

Biomechanics of red blood cells in human spleen and consequences for physiology and disease

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Red blood cells (RBCs) can be cleared from circulation when alterations in their size, shape, and deformability are detected. This function is modulated by the spleen-specific structure of the interendothelial slit (IES). Here, we present a unique physiological framework for development of prognostic markers in RBC diseases by quantifying biophysical limits for RBCs to pass through the IES, using computational simulations based on dissipative particle dynamics. The results show that the spleen selects RBCs for continued circulation based on their geometry, consistent with prior *in vivo* observations. A companion analysis provides critical bounds relating surface area and volume for healthy RBCs beyond which the RBCs fail the “physical fitness test” to pass through the IES, supporting independent experiments. Our results suggest that the spleen plays an important role in determining distributions of size and shape of healthy RBCs. Because mechanical retention of infected RBC impacts malaria pathogenesis, we studied key biophysical parameters for RBCs infected with *Plasmodium falciparum* as they cross the IES. In agreement with experimental results, surface area loss of an infected RBC is found to be a more important determinant of splenic retention than its membrane stiffness. The simulations provide insights into the effects of pressure gradient across the IES on RBC retention. By providing quantitative biophysical limits for RBCs to pass through the IES, the narrowest circulatory bottleneck in the spleen, our results offer a broad approach for developing quantitative markers for diseases such as hereditary spherocytosis, thalassemia, and malaria.

erythrocytes | microcirculation | spleen clearance | malaria | spherocytosis

The spleen, about 10–12 cm in length with a mass of 100–200 g and located in the left superior abdomen, is the largest lymphatic organ in the human body (1, 2). The spleen plays a key role in the human immune system by protecting the body from pathogenic microorganisms reaching the bloodstream, through innate phagocytosis or adaptive responses operated by lymphocytes and antibodies. The spleen also serves as a filter that can remove red blood cells (RBCs) from circulation because of either physiological senescence or pathological alterations.

RBCs can be recognized as altered when changes in their shape, size, or surface are detected or their deformability is impaired as a consequence of such changes. Archetypical surface alterations are externalization of phosphatidyl-serine on the outer leaflet of the phospholipid bilayer of the RBC membrane or fixation of antibody on surface antigens, as occurs in transfusion mismatch. Sensing of these alterations by macrophages can occur all over the body with strong predominance in the spleen and liver (3). By contrast, recognition of altered size, shape, and deformability is considered a spleen-specific function. Surface area loss and reduced deformability also occur during aging of healthy RBCs (4). The archetypical disease where mechanical retention of RBCs in the spleen is the central pathogenic process is hereditary spherocytosis (HS). HS is a genetic disorder resulting in dysfunctional membrane proteins that play a role in transforming the shape of the RBC from a normal discocyte to a sphere. HS occurs at a frequency of 1 in 5,000 births in the Caucasian population and is the most common origin

of hereditary intravascular and extravascular hemolysis. In HS, defects in band 3, ankyrin and spectrin membrane proteins connecting the RBC membrane to the spectrin network can lead to the vesiculation of unsupported lipid bilayer. Such vesiculation causes a gradual reduction in cell surface area by as much as 20% compared with that of a healthy RBC (5). This reduction, in turn, can significantly increase the retention rates of RBCs in the spleen because of their increased sphericity (6). Severe reduction in RBC deformability attributable to spherocytosis results in the flow of RBCs to be obstructed as they pass through the spleen (7, 8). Consequently, the RBCs are phagocytosed causing hemolytic anemia and splenomegaly (i.e., enlarged spleen). The fact that surgical removal of the spleen (splenectomy) alleviates anemia to a large extent in patients with severe HS lends support to the argument that recognition of altered deformability of RBCs is a specific function of the spleen. Increased *in vitro* mechanical retention of RBCs collected in splenectomized subjects further supports this inference (9). RBC deformability is impaired in several other conditions including thalassemia (10), burns (11), and *Plasmodium falciparum* malaria (12). During its 48-h life cycle, the *P. falciparum*-infected RBC progressively increases in stiffness. During the so-called “ring” stage (i.e., within 24 h of host cell invasion by the parasite), the infected RBCs (iRBCs) undergo ~9.6% surface area loss (13) and reduced deformability arising from up to a fourfold increase in membrane shear modulus (14). Innate mechanical retention of a proportion of normally circulating ring-iRBCs has been observed *ex vivo* in a

Significance

The 3D opening of the interendothelial slit in human spleen creates a physical fitness test for red blood cells (RBCs) and clears them from circulation if their geometry and deformability are altered. We present a unique computational framework for the development of prognostic markers for diseases that alter RBC physical characteristics and identify quantitative limits for splenic slit clearance. Our work shows how the splenic slit determines distributions of size and shape of healthy RBCs. Our work lays the groundwork for systematic reconstruction of RBC navigation in the human spleen with consequences for a variety of acute and chronic medical conditions associated with hereditary alterations of RBCs, infectious diseases, and cancers.

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model that provides the overall trends and bounds for comparison with computations and experiments so as to determine how the spleen influences the size and shape distributions of RBCs.

Results and Discussion

Computational Framework. We use the DPD-based RBC model (25, 26) for simulations. The details can be found in *Methods* and *SI Text*.

Fig. 1A is a schematic illustration of a venous sinus located in the cords in the red pulp of the human spleen. The sinuses comprise aligned endothelial cells that are connected by stress fibers to annular fibers. From such geometrical considerations and from detailed structural studies of the sinus wall in the TEM (20), we constructed the computational model for RBC traversal through the IES (Fig. 1B). The average height of the slits, $H_s \sim 0.65 \mu\text{m}$ with a range from 0.25 to 1.2 μm and an SD of 0.5 μm , and average length (the thickness of the slit wall) $L_s \sim 1.89 \mu\text{m}$ with a range from 0.9 to 3.2 μm and an SD of 0.9 μm . The width of the slit $W_s \sim 2\text{--}3 \mu\text{m}$ and the stress fiber width $W_f \sim 1 \mu\text{m}$ (27). Unless otherwise noted, we use the following reference geometry of the IES for all simulations in this study: height $H_s = 1.2 \mu\text{m}$, width $W_s = 4.0 \mu\text{m}$, and length $L_s = 1.89 \mu\text{m}$. The stress fiber width is $W_f = 1 \mu\text{m}$. These geometrical parameters were chosen from the upper bound value for slit height from TEM ultrastructural characterization experiments (20) for two reasons. First, because of the observation angle, the IES dimensions measured experimentally might have been underestimated. Second, the actual IES opening shape is not a rectangle with sharp corners. The opening is likely closer to an elongated ellipse, and the cross-section observed in the experiment might not exceed the maximum dimension in the height direction. Note that the effective stiffness of endothelial cells is ~ 100 times higher than that of RBCs (28); the slit walls were thus modeled as rigid side walls of circular cylinders, comprising endothelial cells, and thin slabs, representing annular stress fibers (Fig. 1B).

Analytical Framework. A simplified axisymmetric model amenable to theoretical analysis is first considered here (Fig. 2) to elucidate the effects of geometric constraints on RBC–IES interactions, which are further taken up for much more detailed investigation in our computational simulations that relax many of these geometric constraints.

We assume that the slit cross-section in the y - z plane is circular (Fig. 2) instead of rectangular (Fig. 1B), but with the same cross-sectional area such that

$$D_s = 2\sqrt{H_s W_s / \pi}. \quad [1]$$

The 3D slit geometry (Fig. 1B) is therefore approximated by the surface of a torus (Fig. 2). The deformed RBC at the critical condition consists of three parts: two spherical balls connected by a central connecting part (torus). The area and volume of the RBC are given, respectively, as

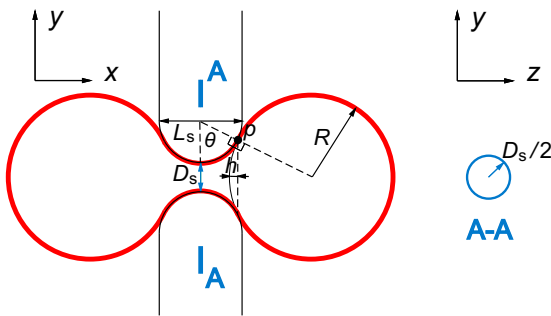


Fig. 2. Schematic illustration of the limiting geometry considered in the theoretical framework. The red solid line represents the RBC. At point p , the RBC surface is tangential to the surfaces of the endothelial cells. The cross-section A-A is shown on the right, where the solid line circle represents the slit cross-section. We approximate the real rectangular slit with the circular slit with the same cross-sectional area.

$$A = 4(2\pi R^2 - \pi R h) + 2\pi L_s \theta \left(\frac{D_s}{2} + \frac{L_s}{2} - \frac{L_s \sin \theta}{2\theta} \right) \quad [2]$$

and

$$V = 2 \left[\frac{4}{3} \pi R^3 - \frac{4}{3} \pi h^2 (3R - h) \right] + 2\pi A_c y_c, \quad [3]$$

where

$$h = R - R \sqrt{1 - \frac{\left(\frac{D_s}{2} + \frac{L_s}{2} \right)^2}{\left(R + \frac{L_s}{2} \right)^2}} \quad [4]$$

is shown in Fig. 2, which is the height of spherical cap cut by the y - z plane intersecting point p ,

$$y_c = \frac{D_s}{2} + \frac{L_s}{2} - \frac{L_s \sin \theta}{3\theta} \quad [5]$$

is the y coordinate of the centroid of the upper one-half cross-section of the torus part,

$$A_c = \frac{L_s \sin \theta}{2} \left[D_s + L_s - L_s \left(\frac{D_s}{2} + \frac{L_s}{2} \right) / \left(R + \frac{L_s}{2} \right) \right] - \frac{L_s^2}{2} (2\theta - \sin 2\theta) \quad [6]$$

is one-half of the cross-section area of the torus part in the x - y plane, and

$$\theta = \arccos \left[\left(\frac{D_s}{2} + \frac{L_s}{2} \right) / \left(R + \frac{L_s}{2} \right) \right] \quad [7]$$

is an angle of the torus part as shown in Fig. 2. The area and volume of the middle part are calculated based on Pappus's centroid theorem. Given the radius of the slit opening, D_s , and the thickness of the sinus wall, L_s , the axisymmetric theory thus provides the maximum volume at which the RBC with fixed surface area will be able to cross the slit of a given geometry. If Eq. 3 is rewritten as $V = f(R)$ and its inverse function is given by $R = f^{-1}(V)$, Eq. 4 and Eq. 7 can be expressed as $h = h(R) = h(f^{-1}(V)) = g_1(V)$ and $\theta = \theta(R) = \theta(f^{-1}(V)) = g_2(V)$. The relationship between the surface area A and the volume V is given by

$$A = 4 \left\{ 2\pi [f^{-1}(V)]^2 - \pi f^{-1}(V) g_1(V) \right\} + 2\pi L_s g_2(V) \left\{ \frac{D_s}{2} + \frac{L_s}{2} - \frac{L_s \sin[g_2(V)]}{2g_2(V)} \right\}. \quad [8]$$

Comparison with Experiments. The bounds predicted by the foregoing analysis can be compared with experimental observations in the literature (23) as well as with our computational simulations. These comparisons benefit from further insights gained through ex vivo experiments performed on isolated perfused human spleen (16) as well as on a synthetic spleen comprising a microsphere filtration system (20). The latter work suggests that the geometric characteristics obtained from spleen experiments could be recapitulated using micrometer-sized metal spheres. The microsphere filtration device was designed based on the observed slit shape and dimensions, with a 5-mm-thick layer of mixture of spheres, 5 to 15 μm in diameter. The pressure difference in the experiments where the RBC suspension was pushed through the device was held at 63.2 cm H_2O , giving an average pressure gradient of 1 Pa/ μm inside the filter (20). These experiments reveal that RBCs with

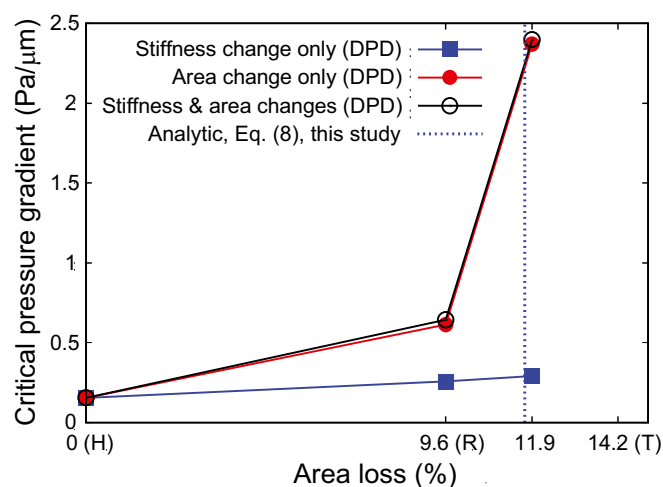


Fig. 5. The critical minimum pressure gradient for *P. falciparum*-infected RBCs in different stages to pass through the IES, as predicted by DPD. The blue dashed line is the limiting condition predicted by theory, Eq. 8, for critical area loss at fixed cell volume. Beyond this limiting geometry, an infinitely high pressure gradient is required for the RBCs to clear the splenic slit. The surface area loss for healthy (H), ring (R), and trophozoite (T) RBCs are taken to be 0%, 9.6%, and 14.2%, respectively, from the experiments of Safeukui et al. (13).

for the cell of about 2–4%, the critical pressure gradient in the spleen would be predicted, from our DPD simulations, to be between 1.42 and 0.64 Pa/μm. This value is close to 1.0 Pa/μm, derived from the microsphere filtration experiments, which were also compared with those from isolated perfused spleen experiments (13, 20). Under this pressure gradient of 1.0 Pa/μm, the retention rate in the spleen of ring-stage iRBCs was about 50%, which implies that this pressure gradient was the critical value for an average ring-stage iRBC to pass through the splenic slit.

Effects of Cell Surface Area and Stiffness. We now consider healthy, ring-, and trophozoite-stage RBCs invaded by *P. falciparum* merozoites; the surface area of the RBCs in these three cases, respectively, was taken to be 135, 122.04, and 115.83 μm² (13), and the respective values of the membrane shear modulus were 5.5, 15.5, and 28.9 pN/μm (31). The cell volume was taken to be 94 μm³ for all three cases. For ring and trophozoite cells, with intracellular parasite maturation over 6–24 and 24–36 h, respectively, we performed three simulations: (i) only stiffness change was considered, (ii) only surface area change was considered, and (iii) both stiffness change and surface area change were considered. By changing the magnitude of the pressure gradient, we identified the critical, minimum pressure gradient necessary for the cells to pass through the slit, as shown in Fig. 5.

The simulation results show that the surface area loss arising from RBC parasitization plays a much more important role than the ensuing stiffness increase. There is a significant increase in the critical pressure gradient from the ring to trophozoite stage. For trophozoite, no RBC passed the IES opening even for very high values of applied pressure gradient, if an area loss of 14.2% is considered. Therefore, we used area loss of 11.9% for trophozoite, which is the midpoint between the ring and trophozoite stages and can be considered as young trophozoite (35).

The blue vertical dashed line in Fig. 5 shows the theoretical prediction, Eq. 8, of the maximum area loss allowed for RBCs (at the fixed cell volume) to pass the circular opening with the same surface area as that considered in the DPD simulations. In physical terms, our theory indicates that cells with larger surface area loss than this critical value (at a fixed volume) will require an infinitely large pressure gradient for RBC passage; this prediction is consistent with the results of the computational simulations.

Effects of Cell Volume. Finally, we investigate how the cell volume influences the critical pressure gradient for RBCs to pass through the splenic slit. For these studies, we focus on the ring-stage iRBCs with a surface area of 122 μm² and a membrane shear modulus of 15.5 pN/μm. Fig. 6 shows the effect of the cell volume, for a fixed cell surface area, on the critical minimum pressure gradient for passage through the IES. The critical pressure gradient steeply increases as the change in the RBC volume approaches 20%. Cells with larger volumes were not able to clear the slit in our DPD simulations.

The blue dashed vertical line in Fig. 6 shows the maximum volume increase predicted by our theory for the RBC to clear the circular opening, with the same cell surface area as that considered in the DPD simulations. The theory predicts a maximum value of 19% volume increase (113 μm³) for a standard healthy cell (95 μm³), beyond which the minimum pressure gradient necessary to clear the slit becomes too large. The simulation results (red solid line) are seen to be in agreement with this axisymmetric theory. Hence, the analytical theory could serve as a rough guide for defining the critical RBC geometry in the limiting case.

Conclusions

We have developed computational simulations, coupled with a theoretical analysis, of the mechanics of RBC clearance by the spleen. We have generated a unique quantitative framework for RBCs crossing a narrow slit in the spleen, a process that cannot be dynamically observed in humans. Shape deformation during this most stringent biomechanical challenge on RBCs in circulation (20) appeared very different from the bullet-shaped deformation of RBCs in narrow capillaries (36). Our analyses and simulations provide limiting conditions of surface area and volume beyond which healthy RBCs can cross the spleen; these predictions are in agreement with the distributions of size and shape of healthy RBCs observed in circulation (Fig. 3). These results are also consistent with independent experimental results obtained by challenging human spleens with human RBCs modified either artificially (6) or by infection with the intraerythrocytic parasite *P. falciparum* (13). The present computations do not fully account for the interactions between the lipid bilayer and the cytoskeleton, which will inevitably require a more computationally expensive, two-component model. The computations also do not consider the effects of intrinsic stiffness of the membrane–cytoskeleton complex and viscosity of the cytoplasm on RBC passage through the splenic slits. However, as previously observed experimentally (6) and predicted here (Fig. 3), the surface to volume ratio is by far the most important parameter of RBC deformability.

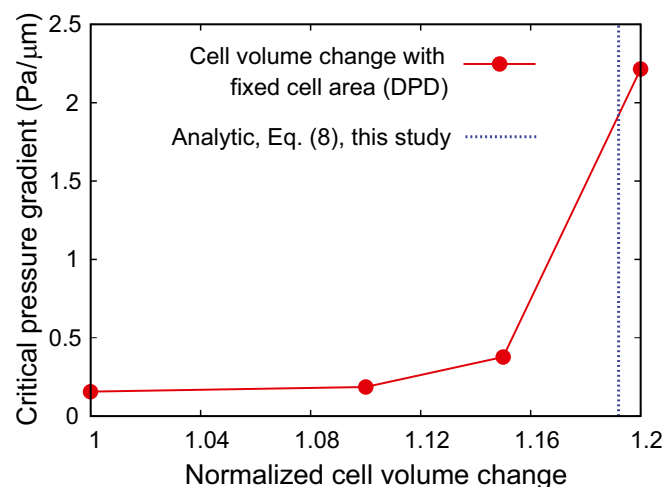


Fig. 6. Dependence of the critical pressure gradient on the cell volume for fixed cell surface area. Blue dashed lines are the limiting geometries predicted by the theory Eq. 8.

Supporting Information

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SI Text

RBC Model. The DPD model (25, 26) mimics the structure of the RBC membrane, which comprises a lipid bilayer and an attached cytoskeleton consisting primarily of spectrin proteins arranged in a network and linked by short actin filaments at junction complexes. There are about 25,000 junction complexes per healthy human RBC. In the computational model, the RBC is represented by a collection of particles. Each particle directly corresponds to a junction complex in the RBC membrane. The particles are connected with links, with each representing a spectrin link in the RBC cytoskeleton. The mechanical properties of the links are described using the worm-like chain model, reflecting the entropic-chain behavior of the spectrin. In addition, viscous components are added to represent the behavior of the RBC membrane. The links form a triangular tessellation of the model surface. For adjacent triangles, bending resistance of the lipid bilayer is added. Two additional constraints are present in the model. The surface area is held constant, taking into account low compressibility of the lipid bilayer. The volume of RBCs is controlled by the osmotic effects and is practically constant even for quite severe deformation. This constraint is reflected by the constant volume in the simulations. Specific details of the RBC model and parameters can be found in refs. 25 and 26.

Ex Vivo and Synthetic Spleen Studies and Structural Characterization.

Clinical interventions associated with left splenopancreatectomy for benign tumors of the pancreas (16) have led to ex vivo experiments in isolated human spleen perfused with healthy RBCs, iRBCs invaded by *P. falciparum* malaria parasites, as well as chemically treated RBCs with controlled surface area loss (6, 13, 16, 20). These studies have also yielded the retention rate of iRBCs by the spleen under different pathological states post-*P. falciparum* invasion. When healthy RBCs were treated with lysophosphatidylcholine (1–18 $\mu\text{mol/L}$) in PBS at 1% hematocrit to induce 18% average surface area loss and more than 27% reduction in surface area-to-volume ratio, they were rapidly and entirely retained within the spleen (6). By monitoring surface area loss in *P. falciparum*-infected RBCs using ImageStream imaging cytometer (Ideas v4.0; Amnis) with at least 10,000 images collected for each sample, the surface area loss and the resultant increase in sphericity were found to be linked to ring-stage iRBC retention, thereby contributing to splenic entrapment of a subpopulation of ring-stage iRBCs (13). Detailed analysis of the structure of the sinus wall in the spleen has also been performed (20), whereby the dimensions of interendothelial slits were determined from observations in the transmission electron microscope.

Geometric characteristics of RBC interactions and trapping from ex vivo human spleen have also been replicated using a “synthetic spleen” made of micrometer-sized metal spheres (20). The microsphere filtration device was designed based on the observed slit shape and dimensions of the human spleen, with a 5-mm-thick layer of a mixture of metal spheres, 5 to 15 μm in diameter. Experiments showed that RBCs with abnormal properties were selectively retained by the microsphere filter with retention rates similar to those observed in the isolated perfused human spleen (16). For the specific questions examined in this study, the experiments (20) also showed that the retention of abnormal RBCs was influenced predominantly by their mechanical properties and that these effects could occur in the absence of ligand–receptor interactions between the surfaces of the RBC external surface and the relevant splenic structures.

DPD Model and Scaling of Physical Units. Each particle in DPD represents a cluster of atoms or molecules (24). The DPD particles interact through pairwise forces: conservative, random, and dissipative. Every particle in the RBC membrane becomes a DPD particle in simulations. The RBC is immersed into and filled with the DPD fluid. The RBC particles interact with the fluid particles through the DPD forces, and the motion of the RBC membrane is fully coupled with the motion of the fluid. Specific details of the DPD method implementation can be found in ref. 26.

When setting up the DPD model, reduced units are implemented for the mass, length, and energy. In the following, the scaling relationships between model units and physical units are developed (26).

If $r = 1$ m represents the length scale in the physical system in SI units and r' represents the length scale of the DPD model, then the same initial diameter ($D_0 = 7.82 \mu\text{m}$) of the RBC can be expressed in both the DPD system and the physical system as

$$D_0 = D_0^M \cdot r' = D_0^P \cdot r = 7.82 \times 10^{-6} \text{ m}, \quad [\text{S1}]$$

where $D_0^P = 7.82 \times 10^{-6}$ and m is meter. The variables with upper index “P” (e.g., D_0^P) are values (numbers without units) of the quantities (e.g., D_0) in the physical system with SI units, whereas the variables with upper index “M” (e.g., D_0^M) are values (numbers without units) of the quantities (e.g., D_0) in the DPD system. We can choose the length scale r' of the DPD system, and usually specific values of r' and D_0^M depend on the size of the DPD system.

Because $\left[\frac{k_B T}{\mu_s}\right] = \text{length}^2$, where $[\cdot]$ denotes the dimension of a quantity, μ_s is the shear modulus, k_B is the Boltzmann constant, and T is the temperature, we should have

$$\frac{k_B T}{\mu_s} = \frac{(k_B T)^M}{\mu_s^M} (r')^2 = \frac{(k_B T)^P}{\mu_s^P} r^2. \quad [\text{S2}]$$

Plugging Eq. S1 into Eq. S2, we get

$$(k_B T)^M = \frac{\mu_s^M}{\mu_s^P} \left(\frac{D_0^M}{D_0^P}\right)^2 (k_B T)^P. \quad [\text{S3}]$$

Similarly for the force N , because $\left[\frac{k_B T}{N}\right] = \text{length}$, we have

$$N^M = \frac{(k_B T)^M}{(k_B T)^P} \frac{r^M}{r^P} N^P = \frac{(k_B T)^M}{(k_B T)^P} \frac{D_0^M}{D_0^P} N^P = \frac{\mu_s^M}{\mu_s^P} \frac{D_0^M}{D_0^P} N^P. \quad [\text{S4}]$$

For time scaling, because $\left[\frac{\eta D_0^2}{N}\right] = \text{time}$, where η is a characteristic viscosity, we have

$$\frac{\eta D_0^2}{N} = \frac{\eta^M (D_0^M)^2}{N^M} \tau' = \frac{\eta^P (D_0^P)^2}{N^P} \tau. \quad [\text{S5}]$$

where $\tau = 1$ second is the time scale in the physical system with SI units, and τ' is the time scale in the DPD system. Plugging Eq. S4 into Eq. S5, we get the time scale of the DPD system as

$$\tau' = \frac{D_0^P}{D_0^M} \frac{\eta^P}{\eta^M} \frac{\mu_s^M}{\mu_s^P} s, \quad [\text{S6}]$$

where s denotes second.

Correction

BIOPHYSICS AND COMPUTATIONAL BIOLOGY, ENGINEERING

Correction for “Biomechanics of red blood cells in human spleen and consequences for physiology and disease,” by Igor V. Pivkin, Zhangli Peng, George E. Karniadakis, Pierre A. Buffet, Ming Dao, and Subra Suresh, which appeared in issue 28, July 12, 2016, of *Proc Natl Acad Sci USA* (113:7804–7809; first published June 27, 2016; 10.1073/pnas.1606751113).

The authors note that on page 7806, the expression for red blood cell (RBC) volume (Eq. 3) appeared incorrectly. The correct exact expression for RBC volume, to be taken within Eq. 8 corresponding to the dotted line in Fig. 3, should be given as

$$V = 2 \left[\frac{4}{3} \pi R^3 - \frac{1}{3} \pi h^2 (3R - h) \right] + \frac{\pi L_s}{2} \left(R + \frac{L_s}{2} \right) \cos \theta (R \sin 2\theta - L_s \theta) + \frac{\pi}{4} \left(L_s^3 \sin \theta - \frac{L_s^3 \sin^3 \theta}{3} \right) \quad [3]$$

In this case, there is no longer a need for Eqs. 5 and 6. The dotted curve in Fig. 3 was actually plotted with the exact analytical solution using the correct Eq. 3 above for the RBC volume. All results presented originally in Fig. 3 are correct with the above Eq. 3.

The authors further note that the originally printed expression for RBC volume (Eq. 3) on page 7806, in conjunction with the original Eqs. 5 and 6, should constitute an approximate solution for RBC volume. After correcting a typo, the correct approximate expression for RBC volume (Eq. 3) would appear as $V = 2 \left[\frac{4}{3} \pi R^3 - \frac{1}{3} \pi h^2 (3R - h) \right] + 2\pi A_{cyt}$. When taking this corrected approximate solution for RBC volume in Eq. 8, the (approximate) analytical solution would not match exactly (i.e., being slightly lower than) the dotted curve shown in Fig. 3.

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