Full dynamics of a red blood cell in shear flow

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At the cellular scale, blood fluidity and mass transport depend on the dynamics of red blood cells in blood flow, specifically on their deformation and orientation. These dynamics are governed by cellular rheological properties, such as internal viscosity and cytoskeleton elasticity. In diseases in which cell rheology is altered genetically or by parasitic invasion or by changes in the microenvironment, blood flow may be severely impaired. The nonlinear interplay between cell rheology and flow may generate complex dynamics, which remain largely unexplored experimentally. Under simple shear flow, only two motions, “tumbling” and “tank-treading,” have been described experimentally and relate to cell mechanics. Here, we elucidate the full dynamics of red blood cells in shear flow by coupling two videomicroscopy approaches providing multidirectional pictures of cells, and we analyze the mechanical origin of the observed dynamics. We show that contrary to common belief, when red blood cells flip into the flow, their orientation is determined by the shear rate. We discuss the “rolling” motion, similar to a rolling wheel. This motion, which permits the cells to avoid energetically costly deformations, is a true signature of the cytoskeleton elasticity. We highlight a hysteresis cycle and two transient dynamics driven by the shear rate: an intermittent regime during the “tank-treading-to-flipping” transition and a Frisbee-like “spinning” regime during the “rolling-to-tank-treading” transition. Finally, we reveal that the biconcave red cell shape is highly stable under moderate shear stresses, and we interpret this result in terms of stress-free shape and elastic buckling.

elastic capsule | low Reynolds number | shape memory | erythrocyte

Blood is a concentrated suspension of cells: 45% in volume is occupied by red blood cells (RBCs). Its fluidity strongly depends on its behavior in flow, which is a key factor of proper tissue perfusion. At the cellular scale, blood flow behavior is affected primarily by the RBC response to the hydrodynamic stress in terms of cell orientation relative to the flow direction and of cell deformation. For example, on one hand, at low shear rates, similar cell orientations may favor the formation of stacks (rouleaux) (1) of RBCs, like rolls of coins, which increases blood viscosity. On the other hand, at high shear rates, the individualization of RBCs, their alignment, and their stretching in the flow (2) decrease blood viscosity (3). The orientation and the deformation in flow of RBCs are governed by their rheological properties. They result from the viscoelastic contributions of all components of the cell composite structure. Moreover, RBC rheological properties also depend on the microenvironment and on metabolic functionality (4). Both local and systemic disturbances of homeostasis (in diabetes mellitus, hypertension) have the potential to induce RBC rheological alterations and consequently to impair blood circulation. It therefore is crucial to understand the relationships between the rheological properties of RBCs and their orientation and deformation in flow. This question is far from trivial because even in a simple shear flow, RBCs present a variety of dynamic states, such as steady tank-treading, swinging, unsteady tumbling, and chaotic motion.

To date, there has been little experimental work on the connection between the mechanical properties of RBCs and their dynamics in shear flow (5–8), compared with the many numerical and theoretical recent studies reported for capsules (9–11) and red blood cells (12–16). Surprisingly, all investigations dealing with RBC orientation in flow focus on a very particular case in

which the axis of symmetry of the cell lies in the shear plane. Some observations, however, suggest that other cellular orientations may be more stable (17, 18). Furthermore, numerical predictions of RBC deformations show significant discrepancies with experimental observations. Indeed, experiments report only the stationary stretched shape of cells steadily aligned in the flow at high shear rates, whereas recent numerical studies predict “breathing” dynamic states with strong shape deformations at low shear rates for both RBCs and elastic capsules (9–16).

Here, we couple two videomicroscopy approaches providing multidirectional pictures of RBCs to elucidate the full dynamics of an RBC in shear flow, and we analyze the mechanical origin of the observed dynamics (shapes and regimes of motion).

Under physiological conditions, a mature cell is a biconcave disk about 6–8 μm in diameter and 2 μm thick. Its membrane consists of a fluid lipid bilayer and an elastic spectrin network lying just beneath the bilayer and attached to the membrane integral proteins. The inner cell volume is filled with a solution of hemoglobin. Although the structure of the RBC is one of the simplest among cells, it nevertheless involves several mechanical parameters: viscosities of the hemoglobin solution and of the lipid bilayer, incompressibility and bending elasticity of the lipid bilayer, and compressibility and shear elasticity of the spectrin cytoskeleton. The nonspherical biconcave shape of RBCs enables shape changes at constant volume and area. Moreover, the membrane has a memory of its shape (19): after a shape deformation induced by an external force, the membrane returns to its initial biconcave shape and the membrane elements return to their initial position after removal of the force. The rim, for instance, is always formed by the same membrane elements. However, the actual deformation state of the membrane, even in the biconcave state, is not known because the stress-free shape of the membrane for which the strain energy vanishes, has not been determined.

For many years, the viscosity ratio λ , where λ is the viscosity inside the cell relative to the viscosity of the suspending solution, has been the only mechanical parameter used to describe the behavior of RBCs in shear flow observed in pioneering studies (2, 20): at high λ , RBCs have been reported to tumble (T), referred to here as the particular unsteady flipping motion (F) when the cell axis of symmetry rotates in the shear plane. The nature of this motion (rigid-body-like or with membrane movement) and its stability are not experimentally known. At low λ values and high shear rates, RBCs have a “fluid-like” tank-treading movement in which the membrane rotates around the center of mass of the cell and has a quasi-stable inclination (TT). The Keller and Skalak (KS) analytical model (21), which describes an RBC as a viscous ellipsoid of fixed shape, qualitatively recovers (T), (TT) as a function of λ . However, recent experiments revealed new dynamic states specifically due to the shear elasticity and the shape memory of the red cell membrane (7, 8): (i)

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swinging superimposed to tank-treading, in which the cell's inclination angle changes periodically relative to the direction of flow (S); (ii) *intermittency*, in which the cell alternately Ts and TTs when it undergoes a TT-to-T transition driven by the shear rate, $\dot{\gamma}$; and (iii) *chaotic motion* in a time-periodic flow (8). The KS model modified to account for membrane elasticity and shape memory, developed by Abkarian et al. (AFV) (7) and by Skotheim and Secomb (SS) (14), semiquantitatively recovers these dynamic states and their transition. Since then, this field has been very active and many theoretical and numerical approaches have been developed (9–16), which account for the most important cell mechanical parameters and allow shape deformation. They recover the various regimes of motion, except intermittency, which is still controversial, but also predict new regimes associated with strong unsteady deformations that have never been seen experimentally. Moreover, probably for the sake of simplicity, all studies ignore the motion of the cell when its initial orientation is random with respect to the shear plane.

In this paper, we report the following: (i) In contrast to the common belief that the stable RBC motion at low $\dot{\gamma}$ is tumbling, we show that the RBC orientation in flow changes when $\dot{\gamma}$ increases, until the cell rolls steadily (R), similar to a wheel on a road. This dynamic state preserves the cell from energetically costly deformations and is a true signature of the elasticity of the cytoskeleton. (ii) The RBC dynamic states display a hysteresis with $\dot{\gamma}$. Upon decreasing $\dot{\gamma}$, the tank-treading-to-flipping transition occurs via a transient intermittent regime. Upon increasing $\dot{\gamma}$, flipping evolves to rolling and the transition to tank-treading occurs via a transient Frisbee-like spinning motion, which we describe, for the first time. (iii) Contrary to most numerical simulations, RBC shapes do not present strong periodic “breathing” deformations in the tank-treading/swinging regime at moderate shear stress and remain biconcave. We discuss the stress-free shapes of RBCs that might explain the stability of their biconcave shape in terms of elastic buckling. This shape stability suggests that the shape-preserving analytical model (AFV-SS) is suitable to describe the dynamics of RBCs in the tank-treading/swinging regime. It opens the way to observations that allow simultaneous assessment of membrane shear modulus and viscosity, and hold promise for applications in cellular-scale diagnostics in clinical hemorheology.

Results

We first describe the dynamics of RBCs in the flipping rigid-body-like regime. We explore regimes of motion, orbits, and deformations. We describe the cell dynamic states during the two transitions toward the fluidized dynamic state and toward the solid dynamic state. We finally discuss the role of the cell mechanics on the observed dynamics.

Nonfluidized Regimes: From Tumbling to Rolling. RBCs are diluted in 9% (wt/wt) PBS–dextran solutions for two molecular weights of dextran, 10^5 g/mol (of viscosity $\eta = 7.15$ mPa·s) and 2×10^6 g/mol ($\eta = 28.5$ mPa·s). Some additional experiments are done with RBCs diluted in 7.5% (wt/wt) ($\eta = 22.2$ mPa·s) and 4% (wt/wt) ($\eta = 7.8$ mPa·s) solutions of PBS and 2×10^6 g/mol dextran. Individual RBCs are first introduced in a flow chamber, then are subjected to a simple shear flow and observed. The shear rate $\dot{\gamma}$ is increased by steps from 0 to 15 s^{-1} for cells suspended in dextran 10^5 g/mol and to 2.7 s^{-1} for cells suspended in 9% (wt/wt) dextran 2×10^6 g/mol. In this case, above $\dot{\gamma} = 2.7 \text{ s}^{-1}$, cells tank-tread. Initially, RBCs present various initial orientations with respect to the flow direction, but we focus more specifically on the cells for which the initial angle of the cell axis of revolution is in the shear plane. The cell motion, although periodic, at first sight appears rather complex and similar to that described by Jeffery (22) for rigid ellipsoids. We characterize the cell orientation by two angular variables: the *orbit angle*, ϕ , between the orthogonal projection of the cell axis of revolution along the flow gradient and the flow direction, and the *cell inclination angle*, θ , between the orthogonal projection of the cell axis of revolution onto the shear plane and the flow gradient direction (Fig. 1A).

Cell orbit. The orbit angle is best detected by observations along the flow gradient (z-view). The cell rocks to and fro between the orbit angles $\pm \phi$ observed when the cell is on the edge (axis of revolution perpendicular to the flow gradient). At low shear rates and starting from low ϕ values (close to tumbling motion), the first striking result is that ϕ does not remain constant. It fluctuates between 0° and 40° but never exceeds 40° . When the shear rate increases, the orbit of the cell drifts and ϕ increases and reaches a well-determined limiting value, which depends on the shear rate. This result is illustrated in Fig. 1B and Movie S1. It is shown in Fig. 2 on 18 different cells, whose orbit has been measured at several increasing shear rates. The cell spins around

Fig. 1. Orbit change of flipping RBCs in a shear flow when the shear rate increases. Observations along the flow gradient (z-view). (A) Schematic drawing. (B) Flipping of one RBC in dextran solution [10^5 g/mol, concentration (c) = 9% (wt/wt)]. (B, 1) $\dot{\gamma} = 2 \text{ s}^{-1}$, time sequence of 10.6 s. (Scale bar, $10 \mu\text{m}$.) (B, 2) $\dot{\gamma} = 6 \text{ s}^{-1}$, time sequence of 3.72 s. (B, 3) $\dot{\gamma} = 12 \text{ s}^{-1}$, time sequence of 3.84 s. (C) Flipping of a hardened RBC at $\dot{\gamma} = 15 \text{ s}^{-1}$, time sequence of 1.2 s (DIC image). (D) Temporal increase and stabilization of the angle ϕ when the shear rate is increased by step, observed on two RBCs (blue and red symbols, respectively) in dextran solution (10^5 g/mol, c = 9% (wt/wt)). The horizontal bars are the limit value of ϕ reached for a given shear rate. (D, Inset) Perturbation and recovery of the axis of revolution of an RBC. $\dot{\gamma} = 12 \text{ s}^{-1}$.

Fig. 2. Variation of the limit value of ϕ vs. the shear stress $\dot{\gamma}$. Each symbol refers to a single cell suspended in solution [9% (wt/wt)] of dextran of molecular weight 10^5 g/mol (blue) or $2 \cdot 10^6$ g/mol (red).

its axis of revolution, which has a precession movement when the orbit departs from pure tumbling ($\phi = 0^\circ$). When the cell orbit is perturbed, the angle ϕ recovers its stable value spontaneously (Fig. 1D, *Inset*). Upon further $\dot{\gamma}$ increase, the cell lies in the shear plane and rolls ($\phi = 90^\circ$). This motion corresponds to a steady spin of the axis of symmetry about the vorticity axis (Fig. 1B). The variation of ϕ with the shear stress is shown in Fig. 1D, *Inset*. A small shift exists between the angle ϕ observed for RBCs

suspended in dextran $2 \cdot 10^6$ g/mol and in dextran 10^5 g/mol, which we do not explain here.

To determine the mechanical origin of the phenomenon, we stiffened RBCs by incubating them in a solution of glutaraldehyde, which cross-links the proteins of the cell membrane. In this case, tumbling is stable even for $\dot{\gamma} = 15 \text{ s}^{-1}$ (Fig. 1C). ϕ may fluctuate but remains less than 40° , and returns to zero from time to time. When the orbit is perturbed externally up to $\phi = 50^\circ$, ϕ returns to zero, clearly demonstrating that finite membrane elasticity promotes rolling whereas rigidity induces tumbling.

Cell inclination. The temporal evolution of the inclination angle θ of the cells imaged in the shear plane (y-view) is shown in Fig. 3B. Although the orbit changes when $\dot{\gamma}$ increases, θ is determined easily as long as the cell does not fully roll in the shear plane. The temporal variation of θ for a flipping rigid ellipsoid, derived from Jeffery (22), is given by the equation

$$\tan\theta = r.\tan\frac{\dot{\gamma}t}{r+r^{-1}}, \quad [1]$$

where r is the cell axis ratio (length of the axis of symmetry to the length of the diametrical axis). We fit the experimental time variations of θ by the two-parameters equation $\tan\theta = A \tan(B\pi t)$, where A characterizes the variations of the flipping velocity during the motion and $1/B$ is the period for one half-rotation from $\theta = -90^\circ$ to 90° . Fits are very satisfactory for all studied cells, as

Fig. 3. Flipping of RBCs observed in the shear plane (*y*-view). (A) Experimental time variation of the inclination angle θ ($\dot{\gamma} = 9 \text{ s}^{-1}$) and fit by the function $\tan\theta = A \tan(B\pi t)$ (solid curve). (B) Variation of the orbit of one flipping RBC with increasing shear rate [dextran $2 \times 10^6 \text{ g/mol}$, $c = 9\%$ (wt/wt)]. (B, 1) $\dot{\gamma} = 2 \text{ s}^{-1}$, time sequence of 10.6 s. (B, 2) $\dot{\gamma} = 6 \text{ s}^{-1}$, time sequence of 13.72 s. (B, 3) $\dot{\gamma} = 12 \text{ s}^{-1}$, time sequence of 3.84 s. (C) Variation of the product $\pi B(A+1/A)$ with the shear rate, where A and B are determined from the fit: $\tan\theta = A \tan(B\pi t)$. The variation is in agreement with Jeffery and KS laws (solid line: bisector). (C, *Inset*) Histogram of values of A obtained by the fits for RBCs in various conditions. Stiffened and normal cells in dextran 10^5 g/mol , $c = 9\%$ (wt/wt) (green) $\dot{\gamma} < 5 \text{ s}^{-1}$, (blue) $\dot{\gamma} > 5 \text{ s}^{-1}$. Cells in dextran $2 \times 10^6 \text{ g/mol}$, $c = 9\%$ and 7.5% : (green) $\dot{\gamma} < 1.34 \text{ s}^{-1}$, (blue) $\dot{\gamma} > 1.34 \text{ s}^{-1}$.

According to their results, the stress-free shape of the RBC membrane is an ellipsoid of small eccentricity, with an area equal to that of an RBC and a volume, V , close to that of a sphere: the ratio V/V_{sphere} is in the range 0.989–0.95. Reducing the volume to the actual physiological RBC volume buckles the initial ellipsoid while keeping the area constant with apparition of two dimples in the polar regions. The material elements located at the initial equatorial region likely are located preferentially at the rim of the buckled biconcave shape, which then has a shape memory. However, because the anisotropy of the initial stress-free shape is small, the barrier of strain energy to displace the material elements from the rim to the dimple is low and will be overcome by providing a small hydrodynamic energy (low shear rates). Within this framework, a normal biconcave RBC shape may be considered as a buckled shell, in which the material elements of the membrane are strained at rest and have a small shape memory. We believe this biconcave shape remains that of lowest strain energy upon TT because of the low variation of strain energy upon displacement of the material elements, as long as the shear stress remains moderate. This shape will change progressively into an ellipsoidal shape at higher shear stresses, as observed by Fischer et al. (2).

We have shown that the shear elasticity of the RBC membrane is of foremost importance for cell deformation, motion, and orientation in shear flow. We observe that close to the transition $\dot{\gamma}_c$, the tumbling regime is unstable and we highlight the stable regime of rolling, in which the cell spins in the shear plane and rolls on its edge. Our results raise new questions about the behavior of viscoelastic particles in a viscous fluid at very low Reynolds number. Future models and simulations on RBCs should consider that the axis of revolution of the cell does not necessarily lie in the shear plane. Whether the observed orientational behavior can be explained by the minimum energy dissipation, as speculated by Jeffery, is still an open question, and we hope our work will stimulate new theoretical numerical studies. We also hope it will generate works on the strain energy of tank-treading buckled shapes of elastic capsules, starting from a quasi-spherical spheroid stress-free shape. Finally, the high stability of the biconcave RBC shape makes analytical shape-preserving models (AFV, SS) very attractive. Such models may be used to determine the viscosity and shear elasticity of RBCs at very low shear stresses, by fitting the characteristics of their

dynamics in shear flow: swinging or TT frequency, critical shear rate $\dot{\gamma}_c$, or orbital angle ϕ . Such an approach, which needs an infinitesimal amount of blood, requires only microscopic observation of flowing cells without any single-cell manipulation.

Materials and Methods

RBC and Solution. The solutions of dextran (from *Leuconostoc* spp., Sigma-Aldrich) were prepared by diluting the dextran powder into PBS prepared using Gibco PBS pH7.4 10× stock solution. Osmotic pressure and pH were adjusted to 290 mOsm and 7.4, respectively. Two dextran polymers, one of 2,000 kDa and the other of 100 kDa molecular weight, were used. The dextran concentrations used in this study were 4% (wt/wt), 7.5% (wt/wt), and 9% (wt/wt) for dextran 2 10⁶ g/mol and 9% (wt/wt) for dextran 10⁵ g/mol. The corresponding range of viscosity was 2–34 Pa·s. At the 9% (wt/wt) concentration, the buoyancy effects on the cell were reduced. Dissolution was done at room temperature during all-night stirring. Before each experiment, 1 μ L of blood was extracted via fingertip needle prick and diluted in 10 mL of PBS–dextran solution. All observations were carried out within 2 h.

Attachment of beads (carboxyl latex, 0.8 μ m; Invitrogen) onto the RBC membrane was achieved by first washing 1 μ L of blood in 1 mL of PBS. The cells then were incubated in 1 mL of PBS + beads solution at 4 °C for 1 h before being washed and resuspended in the PBS–dextran solution.

RBC stiffening was achieved by cell incubation in 1 mL of 20 μ g/ μ L glutaraldehyde solution at room temperature for 1 h. Incubated cells were then washed and resuspended in the PBS–dextran solution.

Flow and Microscopy. Fluid was driven by a syringe pump (11 Plus series, Harvard Apparatus) at a constant flow rate ranging from 100 to 1200 μ L/min through a parallelepiped glass chamber (1 $\times$ 10 $\times$ 50 mm). The wall shear rate ranged between 1 and 12 s^{−1}. Cells were visualized by 40× (DIC) and 50× (brightfield) objectives on a Leica DMIRB microscope within 50 μ m of the 10 $\times$ 50-mm wall. Experiments referred as “z-view” were done with the direction of observation along the flow gradient, the objective being oriented perpendicular to the 10 $\times$ 50-mm side of the flow chamber. So-called y-view observations were made with the direction of observation perpendicular to the shear plane (through the 1 $\times$ 50-mm side of the flow chamber, tilting the microscope 90°). Images were recorded with a Cohu video camera at 25 frames per second. Movies finally were processed either manually or using a Matlab routine to obtain the angular variables related to the cell position.

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