The plasma protein fibrinogen stabilizes clusters of red blood cells in microcapillary flows

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The supply of oxygen and nutrients and the disposal of metabolic waste in the organs depend strongly on how blood, especially red blood cells, flow through the microvascular network. Macromolecular plasma proteins such as fibrinogen cause red blood cells to form large aggregates, called rouleaux, which are usually assumed to be disaggregated in the circulation due to the shear forces present in bulk flow. This leads to the assumption that rouleaux formation is only relevant in the venule network and in arterioles at low shear rates or stasis. Thanks to an excellent agreement between combined experimental and numerical approaches, we show that despite the large shear rates present in microcapillaries, the presence of either fibrinogen or the synthetic polymer dextran leads to an enhanced formation of robust clusters of red blood cells, even at haematocrits as low as 1%. Robust aggregates are shown to exist in microcapillaries even for fibrinogen concentrations within the healthy physiological range. These persistent aggregates should strongly affect cell distribution and blood perfusion in the microvasculature, with putative implications for blood disorders even within apparently asymptomatic subjects.

The physical mechanism of the plasma protein-induced aggregation process is controversial1–5. Although the first studies favoured a model based on bridging6–9, a model based on depletion was also introduced10–13. The strength of aggregation depends on the physical properties of red blood cells (RBCs), such as deformability14,15 and surface charge16. In blood plasma, fibrinogen has been identified already in the 1960’s as the main protein responsible for aggregation7,17–19. In pathological cases such as cardiac diseases20 or sepsis21, both an increased level of fibrinogen and an accumulation of RBC aggregates with more robust clusters have been found. In addition to fibrinogen, model polymers such as dextran can be used to induce aggregation. Recently, the interaction strength between two RBCs was determined at various dextran concentrations using single cell force spectroscopy13. An increase in dextran concentration has been found to initially induce an increase in the interaction strength among RBCs, which is abolished at high concentrations.

Because the viscosity of blood from healthy donors exhibits strong shear thinning up to shear rates of approximately 100 s⁻¹, when all aggregates are broken up12, one could expect aggregation to be negligible in the microcirculation where average shear rates are higher. Nevertheless, about 80% of the total pressure drop in blood circulation occurs in the vascular network8 that irrigates organs in which oxygen and nutrients are exchanged, and contradictory results have been reported on the effect of aggregation in glass capillaries of 30 to 130 μm diameter23,24 and in vivo vascular networks; both higher and lower flow resistances are reported after the addition of aggregating agents22. Two effects appear to counteract each other: first, the aggregated cells appear to align more strongly along the centre line, thus decreasing the flow resistance; however, blocking might occur at the entrance of the capillary network, thereby increasing the pressure drop. Interestingly, athletic mammalian species exhibit higher RBC aggregability1,25, which raises the question whether aggregation might indeed be a way to enhance RBC throughput in the circulation and thus improve the oxygen supply in the body. Theoretical descriptions of the flow of blood or suspensions of vesicles in microcapillaries have improved considerably in...
In numerical simulations of interacting RBCs in capillaries, we considered a two-dimensional geometry, which has proven to capture many of the behaviours of RBCs in three dimensions. The cell model comprises closed inextensible membranes (giant vesicles) filled with fluid (modelling haemoglobin) and suspended in a different fluid (modelling plasma). The vesicles can freely deform in response to the imposed Poiseuille-type flow. Cells can interact by both hydrodynamic forces and by an additional interaction potential with interaction energies that were taken from single cell force spectroscopy measurements with fibrinogen. We take an initial set of five cells in two channels of widths 4.5 and 12 µm, which are typical values in human capillaries (4.5 µm corresponds to the height of our experimental channels and 12 µm where we found that the clusters persist even though they could experience stronger shear stresses out of the center and pass on top of each other). The velocities explored at the centre of the channel are approximately 1 mm/s. Cells are disposed either randomly in the channel or in a line. We progressively increase the interaction energy and analyse the subsequent behaviour of the initial configuration. Fig. 1c–d summarises our findings. The interaction energy is increased from top to bottom in the figure. At low interaction energies, the cells that are initially arranged in a cluster are quickly separated by the flow. Interestingly, when the energy reaches the range corresponding to physiological concentrations, small clusters of two (and occasionally three) cells begin to form and persist. When the energy is such that the concentration approaches the maximal physiological concentration, five cells form a stable cluster. The evolution of the stable cluster size vs. interaction energy is summarised in Fig. 2c–d. Both the experimental and the numerical results show clustering transitions at the same interaction energies.

To measure the interaction energies between two discocytic RBCs in a rouleau at rest (Fig. 3a–b), we used single cell force spectroscopy with an atomic force microscope. A RBC was attached to a cantilever and brought into contact with another RBC resting on the cover slip; the work needed to separate the two RBCs was then measured at various dextran and fibrinogen concentrations (Fig. 3c). The interaction energy was then determined from the force-distance curve. For small and large dextran concentrations, the measured interaction energy goes to zero; a maximum is found at intermediate concentrations. For fibrinogen, the interaction energy is found to increase monotonically. This dependency is possibly explained by a model based on depletion forces. This means that there is a depletion layer between the adhering cells with no macromolecules and the surrounding macromolecules cause an osmotic pressure that pushes the two cells together. It is furthermore assumed that the dextran can penetrate the glyocalyx at higher concentrations, thus cancelling the effect of depletion. Due to its negative charges, fibrinogen is assumed to remain outside the glyocalyx layer and thus the interaction energy continuously increases with the concentration.

The shape of the interaction energy vs. concentration curves (Fig. 3c) is remarkably consistent with the statistics of cluster sizes in the microfluidic experiments (Fig. 2a–b). The same behaviour was found when measuring the erythrocyte sedimentation rate (Fig. 3d), a quantity that is largely used in blood diagnostics to characterise RBC aggregation and indicate inflammatory states.

This ensemble of results leads to the conclusion that the relatively weak interaction forces between cells do indeed have a strong impact on blood flow in the microvasculature by enhancing clustering, regardless of the high wall shear rates present in our experiments and in capillaries in vivo, which were suspected to prevent the formation of clusters. Indeed, the configuration of RBCs in capillaries decreases the extensional stresses between cells that tend to separate them from one another in bulk shear flow. To further stress this major difference with the macrocirculation, we conducted further experimental investigations using classical shear rheometry. The well-known shear-thinning behaviour of blood due to the

Figure 1 | (a) A cluster of RBCs (above) and a single RBC (below) passing through a capillary of a mouse (see SM1 for an online video). The RBCs are imaged in a dorsal skinfold chamber using intravital fluorescence microscopy. To enhance the contrast, 0.05 ml 5% fluorescein-isothiocyanate (FITC)-labelled dextran was injected in vivo via the retrolbulbar space. The effective dextran concentration in the vascular network is well below any significant effect of aggregation. The images are colourised. (b) A schematic drawing of the microfluidic device and shadowgraph snapshots of clusters of RBCs in the microcapillaries. The microfluidic device includes 30 parallel channels of rectangular cross-section 8.5 µm (width) and 4.5 µm (height). The images are recorded using a high-speed camera, and the flow velocity and distribution of cells flowing through the observed section of the channels are determined (i.e., the numbers of single cells or clusters of different lengths (2, 3, 4, 5 or more cells) are counted). (c) A snapshot of the steady-state numerical results regarding the effect of intercellular surface energy due to fibrinogen on RBC organisation in a channel with a width w = 4.5 µm. From top to bottom: ε = 0, ε = 1.78, ε = 3.56, and ε = 4.89 µm² correspond to fibrinogen concentrations of 0, 1, 4 (still in the physiological range) and 6.5 mg/ml, respectively. (d) The same as in (c), but a channel of width w = 12 µm was used.

Recent years. For instance, numerical studies have shown that even very weak hydrodynamic interactions are sufficient to stabilise clusters in capillaries. The viscosity of blood has been numerically calculated, and a comparison of this value with experimental rheological data led to a predicted value for the effective interaction force between two RBCs of approximately 5 pN, a value that is considerably lower than that found here.

Results

Intravital microscopy observations of the microcirculation in mice (Fig. 1a, a movie is available in the online supplementary material) show that RBCs can indeed flow as isolated cells or as clusters in capillaries, but the relative influence of hydrodynamic and aggregation forces remains unclear. We carried out in vitro investigations (Fig. 1b) and numerical simulations (Fig. 1c–d) to quantify this phenomenon. In the experiments, RBCs at a very low haematocrit (less than 1%) are pumped through a microfluidic device. At a typical flow velocity of v = 1 mm/s, which is comparable to physiological values and corresponds to a wall shear rate of approximately 500 s⁻¹, we find that the number of clusters strongly depends on the dextran or fibrinogen concentration. A bell-shaped dependency curve of the interaction energy vs. concentration is observed. Remarkably, robust clusters form within the range of healthy human physiological concentrations of fibrinogen (1.8–4 mg/ml).

In numerical simulations of interacting RBCs in capillaries, we considered a two-dimensional geometry, which has proven to capture many of the behaviours of RBCs in three dimensions. The cell model comprises closed inextensible membranes (giant vesicles) filled with fluid (modelling haemoglobin) and suspended in a different fluid (modelling plasma). The vesicles can freely deform in response to the imposed Poiseuille-type flow. Cells can interact by both hydrodynamic forces and by an additional interaction potential with interaction energies that were taken from single cell force spectroscopy measurements with fibrinogen. We take an initial set of five cells in two channels of widths 4.5 and 12 µm, which are typical values in human capillaries (4.5 µm corresponds to the height of our experimental channels and 12 µm where we found that the clusters persist even though they could experience stronger shear stresses out of the center and pass on top of each other). The velocities explored at the centre of the channel are approximately 1 mm/s. Cells are disposed either randomly in the channel or in a line. We progressively increase the interaction energy and analyse the subsequent behaviour of the initial configuration. Fig. 1c–d summarises our findings. The interaction energy is increased from top to bottom in the figure. At low interaction energies, the cells that are initially arranged in a cluster are quickly separated by the flow. Interestingly, when the energy reaches the range corresponding to physiological concentrations, small clusters of two (and occasionally three) cells begin to form and persist. When the energy is such that the concentration approaches the maximal physiological concentration, five cells form a stable cluster. The evolution of the stable cluster size vs. interaction energy is summarised in Fig. 2c–d. Both the experimental and the numerical results show clustering transitions at the same interaction energies.

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This ensemble of results leads to the conclusion that the relatively weak interaction forces between cells do indeed have a strong impact on blood flow in the microvasculature by enhancing clustering, regardless of the high wall shear rates present in our experiments and in capillaries in vivo, which were suspected to prevent the formation of clusters. Indeed, the configuration of RBCs in capillaries decreases the extensional stresses between cells that tend to separate them from one another in bulk shear flow. To further stress this major difference with the macrocirculation, we conducted further experimental investigations using classical shear rheometry. The well-known shear-thinning behaviour of blood due to the
destruction of aggregates is recovered here with RBC suspensions in the presence of various concentrations of dextran (Fig. 4a). When computing the relative viscosity (the ratio of the viscosity of the RBC suspensions to that of the suspending medium) to extract the effect of the aggregates on the viscosity (Fig. 4c), the bell-shaped dependency of the viscosity is revealed. Again, this is different for fibrinogen, which is charged (Fig. 4b,d). For fibrinogen, the relative viscosity monotonically increases with concentration. However, for both macromolecules, a negligible effect of aggregation is found at higher shear rates (greater than 10 s$^{-1}$ for fibrinogen and greater than 50 s$^{-1}$ for dextran), in a marked contrast with the present microfluidic experiments where shear rates are as high as 500 s$^{-1}$.

Indeed, in the situation of highly confined microfluidic channel flow, the red blood cells are kept in the middle of the Poiseuille profile and therefore experience lower overall hydrodynamic stresses compared to the bulk rheology where clusters are exposed to simple shear that sufficiently stretches them to break them at equivalent shear rates.

Discussion
A microfluidic device was used to study the dextran and fibrinogen induced aggregation of red blood cells in microcapillaries. We used quantitative data from single cell force spectroscopy on the interactions energies of fibrinogen induced aggregates of red blood cells to perform numerical simulations of the cluster formation. The results are in excellent agreement with the experimental results. This finding suggests that robust clusters can survive the relatively high shear stresses experienced in the microvasculature and they might survive over quite a long distance in the microvasculature, despite the large shear rates. This should affect not only the local rheology of blood but also the RBC distribution in the microcirculatory network and the related oxygen delivery to tissues through the potential impact of clusters on cell distribution at bifurcations. Cluster formation should strongly influence the entrance of RBCs into and their distribution within capillaries and their effect on oxygen delivery. RBC aggregation is generally known to increase microvascular flow resistance and consequently reduce blood perfusion to the organs. An extended lack of perfusion over time causes capillaries to become dysfunctional, a first stage towards ischemia. Patients suffering ischemic stroke exhibit high concentrations of fibrinogen (3.8–6.2 mg/ml). Understanding the long-term consequences of persistent cluster formation is therefore crucial to propose new predictive markers and treatment strategies.

Methods
Imaging of RBCs in vivo. Animal experiments were approved by the governmental animal care committee of the Saarland, Germany, and were conducted in accordance with German legislation on the protection of animals and NIH Guidelines for the Care and Use of Laboratory Animals (NIH Publication #85-23 Rev. 1985).

Circulating RBCs were imaged in the dorsal skinfold chamber of BALB/c mice (22–25 g) using intravitral fluorescence microscopy. The dorsal skinfold chamber and its implantation procedure have been described previously in detail. After surgery, the mice were allowed to recover for 72 h. Subsequently, the animals were anaesthetised by i.p. injection of ketamine (75 mg/kg body weight; Ursotamin®; Serumwerk Bernburg, Bernburg, Germany) and xylazine (15 mg/kg body weight; Rompun®; Bayer, Leverkusen, Germany) and fixed in the right lateral decubital position on a
Plexiglas stage. For the visualisation of microvessels by the contrast enhancement of blood plasma, 0.05 ml 5% fluorescein-isothiocyanate (FITC)-labelled dextran (molecular weight: 150,000 Da; Sigma-Aldrich, Taufkirchen, Germany) was injected i.v. via the retrobulbar space. The dorsal skinfold chamber was positioned under an upright microscope (E600; Nikon, Tokyo, Japan) equipped with a 100×, NA 1.2, water immersion objective and a halogen lamp attached to a FITC filterset. The microscopic images were recorded using a charge-coupled device video camera (iXon Ultra; Andor, Belfast, UK) connected to a PC system. For image processing, the black and white pictures were changed into red (original: black) and black (original: white) pictures using look-up tables.

Sample preparation. The study was approved by the ethics committee of the Medical Association of the Saarland (reference No 24/12). We obtained informed consent from the donors after the nature and possible consequences of the studies were explained.

Microfluidics and AFM measurements: A droplet of blood was collected via finger prick from a healthy donor and diluted in 1 ml of physiological buffer solution (phosphate buffered saline, PBS; Invitrogen, Darmstadt, Germany). To remove all substances other than red blood cells (RBCs), the sample was centrifuged at 3,000 rpm for 3 minutes, and the liquid phase and buffy coat were removed completely; the sample was then rediluted with 1 ml of PBS. To measure cluster size in microfluidic channels, 1 mg/ml of bovine serum albumin (BSA; Polysciences, Warrington, USA) was added to the buffer solution in this last step. In the AFM experiments, BSA was added after a cell had been glued to the cantilever. CellTak (BD Biosciences, Franklin Lakes, USA) was used for the functionalisation step. A cuvette is filled with a suspension of washed RBCs at a density of 45 vol% (haematocrit) with various concentrations of dextran or fibrinogen. The beam from the UV-Vis spectrometer is aligned 3 mm below the meniscus. When the RBCs sediment, the opacity of the suspension decreases. Inset: Images of the cuvettes at 0 and 30 mg/ml dextran 11 minutes after filling. Aggregation of the cells leads to more rapid sedimentation.

Rheometry. Rheological measurements of blood cell suspensions in the dextran solutions were carried out using a commercial constant shear rheometer (Mars II, Thermo Scientific, Karlsruhe, Germany) equipped with a cone-plate geometry (diameter: 60 mm, angle: 1 °) at a temperature of 23 °C. Samples were pre-sheared at 100 s⁻¹ for one minute to homogenise the samples, limit sedimentation and obtain comparable initial conditions. Consequently, the viscous stress was determined at 21 different shear rates between 10 and 100 s⁻¹ (these values correspond to the regions where correct viscosities can be obtained). For each measurement point, we ensured that the cone was rotating first for at least one and a half revolutions; then, the torque was averaged over at least one revolution to obtain a good resolution at low shear rates. Measurements with fibrinogen were carried out on a low-shear Contraves LS-30 rheometer (pronTech GmbH, Althengstett, Germany) for greater sensitivity using a Couette geometry (outer radius 5.5 mm, gap 0.5 mm, height 20 mm). For each value of the shear rate, a steady value of the torque was obtained after a rotation period of 15 s.

Single cell force spectroscopy. An AFM (JPK Instruments, Berlin, Germany) was used in single cell force spectroscopy measurements to determine the interaction energies between RBCs in the presence of dextran or fibrinogen. The AFM was equipped with a special piezocontroller (CellHesion. JPK Instruments, Berlin, Germany) that allowed large vertical displacements (up to 100 μm). The cantilevers used (MLCT-O, no tip, Bruker, Billerica, USA) had a very low spring constant.
Figure 4 | (a) Bulk viscosity of RBC suspensions at 45% haematocrit as a function of shear rate for various dextran concentrations. Error bars are only shown for the highest dextran concentration (b) the same as (a) but for different concentrations of fibrinogen. In both cases, the viscosity at low shear rates and the effect of shear thinning increases with macromolecule concentration. The viscosity of the solvent increases strongly with dextran concentration but is independent of fibrinogen concentration within the instrumental resolution. To separate the effect of aggregation in (c) and (d), the relative viscosity (the ratio of the viscosity of the RBC suspension and the viscosity of the solvent with dextran or fibrinogen only) is shown. Lines are drawn as guides for the eye.

Sedimentation measurements. Absorption measurements at various concentrations of dextran and fibrinogen were performed to determine the sedimentation speed at 23°C. A cuvette was filled with a solution of washed RBCs at a density of 10 vol% (haematocrit) at various concentrations of dextran (MW = 70,000 kDa) or fibrinogen (both in powder form, Sigma Aldrich, Taufkirchen, Germany). The xenon lamp of the UV-Vis spectrophotometer Genesys 6 (Thermo Spectronic, Rochester, USA) was aligned 3 mm below the meniscus and adjusted to a wavelength of 940 nm. The cuvette (QS 170, quartz glass, Hellma analytics, Müllheim, Germany) had a layer thickness of 1 mm, a filling volume of 120 μl and dimensions of 35 × 12.5 × 12.5 (h × w × d in mm). Before each measurement, the cuvettes were cleaned with distilled water and ethanol.

Microfluidic measurements. Microfluidic devices were produced using the classic soft-lithography technique: PDMS was cast over a master obtained by photolithography of a SU8 photoresist over a silicon wafer and glued to a glass slide after plasma treatment. The microfluidic chip comprised 30 parallel channels with a rectangular cross-section of 8.5 μm in width, 4.5 μm in height and 5 cm in length. The channels were connected to a large inlet chamber where the blood sample was injected using a syringe pump. Images of the flowing cells at different locations along the channels were taken with a high-speed camera through an inverted microscope with a 10× objective lens. Nine channels were observed simultaneously. We ensured that the experimental conditions remained constant by taking several movie sequences (100 s each) at each location. Image processing allowed the detection of individual cells and clusters. Two cells were considered as part of the same cluster when their membrane-to-membrane distance was smaller than 0.6 times their length.

Numerical method. The surface to volume ratio (or the enclosed area A to the perimeter l, ratio in two dimensions) is taken to be that of a human RBC; more precisely, \( \frac{A}{l} = \frac{1}{2\pi r^2} \approx 0.65 \). The fluid inside and outside of the cell model (CM) obeys the Stokes equations. Blood plasma viscosity is taken to be equal to that of water, whereas inside the CM, the viscosity is taken to be 5 times that of the plasma, a value that is consistent with available data on haemoglobin solutions. The velocity at the walls (mimicking blood vessels) is taken to be zero (the no-slip boundary condition). At the CM, the velocity is continuous, and the resulting hydrodynamic forces are balanced by the membrane force (a local, instantaneous mechanical equilibrium due to the weakness of inertial forces). The membrane force is composed of three contributions: (i) the bending force, (ii) tension forces that maintain local membrane incompressibility, and (iii) the net depletion force that has a repulsive part
at short distances, taking into account the steric repulsion between cells (and possible electrostatic force), and an attractive long range force modelling the depletion mechanism, consistent with the classical model of Neu and Meiselman [25]. The depletion energy is chosen as \( \phi = 4 \epsilon [(\sigma/w)^{12} - (\sigma/w)^6] \). This expression has a minimum at \( r = 2\epsilon/\sigma \), where the energy has the value \( -\epsilon \); these are the only two parameters specifying the interaction energy. The depletion force is simply the derivative of the energy \( \phi \), and each given point on CM interacts with all other points belonging to any other membrane. The CM can resist the flow due to the membrane bending force, and the membrane is locally inextensible. Simulations were performed using the Green’s function technique with an attractive long-range force between the vesicles modelling the depletion mechanism. Using Green’s function techniques, we can express the velocity of each point of any given cell as an integral over all boundaries (the cell boundaries plus the wall boundaries). We used a specific Green’s function (which is distinct from the classical Oseen tensor, albeit several preliminary results were obtained using the Oseen tensor), which offers the great advantage of automatically incorporating the presence of the boundaries (mimicking the blood vessels) in that we do not need to integrate over the boundaries where the stresses are not known a priori. This provides us with a powerful and precise computation. Technical details on the method and the numerical implementation will be given elsewhere.

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