caused by the dilution of the blood by the presence of the growth media and/or detached endotheliocytes since CSS does not depend on the hematocrit [14]. Hence, the role of variations in the number of RBC within the blood samples can be disregarded.

Another possible mechanism might be the decrease in the height of the lumen of the microchannel upon culturing HUVEC inside. The thickness of HUVEC monolayer is generally does not exceed 1–2 μm [25]. The RheoScan AnD-300 device is operated with the software, which considers the role of variations in the channel height dimensions in the value of wall shear stress [20]. According to the calculation formula, described in [20], the presence of HUVEC monolayer in the channel with 200 μm height would lead only to 1%-variation in values of wall-shear stress. However, one still should consider the role of the endothelium thickness when experimenting with endothelialized microchannels with higher thickness of the endothelial monolayer.

We also analyzed separately the regions of syllectogram, corresponding to disaggregation (Figure S3A) and re-aggregation (Figure S3B) of RBC upon the blood flow in the fluidic channel. It is seen that during RBC disaggregation, the influence of endothelium coverage is pronounced during first stage of the measurements when the shearing rates are high (first 4 s). It should be noted that activation of endothelium did not change the profile of the backscattered light during the disaggregation stage. At lower shearing rates one can observe the slight impact of endothelium activation even earlier, during first 2–3 s of the re-aggregation. This stage is usually associated with the formation of linear aggregates [26]. Since these are very short-scale time periods, the influence of endothelium in terms of releasing gas transmitters and other substrates is negligible. However, the rheological impact of some substances, secreted by endothelial cells, should be considered in specific cases, when rheological measurements are performed on the longer time scales. For instance, arachidonic acid derivatives prostaglandins PGE1 and PGE2, and prostacyclins PGI2 not only modulate vascular permeability and promote fluid extravasation into tissues to mediate inflammatory processes and allergic reactions, but also exert microrheological effects, influencing the aggregation and deformability of RBC [27].

We suppose that the nature of the observed decrease in CSS is associated with the changes in the velocity profile, and hence the shear stress profile, of the flow caused by the presence of endothelium and its glycocalyx. The results of the several in silico investigations [28–31] suggested that endothelialization of microchannels leads to reduced mean blood flow velocity and, importantly, increased wall shear stress. In particular, according to the blood flow modeling performed in [29], decrease in mean blood flow velocity in endothelialized channels compared with a smooth surface (i.e., glass or plastic) is caused by the roughness of endothelium. Authors suggested that the mean shear stress in a fluidic channel with endothelial cells coverage also increases with the magnitude of the endothelium shape fluctuations. We suppose these observations underlie the mechanisms of the observed CSS decrease: when endothelium is cultivated in the slit microchannel, it dominantly increases the wall shear stress, therefore supporting an addition to the shearing force, aiding to disperse RBC aggregates.

We performed calculations of the shear stress in the slit channel, which geometry parameters are identical to those of K-01 microchip, with smooth surface and with endothelial-like surface, using COMSOL Multiphysics software Version 6.1 (COMSOL Inc., Sweden), in which the solutions to

Navier–Stokes equations for steady flow were obtained. The detailed description of the modelling and its results are provided in the supporting information (Figure S4). It is important to note that the modeling did not consider the interaction of cells with the wall/endothelium-like surface. It can be seen that while the mean average values of the shear stress were the same for both smooth and endothelial-like surface, the variations in the wall shear stress were 2–2.5 times higher in case of rough endothelial-alike surface in comparison with smooth surface. Thus, the presence of the rough endothelium led to the increased wall shear stress in certain points of the surface. In [32], it was shown that backscattering of light is predominantly affected by wall shear stress rather than bulk volume effects. This means that in the experimental setup implemented in the RheoScan And-300 with the K-01 chip, the main contribution to the scattering pattern comes from the RBC near the top surface of the chip. Thus, the presence of endothelium at the upper wall of the channel contributes to multiple regions of high and low shear stress, which apparently aids to disperse RBC aggregates under the flow. The latter resulted in the decreased CSS values, observed in the experiment for both healthy norm and arterial hypertension groups. Further studies are needed for the detailed investigation of this phenomenon.

Since the endothelization of the fluidic channels is capable of affecting the microrheological behavior of blood, the role of the direct cell–cell interaction between endothelial cells and RBC should not be neglected. Series of the experimental evidence demonstrated an increased RBC adherence to the endothelium in many diseases, such as, diabetes mellitus, hypertension, hemoglobinopathies, inflammation, sickle cell disease, malaria, and hereditary hematological pathologies [32–36]. To date the molecular mechanisms of the RBC adhesion are far from being understood completely. Enhanced RBC adhesion can be driven selectively by specific metabolic cascades, in particular, adenylyl cyclase signaling cascade can selectively promote RBC adhesion by upregulating Lu/BCAM (Lutheran [Lu] blood group Basal Cell Adhesion Molecule) [37] or can be mediated non-selectively by the blood plasma proteins, in particular, fibrinogen [38]. In our previous works [39, 40], it was demonstrated that the RBC-endothelial cells interaction force, measured by optical tweezers, depends on the concentration of fibrinogen in the blood plasma. At present, the impact of the enhanced RBC-endothelial cells interaction on the blood microrheology is poorly investigated neither in the case of endothelium activation nor in the case of stimulation of RBC intracellular signaling cascades.

## 5 | Conclusion

Cultivation of endothelial cells provides novel opportunities for the laser aggregometry in vitro assessment of the blood microrheological behavior using fluidic biochips. The complex approaches of culture and maintaining endothelium cells in the fluidic channels allow to investigate how the local environment affects the blood flow in physiologically more relevant scenarios and therefore opens new possibilities in studies of the blood microrheology in norm and pathology.

Modulating the interactions between blood cells and endothelial cells can significantly influence blood rheology, potentially affecting flow velocity, shear stress, and inducing spatial variations in the formation of the marginal cell-free layer near blood vessel walls. Understanding the diverse molecular mechanisms of interaction between RBC and endothelium cells enables the manipulation of blood cell adhesion levels in vitro, ranging from physiological to highly elevated conditions. This capability, facilitated by microfluidics, offers unique opportunities to model microrheological abnormalities in physiologically more relevant environment.

### Author Contributions

**Alexey N. Semenov:** conceptualization, investigations, data analysis, and original draft writing. **Andrei E. Lugovtsov:** project management and data curation. **Petr B. Ermolinskiy:** investigations, statistical analysis, computing and modeling, and draft editing. **Alexander V. Priezzhev:** conceptualization, project management, writing – review, and editing.


### Acknowledgments

This work was supported by the Russian Science Foundation Grant No. 22-15-00120. The authors are grateful to Dr. Ekaterina Kiseleva from the Koltsov Institute of Developmental Biology (RAS, Moscow, Russia) for the endothelial cells supply and Arseniy Kapkov for his help in experimental measurements. Open Access funding enabled and organized by Projekt DEAL.


### Conflicts of Interest

The authors declare no conflicts of interest.

### Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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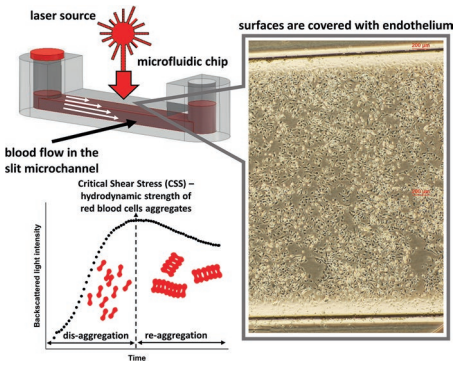


Microrheological behavior of blood flow in the slit channel of the plastic microfluidic chip with endothelium (HUVEC)-covered surfaces is investigated. The channel is illuminated with the laser light. The time-dependence of backscattered light intensity is measured. The critical point between disaggregation and reaggregation of erythrocytes corresponds to the critical shear stress (CSS), that is, the hydrodynamic strength of erythrocytes aggregates. Presence of endothelial cells decreased CSS, demonstrating its impact on blood microrheology.