Bio-Medical Materials and Engineering 27 (2016) 405–412
DOI 10.3233/BME-161594
IOS Press

Differences in bead-milling-induced hemolysis of red blood cells due to shape and size of oscillating bead

Kenneth M. Alfano [a,b,*], Sumita Chakraborty [b] and Michael Tarasev [b]

[a] *University of Michigan, Ann Arbor, MI 48109, USA*

[b] *Blaze Medical Devices, 330 E. Liberty, LL, Ann Arbor, MI 48104, USA*

E-mails: kalfano@blazemedicaldevices.com, sumita@blazemedicaldevices.com, mtarasev@blazemedicaldevices.com

Received 16 May 2016
Accepted 9 August 2016

Abstract.
BACKGROUND: Red blood cell (RBC) susceptibility to hemolysis – or fragility – can be profiled by subjecting a sample to progressive durations of mechanical stress and measuring hemolysis upon each. The ability to control stress application with multiple variable parameters can be useful in various areas of research. Bead milling, by oscillating an object in a blood sample, can offer control of parameters including oscillation force and frequency.
OBJECTIVE: This work addresses the role of bead shape and size, for a given container, in potentially creating qualitatively as well as quantitatively different fluidic stresses in the sample.
METHODS: Identical, diluted RBC samples were stressed via bead milling using different beads, with other parameters the same. Resulting hemolysis was plotted for several time increments in each case.
RESULTS: For a cylindrical bead oscillating at a given frequency and force, bead length was a determinant of albumin's protective effect on RBC, as reflected by mechanical fragility. Compared to a sphere of same diameter, the protective effect was absent with shorter cylinders, whereas for longer ones it appeared enhanced.
CONCLUSIONS: Bead milling based RBC fragility testing could present a useful tool for creating, and studying effects of different shear stress types in inducing hemolysis.

Keywords: RBC, hemolysis, fragility, flow, shear, bead mill

1. Introduction

Bead milling, utilizing mechanical stress from one or more objects oscillating in a container, is commonly used for disrupting biological samples. One application of bead-based disruption is in testing red blood cell (RBC) mechanical fragility, which represents RBC susceptibility to hemolysis under mechanical stress. RBC mechanical fragility is known to be a more sensitive metric of blood damage than hemolysis alone, as well as a critical parameter in calibrating hemolysis measurements [1,2]. If a sample containing RBC is subjected to a range of durations of mechanical stress, e.g. by oscillating a bead in

*Corresponding author. E-mail: kalfano@blazemedicaldevices.com.

the sample, with induced hemolysis measured upon each such duration, a fragility "profile" can be generated. In addition to the duration of the stress, overall stress magnitude can also be varied through the intensity of the stress; in the case of a bead mill, this can involve stress parameters such as oscillation frequency and force, as well as relative dimensions of the bead and container. A bead-mill-based system to facilitate profiling of RBC fragility has recently been reported [3].

Spherical beads are often used to create the mechanical stress, and cause the RBC hemolysis by oscillation, in mechanical fragility testing (e.g. [4]). In liquid media, cell disruption results primarily from turbulent flow in a bead's wake. It was demonstrated with spherical beads that magnitudes of resultant RBC hemolysis can be significantly affected by cell environment; in particular, albumin in a wide range of concentrations (including physiological) protected RBC against mechanical damage [5,6]. Bead milling with non-spherical beads remains largely unexplored in general. Here, in the context of stressing RBC, we explore potential use of *cylindrical* beads for inducing both quantitatively and qualitatively different stress (relative to spherical beads, as well as other cylindrical ones of different lengths, for a given container).

2. Materials and methods

Packed RBC samples were obtained from the University of Michigan's blood bank. Samples were diluted to a total hemoglobin (Hb) concentration of 1.2 g/dl (except where otherwise indicated), corresponding to about 4% hematocrit, verified by a Hemoglobin 201 system from HemoCue (Angelholm, Sweden), with AS-3 storage buffer, pH 5.75, containing when necessary 40 g/l albumin (bovine serum albumin, BSA, from RPI (IL, USA); BSA had been shown to affect RBC mechanical fragility similarly to human serum albumin [6]). Diluted sample was gently agitated and aliquoted into 2 ml low-retention centrifuge tubes at 350 µl per tube.

Mechanical stress was applied with a TissueLyser LT (Qiagen, Dusseldorf, Germany) cam-based vertical bead mill, at an oscillation frequency of 50 Hz, using a single stainless steel spherical or longitudinally-oriented cylindrical bead. Cylindrical beads were custom machined to desired dimensions. Hemolysis profiles were obtained by subjecting sample aliquots to a range of stress durations, in this case by splitting samples for separate stressing at the respective different durations. All tests were performed at 6 °C, with the container tube holder of the TissueLyser modified to allow air ventilation while in operation, resulting in sample temperature stabilization to within 2 degrees of start temperature.

Un-lysed cells were precipitated by centrifuging the samples for 5 minutes at 5000 rpm on an Eppendorf 5417C centrifuge (Hauppauge, NY). Aliquots of supernatant were collected and their spectra recorded. Hemolysis (Hem) induced by the bead mill was determined based on the difference in absorbance at 576 nm, a wavelength of oxygenated Hb maximum, and at 685 nm, local minimum, and expressed as a fraction of free hemoglobin (HB^F) relative to total hemoglobin concentration (Hb^T) according to Formula (1), including correction for sample hematocrit [7]. Spectroscopic measurements were performed with a NanoDrop N100 spectrophotometer (Thermo Scientific, Waltham, MA)

$$\text{Hem} = \frac{Hb^F_{576} - Hb^F_{685}}{Hb^T_{576} - Hb^T_{685}} * (1 - \text{Hematocrit}). \qquad (1)$$

3. Results and discussion

3.1. Impact of bead shape and length on efficiency of induced hemolysis

A cylindrical bead, compared to spherical, can result in markedly different hemolysis efficiency when tested under the same conditions (e.g. oscillation parameters; container and bead diameters). This difference depended significantly on bead length (Fig. 1). For testing parameters as for Fig. 1, lysing efficiency with a sphere was comparable to that with a cylinder having both the same length and width as the sphere's diameter. The lysing efficiency increased with the length of the cylinder, plateauing in this case at about 60% bead-to-tube length ratio before declining for longer beads (data not shown).

Flow patterns for a sphere/ball moving through a medium are a function of medium viscosity and ball speed. Reynolds numbers characterize turbulent flows in the wake, which for spheres in non-oscillating contexts are well understood (e.g. [8]). Cylindrical beads, oriented and oscillating longitudinally, would also generate such bulk fluid turbulence, but with boundary conditions involved (as in a tube with a diameter not much greater than that of the bead), flow through the annulus should be considered. Cylinders moving relative to fluid, all else being equal, would provide relatively more fluid-surface interaction than spheres of similar diameter – the latter having a vanishingly short annular length – and increased interaction between fluid layers in the annulus.

With increasing length of the cylinder, volume of media in the annulus and surface area of the annulus would both increase. Thus, stresses due to annular flow would be manifestly larger for cylinders, while the wake turbulence and associated stresses (assuming comparable flow velocities) would not be as much affected. It was first established in the context of cylindrical objects in pipes with liquid flow, that for sufficiently high ratios of cylinder-to-tube diameters, turbulence in the annulus can be suppressed sufficiently to establish laminar flow [9]. With regard to inducement of RBC hemolysis, mechanical stress can be generated by both turbulent [10,11], and laminar flows, with cell damage induced "in bulk" from the flow itself [12,13], and from cell interaction with the surface [14]. It had been suggested that causes for mechanical hemolysis could be divided into three broad classes: one surface-induced and two

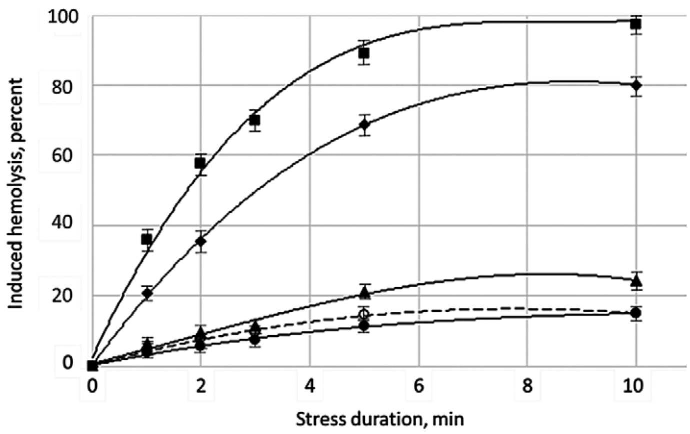

Fig. 1. Dependence of induced hemolysis on bead length. Shown are mechanical fragility profiles of packed RBC diluted with Additive Solution 3 (AS3) to a hemoglobin concentration of 1 g/dL, tested at 50 Hz bead oscillation frequency. Stress was provided using: a 7 mm ball/sphere (○); or a cylinder 7 mm in diameter with length of 6 mm (●), 12 mm (▲), 16 mm (◆), and 22 mm (■). Tube was 8.8 mm in diameter and 36 mm in length, with bead-to-tube length rations of 0.17, 0.33, 0.44 and 0.61 for the 6, 12, 16 and 22 mm beads correspondingly. Error bars are ± SD. Fits are for illustration only, with dashes marking the fit for the profile obtained using the ball.

"in-bulk" – likely differing in types of flow [15]. For those bulk stress types, one would be characteristic of high intensity stress, and would induce instantaneous tensile failure of the RBC membrane. The other would be characteristic of medium intensity stress, and would induce gradual membrane fragmentation.

Data presented in this work (Fig. 1) show that hemolysis induced under the same conditions can be much higher for a cylindrical bead as compared to spherical. However, induced hemolysis for a cylinder with length and diameter the same as the spherical bead, under the conditions used, was found to be less than that for the spherical bead – likely due to decreased speed of the cylinder from higher resistance to its movement through the medium. Efficiency of hemolysis increased with increased bead length – up to a point, as noted above. Such an increase could be expected where any increase in hemolysis due to annular length (which can increase with increased bead length) outweighs any decline due to decrease in flow speed (which can decrease with increased bead length). For a very long bead, hemolysis becomes more dependent upon bead speed than annular length (e.g., a bead and tube of equal lengths would of course result in zero hemolysis); such a decrease in hemolysis was indeed observed in this work for very long beads (as noted). These effects suggest the potential of annular – possibly laminar – flow to stress and hemolyse RBC to a greater extent than turbulent flow in a cylindrical bead's wake.

3.2. Impact of bead shape and length on dependency of hemolysis on cell environment

With a spherical bead, the amount of hemolysis observed at a given bead oscillation frequency and duration depends on bead environment; in particular, lysis propensity was significantly reduced in the presence of physiological concentrations of albumin. Although for a spherical bead, albumin provides significant protection against the mechanical stress, for a cylindrical bead of comparable linear dimensions, albumin notably provides no protection (Fig. 2). Moreover, for relatively short cylindrical beads (here 6–8 mm in length) under the stress conditions involved, hemolysis observed in media without albumin was about 10% less than when albumin was present (Fig. 3). However, with increasing bead length, hemolysis induced by shear stress due to the bead's longitudinal oscillations became progressively more

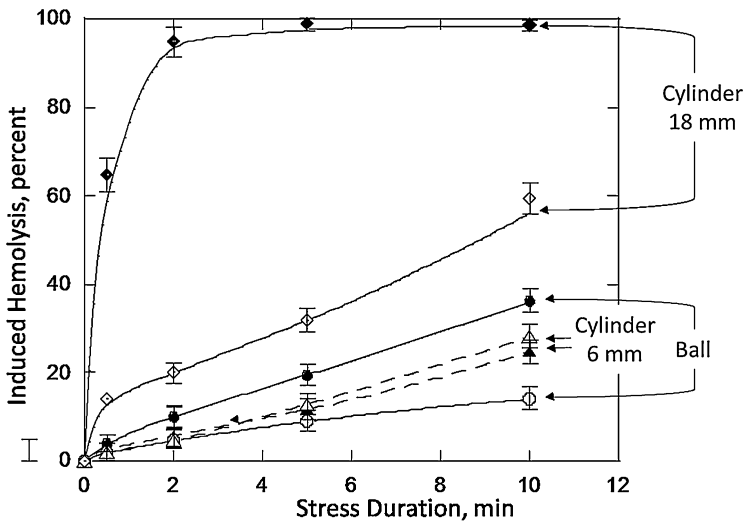

Fig. 2. Impact of bead characteristics upon albumin's protection of RBC from bead-milling-induced hemolysis. Samples of RBC were prepared as described in Materials and Methods. Shown here are hemolysis profiles for samples diluted with AS3 not supplemented (filled markers) and supplemented with 4 g/L albumin (open markers) for a 7 mm ball (●, ○), and for 7 mm diameter cylinders with lengths of 6 mm (▲, △) and 18 mm (◆, ◇). Fits are for illustration only. Error bars are ±SD.

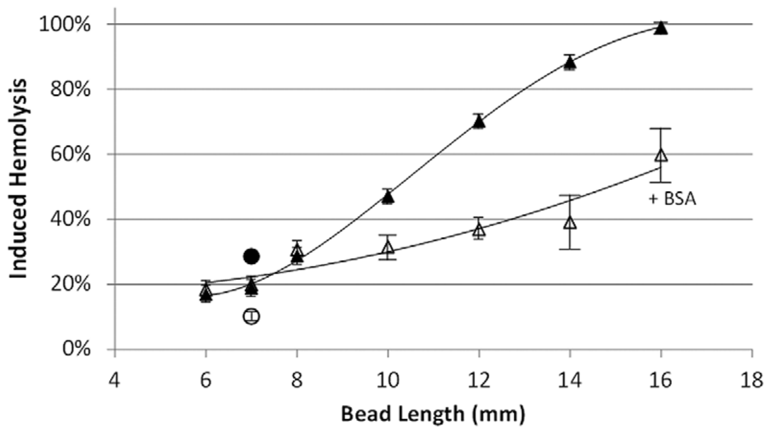

Fig. 3. Effect of albumin on hemolysis induced by mechanical stress through bead milling, for a sphere and for cylinders of varying lengths. Samples of RBC were prepared as described in Materials and Methods. Shown here are hemolysis results for samples diluted with AS3 not supplemented (filled markers) and supplemented with 4 g/L albumin (open markers) for a 7 mm ball (●, ○), and for 7 mm diameter cylindrical beads of the various lengths (▲, △). Fits are for illustration only. Error bars are ±SD.

affected by the presence of albumin (Fig. 2). For longer beads, the relative magnitude of such protective effect can markedly exceed that for a spherical bead (Fig. 3).

Decreased hemolysis under mechanical stress in the presence of albumin had been reported previously, attributed to proteins' ability to bind with the RBC membrane – providing the cell with a measure of protection in a turbulent wake flow [5,6]. (Notably, the protective effect of plasma proteins on RBC can potentially differ depending on the type of mechanical stress employed in a given fragility testing approach [15].) Prior work involving bead oscillation employed spherical beads, but the wake turbulence and associated hemolysis likewise occurs with a cylinder (with the latter less streamlined). For that associated hemolysis, there would be expected similar protection from plasma proteins as with a sphere. Magnitudes of the "wake hemolysis" would likely tend to be positively correlated with bead and flow speed, as well as with duration of bead motion per oscillation cycle, and thus for the present setup should be negatively correlated with bead length.

In light of the above-noted potential of cylindrical objects in tube flow to sometimes suppress turbulence in an annulus, the results here could be interpreted as indicative of such a phenomenon.

For the protective effect to essentially disappear when using comparably sized beads of cylindrical shape instead of spherical, it may be inferred that some effect apart from wake-turbulence, likely relating to the annulus, works in the opposite direction (i.e., a stress from which cells are more susceptible to hemolysis in the presence of plasma proteins, in contrast to the stress from wake turbulence). The slightly higher hemolysis observed for such beads in the presence of albumin (Fig. 3), as compared to un-supplemented AS3, seems to support this possibility.

The observation that, as bead length increases, albumin again starts to provide increasingly greater protection against hemolysis (Fig. 3), may suggest another annulus-associated stress, which (unlike the one discussed above) is positively correlated with cell resistance to shear-induced hemolysis in the presence of the proteins. (It further could be hypothesized that one of these annular stresses could be a wall-associated stress (e.g., through cell adhesion to the chamber wall, with follow-on dragging from development of boundary layer turbulent eddies), while the other could possibly be laminar "in bulk" stress.) If this is the case, the overall effect of plasma proteins on RBC hemolysis when stress is applied via a cylindrical bead would encompass the impacts of all three respective effects, with the contribution of each depending significantly on bead length, which also determines the length and thus the surface area of the annulus, as well as being one of the determinants of bead speed, which can also be affected by the fill-fraction of the tube with sample as well as oscillation force and frequency. Notably, although some positive correlation between bead speed and the extent of albumin's protective effect on RBC seems plausible (e.g., considering a sharp decline of the protective effect for slower-moving very long beads), a negative correlation cannot be ruled out (e.g., possibly through loss of flow-speed-induced laminarity in the annular channel), particularly as it is conceivable that flow speed could have different respective impacts on albumin's protective effect for different stress types (e.g. annular bulk stress vs. annular wall stress).

Additionally, flow perturbations from the bead reaching the ends of the tube during oscillation, and/or non-ideal oscillation behavior (e.g. bead "wobbling" within tube, colliding with tube wall, or having incomplete oscillation cycles) could play a role and be affected by bead speed/length.

Preliminary computational fluid dynamics analysis (not shown) of fluid movement relative to different beads was initiated to begin exploring how a cylindrical bead can provide different flow overall than a spherical bead even when of comparable linear dimensions. At this time, modeling is performed under simplifying assumptions (e.g. one-directional fluid movement rather than actual bead oscillation; infinite tube length; 2-dimensional approach). Initial results seem to support an assertion that the cylinder, similar to sphere, creates turbulence in its wake, but also has significant high-speed laminar flow proximate to the lateral surface of the bead. Also, for a given flow velocity, stresses seem greater around the cylinder, compared to the sphere of the same diameter (due to the cylinder's longer annulus). Models of the corresponding pressure gradients indicated that they dropped precipitously at annulus entry for both beads, with the change significantly more abrupt for a blunt-end bead than for round (and graduality increased when flow speed declined).

4. Conclusion

The RBC membrane is a complex structure of lipid bilayers, transmembrane complexes, and associated underlying protein skeleton. In circulation, RBCs are subjected to multiple and variable mechanical stresses that constantly test the membrane's ability to adapt and respond without plastic or insufficiently reversible deformation. When implantable or extracorporeal blood-handling devices are used, mechanical stresses on RBC could be further amplified, and could result in both immediate and delayed cell destruction. Induced hemolysis is commonly used as a measure of lethal cell damage; however, sublethal damage, responsible for potential degradation of a cell's physiological performance and delayed hemolysis, is not yet sufficiently explored. As noted, both turbulent and laminar flows, with bulk and surface effects, can contribute to the resultant damage, which makes RBC mechanical fragility (MF) results significantly dependent on the conditions and method of mechanical stress application used. It can be hypothesized that a sample's response to different types of mechanical stress could be driven by each stress's respective effects on different membrane components – possibly similar to how different skeleton protein associations regulate RBC fragility and deformability [16].

Enhancing understanding of effects of different flow types, and mechanical stresses they generate, on RBC hemolysis and susceptibility thereto could be beneficial in areas such as diagnostic research, hemocompatibility of blood-contacting materials, or assessment of cell damage in artificial organs, implants, pumps and other mechanical circuits – particularly in light of the fact that RBC can have fragility parameters that vary significantly from person to person [17].

The present work, employing bead milling to stress RBC samples, shows that replacement of spherical beads with cylindrically shaped beads can cause a commonly observed protective effect of albumin on RBCs to disappear when the bead is of similar linear dimension (given fixed stress parameters otherwise). Albumin's protective effect, as well as general lysing efficiency, can be enhanced with increased bead length – until bead length approaches tube length.

Bead milling using a cylindrical bead seems to facilitate inducing qualitatively different types of flow and thus associated types of mechanical stress. If effects of particular beads on flows and stresses during oscillation can be sufficiently understood, then selection of beads with particular characteristics (e.g. shape, size, surface type) could establish key flow – and thus stress – parameters. This could support some particularly useful applications for qualitatively differentiated RBC fragility testing. Albumin's or other plasma proteins' presence in a testing medium, and the impact on RBC resistance to hemolysis, could serve to differentiate types of mechanical stresses that may differently stress the cell membrane – facilitating assessment of different aspects of membrane structure.

Further computational fluid dynamics modelling, including 3-dimensional, could help elucidate effects of acceleration/velocity profiles during oscillation on aggregate shear, Reynolds numbers, end effects, etc. – and generally enable better understanding of the flows and stresses involved.

Next steps would involve further exploring the present findings, as well as similar RBC effects using other sorts of bead mill configurations (such as an integrated MF test under development [3]) and other combinations of stressing parameters (including use of other bead shapes/sizes as well as other bead modifications). Select methods and systems that could facilitate RBC fragility testing with different types of fluid stresses are under development [18]. Specific factors to be investigated include the roles and interrelationships of stress intensity, bead and cartridge dimensions, and sample/fluid characteristics – particular combinations of which may alter the phenomena observed. Finally, potential clinically-relevant correlations could be explored for a range of RBC MF testing parameters.

Acknowledgements

The authors thank Dr. Robertson Davenport of the University of Michigan for donating RBC samples for testing and also for useful discussion. The fluid modelling referred to was done by Gene Parunak and Aaron Kehrer of in2being, LLC. Blaze employees Marina Muchnik and Hsin-Hung Huang assisted with lab experiments.

Conflict of interest

See disclosure below.

Funding/Disclosure

The presented work was funded by Blaze Medical Devices, an early stage company developing blood analysis technologies and applications. Aspects of the presented subject matter were also described in patent material of the company. The authors disclose having equity in, as well as employment with, Blaze Medical Devices.

References

[1] M.V. Kameneva and J.F. Antaki, Mechanical trauma to blood, in: *Handbook of Hemorheology and Hemodynamics*, O.K. Baskurt, ed., IOS Press, Amsterdam, The Netherlands, 2007, pp. 206–227.

[2] L. Gu, W.A. Smith and G.P. Chatzimavroudis, Mechanical fragility calibration of red blood cells, *ASAIO J.* **51** (2005), 194–201. doi:10.1097/01.MAT.0000161940.30190.6D.

[3] K.M. Alfano, M. Tarasev, S. Meines and G. Parunak, An approach to measuring RBC haemolysis and profiling RBC mechanical fragility, *J. Med. Eng. Technol.* **40** (2016), 162–171. doi:10.3109/03091902.2016.1153741.

[4] J.S. Raval, J.H. Waters, A. Seltsam, E.A. Scharberg, E. Richter, A.R. Daly et al., The use of the mechanical fragility test in evaluating sublethal RBC injury during storage, *Vox Sang.* **99** (2010), 325–331. doi:10.1111/j.1423-0410.2010.01365.x.

[5] M.V. Kameneva, J.F. Antaki, K.K. Yeleswarapu, M.J. Watach, B.P. Griffith and H.S. Borovetz, Plasma protective effect on red blood cells exposed to mechanical stress, *ASAIO J.* **43** (1997), M571–M575. doi:10.1097/00002480-199709000-00046.

[6] M. Tarasev, S. Chakraborty, L. Light and R. Davenport, Impact of environment on red blood cell ability to withstand mechanical stress, *Clin. Hemorheol. Microcirc.* (2016). doi:10.3233/ch-152037.

[7] S.O. Sowemimo-Coker, Red blood cell hemolysis during processing, *Transfus. Med. Rev.* **16** (2002), 46–60. doi:10.1053/tmrv.2002.29404.

[8] G. Constantinescu and K. Squires, Numerical investigations of flow over a sphere in the subcritical and supercritical regimes, *Physics of Fluids* **16** (2004), 1449–1466. doi:10.1063/1.1688325.

[9] M.E. Charles, The pipeline flow of capsules: Part 2: Theoretical analysis of the concentric flow of cylindrical forms, *The Canadian Journal of Chemical Engineering* **41** (1963), 46–51.

[10] M.V. Kameneva, G.W. Burgreen, K. Kono, B. Repko, J.F. Antaki and M. Umezu, Effects of turbulent stresses upon mechanical hemolysis: Experimental and computational analysis, *ASAIO J.* **50** (2004), 418–423. doi:10.1097/01.MAT.0000136512.36370.B5.

[11] J.H. Yen, S.F. Chen, M.K. Chern and P.C. Lu, The effect of turbulent viscous shear stress on red blood cell hemolysis, *J. Artif. Organs* **17** (2014), 178–185. doi:10.1007/s10047-014-0755-3.

[12] S.P. Sutera, Flow-induced trauma to blood cells, *Circ. Res.* **41** (1977), 2–8. doi:10.1161/01.RES.41.1.2.

[13] J. Dupire, M. Socol and A. Viallat, Full dynamics of a red blood cell in shear flow, *Proc. Natl. Acad. Sci. USA* **109** (2012).

[14] R.D. Offeman and M.C. Williams, Material effects in shear-induced hemolysis, *Biomater. Med. Devices Artif. Organs* **7** (1979), 359–391. doi:10.3109/10731197909119386.

[15] P.L. Blackshear, Mechanical hemolysis in flowing blood, in: *Biomechanics: Its Foundations and Objectives*, Y.C. Fung, ed., Prentice-Hall, Englewood Cliffs, N.J., 1972, pp. 501–528.

[16] J.A. Chasis and N. Mohandas, Erythrocyte membrane deformability and stability: two distinct membrane properties that are independently regulated by skeletal protein associations, *J. Cell Biol.* **103** (1986), 343–350. doi:10.1083/jcb.103.2.343.

[17] M. Tarasev, K. Alfano, S. Chakraborty, L. Light, K. Doeden and J.B. Gorlin, Similar donors-similar blood?, *Transfusion (Paris)* **54** (2014), 933–941. doi:10.1111/trf.12457.

[18] K. Alfano, M. Tarasev and S. Chakraborty, Method and system for inducing controlled and varied fluid stresses by bead oscillation. U.S. Patent Application No. 15/098,228; Filed: April 13, 2016.