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Mehdi Abbasi, Alexander Farutin, Hamid Ez-Zahraouy, Abdelilah Benyoussef, Chaouqi Misbah. Erythrocyte-erythrocyte aggregation dynamics under shear flow. *Physical Review Fluids*, 2021, 6 (2), 10.1103/PhysRevFluids.6.023602 . hal-03450371

HAL Id: hal-03450371

<https://hal.science/hal-03450371>

Submitted on 26 Nov 2021

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Erythrocyte-erythrocyte aggregation dynamics under shear flow

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Red blood cells (RBCs) –erythrocytes– suspended in plasma tend to aggregate and form rouleaux. During aggregation the first stage consists in the formation of RBC doublets [Blood cells, molecules, and diseases 25, 339 (1999)]. While aggregates are normally dissociated by moderate flow stresses, under some pathological conditions the aggregation becomes irreversible, which leads to high blood viscosity and vessel occlusion. We perform here two-dimensional simulations to study the doublet dynamics under shear flow in different conditions and its impact on rheology. Few illustrative results obtained here in 3D agree with 2D results. We sum up our results on the dynamics of doublet in a rich phase diagram in the parameter space (flow strength, adhesion energy) showing four different types of doublet configurations and dynamics. We find that membrane tank-treading plays an important role in doublet disaggregation, in agreement with experiments on RBCs. A remarkable feature found here is that when a single cell performs tumbling (by increasing vesicle internal viscosity) the doublet formed due to adhesion (even very weak) remains stable even under a very strong shear rate. It is seen in this regime that an increase of shear rate induces an adaptation of the doublet conformation allowing the aggregate to resist cell-cell detachment. We show that the normalized effective viscosity of doublet suspension increases significantly with the adhesion energy, a fact which should affect blood perfusion in microcirculation.

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I. INTRODUCTION

The distribution of nutrients and oxygen to tissues and organs is ensured by red blood cells (RBCs). Several cardiovascular dysfunctions and RBC anomalies may impair proper blood perfusion of the organism. For example, drepanocytosis [1, 2], which results in a stiff cytoplasm of RBCs under low oxygen conditions, compromises a proper RBC flow in small blood vessels due to a formation of occlusions. In several blood diseases, such as diabetes and hypercholesterolemia, as well as coronary heart disease, an enhanced tendency of RBCs to form aggregates [3–10] has been reported. Fibrinogen (a protein contained in plasma) is the main cause of adhesion between RBCs [11]. Under physiological conditions the range of human fibrinogen level is approximately 1.8 - 4 mg/ml [12], and RBCs aggregate and disaggregate reversibly under flow. In contrast, under pathological conditions the level of fibrinogen may become high enough leading to more stable aggregates [9, 13]. Foresto et al [14] compared the aggregability of RBCs from diabetic and healthy patients, using direct microscopic observation and numerical processing. They found that the RBCs aggregation is highly enhanced in the case of diabetic patients compared to healthy patients. It has been documented [15, 16] that within these patients a degradation of the glycoprotein surface of the RBC membrane (which controls the electrical as well as steric repulsion between RBCs) favors aggregation. In addition, it has been reported that under pathological conditions, such as diabetes and drepanocytosis, the fibrinogen concentration is higher [8, 9, 13]. It is to be noted also that blood from pregnant women shows an important aggregability level which is correlated with a high fibrinogen concentration [17]. It seems thus that enhanced adhesion can result both from an increase of fibrinogen level and alteration of RBCs surface properties (within diabetic patients, for example).

In a completely different context, that of microgravity during space missions, it has been reported that the analysis of blood from cosmonauts [18] showed an increase of amylase activity. This enzyme is known to digest partially the glycocalyx on the surface of cells, such as RBCs and endothelial cells. This degradation promotes RBC-RBC aggregation [19] and their adhesion to macrophages. This highlights the potential impact of long term space missions on cardiovascular dysfunctions.

Theoretically, two models are evoked to describe the mechanism of adhesion between RBCs. The first one, which was prevailing for a long time, is the bridging model and has been adopted to account for fibrinogen and neutral dextran macromolecule-induced RBCs aggregation [20]. This model assumed that the proteins adsorb onto the RBC membrane and form a cross-link to the nearby RBC [21]. The second model is the depletion one, stating that configurational entropy of the suspended molecules (e.g. fibrinogen) is lowered close to RBC surface, leading to a depletion layer, so that when the gap between two RBCs becomes of the order of depletion layer, the gap becomes less populated by fibrinogen molecules than elsewhere, and this results into osmotic attraction between RBCs.

The erythrocyte doublet aggregation is the primary process in the formation of erythrocyte aggregates in blood flow. Bertoluzzo et al [22] studied the erythrocytes aggregation, using light transmission through blood sample and observed that the aggregation starts by the formation of a collection of RBC doublets. The doublet formation is a first basic building block for aggregation that should be clarified. Ju et al [23] found numerically, using a Morse potential to account for cell-cell interaction (representing depletion forces), that RBC doublet with a homogeneous deformability sustains the adhesion, while an increased deformability difference between the two RBCs forming the doublet favors the doublet dissociation. In another numerical study, Bagchi et al [24] modeled adhesion between RBCs by the ligand-receptor model (the bridging model). They analyzed the dependence of dynamics of RBCs aggregates on the adhesion energy. They found that the shearing force due to an imposed flow is more efficient to break the bonds than the normal pulling force. Wang et al [25] observed numerically that the doublet performs rotation or undergoes dissociation depending on the strength of inter-cellular force, the membrane deformability and the shear stress. Recently, Flormann et al [26] analyzed the doublet shape in a quiescent fluid *in vitro* and *in silico*, and observed that the contact surface of the doublet is flat for weak adhesion and becomes of sigmoid-type upon an increase of the adhesion energy (the protein concentration). Their results [26] show a good agreement between 2D and 3D simulations and with *in vitro* experiments. Hoore et al. [27] studied the effect of the spectrin network elasticity on the doublet shapes in equilibrium, and found qualitatively the same doublet shapes found in 2D [26].

More recently Quaife et al. [28] studied dynamics of a doublet under extensional and shear flows by using a 2D vesicle model. Under a linear shear flow (which is of interest to our study) they observed that the doublet undergoes a tumbling regime, that we shall refer to as *rolling* (for the sake of distinction with the classical single cell tumbling). By analyzing systematically dynamics of 2D vesicle doublets under various conditions, we find besides rolling, three other distinct dynamics: flexible rolling (*FR*), rolling-sliding (*RS*) and flow alignment (*FA*). The *FR* motion corresponds to a situation where the two vesicles undergo global tumbling (rolling) but the contact interface between the two vesicles oscillates between flat and sigmoid shape. In the *RS* regime the two vesicles slide with respect to each other during rolling. The *FA* regime corresponds to the situation where the two vesicles align with the flow. We present a general phase diagram of different phases of doublets. We shall also analyze the overall rheology. RBCs aggregation controls the rheological properties of blood which may constitute a promising diagnosis for cardiovascular diseases [29]. We will also present few illustrative simulations in 3D in the presence of shear elasticity. We find occurrence

of R , FR and RS in the same regions of flow and adhesion strength as in 2D. While a systematic analysis is under investigation, the preliminary results lend support to the general character of the results.

II. MODEL AND SIMULATION METHOD

A. Membrane model

We consider a 2D model, namely phospholipid vesicles. The two-dimensional vesicle model has proven to capture several features known for RBCs. Shapes like parachute and slipper [30, 31], dynamics, such as tumbling and tank-treading [32, 33] are also manifested by both systems. We consider a set of phospholipid vesicles inside a straight channel, bounded by two rigid walls located at $y = 0$ and $y = W$, where W is the channel width. The vesicles are subject to a linear shear flow $v_x^\infty(y) = \dot{\gamma}y$ where $\dot{\gamma}$ is the shear rate. Periodic boundary conditions are used along x axis (the flow direction).

The force applied by the membrane on the surrounding fluid is obtained from the functional derivative of the following energy, which is the sum of three terms: the bending energy (Helfrich energy [34]), the membrane incompressibility contribution and the adhesion energy (Lennard-Jones potential) between two vesicles:

$$E = \sum_i E_i^b + \sum_{i \neq j} E_{i,j}^{adh}, \quad (1)$$

where

$$E_i^b = \frac{k}{2} \oint_{m_i} c^2 ds + \oint_{m_i} \zeta ds \quad (2)$$

is the bending and incompressibility energy of the i -th vesicle and

$$E_{i,j}^{adh} = \varepsilon \oint_{m_i} ds(\mathbf{X}_i) \oint_{m_j} ds(\mathbf{X}_j) \phi(|\mathbf{X}_i - \mathbf{X}_j|) \quad (3)$$

is the energy of adhesion between i -th and j -th vesicle. The variable s represents the curvilinear coordinate on the vesicle contour, c is the local curvature of the membrane, k is the membrane bending rigidity, ζ is a local Lagrange multiplier associated with the constraint of local perimeter inextensibility, $\phi = -2 \left(\frac{h}{r_{ij}}\right)^6 + \left(\frac{h}{r_{ij}}\right)^{12}$ is Lennard-Jones potential which describes attractive interaction at long ranges and repulsive interaction at short ranges. Here $\mathbf{r}_{ij} = \mathbf{X}_i - \mathbf{X}_j$, where \mathbf{X}_i and \mathbf{X}_j are two position vectors of two material points on two different vesicles i and j . h is the equilibrium distance between two points of the i -th and j -th vesicle and ε is the minimum energy associated to this distance. The functional derivative (providing the force) of the bending energy the membrane incompressibility contribution can be found in [35]. The total force including vesicle-vesicle interaction has the following form :

$$\mathbf{f}(\mathbf{X}_i) = \mathbf{f}(\mathbf{X}_i)^b + \mathbf{f}(\mathbf{X}_i)^{adh} \quad (4)$$

Where

$$\mathbf{f}(\mathbf{X}_i)^b = k \left[\frac{d^2 c}{ds^2} + \frac{c^3}{2} \right] \mathbf{n} - c \zeta \mathbf{n} + \frac{d\zeta}{ds} \mathbf{t} \quad (5)$$

and

$$\mathbf{f}(\mathbf{X}_i)^{adh} = -\varepsilon \sum_{j \neq i} \int_{m_j} \left[\frac{d\phi(r_{ij})}{dr_{ij}} \left(\frac{\mathbf{r}_{ij}}{r_{ij}} \cdot \mathbf{n}(\mathbf{X}_i) \right) + c(\mathbf{X}_i) \phi(r_{ij}) \right] \mathbf{n}(\mathbf{X}_i) ds(\mathbf{X}_j). \quad (6)$$

\mathbf{n} and \mathbf{t} are the normal and tangential unit vector respectively. The force can be rewritten in a dimensionless form:

$$\bar{\mathbf{f}}(\mathbf{X}_i)^b = \left[\frac{d^2 \bar{c}}{d\bar{s}^2} + \frac{\bar{c}^3}{2} \right] \mathbf{n} - \bar{c} \bar{\zeta} \mathbf{n} + \frac{d\bar{\zeta}}{d\bar{s}} \mathbf{t} \quad (7)$$

and

$$\bar{\mathbf{f}}(\mathbf{X}_i)^{adh} = -\bar{\varepsilon} \sum_{j \neq i} \int_{m_j} \left[\frac{d\bar{\phi}(\bar{r}_{ij})}{d\bar{r}_{ij}} \left(\frac{\bar{\mathbf{r}}_{ij}}{\bar{r}_{ij}} \cdot \mathbf{n}(\mathbf{X}_i) \right) + \bar{c}(\mathbf{X}_i) \bar{\phi}(\bar{r}_{ij}) \right] \mathbf{n}(\mathbf{X}_i) d\bar{s}(\mathbf{X}_j) \quad (8)$$

where dimensionless variables are defined as follows:

$$\bar{f} = \frac{R_0^3 f}{k}, \quad \bar{\varepsilon} = \frac{R_0^2 \varepsilon}{k}, \quad \bar{c} = cR_0, \quad \bar{s} = \frac{s}{R_0}, \quad \bar{r}_{ij} = \frac{r_{ij}}{R_0}, \quad \bar{\phi}(\bar{r}_{ij}) = \phi(\bar{r}_{ij}R_0). \quad (9)$$

$R_0 = \sqrt{A/\pi}$ is the effective radius of the vesicle and A is the enclosed vesicle area.

Fibrinogen concentration mg/ml —	0.898	2.391	4.197	5.402	6.597	8.098
Interaction energy $\mu J/m^2$ —	-1.884	-2.719	-3.748	-4.655	-4.922	-6.566
Dimensionless macroscopic adhesion energy $\bar{\varepsilon}_{adh}$ —	42.38	61.17	84.33	104.73	110.74	147.73

TABLE I. Fibrinogen level versus Interaction energy between two RBC measured using atomic force microscopy [11]

Previously, Brust et al [11] quantified the interaction energy between two RBCs at various fibrinogen and dextran levels using single cell force microscopy. From their data (table I), we can estimate which range of protein level corresponds to our simulation condition. When the contact length of two adhering vesicles is larger than the equilibrium distance h , the interaction energy per unit surface is practically the energy of adhesion between two infinite plates. The distance h corresponds to the minimal energy between two points, and does not necessary correspond to the minimal energy for two planar surfaces. Let us denote that distance h_p (see Fig.1). We first calculate the energy for a given separation h_p

$$\varepsilon_{adh} = -\varepsilon \int_{-\infty}^{\infty} \phi(\sqrt{x^2 + h_p^2}) dx = \varepsilon \int_{-\infty}^{\infty} \left[2 \left(\frac{h^2}{h_p^2 + x^2} \right)^3 - \left(\frac{h^2}{h_p^2 + x^2} \right)^6 \right] dx = \frac{3\pi h^6}{16h_p^5} - \frac{63\pi h^{12}}{256h_p^{11}}, \quad (10)$$

where x is the coordinate along the vesicle-vesicle contact line. We easily find that this energy has a minimum for $h_p = (231/320)^{1/6}h$, and is equal to

$$\varepsilon_{adh} \simeq 1.6862h\varepsilon. \quad (11)$$

This will define the adhesion energy of the doublet. The dimensionless macroscopic adhesion energy (to be referred to in all following results) is defined as

$$\bar{\varepsilon}_{adh} = \frac{\varepsilon_{adh} R_0^2}{k} \quad (12)$$

We will take typical values of RBC membrane rigidity $k = 4 \cdot 10^{-19}$ J and radius $R_0 = 3 \mu m$.

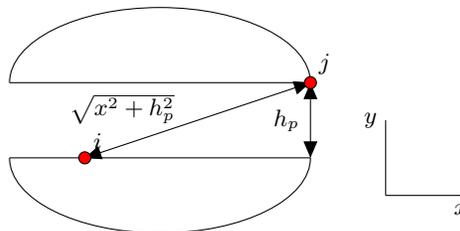


FIG. 1. Notations

B. Boundary integral formulation

The RBCs velocity and size are very small corresponding to a small Reynolds number (in the range 10^{-4} to 10^{-2}). Here we consider the limit of vanishing Reynolds number. In this case the fluid velocity inside and outside the vesicles is described by the Stokes equations:

$$-\nabla p + \eta_i \Delta \mathbf{v} = 0 \quad (13)$$

$$\nabla \cdot \mathbf{v} = 0, \quad (14)$$

where η_i , with $i = 0, 1$, is the viscosity of the internal (1) or the external (0) fluid, p is the pressure and \mathbf{v} is the velocity field. Due to the linearity of Stokes equations we can transform the set of fluid equations into an integral equation. This is based on the use of Green's function techniques[36], and is a quite accurate method for interface problems. More precisely, for a point \mathbf{r}_0 which belongs to a membrane, the velocity $\mathbf{v}(\mathbf{r}_0)$ of that point has the following dimensionless expression:

$$\mathbf{v}(\mathbf{r}_0) = \frac{2}{1+\lambda} \mathbf{v}^\infty(\mathbf{r}_0) + \frac{1}{2\pi C_a(1+\lambda)} \oint_m \underline{\underline{G}}(\mathbf{r} - \mathbf{r}_0) \cdot \mathbf{f}(\mathbf{r}) ds(\mathbf{r}) + \frac{(1-\lambda)}{2\pi(1+\lambda)} \oint_m \mathbf{v}(\mathbf{r}) \cdot \underline{\underline{T}}(\mathbf{r} - \mathbf{r}_0) \cdot \mathbf{n}(\mathbf{r}) ds(\mathbf{r}), \quad (15)$$

where $G_{ij}(\mathbf{r} - \mathbf{r}_0)$ and $T_{ijk}(\mathbf{r} - \mathbf{r}_0)$ are the Green's functions corresponding to the two-dimensional channel bounded by two rigid walls [37] (G_{ij} refers to the so-called single-layer contribution, while T_{ijk} accounts for the double-layer contribution). The Green's functions satisfy the no-slip boundary condition at rigid walls. C_a is the capillary number and λ the viscosity contrast, to be defined below.

We will analyze below the effect of the relevant blood flow and geometrical parameters on the rheological behavior of vesicle doublet and discuss the mechanism of separation. In order to preserve high accuracy we use Fourier basis discretization of all functions and compute all derivatives in Fourier domain [38, 39].

At high shear rates numerical stability problems may arise. In order to ensure long-term stability of the simulations, we keep carefully the vesicle perimeter and surface fixed. Normally, fluid incompressibility and membrane impermeability should keep the inner area of the vesicle constant. However, a small drift due to numerical errors can not be fully excluded. We compensate this drift by reinflating or deflating the vesicle through homogeneous normal deformation. Several benchmark simulations were performed with more refined meshes and time steps, higher number of Fourier harmonics, sampling points and reduced critical inter-particle distance so as to avoid numerical artifacts. Based on this verification and to ensure the numerical convergence/accuracy, each vesicle was characterized by 63 Fourier Harmonics. 512 sampling points were used to resolve the short-range hydrodynamics interactions.

C. Dimensionless parameters

Dimensionless numbers are used to describe the vesicle and the flow characteristics:

- The capillary number: allows to quantify the flow strength over bending rigidity of the membrane

$$C_a = \frac{\eta_0 \dot{\gamma} R_0^3}{k} \equiv \dot{\gamma} \tau_c \quad (16)$$

- The confinement: describes the ratio between the effective diameter of the vesicle and the channel width

$$C_n = \frac{2R_0}{W} \quad (17)$$

- The viscosity contrast: the ratio between the viscosities of the internal and external fluids

$$\lambda = \frac{\eta_1}{\eta_0} \quad (18)$$

- The reduced area: combining the vesicle perimeter L and its enclosed area A

$$\tau = \frac{(A/\pi)}{(L/2\pi)^2} \quad (19)$$

This value will be set to 0.65 (inspired by that of human RBCs). Throughout this paper, we will use the following scales: R_0 for the distance, τ_c for the time and η_0 for the viscosity.

III. RESULTS

The strategy followed in this work consists in preparing initially two vesicles in the middle of the channel, separated by a small distance that allows them to adhere to each other in the absence of an applied flow. Once they adhere to each other and reach a steady state configuration, which depends on the adhesion strength, a shear flow is applied. The study of conformation of vesicle doublet in the absence of flow allowed us to perform benchmarking of our code by reproducing previous results [26, 27].

A. Effect of viscosity contrast, flow strength and adhesion on the phase diagram of doublet

We first analyzed the effect of the viscosity contrast λ , the capillary number C_a and the adhesion energy ε_{adh} on the dynamics of the doublet. The confinement parameter is set to $C_n = 0.4$. We explored two values of viscosity contrast, $\lambda = 1$ and $\lambda = 10$. It is to be noted (for later purposes) that a single vesicle exhibits a tank treading motion in the range from $\lambda = 1.0$ (and also below that value) to approximately 12 (when $C_n = 0.4$ and $\tau = 0.65$), beyond which it undergoes tumbling. This means that for both viscosity contrasts a single vesicle shows tank-treading. The critical value for transition from tank-treading to tumbling also depends on C_n . We will explore a less confined situation where a vesicle shows tumbling for $\lambda = 10$. We will see in the next section that having tumbling will dramatically change our conclusion on the doublet separation process.

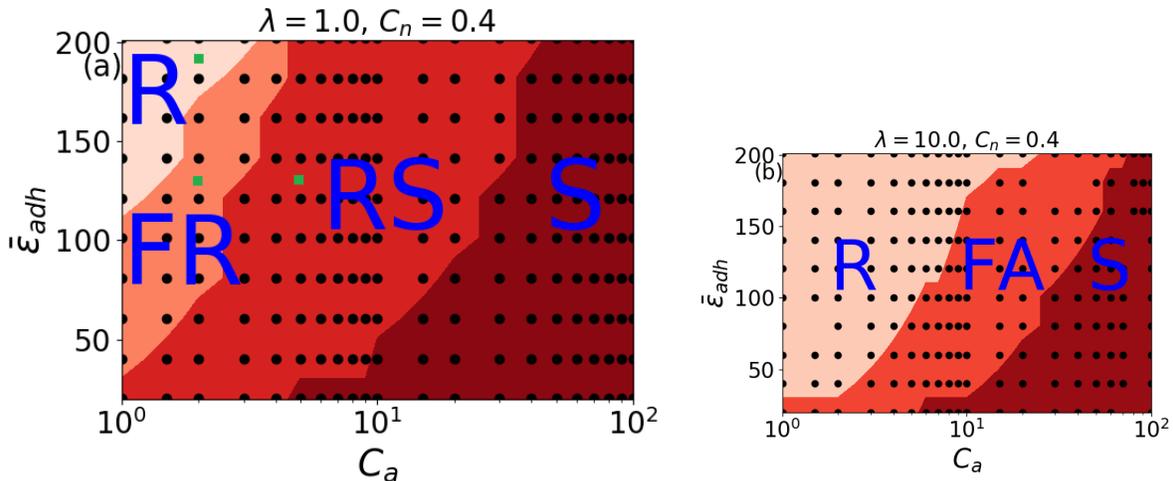


FIG. 2. Phase diagrams showing the different behaviors of doublet in the parameter space of the capillary number and the dimensionless macroscopic adhesion energy. The simulation data are shown as dots. (a) $\lambda = 1.0$, (b) $\lambda = 10.0$. In (a) the square symbol represents 3D simulations which are shown in the supplementary material [48] (R.avi, RR.avi and RS.avi)

Once the doublet reaches a steady-state configuration at equilibrium (i.e. in the absence of external flow), we applied a shear flow with different values of shear rate and analyzed the dynamics exhibited by the doublet. We found a quite rich phase diagram (Fig. 2-a) in a wide range of capillary number C_a and dimensionless macroscopic adhesion energy $\bar{\varepsilon}_{adh}$. Before giving a theoretical foundation to each phase exhibited by the doublet, let us first describe the phase diagram. Three regimes have been identified in the case of $\lambda = 1$: (i) at low capillary number the rolling phase: the two vesicles remain attached with constant contact length and show a rolling motion (akin to tumbling; see Fig. 3-a). This regime is denoted as R in Fig. 2-a. (ii) When the adhesion energy decreases the contact interface shape transits from sigmoid to a flat interface during time as shown in the snapshots of (Fig. 3-b). (iii) Increasing the capillary number the phase (FR) undergoes a transition towards a phase of rolling+sliding (RS ; the doublet shows rolling accompanied with a sliding between the vesicles; the length contact changes during time by oscillating between two values (Fig. 3-c)). The sliding of the two vesicles on each other is due to the competition between the aggregation and dis-aggregation (flow) forces, as well as due to tank treading of each membrane. This regime is denoted as RS in (Fig. 2-a). (iv) At high capillary number the separation between the vesicles takes place, meaning the dis-aggregation force due to flow is high enough to overcome the adhesion between vesicles. This regime is denoted as S in Fig. 2-a. We will see below situations where a doublet may persist whatever the magnitude of the shear flow is.

In order to check the robustness of our results we have briefly analyzed the doublet in 3D by adopting a capsule model with shear elasticity (see [26] for a brief description of the model). We present here three typical phases exhibited by 3D doublet: R , FR and RS . The green squares in Fig. 2 (left panel) show the values of capillary number and adhesion energy corresponding to 3D simulations. We have taken the same bending rigidity as in 2D and the capillary number associated with shear elasticity is defined as $C_s = \eta_0 \dot{\gamma} R_0 / \mu$ (where μ is cytoskeleton shear modulus, $\mu \simeq 4 \mu N/m$). The ratio between this number and bending one is $\mu R_0^2 / \kappa \simeq 100$. This allows, if need be, to convert the data in Fig. 2 in terms of C_s (we find 0.03 and 0.06). For these values usually RBCs undergo tumbling due to shear elasticity [41, 42]. In the supplementary material we show movies of the 3D simulations. We leave the exploration of the full phase diagram in 3D for future works.

1. Theoretical foundation to the phase diagram

We have seen in [26] that at equilibrium (absence of flow) at small adhesion the cell-cell interface is flat while at large enough adhesion energy the interface is deformed. The passage from the flat to the deformed configuration is a supercritical bifurcation. The shape of the cell-cell interface is characterized by the value of the amplitude of deformation of the contact zone between cells A_s (when the contact interface is flat $A_s = 0$, and when the interface has a sigmoid form $A_s \neq 0$). Figure 4 (blue symbols) show the supercritical bifurcation that we have reproduced here. After the bifurcation there are two states (the upper and lower branches) where the two corresponding doublet shapes (called state 1 and state 2 in Fig. 5) are mirror-symmetric with respect to a horizontal mirror.

In the presence of flow the sigmoid interface confers chirality to the doublet, as explained in Fig. 5. The order parameter A_s is a measure of the chirality of the doublet. Hence we conclude that the transition from a straight doublet interface at low adhesion to a sigmoid interface at high adhesion, as observed without flow, is a transition from achiral to chiral states. The shear flow is chiral as well: Writing the shear flow as $(\dot{\gamma}y, 0)$, we see that it can be represented as a sum of an elongational flow $(\dot{\gamma}y/2, \dot{\gamma}x/2)$ and a rotational flow $(\dot{\gamma}y/2, -\dot{\gamma}x/2)$. While the elongational flow is achiral, the rotational flow is clearly chiral. It is also easy to check directly that no rotation of coordinates can transform flow $(\dot{\gamma}y, 0)$ into flow $(-\dot{\gamma}y, 0)$. Because the shear flow is chiral, a doublet subject to this flow is chiral regardless of the adhesion energy. Indeed, if the shape of the doublet under shear flow were achiral, the membrane and adhesion forces would also be achiral, while the viscous forces of the chiral flow applied to an achiral shape are chiral. It would thus be impossible for the two to balance each other (and to respect a force-free condition).

Let us provide a qualitative picture of the imperfect bifurcation. We have seen in [26] that the energy associated with interface deformation (with amplitude A_s) can be written as $a(\beta - \beta_c)A_s^2 + bA_s^4$, with $\beta \sim \epsilon_{adh}/\kappa$, β_c the critical value for bifurcation, and a and b are positive constants. In the presence of flow the work due to interface deformation is proportional to CaA_s (changing the direction of flow changes sign of deformation, leaving CaA_s invariant). The total energy takes thus (apart from numerical prefactors) the form

$$E = CaA_s + a(\beta_c - \beta)A_s^2 + bA_s^4 \quad (20)$$

If $Ca = 0$ the energy is minimal for $A_s = 0$ if $\beta < \beta_c$ (low adhesion), and is minimal for $A_s \neq 0$ when $\beta > \beta_c$. In the presence of flow the energy has a typical form of imperfect bifurcation. Setting derivative of E with respect to A_s to zero yields a cubic equation for A_s which has either a single (non zero) real solution or three real solutions depending on β (for a given Ca). This is nothing but the bifurcation represented in red in Fig. 4 showing a single solution with $A_s \neq 0$ at small adhesion and three solutions at large adhesion.

The transition from FR to RS corresponds to a sudden relative complete sliding of the two cells with respect to each other. By complete we mean that the surfaces slides from one end to the other where the two cells remain pinned by a very small area (Fig. 3c); the two cells slide back and forth during time. Figure 6 shows the minimal area of contact as one crosses the boundary $FR - RS$ showing a sudden jump. Increasing Ca may either lead to FA or S phase. In the FA phase the two cells align steadily with the flow, where each cell exhibits tank-treading (Fig. 3d). Finally the S phase corresponds to the situation where the two cells cease to be bound to each other.

2. Effect of viscosity contrast

Subsequently we have evaluated the effect of the viscosity contrast on the phase diagram discussed above. The results are shown in Fig. 2-b for $\lambda = 10$. Several observations are made. First, the rolling region becomes wider if viscosity contrast is increased. Second, the rolling-sliding phase is absent for this viscosity contrast, in favor of a flow-alignment (FA) phase (Fig. 3-d)). In this phase the vesicles align with the flow direction, remain attached and they show a tank treading motion of their membrane. In order to shed some light on the origin of the flow alignment at high viscosity contrast, we performed simulations on a single vesicle at fixed values of the capillary number Ca , the confinement C_n and we varied only the viscosity contrast λ . Figure 7 shows that the inclination angle Ω of a single vesicle (the angle between the long axis of the vesicle and the flow direction) decreases with the viscosity contrast, until it aligns with the flow direction at high viscosity contrast. In this configuration (flow alignment) the doublet is in an orientation with a small extensional tension which is not efficient to enforce the two vesicles to slide with respect to each other.

B. The mechanism of doublet separation

One major question is whether or not doublet formation is reversible *in vivo*, and whether there is any simple criterion (or hint) to answer this question. With this regard, Chien et al [40] investigated experimentally the dis-

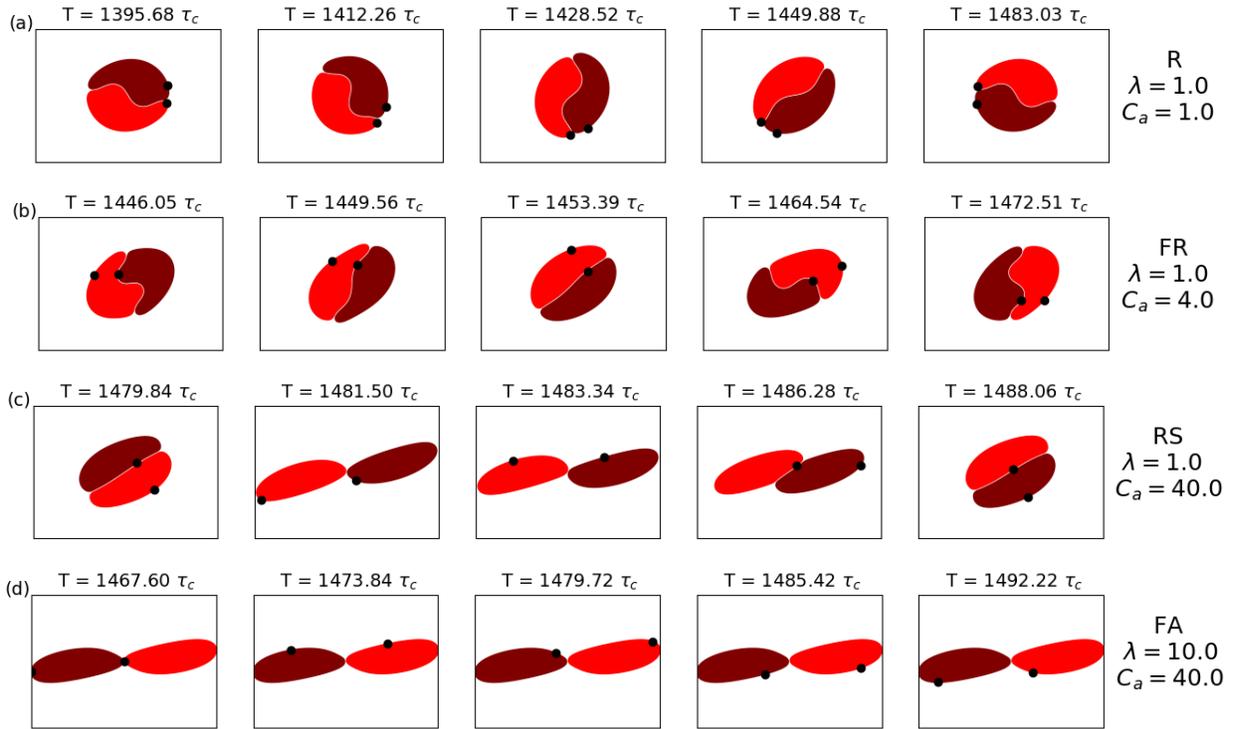


FIG. 3. Snapshots showing the dynamics of vesicle doublet for different capillary number C_a and viscosity contrast λ . Here $\bar{\epsilon}_{adh} = 202.00$ and $C_n = 0.4$. The snapshots are taken over one period of the doublet dynamic.

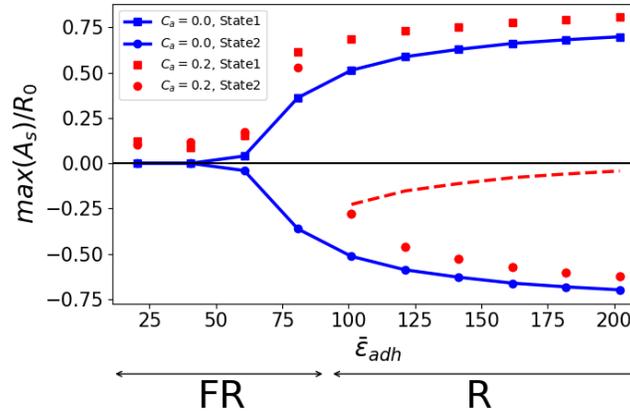


FIG. 4. Bifurcation diagram in the absence of flow (blue) and in the presence of flow (red) showing maximum deformation amplitude of the contact line. Dashed line is the unstable branch (drawn by hand since the unstable branch can not be captured by dynamics). The red branches are captured by starting from two different initial conditions (state 1 and 2, shown in Fig.5). Note that if for state 1 the amplitude of the contact line deformation is counted positive, then it is negative for state 2, and vice versa. Below the turning point we have FR phase and beyond we have R phase.

aggregation of RBC doublets under oscillatory shear flow. They prepared two adhered cells in a flow channel where the bottom cell adheres to a fixed plane and a polystyrene latex particle is used as a marker on the top cell. By applying an oscillatory shear flow they observed that the velocity of the latex bead is twice the velocity of the upper cell. From this it was concluded that the detachment occurs as rolling of the top cell along the bottom one and not as sliding. In other words, the bottom surface of the top cell remained stationary in the lab frame except at the point where the detachment occurred, while the velocity of the top surface of the top cell was twice that of its center of mass. This motion is similar to tank-treading in the reference frame co-moving with the top cell, where its top and bottom surfaces move with opposite velocities, while the overall shape of the cell remains virtually unchanged. Thus

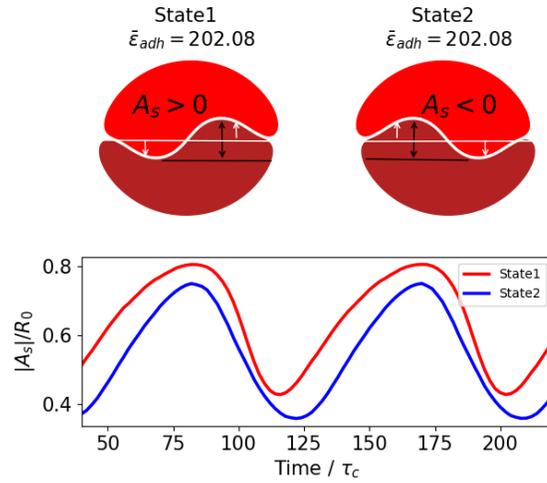


FIG. 5. (a) The two states 1 and 2. (b) The time evolution of the amplitudes of the interface between the two cells for a given situation corresponding to the two branches in Fig.4 in the regime of R phase. A_s is defined as the algebraic amplitude measuring maximum algebraic values. More precisely, the amplitude is positive if the interface is displaced counterclockwise (see upward and downward white arrows in left panel) from the straight line segment joining the two endpoints of the interface (shown as while horizontal line) and negative if displaced clockwise (right panel). For example it is positive in a) and negative in b). The black arrow shows the value of A_s . c) We see from amplitude evolution an asymmetry between the two branches confirming the imperfect bifurcation in Fig.4.

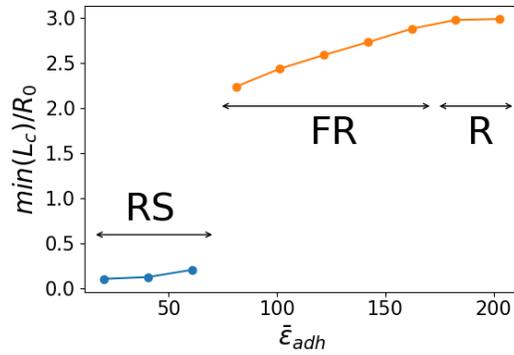


FIG. 6. Minimal contact length between the two cells as a function of adhesion energy showing its collapse when passing FR to RS .

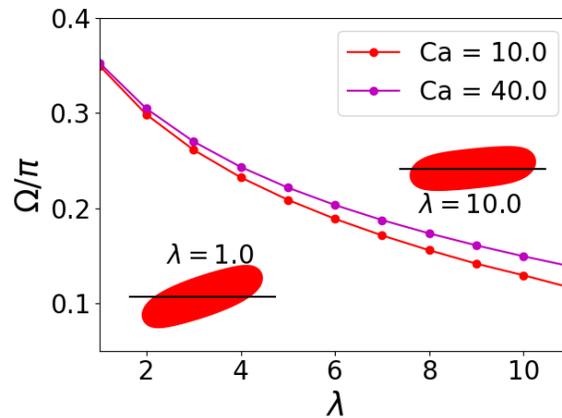


FIG. 7. Inclination angle of a single vesicle as a function of viscosity contrast λ . The angle decreases with λ . The snapshots show the vesicle under shear flow of $Ca = 10.0$.

the ability of the cell membrane to tank-tread is essential for the doublet separation. We now use our model to see whether preventing the membrane from tank-treading would have an effect on doublet dissociation.

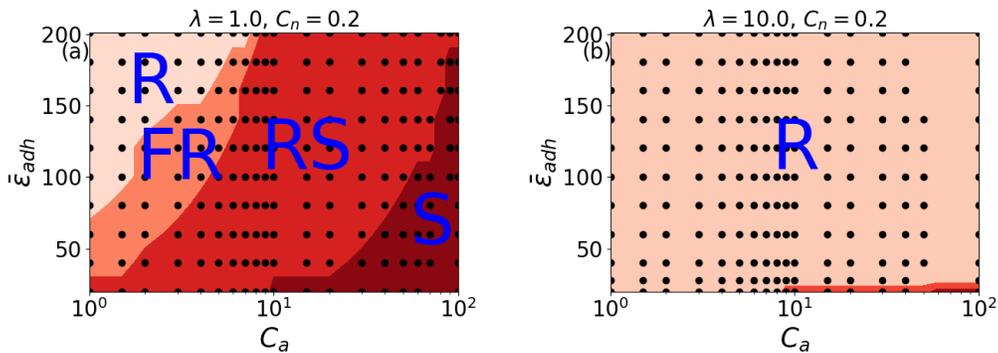


FIG. 8. Phase diagrams showing the different behaviors of doublets in the parameter space of the capillary number and the dimensionless macroscopic adhesion energy. The simulation data are shown as dots. (a) $\lambda = 1.0$, (b) $\lambda = 10.0$.

1. Suppression of doublet separation

We have seen above that there exists (Fig. 2) a region of separation (region S) for a given set of parameters. In both diagrams of Fig. 2 the separation phase (S) is preceded either by RS or FA phase. In both of the latter two phases the membrane undergoes tank-treading. The question naturally arises of whether or not the separation phase is associated with the existence of membrane tank-treading. We have thus investigated if the suppression (or a significant reduction) of membrane tank-treading may affect separation. For that purpose we have chosen a wide enough channel ($C_n = 0.2$) and only varied the viscosity contrast λ in order to reduce membrane tank-treading. As a guide, we have first analyzed the case of a single vesicle and determined the critical λ for the transition from tank-treading to tumbling, and found $\lambda \simeq 7.0$ (note that when $C_n = 0.4$ this transition takes place at about $\lambda = 12$). This means that for $C_n = 0.2$ a single vesicle shows tank-treading for $\lambda = 1$ and tumbling for $\lambda = 10$. We have analyzed the phase diagram for these two values and the results are shown in Fig. 8. For $\lambda = 1$ the same overall picture is found as in Fig. 2a. However, the situation is drastically different when $\lambda = 10.0$ (Fig. 8-b). Indeed, we see that RS and S phases are almost absent, and they appear only for extremely weak adhesion, far below physiological ranges. In other words, the doublet seems to be very robust even for a very large capillary number. We have attempted to dig further into this result. The doublet precise shape is a compromise between adhesion, which tends to increase vesicle-vesicle interface (like sigmoid shape) and bending energy, which tends to minimize deformation, favoring a flatter interface. Deformation ability can be measured by C_a , and dimensionless macroscopic adhesion energy by $\bar{\epsilon}_{adh}$. An enlightening representation of our result Fig. 8 is to plot $\bar{\epsilon}_{adh}$ as a function of $C_a/\bar{\epsilon}_{adh}$. The results are shown in Fig. 9. This clearly shows that the shape adapts itself to shear flow. Indeed, if that were not the case, namely that the doublet conformation were independent of C_a for given adhesion energy, then the R/RS and RS/S phase borders observed for the lowest dimensionless macroscopic adhesion energies would continue vertically to high dimensionless macroscopic adhesion energies (in the representation of Fig. 9). In other words, the transition value of $C_a/\bar{\epsilon}_{adh}$ would be independent of adhesion energy. Indeed, if bending energy saturates, the only remaining two energy scales are shear and adhesion ones, so that the critical $C_a/\bar{\epsilon}_{adh}$ would have been a numerical constant. The fact that the phase diagram in the new representation shows the same trend as in Fig. 8-b is a clear indication that the doublet adapts its shape in order to escape dissociation. Figure 10 shows some doublet shapes highlighting adaptation to shear flow. Our results are consistent with the work of Chien et al. [40], in that membrane tank-treading plays an important role in doublet dissociation. For a single RBC the possibility of membrane tank-treading depends on capillary number, defined by $C_s = \eta \dot{R}_0 \gamma / \mu$, where we recall μ is the cytoskeleton shear modulus (typically $\mu \simeq 4 \mu N/m$). For low enough C_s tumbling prevails whereas membrane tank-treading is possible at high C_s [41, 42]. Taking for η the value of water viscosity (which is close to the plasma one) and $R_0 \sim 3 \mu m$, we obtain $C_s \sim 10^{-3} \dot{\gamma}$ (with $\dot{\gamma}$ in unit of s^{-1}). For healthy RBCs the transition between tumbling and tank-treading takes place at about $C_s \sim 0.1$ [41, 42]. In human vascular networks we expect membrane tank-treading to take place in arterioles only (the only vasculature site where shear rate can reach values of about few $10^3 s^{-1}$). In some RBCs diseases, such as thalassemia [43], sickle-cell disease [44] and malaria [45], the membrane shear modulus as well as cytoplasm viscosity may be significantly higher than healthy ones. The corresponding shear rate beyond which membrane tank-treads may become significantly larger for

pathological cells [41] so that its occurrence in vivo becomes unlikely. We can speculate that in this case RBC doublets and larger aggregates become irreversible, compromising thus a proper blood perfusion to tissues and organs.

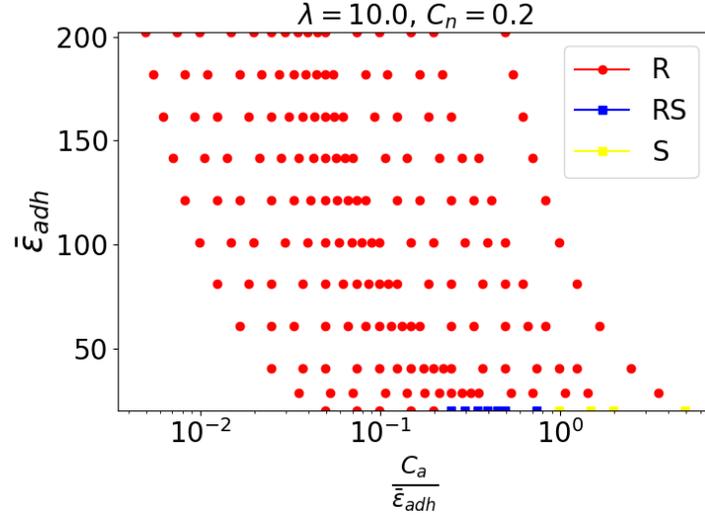


FIG. 9. Phase diagrams showing the different behaviors of doublets in the parameter space of the dimensionless macroscopic adhesion energy and the ratio of capillary number over dimensionless macroscopic adhesion energy. The simulation data are shown as dots.

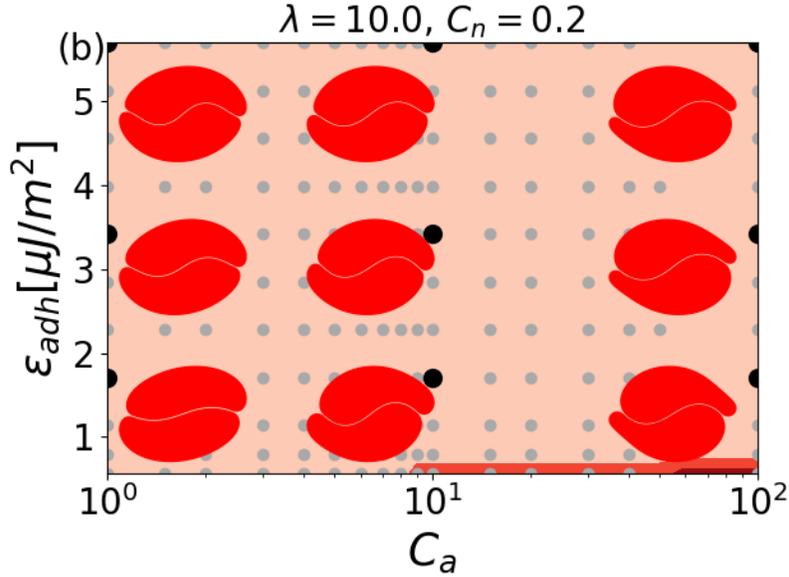


FIG. 10. Phase diagrams (same as in Fig.2) showing few shapes of doublet in the parameter space of the capillary number and the dimensionless macroscopic adhesion energy. The simulation data are shown as dots. The darkest spots indicate the pairs of parameters for which the shape is shown. We clearly see shape adaptation as C_a increases.

C. The effect of adhesion energy on the instantaneous normalized viscosity.

The goal of the next two paragraphs is to explore the effect of doublet dynamics discussed above on the rheological behaviors of a doublet suspension. In order to quantify rheology we analyze the normalized viscosity. Here we consider the very dilute regime. The effective viscosity can be written in the following form:

$$\eta = \eta_0(1 + [\eta]\varphi) \quad (21)$$

where φ is the vesicle concentration, and $[\eta]$ is the normalized viscosity (called also the intrinsic viscosity), representing the doublet contribution to the viscosity. The effective viscosity is the ratio of the xy component of stress tensor to the applied shear rate:

$$\eta = \frac{\langle \sigma_{xy} \rangle}{\dot{\gamma}} \quad (22)$$

Where bracket $\langle \dots \rangle$ means a surface average (i.e. average over the simulation area). Following Batchelor [46], the normalized viscosity is given by:

$$[\eta] = \frac{\eta - \eta_0}{\eta_0 \varphi} = \frac{1}{\eta_0 A \dot{\gamma}} \sum_i \left[\int_{m_i} y f_x ds + \eta_0 (\lambda - 1) \int_{m_i} (n_x v_y + n_y v_x) ds \right] \quad (23)$$

The first term of the normalized viscosity describes the dynamical contribution which is due to the membrane force, whereas the second term represents the kinematic contribution of the vesicle (the membrane velocity).

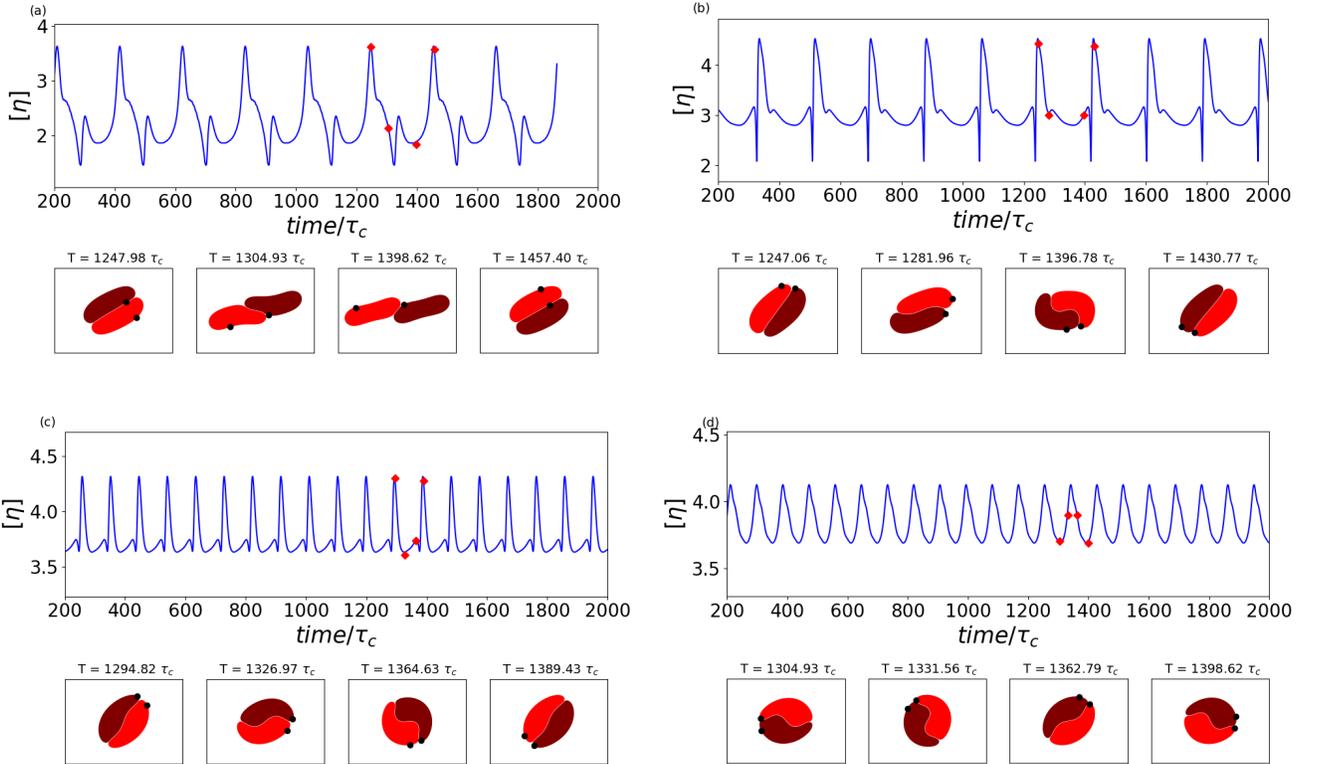


FIG. 11. Evolution of the normalized viscosity $[\eta]$ as function of time. The snapshots are taken over one time period as shown with the red dots. Here $\lambda = 1.0$, $C_n = 0.4$ and $C_a = 1.0$ (a) $\bar{\epsilon}_{adh} = 20.20$, (b) $\bar{\epsilon}_{adh} = 40.40$, (c) $\bar{\epsilon}_{adh} = 80.83$, (d) $\bar{\epsilon}_{adh} = 202.00$

We first analyzed how the normalized viscosity $[\eta]$ changes during time for increasing adhesion energy with a viscosity contrast $\lambda = 1.0$ and low capillary number $C_a = 1.0$. Figures 11a-d show that the viscosity of the suspension is periodic with time. At very low adhesion strength the doublet shows *RS* regime (Fig. 11a). This state undergoes a transition towards the *R*-regime at high adhesion strength ((Fig. 11d). This transition is accompanied by an increase of the amplitude of the normalized viscosity. In the *R*-phase the contact interface of the doublet does not evolve with time. The amplitude as well as the period of the normalized viscosity oscillation decreases with the adhesion strength. This is attributed to the fact that the two vesicles become more and more pinned to each other (Fig. 11d) as the adhesion energy increases, so that the overall cross section of the doublet which is exposed to the flow decreases, opposing thus less resistance.

D. Time average rheology of doublet suspension

As seen above a single vesicle is known to exhibit both tank-treading motion (at low viscosity contrast) and tumbling motion (at high viscosity contrast). A suspension of vesicles exhibit both shear thinning and shear thickening depending on viscosity contrast[47]. Here we find that the doublet suspension always exhibits shear thinning for the set of parameters explored so far. The results are shown in Fig. 12 (note that capillary number axis in Fig. 12 is shown in logarithmic scale). In all the three studied cases (Fig. 12a,b,c) the viscosity collapses by about 50%. A common feature shown in Fig. 12a,b,c is that all curves (obtained for different adhesion energies) collapse on the same curve for high enough capillary number. This collapse corresponds to the situation where all doublets are dissociated.

The persistence of shear thinning here is quite natural. Indeed, as Ca increases we have succession of phases that tend to lower cross section of the doublet against the flow. At low Ca the doublet shows a rigid-like rolling (R). Increasing Ca leads to FR . Because in this case the doublet flattens during time (due to interface flexibility), we expect that the doublet offers less flow resistance. Similarly, in the RS and FA regimes the doublet spends some time (RS) in the aligned direction with the flow, and this should lead to viscosity reduction. In the FA regime the doublet viscosity is constant with shear rate since the FA configuration remains steady during time. Since our preliminary simulation in 3D shows the existence of R , FR and RS upon increasing Ca , we expect the same impact on rheology as in 2D.

Figure 12d shows a peculiar behavior, in that the curves do not collapse at large capillary number, due to the absence of dissociation. This figure shows a peculiar behavior: at low capillary number the suspension with high dimensionless macroscopic adhesion energy has a higher normalized viscosity (which is quite intuitive), but at higher capillary number the opposite is found. Let us provide an argument for this behavior. For a low dimensionless

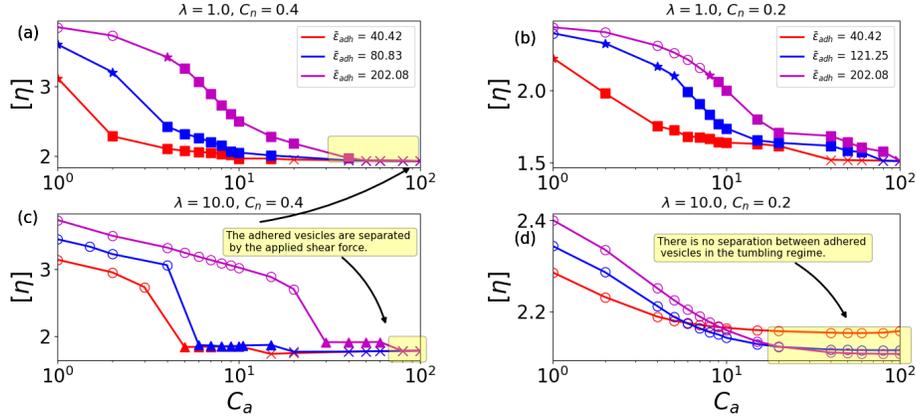


FIG. 12. The normalized viscosity $[\eta]$ as a function of capillary number Ca for different dimensionless macroscopic adhesion energy $\bar{\epsilon}_{adh}$. The simulation data are shown as dots. (a) $\lambda = 1.0$ and $C_n = 0.4$, (b) $\lambda = 10.0$ and $C_n = 0.4$, (c) $\lambda = 1.0$ and $C_n = 0.2$, (d) $\lambda = 10.0$ and $C_n = 0.2$. Circle symbol represents the rolling phase, star: flexible rolling, square: rolling + sliding, triangle: flow alignment, cross: separation.

macroscopic adhesion energy (say the red curve in Fig. 12d) and at low capillary number the doublet shows the R motion where the contact length between the two vesicles oscillates in time while keeping sigmoid shape (not to be confused with FR ; see Movie 1 in the supplemental material [48]). For a higher adhesion (say the blue curve in Fig. 12d), and at low capillary number, the contact surface has a persistent sigmoid shape. In other words, for a high enough adhesion the doublet is quite rigid in its configuration yielding a higher viscosity, as intuitively expected. When the capillary number increases, for the low adhesion case (the red curve in Fig. 12d), the shear stress is strong enough to pull on the doublet leading to a peeling off the tail of each vesicle at the poles (see Movie 3 in the supplemental material [48]). The extra tails that stick out of the vesicles increase the cross section of the doublet, leading to a higher viscosity than the case where adhesion is stronger (preventing tails to stick out of the doublet, see Movie 4 in the supplemental material [48]). This explains why, at high capillary number, for weak adhesion the viscosity is higher than for strong adhesion (the blue and red curves intersect).

Finally, let us quantify the effect of the dimensionless macroscopic adhesion energy on the normalized viscosity. Note that the range of the dimensionless macroscopic adhesion energy from 5.0 to 90.0 corresponds to the physiological conditions of fibrinogen level (these values are estimates from [11]). Pathological conditions correspond to a value of dimensionless macroscopic adhesion energy greater than 90.0. Figure 13 shows that $[\eta]$ increases monotonically

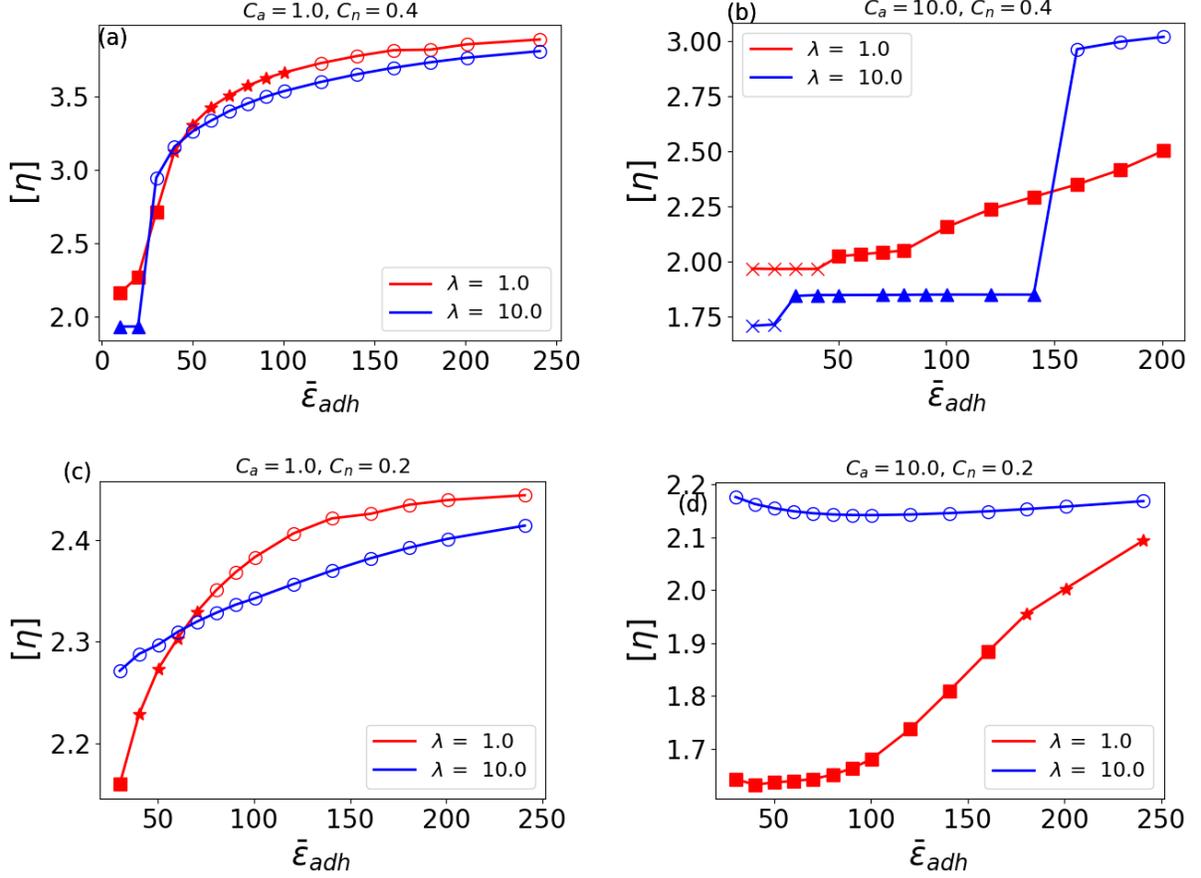


FIG. 13. The normalized viscosity $[\eta]$ as a function of the dimensionless macroscopic adhesion energy $\bar{\epsilon}_{adh}$ for different values of viscosity contrast and confinement. The simulation data are shown as dots. (a) $C_a = 1.0$ and $C_n = 0.4$, (b) $C_a = 100.0$ and $C_n = 0.4$, (c) $C_a = 1.0$ and $C_n = 0.2$, (d) $C_a = 100.0$ and $C_n = 0.2$. Circle symbol represents the rolling phase, star: flexible rolling, square: rolling + sliding, triangle: flow alignment, cross: separation.

with $\bar{\epsilon}_{adh}$, except for the case of Fig. 13d with $\lambda = 10$ which has been discussed in Fig. 12d where we have seen an inversion of normalized viscosity behavior as a function of the dimensionless macroscopic adhesion energy. Note also that the range of variation of normalized viscosity can be ample enough (it can attain a factor of two; see Fig. 13a). Figure 13b shows that (for $\lambda = 10$) the normalized viscosity exhibits two jumps, one at about $\bar{\epsilon}_{adh} = 30$, which is due to a sudden transition from S to FA when the adhesion energy decreases, and a second jump at $\bar{\epsilon}_{adh} = 150$, due to a transition from FA to R . The plateau shown in Figure. 13b (triangles) correspond to FA regime where the doublet configuration remains the same upon increasing adhesion energy.

IV. SUMMARY AND CONCLUDING REMARKS

To summarize, in this paper we employed a boundary integral method and vesicle model to study the dynamics and the rheology of vesicle doublet under shear flow. We varied the dimensionless macroscopic adhesion energy in this study from 5.0 to 250.0, a range which corresponds to physiological and pathological conditions. We found that the doublet can exhibit rolling, flexible rolling, rolling+sliding, aligned vesicles and that separation depends on several parameters (capillary number, adhesion energy and viscosity contrast). A remarkable feature is that when each single cell exhibits tumbling (for example due to a high enough internal viscosity), the doublet becomes quite stable even for an extremely large shear stress (the separation region is almost absent in the phase diagram, Fig.8). Indeed, the doublet adapts its spatial configuration to the applied flow in a way to escape dissociation. In vivo RBC membrane may perform tank-treading in arterioles only. However, several RBCs pathologies are associated with increased membrane rigidity or cytosol viscosity. This may result in a collapse of the membrane tank-treading ability (even in arterioles) causing a stability of doublets, and impairing blood perfusion in microcirculation. Another

important observation is that there are two stable configurations of the doublet at high enough adhesion. These two states have quantitatively different dynamical signatures under shear flow. Lowering the adhesion strength results in a loss of one of the configurations. This bifurcation marks the transition from rolling to flexible rolling dynamics, due to the presence of a saddle node (turning point of the imperfect bifurcation).

The rheological study showed shear thinning, and a quite significant increase of viscosity with adhesion energy. This increase with energy is *a priori* expected, since the doublet becomes more and more robust, and its higher cross section opposes more flow resistance. Rheology may be an interesting alternative for a systematic blood diagnosis and estimate of adhesion energy. A systematic numerical study in 3D including cytoskeleton is necessary before drawing more practical conclusions. Our preliminary simulations in 3D presented here support phases like R , FR and SR in the same range of flow strengths explored in 2D. It is hoped to investigate this matter further in a future work.

ACKNOWLEDGMENTS

We acknowledge financial support from CNES (Centre National d'Etudes Spatiales) and for having access to data, and the French-German University Programme "Living Fluids" (Grant CFDA-Q1-14). The simulations were performed on the Cactus cluster of the CIMENT infrastructure, which is supported by the Rhône-Alpes region (Grant No.CPER07.13 CIRA).

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