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Morphology of small aggregates of red blood cells ☆

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Abstract

Blood can be considered a two-phase liquid composed of plasma as well as cells and cell aggregates. The degree of cell aggregation is an important determinant of blood rheology: The size and shape of the aggregates affect blood viscosity. The microscopic mechanisms of red blood cell adhesion involve a complex interplay of electrostatic, van der Waals, and a range of specific biochemical inter-membrane interactions. Here we use an effective model of these interactions combined with the membrane elasticity theory to calculate the equilibrium shape of a red blood cell doublet and compare it with the experimentally observed red blood cell aggregates both *in vitro* and *in vivo*. Special attention is devoted to the shape of doublets formed by dissimilar cells. A possible effect of doublet shape on pathways of the formation of multicellular aggregates is discussed. Red blood cell rouleau formation is expected to take place at intermediate adhesion strengths where the outer doublet surfaces are either concave or flat, whereas in the strong-adhesion regime where the outer doublet surfaces are convex the cells should form rounded clump-like aggregates.

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Keywords: Erythrocyte; Aggregation; Doublets; Rouleau

1. Introduction

If the blood flow is slow enough such that the hydrodynamic shear stress in plasma is small, red blood cells form a tightly packed linear stack of cells called a rouleau. This normal physiological process has been studied in considerable detail [1] (and recently also directly observed *in vivo* in post-capillary venules [2]) primarily because of its relevance for erythrocyte aggregation and the related erythrocyte sedimentation rate measurement as a non-specific inflammation test routinely used in medical screening. An inflammation process increases the concentration of fibrinogen and this induces the formation of large aggregates which sediment faster than individual erythrocytes due to a decreased viscous drag force per erythrocyte, and so the sedimentation rate can be used as a measure of the fibrinogen presence. The importance of this experiment for hematology is one of the reasons why erythrocyte aggregation can be considered a prototypal biophysical problem: It is directly related to medical practice but it involves biological objects which can be described by tractable physical models [3,4] although they exhibit an extremely broad range of morphological variability depending on conditions [5].

Theoretical efforts to understand erythrocyte aggregation from a mechanical perspective build on Helfrich's elastic theory of lipid bilayer membranes [6] which proved quite useful in the interpretation of several mechanical aspects of simple biological cells [7]. Studies of aggregates were pioneered by Skalak et al. [8] who extended the elastic theory of lipid membranes by the shear elasticity of the erythrocyte membrane and by the minimal, coarse-grained model of adhesion where the adhesion energy is proportional to the area of the contact zone. The first analyses of aggregates resorted to the approximation that worked rather well for free cells: The equilibrium, minimal-energy structures were assumed to belong to the class of axisymmetric vesicles. Furthermore, Skalak et al. [8] restricted their candidate solutions by the additional symmetry constraint of a planar contact zone.

☆ This paper was inspired by the work of William Terence Coakley who passed away on November 18 2006, and we dedicate it to his memory.

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But they noted that at small adhesion strengths, the observed rouleaux are typically characterized by a zig-zag stack of cells as if the discocytic shape of the erythrocytes were not distorted by aggregation and each erythrocyte would partly fit in the concave part of its neighbor's membrane. This tentative description is supported by the concave shape of the outer, non-adhering part of the cap cells in a rouleau, which appears to be quite similar to concave part of a free discocyte. These features of rouleaux were inconsistent with the flat-contact zone hypothesis, and one of the conclusions of Ref. [8] is that their model could not account for all observed rouleau morphologies.

The attempts to use models such as that explored in Ref. [8] to resolve these issues have been impeded by technical difficulties. Nonetheless, the detailed and insightful results of work on vesicle–substrate adhesion [9,10] paved the way to the theoretical analysis of axisymmetric infinite rouleau [11] which predicted that a weak-adhesion stack of cup-like erythrocytes should transform into a stack of flat-contact shapes as the adhesion strength is increased. The flat-contact cell shapes appear to describe the rouleaux in the strong-adhesion regime rather accurately, whereas the weak-adhesion rouleau structure, and thus the aggregation threshold, is still not understood theoretically. The related open questions of the mechanics of rouleau formation are: i) why is the length of the experimentally observed rouleaux finite, ii) what is the role of erythrocyte polydispersity in aggregation, and iii) what are the possible pathways of rouleau formation.

A large part of theoretical as well as experimental work on erythrocyte aggregates points to the limitations of the symmetry restrictions imposed in the theoretical studies. In free vesicles, the non-axisymmetric shapes can be stable in a relatively narrow although not negligible part of the phase diagram [12]; in aggregates, they may be more prominent if not essential. To explore this possibility, we have recently used a numerical approach to explore the morphologies of vesicle doublets [13] thus following Ref. [8] in terms of the type of aggregates studied. In the absence of any symmetry restrictions, we found that at moderate reduced volumes the dominant morphologies are the axisymmetric flat-contact doublet and the non-axisymmetric sigmoid-contact doublet with a non-planar contact zone [13].

In this paper, we present further theoretical arguments to show that the sigmoid-contact zone may be an essential element of the mechanics of rouleau formation. We first outline the theoretical framework used and introduce the physical parameters that control the morphology of aggregates (Section 2). In Section 3 we first review the main qualitative features of symmetric doublets [13] and then explore the most interesting region of the parameter space in more detail. Section 4 deals with the morphology of asymmetric doublets, Section 5 discusses the modes of cell aggregation, and Section 6 concludes the paper.

2. Theoretical framework

To study the shapes of cell doublets, we resort to the standard continuum theory of vesicle elasticity, the so-called area-difference-elasticity (ADE) theory [14] extended by the minimal model of adhesion. Although it does not include the shear elasticity of the erythrocyte cytoskeleton [4], this approach describes the main features of the large-scale cell deformations rather well [13]. Its main ingredients are the membrane bending energy, non-local bending energy, and the adhesion energy. The bending energy depends on the integrated squared mean curvature:

$$W_{\mathrm{b}} = \frac{k_c}{2}\oint (C_1 + C_2)^2 dA, \tag{1}$$

where k_c is the bending constant, and C_1 and C_2 are the principal curvatures. The non-local bending energy is related to the relative stretching of the two monolayers and reads

$$W_{\mathrm{r}} = \frac{k_r}{2h^2 A_0}(\Delta A - \Delta A_0)^2, \tag{2}$$

where k_r is the non-local bending constant $\approx 3k_c$ [15], h is the separation of the neutral planes of the lipid monolayers in the membrane, A_0 is the membrane area, and

$$\Delta A = h\oint (C_1 + C_2) dA \tag{3}$$

is the actual monolayer area difference and ΔA_0 is its equilibrium value in a free vesicle. The simplest model of the adhesion energy is the contact potential

$$W_{\mathrm{a}} = -\Gamma A_{\mathrm{c}}, \tag{4}$$

where Γ is an effective adhesion strength typically ranging from 10^{-7} to 10^{-5} J/m^2 [16] and A_{c} is the area of the contact zone. The total energy of an aggregate includes the bending energies of all members and the adhesion energies of all pairs of members, and the equilibrium shape of the aggregate is to be sought at fixed volume and fixed area of each member.

The vesicle volume and surface area can be combined in a single quantity, the so-called reduced volume v defined as the ratio of the volume of a given vesicle and the volume of a sphere such that their areas are identical; the radius of this sphere is given by $R_{\mathrm{s}} = \sqrt{A_0/4\pi}$ and the energy of a vesicle is conventionally measured relative to the sphere's elastic energy of $8\pi k_c$. The reduced volume is the dominant morphological determinant: If v is close to 1, the vesicle cannot depart very much from a spherical shape. However, if the reduced volume is not too large, the equilibrium shape can be controlled by the reduced equilibrium monolayer area difference $\Delta a_0 = \Delta A_0/8\pi h R_s$ (where h is the separation of the neutral planes of the two monolayers) provided that the non-local bending constant is large enough: $\Delta a_0 \lesssim 1$ stabilizes cup-like, stomatocytic shapes, for $\Delta a_0 \approx 1$ the free vesicle assumes a biconcave discocytic shape, and for $\Delta a_0 \gtrsim 1$ the vesicles are cigar-like [17]. The reduced actual monolayer area difference

$$\Delta a = \frac{\Delta A}{8\pi h R_{\mathrm{s}}} \tag{5}$$

is defined analogously to Δa_0.

In an aggregate, the set of shape-determining factors is extended by adhesion described by the reduced adhesion strength

$$\gamma = \frac{\Gamma R_{\mathrm{s}}^2}{2k_c}. \tag{6}$$

In red blood cells, the reduced adhesion strength that can be induced experimentally *in vitro* may reach $\gamma \approx 100$ [13].

To expose the mechanisms of the doublet morphology as clearly as possible, we study two limiting cases of the ADE model. If we regard the doublet shape as a result of the interplay of non-local bending elasticity and adhesion as the competing morphological determinants, then it is clear that at small adhesion strengths the shape of a doublet will be dictated by the shape parameters of a free cell. This limit is conveniently described by the *fixed area-difference model*, where we assume that the non-local bending constant is infinite such that the actual monolayer area difference ΔA must be equal to ΔA_0, the monolayer area difference of a free cell. By varying ΔA_0, we scan the full range of possible doublet morphologies needed for any subsequent ADE analysis. In the opposite limit of large adhesion strengths, adhesion is expected to deform the cells such that their contact area is as large as possible, thereby inducing an extension of the lipid monolayers in the membrane and overriding the effect of non-local bending. This behavior is covered by the *relaxed area-difference model* where the non-local bending constant is 0 so that ΔA is effectively unconstrained.

3. Symmetrical doublets

The existence of a curved rather than flat cell–cell contact has been noted experimentally a long time ago: The electron micrograph of an erythrocyte doublet [8] and many of the cell–cell contacts both in doublets and in aggregates of more than two cells [18,19] clearly demonstrate the tendency of cells to form curved or even almost folded contact zones as large as possible at the expense of the non-adhering part of the membrane. Although many of the reported structures do not correspond to cells of identical volume and surface areas and the cross-sections in the micrographs often show a non-representative view of the aggregate, these observations imply that the preferred type of contact is such that the shapes of adhering cells are the same. In a doublet, this invariably means that the contact zone should be either flat or symmetrically curved. The simplest, least bent symmetrically curved contact is characterized by an S-shaped cross-section, and this type of cell–cell contact has been proposed as the salient feature of the limiting doublet shape [8]. To provide theoretical support for the proposed doublet shape, we have used the *Surface Evolver* software package [20] to analyze the stable morphologies. Indeed we found that the axisymmetric flat-contact doublets and the non-axisymmetric sigmoid-contact doublets are stable at small and large adhesion strengths, respectively [13]. In the flat-contact doublet, each cell (in the following, vesicles used as theoretical models of cells are referred to as cells) looks as if it adhered to a solid substrate; it retains the rotational symmetry of a free discocyte but loses the equatorial mirror symmetry. The most notable structural feature of the sigmoid-contact doublet is the S-shaped contact zone with one invagination and a matching evagination. The cells do not sit exactly on top of each other but are displaced alongside the contact zone (Fig. 1).

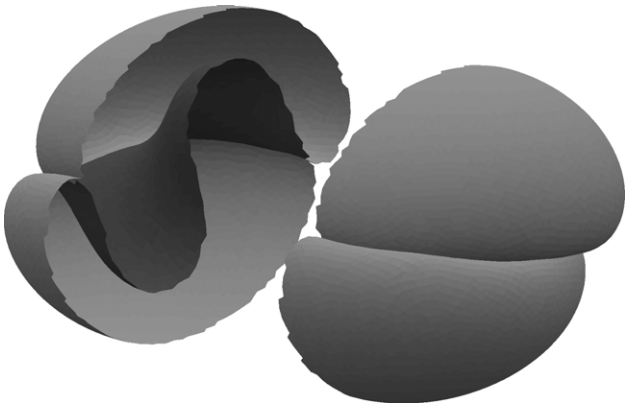

Fig. 1. Exploded view of relaxed sigmoid-contact doublet for $v=0.6$ and $\gamma=9$. The outer, non-contact part of the membrane is quite convex, and the hallmark of the doublet is the S-shaped contact zone with an invagination and a matching evagination.

We explored the morphological sequence of doublets in detail at the reduced volume of $v=0.6$ which corresponds to the normal human erythrocyte, and we first restricted the discussion to doublets formed by cells of identical areas, equilibrium monolayer area differences, and volumes. The morphological sequence of such doublets is as follows: At very small adhesion strengths $\gamma \lesssim 1$, the cells are free as the energy gained upon aggregation is smaller than the elastic deformation caused by aggregation [13]. At moderate adhesion strengths $\gamma \sim 1$, the cells form flat-contact doublets, whereas in the strong adhesion regime with $\gamma \gg 1$ the doublets are characterized by a sigmoidally curved contact zone.

We analyzed the doublet shapes both for cells with fixed reduced monolayer area difference and for relaxed cells, the latter referring to the unconstrained value of Δa. The values of the adhesion strength at the free cell/flat-contact doublet and the flat-contact/sigmoid-contact doublet transition predicted by the relaxed and the fixed Δa models are generally different. However, the two γs characterizing these transitions obtained by the relaxed Δa model coincide rather nicely with their counterparts computed using the fixed Δa model with the physiologically relevant value of $\Delta a = 1.04$ which corresponds to the human erythrocyte: The threshold for doublet formation is at $\gamma \approx 0.3$ for cells with fixed $\Delta a = 1.04$ and at $\gamma \approx 0.4$ for relaxed cells [13]. In the case of cells of membrane area and bending elastic constant of red blood cells ($A_0 \approx 140\ \mu m^2$, $k_c \approx 2 \times 10^{-19}$ J [15]) this suggests that rouleau formation should be induced by an adhesion strength of the order $8\pi k_c / A_0 \approx 4 \times 10^{-8}$ J/m^2. This threshold is well within the range of adhesion strengths that can be induced *in vitro* by a solution of macromolecules [16] so that the whole set of doublet morphologies theoretically discussed here should be observable experimentally.

On the qualitative note, the calculated shapes of doublets do reproduce many features of the observed morphologies of erythrocyte doublets. Especially striking is the agreement of the relaxed model doublets and the erythrocyte doublets in dextran [13,18] which proves that if strong enough, adhesion is the dominant factor determining the shape of an aggregate. In the

limit of $\gamma \gg 1$, the relative stretching of the lipid monolayers in the membrane and the associated non-local bending energy seem to be less important.

To assess the relative effect of adhesion compared to the non-local bending energy, we calculated the doublet energy within the fixed monolayer area difference model for several values of Δa. The total doublet energy as a function of Δa is shown in Fig. 2. At large enough adhesion strengths $\gtrsim 10$, the doublet energy increases steeply as Δa departs from the value it assumes in a relaxed doublet, this value being only weakly dependent of γ and approximately equal to 0.94. Fig. 2 shows that the minimum of the total energy as a function of Δa is more and more pronounced as γ is increased. These results suggest that at large adhesion strengths, any variation of Δa around the relaxed value results in an increase of the combined bending and adhesion energy that is much larger than the associated change of the non-local bending energy. In this regime, the cells in a doublet are best described by the relaxed Δa model where Δa is unconstrained and free to assume the optimal value at a given γ, and the shape of a doublet is determined primarily by adhesion. On the other hand, at small adhesion strengths $\lesssim 3$, the non-local bending deformation plays an important part, and it forces the cell in an aggregate to retain the discocytic free cell shape. In the limit of $\gamma \to 0$, the cells behave as if Δa were fixed to the equilibrium value of a free discocyte ≈ 1.04.

An important determinant of the shape of a free cell is its reduced volume, and the same holds true for cell aggregates. Here we provide a semi-quantitative insight into the dependence of the doublet morphological sequence on the cell volume. The phase diagram shown in Fig. 3 is partly based on the analysis of a vesicle adhering to a flat substrate [10] where the adhesion

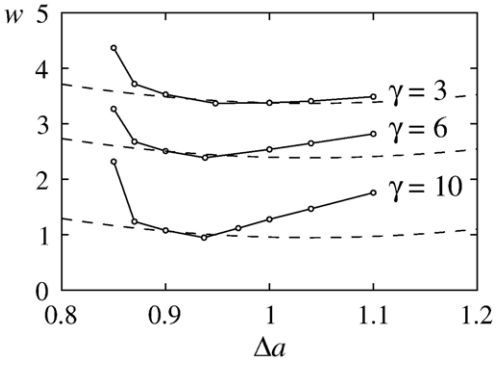

Fig. 2. Doublet energy computed using the fixed monolayer area difference model as a function of Δa for reduced adhesion strengths of $\gamma = 3$, 6, and 10 (open circles; solid lines connecting sets of data corresponding to a certain value of γ indicated in the figure are a guide to the eye; the lowest point of each set was obtained using the relaxed Δa model) compared to the superposition of the energy of a relaxed doublet at $\gamma = 3$, 6, and 10 and the non-local bending energy with $k_r/k_c = 3$ [15] and $\Delta a_0 = 1.04$ (dashed lines). At $\gamma = 3$, the minimum of w obtained by the fixed Δa model at $\Delta a \approx 0.95$ is shallow, and the ADE analysis with $\Delta a_0 = 1.04$ and k_r/k_c (which correspond to a human erythrocyte) would shift it to $\Delta a \approx 0.99$. On the other hand, at large values of adhesion strength (e.g., at $\gamma = 10$) the minimum of the total energy becomes more pronounced and is located at $\Delta a \approx 0.94$ virtually irrespective of γ. For $\gamma \gtrsim 10$, any departure of Δa from the minimal, relaxed value increases the total bending and adhesion energy far more dramatically than the associated variation of the non-local bending energy, and thus the effects of non-local bending are negligible.

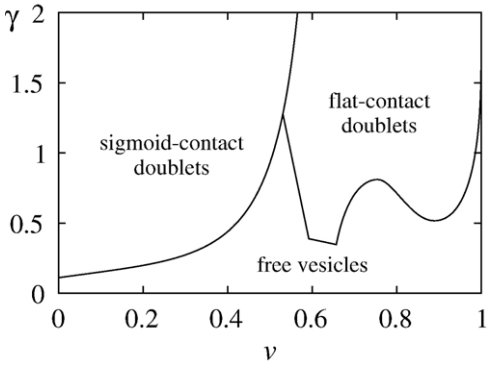

Fig. 3. A semi-quantitative phase diagram of cell doublets: The flat-contact doublets are stable at reduced cell volumes larger than about ≈ 0.5 (provided that the adhesion strength is not too large), whereas at smaller volumes the only stable type of doublet is the sigmoid-contact doublet. The limit of stability of the sigmoid-contact doublet is based on our numerical results at $v = 0.5$ and $v = 0.6$, and the free cell/flat-contact doublet transition line is adapted from Ref. [10].

threshold was found to increase as the reduced vesicle volume is decreased below ≈ 0.5, and partly on our numerical results at $v = 0.5$ and $v = 0.6$ which show that the adhesion strength at which the flat-contact/sigmoid-contact transition takes place decreases rapidly with decreasing volume. These findings imply that at reduced volumes below ≈ 0.5 the flat-contact doublets are not stable at any value of γ and that at small reduced volumes the only stable doublet morphology is the sigmoid-contact doublet.

4. Asymmetrical doublets

Having established that the sigmoid shape of the contact zone is a structural feature of doublets formed by identical cells within a broad range of volumes and other parameters, we now explore how it is affected by a moderate polydispersity of cells. Clearly this particular contact-zone shape cannot be present in aggregates formed by cells of very dissimilar volumes; in this case, the larger cell should either partly or completely envelop the smaller one, which should lead to structures resembling the intermediate stages of phagocytosis. We would expect that in this limit, doublets should bear some similarity to vesicle–colloid complexes [21,22].

On the other hand, it is natural to expect that if the parameters of the cells are not too different, the sigmoid contact should remain qualitatively unchanged. We investigated the effect of dissimilar parameters by studying doublets of cells that differ in volume, whereas the areas and monolayer area differences are identical. We find that the stable sigmoid-contact doublets are generally shifted to larger adhesion strengths compared to completely identical cells but the sigmoid-contact doublet still is the preferred shape at strong enough adhesion. The doublet is, of course, no longer symmetric (Fig. 4a): The contact-zone evagination on the more inflated cell is larger than the evagination on the less inflated one.

In a similar way, the axisymmetric weak-adhesion doublet with a contact zone protruding into the less inflated cell (Fig. 4b) can be regarded as a derivative of the flat-contact doublet. Thus it

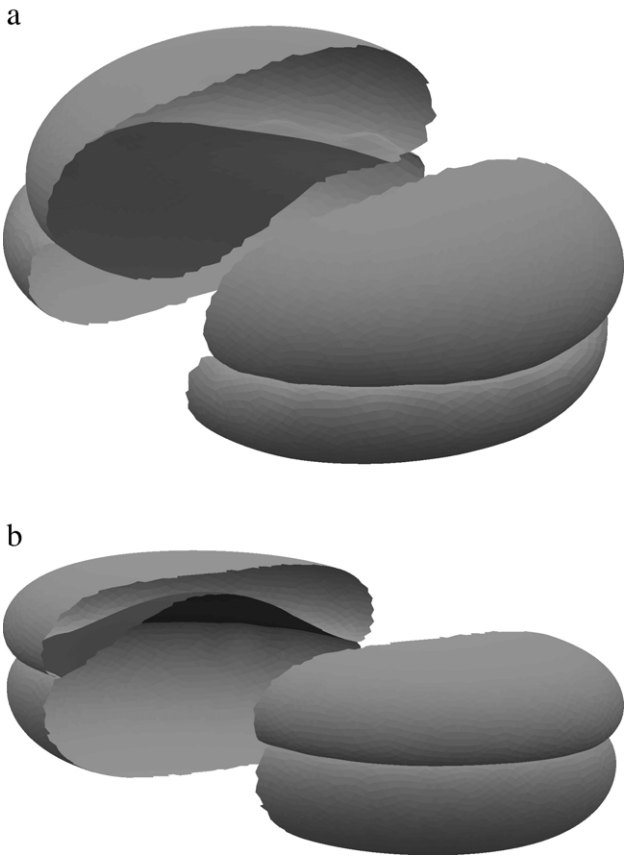

Fig. 4. Exploded view of the sigmoid-contact doublet of cells of identical area, $\Delta a = 1.04$, and $v_1 = 0.4$ and $v_2 = 0.8$. At large adhesion strength the stable morphology is an asymmetric version of the sigmoid-contact doublet (a, $\gamma = 18$), whereas at moderate adhesion strengths (b, $\gamma = 9$), the cells form an axisymmetric male–female doublet.

appears reasonable to divide the doublet shapes into two classes based on the number of invaginations/evaginations on the contact zone of a cell rather than on the symmetry. Such a division puts all male–female axisymmetric doublets with 1 contact-zone invagination and no evaginations (or vice versa) into the same class regardless of the relative cell areas and volumes, the flat-contact doublet being the limiting case where the magnitude of the invagination vanishes. In a similar fashion, all doublets with 1 invagination and 1 evagination on the contact zone fall into the class of sigmoid-contact doublets.

The results discussed above and illustrated by Fig. 4 are consistent with electron micrographs of erythrocytes in dextran [19] which show a certain non-negligible degree of polydispersity of cells. Yet many of the cell–cell contacts are consistent with the sigmoidal shape of the contact zone, especially in view of the fact that the cross-sections captured by the micrograph are likely not the most representative.

5. Modes of aggregation

The above discussion of the doublet shapes carries an important message about the formation of large red blood cell aggregates. In Fig. 3, we have argued that i) the sigmoid-contact doublets are the dominant morphology at volumes comparable to and smaller than the volume of human erythrocyte and that ii) the aggregation threshold decreases with decreasing volume. Given that for small adhesion strengths, the non-local bending energy causes the cells to remain biconcave (as demonstrated in Fig. 2) we conclude that the members of sigmoid-contact doublets at small γ should not be very different from free discocytes. Thus upon adhesion induced by increasing γ beyond the aggregation threshold, which is small for $v \lesssim 0.6$ (Fig. 3), the cells in a doublet retain the invagination on the non-contact, cap part of the membrane because of the non-local bending energy which favors the discocytic shape. This suggests that a third cell could conceivably easily adhere to the doublet cap just like the members of the doublet adhere to each other, i.e., such that the shapes of both contact zones in the linear triplet would be close to the shape of the sigmoidal doublet contact zone. A fourth, fifth, sixth… cell could be appended the same way and with identical energy balance, thereby forming a large rouleau by adding a single cell at a time [23]. Of course, the same final configuration can also be achieved by combining several rouleaux with concave caps end-to-end.

This scenario can be corroborated quantitatively in terms of the cap curvature defined as the reduced mean curvature

$$c_{\mathrm{cap}} = R_{\mathrm{s}} \frac{C_{1\mathrm{cap}} + C_{2\mathrm{cap}}}{2} \tag{7}$$

at the apex of the invagination/evagination on the outer face of a cell in a doublet. This parameter has been first used in Ref. [24] to quantify the shape of end cells in a rouleau. In Fig. 5, cap curvature is plotted as a function of adhesion strength for the flat-contact and sigmoid-contact doublets with $\Delta a = 1.04$ as well as relaxed sigmoid-contact doublets, and compared to the cap curvature of a free cell; the reduced volume is 0.6. The figure clearly shows that the cap curvature c_{cap} of the metastable $\Delta a = 1.04$, $\gamma = 3$ doublet is rather close to that of the free cell, whereas in the stable flat-contact $\Delta a = 1.04$, $\gamma = 3$ the caps are

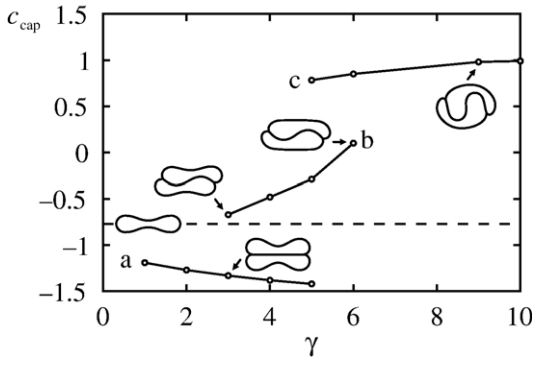

Fig. 5. Cap curvatures versus reduced adhesion strength for $v = 0.6$ cell doublets: a) flat-contact doublet with $\Delta a = 1.04$, b) sigmoid-contact doublet with $\Delta a = 1.04$, c) relaxed sigmoid-contact doublet. The dashed line corresponds to the cap curvature of the free cell. Also shown are a few characteristic cell and cell aggregate cross-sections adapted from Ref. [13]. A free cell is morphologically most compatible with the cap of a sigmoid-contact doublet at adhesion strengths around $\gamma = 3$ where their cap curvatures are very similar. The optimal conditions for rouleau formation thus correspond to adhesion strengths about 10 times larger than the aggregation threshold at $\gamma \approx 0.3$ [13].

much more concave than in the free cell. As the adhesion strength is increased, the cap curvature of the flat-contact doublets decreases. Simultaneously, the caps of the $\Delta a = 1.04$ sigmoid-contact doublets become less concave and eventually flatten at $\gamma \approx 6$. Since at $\gamma = 6$ the fixed Δa model is less realistic than the relaxed Δa model (as shown in Fig. 2, in this regime the effects of non-local bending energy stabilizing the biconcave cell shape are outbalanced by adhesion which favors as large a contact area as possible, and this is best described by the relaxed model), we also plot the cap curvature for the relaxed sigmoid-contact doublets whose outer surface is clearly very convex. Thus the doublet type that might most effectively participate in the rouleau-forming process is the small-γ sigmoid-contact doublet with concave caps.

Based on these results, we can define five distinct adhesion regimes each characterized by a specific type of aggregate: a) free cells; b) threshold regime; c) weak-adhesion regime; d) strong-adhesion regime; and e) very strong-adhesion regime (Fig. 6). For reduced volume of 0.6 (all values listed correspond to cells of $v = 0.6$), free cells are the stable configuration at reduced adhesion strengths no larger than 0.3 [13]. In the threshold regime with $0.3 < \gamma \lesssim 2$ (all values listed except the aggregation threshold of $\gamma = 0.3$ are approximate within 10–20%), the predominant aggregate shape is the flat-contact doublet. Caps of such doublets are considerably more concave than those in free cells and the corresponding elastic energy barrier for aggregation is large. As a result, formation of triplets, quadruplets, etc. is suppressed.

In the weak-adhesion regime ($2 \lesssim \gamma \lesssim 4$), the sigmoid contact is the basic morphological feature that may allow the cells to form a rouleau while keeping the biconcave discocyte shape. We expect that the energy barrier that prevents a free cell from adhering to either end of a rouleau (which could be quantified as the difference between the bending energy of a cell in an aggregate and that of a free cell) is rather small. In contrast, any other type of rouleau growth would involve a much larger deformation of the cell and should be disfavored; the proposed structure of a rouleau in the weak-adhesion regime is shown in Fig. 7a. The equilibrium rouleaux should consist of a large number of cells: Ideally, they would be infinitely long. At the same time, at small adhesion strengths the cells are not bonded very strongly, and thus in this regime the rouleaux may be broken apart by a weak mechanical disturbance such as blood flow.

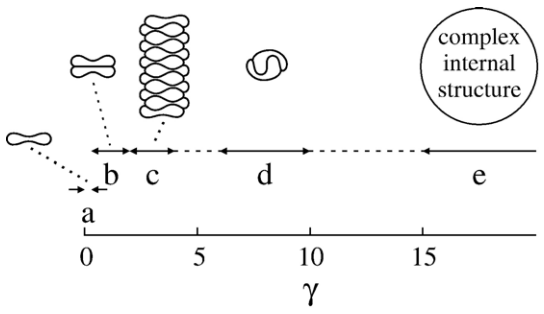

Fig. 6. Adhesion regimes for reduced volume of a normal red blood cell $v = 0.6$: a) free cells; b) flat-contact doublets; c) sigmoid-contact rouleaux; d) sigmoid-contact round doublets; and e) spherical large clumps with an irregular internal structure (not indicated in the figure). Dashed lines indicate that the boundaries between the respective regimes are approximate. See text for detailed explanation. The free cell and doublet cross-sections are based on the numerical minimization of the fixed Δa (a and b) and the relaxed Δa model (d) [13], whereas the tentative structure of the sigmoid-contact rouleau is schematic.

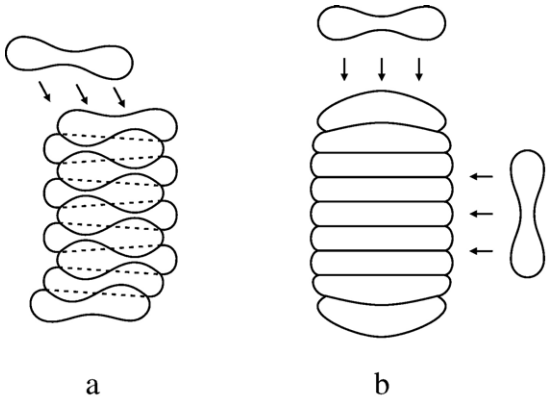

Fig. 7. Schematic of a rouleau cross-section in the weak-adhesion regime (a) where the preferred mode of rouleau growth is linear; both zig-zag and staircase cell sequence should be possible. The dashed lines indicate the rim of the contact zone which is tilted with respect to the lengthwise rouleau axis consistent with the micrographs of rouleaux [8]. In the strong-adhesion regime (b), the contact zones are flat and the rouleau caps are convex. This could conceivably impede end-on growth of the rouleau; instead, the free cells could adhere to it side-on which should result in a more or less spherical clump.

In the strong-adhesion limit ($6 \lesssim \gamma \lesssim 10$), experiments show that the contact zones of cells in a rouleau are most likely flat and the rouleau caps are convex [8]. Upon adhesion at the end of a rouleau, a free cell must be deformed considerably to form the convex cap, and several cells near the end of the rouleau must undergo a certain morphological transformation such that appending an erythrocyte is a non-local process. This suggests that the energy barrier separating a n-member rouleau and a free cell from a $(n+1)$-member rouleau is large and impedes aggregation despite the considerable favorable energy difference. Due to this energy barrier, a free cell is unlikely to stick to either cap of a rouleau. As a result, we expect the rouleaux to be short rather than long; it is possible that the preferred type of aggregate formed in the strong-adhesion regime is a doublet.

If the adhesion is very strong ($\gamma \gtrsim 15$), cells attracted to a rouleau may prefer an energetically less favorable lateral contact (Fig. 7b) and eventually leads to a disordered and predominantly spherical clump rather than to a rouleau. Alternatively, the clump may be formed in a three-stage process where i) the cells initially form sigmoid-contact doublets whose external shape is virtually round in this regime; ii) the spherical doublets pack like hard-sphere particles to form a clump; and iii) the doublet–doublet contacts relax such that the inter-doublet voids within the clump are minimized, thereby compactifying the internal structure of the clump. The clump is presumably closer to the global minimal-energy configuration than the rouleau: We stress that in the above discussion, we considered the most probable way of rouleau growth by adding a single cell at a time, and we looked for the most favorable way of appending it without rearranging the existing aggregate. This means that the

scenarios described are determined kinetically, i.e., by the mode with the fastest growth rate. However, it seems very likely that the rouleau and the (spherical) clump are the equilibrium aggregate morphologies in weak and strong-adhesion regime, respectively, so that the two kinetic pathways do lead to minimal-energy configurations.

The above arguments addressing the rouleau length build on the local morphology of a cell compared to either rouleau or a spherical clump. At this level, they provide a qualitative insight into the preferred growth modes depending on the adhesion strength. A complete description of the ensuing distribution of rouleaux lengths is a considerably more complex problem which has been treated using theories of addition and condensation polymerization [25]. At this level, it is possible to analyze the rouleau structure in terms of the different processes such as the addition of a single cell, the end-to-end merging of two rouleaux, and branching [25].

Our present understanding of the mechanics of rouleau formation relies on the specific morphological features of the sigmoidal contact zone which, in the weak-adhesion regime, is instrumental for keeping the shape of rouleau caps quantitatively very similar to the face of a free cell such that upon adhesion, a cell need not undergo a large deformation. In this context, we stress that the concave shape of a doublet cap does not depend solely on the adhesion strength but also on reduced cell volume. With decreasing reduced volume, the biconcave character of an isolated cell becomes more pronounced, and the concavity of the outer surfaces of the sigmoid-contact doublet also increases simultaneously thereby facilitating the end-on addition of a cell to a doublet. Furthermore, the semi-quantitative phase diagram of a doublet shown in Fig. 3 suggests that the sigmoid-contact doublet is the only stable doublet shape at reduced volumes smaller than ≈ 0.5. Thus we conclude that rouleau formation may also be promoted by decreasing the red blood cell volume at fixed adhesion strength. This mechanism of increasing the rouleau-forming tendency would require a weak intercellular attraction in conjunction with an agent that would reduce the volume of the cells, e.g., by way of osmotic stress in a hypertonic environment. This could be caused by increasing the concentration of a solute in blood plasma, and it is possible that both non-specific molecules that do not affect the cell–cell interaction directly as well as the non-adsorbed fraction of molecules actively involved in inter-membrane adhesion (e.g., fibrinogen and immunoglobulin) may produce a similar effect. This effect has been studied and observed *in vitro* [26].

It is also possible that a similar effect may be caused by a moderately hypotonic environment which increases rather than decreases the cell volume. A normal red blood cell is biconcave, and if its reduced volume is increased to about $v \approx 0.9$ the invaginations disappear and the curvature of the flattened parts of the cell is virtually 0. On the other hand, the threshold for adhesion at a flat substrate is lowest at $v \approx 0.65$ [10]; the invagination of the non-contact cap of the cell is still rather pronounced at this volume. Taken together, these two facts suggest that at a certain volume somewhere between 0.65 and 0.9 the free cell shape needs not be modified dramatically to form a flat-contact rouleau and the aggregation threshold should reach a minimum at this value of v. However, we note that if the cell volume is increased beyond the point where the adhesion threshold is smallest (at $v \approx 0.65$, cf. Fig. 3), aggregation should be suppressed.

6. Conclusions

Based on our theoretical insight into the morphology of vesicle doublets, we have analyzed the possible mechanisms of rouleau formation in red blood cells. We find that the most probable mode of growth of the linear aggregates is by adding a single cell end-on or, alternatively, by an end–end contact of several rouleaux. This mode is active in the weak-adhesion regime where the shape of the rouleau cap is moderately concave. The estimated range of reduced adhesion strengths where rouleau growth is promoted is between $\gamma \approx 2$ and $\gamma \approx 4$; for erythrocytes, this corresponds to adhesion strengths between 80 and 160 nJ/m^2. At larger adhesion strengths, the rouleau growth is impeded or even arrested, and any further aggregation produces more or less spherical clumps of cells rather than rouleaux. This scenario is consistent with the experimental observations of red blood cell aggregates in dextran [18] and *in vivo* [25].

Finally, we stress that the adhesion strength is not the only physical and physiological parameter that controls aggregation: Cell volume is also important, and rouleau growth should be promoted either by deflation (which stabilizes sigmoid-contact rouleaux) or by a moderate inflation (which stabilizes flat-contact rouleaux). Thus we conclude that rouleau formation is not governed solely by the presence of biochemical agents causing inter-membrane attraction but also by any plasma component that affects the osmotic stress. At this point, it would be worthwhile to identify the potentially relevant blood factors such that their physiological concentration varies across a large enough range to affect aggregation by altering red blood cell volume. This would further elucidate the causal relationship between the erythrocyte sedimentation rate and the physiological conditions, which remains an open and clinically relevant problem [27].

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