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The dynamic behavior of chemically "stiffened" red blood cells in microchannel flows

Alison M. Forsyth [a], Jiandi Wan [b], William D. Ristenpart [c,d], Howard A. Stone [b,*]

[a] School of Engineering and Applied Sciences, Harvard University, USA
[b] Department of Mechanical and Aerospace Engineering, Princeton University, USA
[c] Department of Chemical Engineering and Materials Science, University of California at Davis, USA
[d] Department of Food Science and Technology, University of California at Davis, USA

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ABSTRACT

The rigidity of red blood cells (RBCs) plays an important role in whole blood viscosity and is correlated with several cardiovascular diseases. Two chemical agents that are commonly used to study cell deformation are diamide and glutaraldehyde. Despite diamide's common usage, there are discrepancies in the literature surrounding diamide's effect on the deformation of RBCs in shear and pressure-driven flows; in particular, shear flow experiments have shown that diamide stiffens cells, while pressure-driven flow in capillaries did not give this result. We performed pressure-driven flow experiments with RBCs in a microfluidic constriction and quantified the cell dynamics using high-speed imaging. Diamide, which affects RBCs by cross-linking spectrin skeletal membrane proteins, did not reduce deformation and showed an unchanged effective strain rate when compared to healthy cells. In contrast, glutaraldehyde, which is a non-specific fixative that acts on all components of the cell, did reduce deformation and showed increased instances of tumbling, both of which are characteristic features of stiffened, or rigidified, cells. Because glutaraldehyde increases the effective viscosity of the cytoplasm and lipid membrane while diamide does not, one possible explanation for our results is that viscous effects in the cytoplasm and/or lipid membrane are a dominant factor in dictating dynamic responses of RBCs in pressure-driven flows. Finally, literature on the use of diamide as a stiffening agent is summarized, and provides supporting evidence for our conclusions.

Introduction

The rigidity of red blood cells (RBCs) has been correlated with myocardial infarction, diabetes mellitus, essential hypertension, and is a known contributor to the viscosity of whole blood (Simchon et al., 1987; Chien, 1987; Mokken et al., 1992; Ajmani, 1997). Other hematological disorders and diseases effect RBC deformation more directly, such as hereditary spherocytosis, sickle cell anemia, and malaria (Mokken et al., 1992; Shelby et al., 2003). Diamide and glutaraldehyde are two chemicals commonly used to study the rigidity of RBCs. In particular, glutaraldehyde is being investigated for use in medical therapeutics such as cancer treatments in which drugs are cross-linked into the cells (Marczak and Jozwiak, 2008), and for use in blood substitutes made of cross-linked hemoglobin (Eike and Palmer, 2004; Sou et al., 2005). Consequently, characterizing the effects of chemicals associated with the stiffening of cells on the dynamics of RBCs will improve the understanding of blood flow associated with cardiovascular diseases, hematological disorders, and provide insight relevant to the design of medical therapeutics.

The mature RBC has three main structural components: the lipid membrane, the membrane skeleton, and the fluid cytoplasm. The membrane skeleton is directly adjacent to the lipid membrane and is made up of a spectrin–actin protein network attached to the membrane through transmembrane proteins (Marchesi, 2008). The cell membrane is effectively inextensible, and the macroscopic deformation of the cell is typically described in terms of three different properties: viscosity, shear modulus, and bending modulus. The elasticity of the spectrin–actin network is believed to dictate cellular deformation, while the fluid cytoplasm and lipid membrane are considered to have a negligible influence (Evans and Hochmuth, 1976; Hochmuth et al., 1979; Hochmuth and Waugh, 1987). However, dynamic models derived from work with vesicles, which lack membrane skeletons, indicate that phenomena such as tumbling can be achieved by altering the viscosity contrast between the vesicle and suspending phase (Olla, 1997; Abkarian et al., 2002; Kantsler and Steinberg, 2006; Skotheim and Secomb, 2007). This line of work suggests that viscosity can play a major role in dynamics.

Although many chemicals, including diamide, are commonly referred to as "stiffening agents", there are discrepancies in the literature regarding the effect of diamide on cell rigidity. We have summarized several studies on the effect of diamide in Table 1. The most common interpretation is that RBCs exposed to diamide deform less (i.e., are more rigid) under a variety of experimental conditions,

* Corresponding author. Department of Mechanical and Aerospace Engineering, D326 Engineering Quadrangle, Olden Street, Princeton University, Princeton, NJ, 08544, USA. Fax: +1 609 258 6109.
E-mail address: hastone@princeton.edu (H.A. Stone).

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Table 1
Reports in the literature which have tested red blood cell stiffness upon diamide exposure.

A) Diamide reduces RBC deformation		B) Diamide does not reduce deformation	
Authors and year	Technique	Authors and year	Technique
Fischer et al. (1978)	Cone–plate viscometer	Teitel (1981)	Filtrometer
Johnson et al. (1980)	Micropipette	Schmid-Schonbein and Gaehtgens (1981)	*In vivo* capillaries and glass capillaries
Aarts et al. (1984)	Couette-flow viscometer	Driessen et al. (1982)	*In vivo* capillaries
Kon et al. (1987)	Cone–plate viscometer	Ogura et al., 1991*	Filtration
Engelhardt and Sackmann (1988)	Electric field		
Noji et al., 1991	EPR spin-labeling		
Giger et al., 1995	Ecktacyometer		

(A) Papers concluding that diamide stiffens red blood cells. (B) Papers that found contradictory evidence in pressure-driven flows, where cell behavior was not significantly changed by diamide exposure when tested at physiological stress levels. * Ogura et al. found reduced filtration passage times following RBC exposure to 0.2 mM diamide, but not for higher concentrations. Only papers that directly compared the deformation of diamide-treated and healthy RBCs are shown.

including micropipette aspiration and cone–plate viscometry (Table 1A). In contrast, a few groups using other experimental conditions, such as pressure-driven flows in capillaries, report that diamide-exposed cells do not exhibit behavior typical of stiffened cells (Table 1B). Most recent work that utilizes diamide as a chemical agent on RBCs typically assumes that diamide acts as a stiffening agent, which has now become the prevalent theory in the literature (Faris and Spence, 2008). However, note that the experiments in Table 1B are most similar to *in vivo* conditions, which suggests that some conclusions based on experimental techniques that do not probe the response to a pressure gradient are not applicable to *in vivo* dynamics in arteriole-sized flows.

One possible explanation for the discrepancy summarized in Table 1 is that viscous effects produced by the cytoplasm and/or the lipid membrane are a significant contributing factor to the dynamic response of RBCs in microchannel flows (Schmid-Schonbein and Gaehtgens, 1981; Teitel, 1981; Chien, 1987). Diamide is expected to increase the shear modulus and viscosity of the RBC membrane skeleton by creating disulfide bonds preferentially on the spectrin proteins, but it has little effect on the cytoplasmic or lipid membrane viscosity (Fischer et al., 1978; Johnson et al., 1980; Chien, 1987). In support of the argument that the membrane skeleton does not solely govern the dynamic response, it was demonstrated that at physiological pressures diamide-exposed cells had similar passage times to healthy cells in pressure-driven flows (Teitel, 1981; Schmid-Schonbein and Gaehtgens, 1981; Driessen et al., 1982). In comparison, glutaraldehyde is a non-specific fixative that cross-links the membrane skeletal proteins, phospholipids in the membrane, and the cytoplasm, thereby increasing the shear modulus and viscosity of the entire cell including the cytoplasm and lipid membrane (Morel et al., 1971; Noji et al., 1991; Szwarocka et al., 2001).

Here we investigate the effects of diamide and glutaraldehyde on the dynamics of RBCs, by direct visual observation of cells in flow through channels, to determine whether viscous effects play a key role in dynamic behavior. We performed quantitative characterizations of the cellular dynamic response in a microfluidic channel in which the cell deformation is visualized using a high-speed camera and quantified using image-processing techniques. The microchannels had a cross-sectional height of 18 μm, which is comparable to the size of the cell (largest dimension 8 μm); the cells therefore experienced a pressure-driven (nearly parabolic) flow, similar to conditions found in arteriole-sized vessels *in vivo*. The main conclusion from our experimental work is that glutaraldehyde reduces the deformability of RBCs in pressure-driven flows, but diamide has no effect on deformability. This result suggests that the effective cellular viscosity, rather than the shear modulus or viscosity of the membrane skeleton (i.e. the spectrin–actin network), governs the behavior of RBCs in pressure-driven flow, and that care must be taken when comparing cell deformation results obtained from different methods.

Materials and methods

Our experimental approach was similar to that used by Wan et al. (2008). Standard soft photolithographic techniques were used to fabricate microchannels in polydimethylsiloxane (PDMS) (Duffy et al., 1998). All channels were 1.1 cm (l) × 100 μm (w) × 18 μm (h) and contained a single 100 (l_c) × 40 (w_c) × 18 (h_c) μm constriction (Fig. 1A). Human RBCs were extracted from a healthy donor and used within 2 h to prevent reduced deformation (Ogura et al., 1991). Preliminary experiments with RBCs from four donors were performed, all with similar results. However, for better comparison of the effects of chemical treatment on RBCs, data reported here is from a single donor. The cells were diluted into a physiological salt solution (PSS), which was prepared according to a standard protocol (Price et al., 2004): 4.7 mM KCl, 2.0 mM CaCl2, 1.2 mM MgSO4, 140.5 mM NaCl, 21.0 mM tris(hydroxymethyl)aminomethane, and 11.1 mM dextrose with 1 mg/ml bovine serum albumin; the pH was adjusted to 7.4 using 1.0 M HCl. In particular, 6 μl of blood was diluted into 500 μl of PSS, and separated from the other blood components by centrifuging at 3000 RPM for 3 min at room temperature. After aspirating out the buffy coat and plasma, the packed RBCs were then re-suspended and washed three times in PSS with centrifugation as above. At this low hematocrit, cells typically flowed through the constriction individually (one or at most two cells at a time). This procedure allowed the single cell response to be analyzed, without having to consider more complicated interactions between cells.

For RBCs exposed to chemicals, the cells were incubated for 20 min at room temperature (~25 °C) with 38 μM, 0.38 mM, or 3.8 mM diamide (Sigma-Aldrich), or a 0.05% vol/vol glutaraldehyde (Sigma-Aldrich) solution and then used immediately in experiments (different measures of concentration were used based on standards found in the literature). Iodoacetate has been used by other investigators to prevent the consumption of diamide by cytoplasmic glutathione (GSH), and thereby increase the efficacy of diamide (Fischer et al., 1978); we did not use iodoacetate because it has been shown to almost eliminate

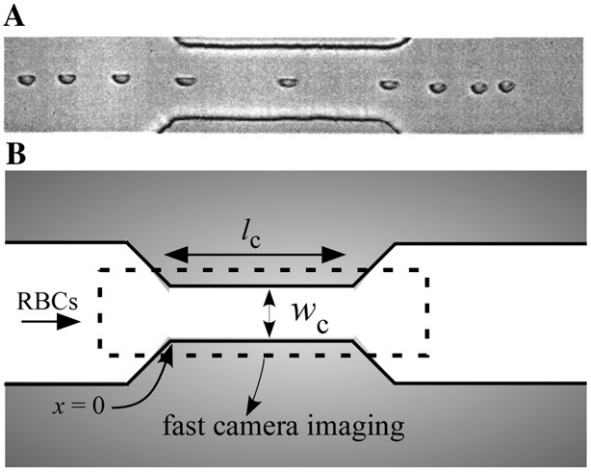

Fig. 1. The microfluidic channel used to quantify RBC deformation and dynamic behavior. (A) A time-lapse image of a single RBC exposed to 38 μM diamide passing through a constriction. (B) A two-dimensional schematic of a constriction in a microfluidic channel through which red blood cells travel. Single RBCs were imaged with a fast camera as they passed through a constriction 100 (l_c) × 40 (w_c) × 18 (h_c) μm. Only cells in the center of the channel were recorded at typical flow rates of 1–3 μl/min.

intracellular ATP (Feo and Mohandras, 1978), which may impact cellular deformation (Gov and Safran, 2005). Instead, our highest concentration of diamide, 3.8 mM, was an order of magnitude larger than those who used iodoacetate, in order to overcome the effects of GSH (Fischer et al., 1978; Teitel, 1981; Schmid-Schonbein and Gaehtgens, 1981).

To begin an experiment, the appropriate suspension of cells was loaded in a 1 ml syringe (Norm-Ject®), which was capped with a 27.5 gauge needle (PrecisionGlide®) and connected to a syringe pump (Harvard Apparatus, PHD 2000). A piece of 0.38 mm I.D. polyethylene (Intramedic®, PE 20) tubing connected the syringe needle to the inlet hole in the device.

Rather than relying on the accuracy of the syringe pump, we instead identified the fastest cell inside the constriction for each experiment and used its velocity as an estimate of the maximum velocity of the flow u_{max}. With this observed maximum velocity, the average flow velocity in the constriction was estimated as $U_c = \frac{2}{3}\left(1-0.627\frac{h_c}{w_c}\right)u_{max}$ which is a good average velocity estimate for pressure-driven flow through a rectangular cross-section in which $\frac{h_c}{w_c}<1$ (Stone, 2007); this value and all subsequent calculations were then applied to all the cells within an experiment. Typical syringe-pump flow rates were 1–3 µl/min, and the calculated average flow speeds were 2–7 cm/s. The flow rate $Q = U_c \cdot h_c w_c$, and average shear rate $\dot{\gamma} = 3U_c / h_c$ inside the constriction were then calculated. The average flow velocity U_c, channel dimension h_c, and Reynolds number for our experiments were all within an order of magnitude of values for human arterioles (Vennemann et al., 2007). Care was taken to only analyze cells that were near the center of the channel, i.e., not in contact or near-contact with the channel walls.

The RBC solution was visualized using a 40× objective on a light microscope (Leica DMIRB, Bannockburn, IL). High-speed video was acquired with a Phantom V9 camera (Vision Research, Wayne, NJ), which was attached to the microscope through a 1× magnification C-mount. Movies were acquired at about 9000 frames per second with a 20 µs exposure time. The resulting movies were analyzed using custom-written image analysis routines in Matlab (Mathworks™).

Results

To determine the effects of pressure-driven channel flow on the dynamic behavior of individual RBCs following exposure to glutaraldehyde or diamide, we used a microfluidic setup in which we monitored the change in length of single RBCs as they passed through a 100 µm long constriction; healthy untreated controls were also used. The constriction was 18 µm high and 40 µm wide, which is enough to cause deformation without the cells interacting with the walls (Fig. 1). Using an image analysis software, we determined the length of the major axis for each cell as it traveled through the constriction and plotted the length of the cell versus position in the channel constriction. We denote the flow direction as x, with $x = 0$ the beginning of the uniform constriction.

The experiments revealed three different types of motion due to the increased shear rate in the constriction: stretching, tumbling and recoiling (Fig. 2). Each behavior is characterized as follows.

1) *Stretching*. The majority of cells exhibited stretching behavior, which is characterized by an elongation with constant orientation along the direction of flow (Fig. 2A). The stretching begins when the channel width narrows at approximately $x = -30$ µm, presumably due to the increased viscous stresses. The cells then relax as they move through the remainder of the constriction. Qualitatively similar stretching was reported by Wan et al. (2008). Note the flow direction, indicated by the arrows, and the orientation of the cells in Fig. 2, which is distinct from the parachute and slipper shapes typically observed in narrower, circular microchannels.
2) *Tumbling*. A sizable fraction of cells, especially at lower shear rates, displayed a tumbling behavior (Fig. 2B). Tumbling is characterized by the cell flipping end-over-end as a rigid body, which is identified both visually and by the appearance of pronounced oscillations in the length versus position data. Note that the tumbling occurs both inside and outside the constriction.
3) *Recoiling*. The final unique motion, which we call "recoiling", is characterized by a sequence of rapid stretching, relaxing, and stretching again, all while the cell is inside the constriction (Fig. 2C). This behavior is markedly different from tumbling because the changing cell length occurs only inside the constriction, where the shear rate is highest. By comparison, cells characterized as tumbling displayed oscillating cell lengths both inside, and outside the constriction. We conclude that the recoiling motion is distinct from tumbling.

The chemical treatment strongly affected the probability of stretching, tumbling or recoiling. For each treatment (control, diamide, and glutaraldehyde) we recorded the motion of $N>33$ cells and determined the probability of tumbling or recoiling (Fig. 3). The probability of stretching is not specifically reported, but is represented by the lack of one of the other two motions. We observed that cells exposed to 3.8 mM of diamide were most likely, at 23%, to recoil inside the constriction, while 9% of control cells recoiled. In contrast, we did not observe recoiling in any glutaraldehyde-exposed cells (Fig. 3A). Glutaraldehyde did increase the probability of tumbling, thereby decreasing the probability of stretching; 52% of glutaraldehyde-exposed cells were found to tumble (Fig. 3B). The key point is that despite both being described as stiffening agents, diamide and glutaraldehyde have markedly different effects on RBC motion in pressure-driven flows.

To gain insight on the effect of chemical treatment on the deformability of RBCs, we grouped the data for each experiment and averaged the normalized cell length according to position. Tumbling cells, which are simply rotating rigid bodies, were excluded. Fig. 4A shows the deformation behavior of treated and control cells at an average shear rate of $4.3\times10^3\ s^{-1}$. The data show that diamide-exposed cells had on average the same deformation behavior as the control cells. On the other hand, glutaraldehyde-exposed cells showed a significantly decreased maximum deformation.

Consideration of the maximum amount of deformation yields a similar picture. Additional experiments at varying shear rates were performed to identify the maximum cell deformation, defined in dimensionless terms as the maximum observed length L_{max}, divided by the average initial length L_{in}, prior to entering the constriction ($x<-30$), i.e. L_{max}/L_{in}. The maximum deformation of the diamide-exposed cells and the control cells were almost identical, $1.11\pm0.01\ L_{max}/L_{in}$, and $1.12\pm0.02\ L_{max}/L_{in}$ respectively (Fig. 4B). The glutaraldehyde-exposed cells, by comparison, exhibited a significantly smaller deformation $1.06\pm0.01\ L_{max}/L_{in}$. Taken together, our results indicate that glutaraldehyde and diamide-exposed cells respond differently to pressure-driven flows.

To further determine how glutaraldehyde, diamide, and control cells differ in their dynamic response under flow we investigated the effective strain rate of individual cells. We estimated this effective strain rate as the slope of the deformation versus position plot as the cell reaches the entrance to the constriction (Fig. 2A). In particular, we calculated each cell's effective strain rate by dividing the change in length ΔL by the initial length L_{in} and the change in time Δt over which the change in length occurred. This value was then normalized by the imposed shear rate $\dot{\gamma}$ to obtain a non-dimensional strain rate, $\acute{Y} = \Delta L / \Delta t L_{in}\dot{\gamma}$. We then averaged the values for all the cells in each cell type: healthy, diamide- and glutaraldehyde-exposed (Fig. 4C). We found that during entrance flow diamide-exposed and healthy cells had similar non-dimensional strain rate values of $\acute{Y} = 2.3\text{–}2.6\times10^{-2}$ while glutaraldehyde-exposed cells have a statistically significant lower value of $\acute{Y} = 1.2\times10^{-2}$ when compared with the other two groups. Increasing the concentration of diamide by an order of magnitude to 3.8 mM did not change the non-dimensional strain rate, suggesting

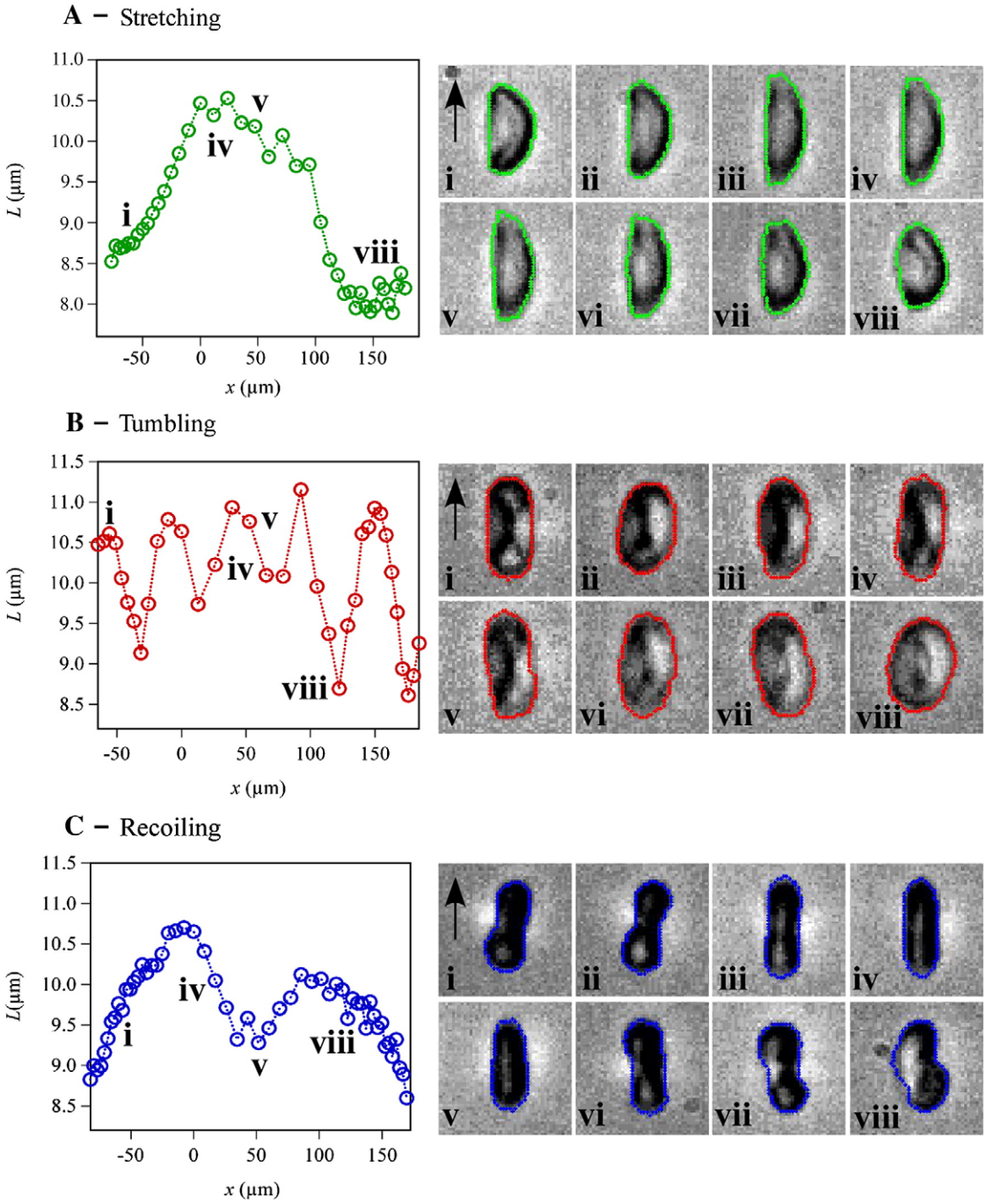

Fig. 2. Dynamic behaviors of chemically exposed RBCs in channel flow. The left-hand side shows the length of the cell versus position, where $x = 0$ is the channel entrance. The right-hand side shows corresponding time-lapse images of the RBC at the labeled positions. The arrow indicates the direction of flow (vertical). (A) Representative example of a "stretching" cell that was exposed to 38 μM diamide, average shear rate of $4.1 \times 10^3\ s^{-1}$. (B) A representative example of a tumbling cell that was exposed to 0.05% glutaraldehyde; average shear rate of $4.7 \times 10^3\ s^{-1}$. (C) A recoiling cell that was exposed to 3.8 mM diamide; average shear rate of $3.4 \times 10^3\ s^{-1}$. Recoiling is characterized by a relaxation and subsequent lengthening inside the constriction at high shear ($x = 0$–100 μm), while tumbling occurs throughout the channel.

that diamide has little influence on the effective strain rate under flow, while glutaraldehyde does have a statistically significant ($p < 0.05$) effect.

Discussion

It is commonly stated that the elasticity of the spectrin–actin network dictates cellular deformation and recovery, while the cytoplasm and lipid membrane have a negligible effect (Evans and Hochmuth, 1976; Hochmuth et al., 1979; Hochmuth and Waugh, 1987). However, dynamic models derived from work with vesicles indicate that dynamic phenomenon, such as tumbling, are controlled by the viscosity contrast between the cell and external solution (Olla, 1997; Abkarian et al., 2002; Kantsler and Steinberg, 2006; Skotheim and Secomb, 2007). Studies of diamide-exposed cells in pressure-driven channel flows have reported similar passage times to healthy controls, suggesting that under certain conditions diamide has little influence on RBCs (Table 1B). However, diamide has been shown to affect the spectrin network, increasing the shear modulus and viscosity of the spectrin–actin network (Fischer et al., 1978; Johnson et al., 1980; Chien, 1987). In contrast, glutaraldehyde cross-links the entire cell, and is known to increase the cytoplasmic and lipid

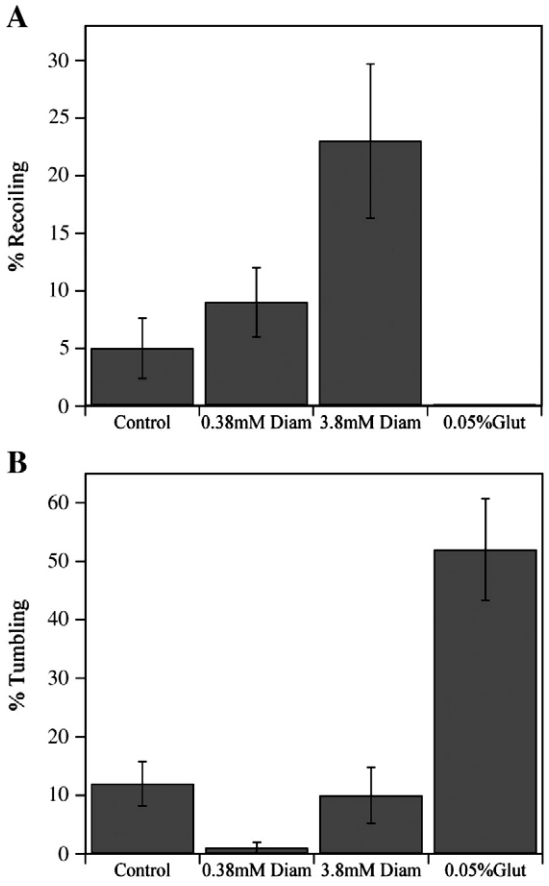

Fig. 3. The percentage of cells tumbling and recoiling. (A) The percentage of cells recoiling for each chemical treatment, average shear rate of $3.5–5.6\times10^3\ s^{-1}$. Diamide-exposed cells were more likely to recoil, while glutaraldehyde-exposed cells did not recoil. (B) The percentage of cells tumbling for each chemical treatment. Glutaraldehyde-exposed cells were more likely to tumble. Number of cells ranged from 33 to 95; error bars indicate one standard error of the mean.

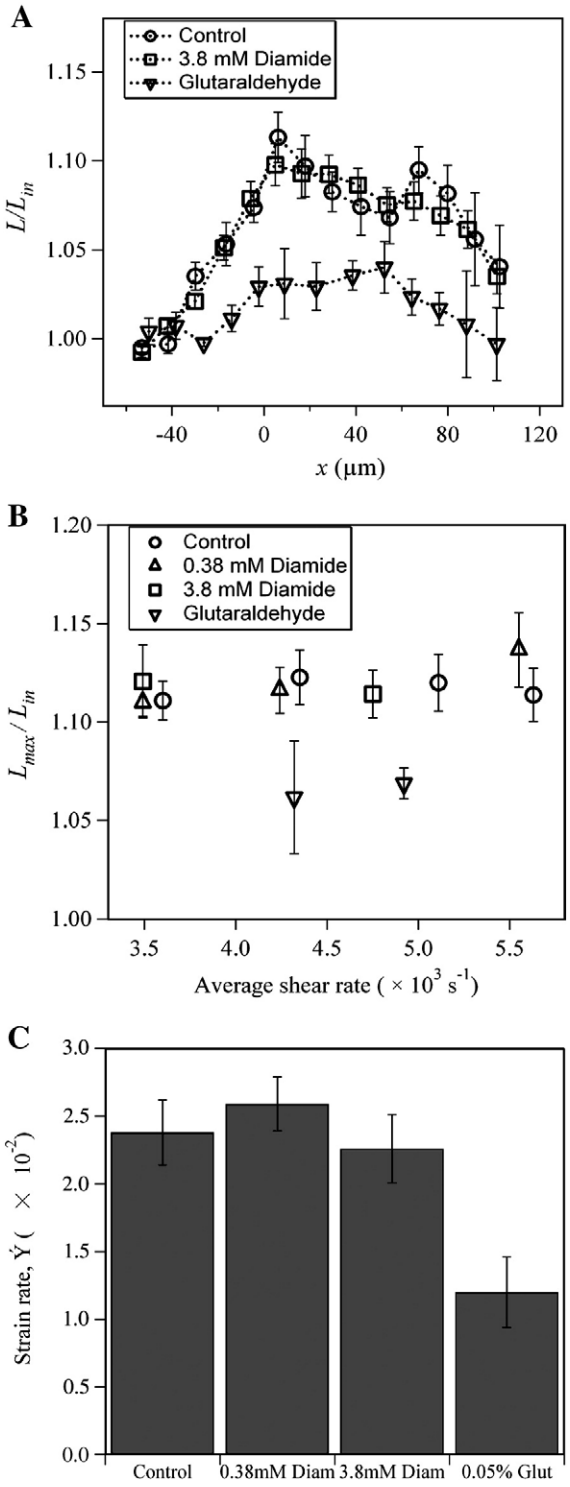

Fig. 4. Analyses of the deformation and relaxation response. (A) Average normalized extent of deformation versus position in the constriction for 3.8 mM diamide-exposed, 0.05% glutaraldehyde-exposed, and control cells at an average shear rate of $4.3\times10^3\ s^{-1}$. The deformation was calculated as the length divided by an average initial length before the cell entered the region of increased shear. (B) The maximum stretch versus shear rate for chemically exposed and control cells. (C) The effective cellular strain rate divided by the average flow shear rate in the entrance of the constriction. Number of cells (N) = 41, 62, 35, and 12 for controls, 0.38 mM, 3.8 mM, and 0.05% glutaraldehyde respectively.

membrane viscosity, and we found reduced total deformations when compared to diamide-exposed cells. Since diamide affects the RBC skeletal membrane and does not change either the lipid membrane or the cytoplasm, our observations that diamide-treated cells are deformed almost identically to control cells (Fig. 4) suggest that the shear elasticity of the skeletal membrane proteins does not dominate the deformation in a pressure-driven flow. On the other hand, glutaraldehyde decreases the fluidity of all components of the cell, including the lipid membrane and cytoplasm (Noji et al., 1991; Szwarocka et al., 2001), and therefore the different dynamic behaviors between diamide- and glutaraldehyde-treated cells indicate that the cytoplasmic and/or the lipid membrane viscosity plays a significant role. However, observations at the whole cell level in our experimental setup make it difficult to differentiate between the contributions of the membrane and cytoplasmic viscosity. Furthermore, increasing the concentration of diamide by an order of magnitude made little difference in the deformation behavior of the RBCs (Fig. 4B). These data show that there is a fundamental difference in the dynamic response of glutaraldehyde and diamide-exposed RBCs.

A natural question to ask is: why do we see no effect of diamide when many other experimental studies have concluded that diamide is a stiffening agent? We propose that the apparent discrepancy is unfounded because different experimental techniques measure different cellular responses. Our conclusions were all based on the use of standard protocols, which have been associated with reports of reduced deformation of diamide-exposed cells in non-pressure-driven experiments (Table 1A). All of the work reporting no reduction of deformation in diamide-exposed cells (i.e., our results and those listed in Table 1B) used pressure-driven flows. In contrast, the work that does show a pronounced effect of diamide on deformation (Table 1A), relied on experiments that used very different stress fields, e.g. micropipette aspiration or cone–plate viscometry, where the

influence of cytoplasmic and lipid membrane viscosity are negligible. The above results therefore do not directly contradict each other, and should instead be seen as offering complementary sets of information. Our results do illustrate, however, the importance of specifying exactly what is meant by the term "stiffening agent." RBCs *in vivo* move via pressure-driven flow, so chemicals shown to stiffen RBCs using other experimental techniques might not necessarily have the intended effect *in vivo*.

The importance of cytoplasmic viscosity and the interaction between hemoglobin and the membrane proteins in cellular deformation was first suggested by Chien (1987), who based this idea on *in vivo* results from separate reports (Driessen et al., 1984; Simchon et al., 1985) in which glutaraldehyde-exposed RBCs were quickly removed from circulation compared with a small loss of diamide-exposed cells. However, Chien does comment that the discrepancy may be due to a difference in the degree of stiffening. In our experiments, in which we have visualized and directly compared the deformation and flow behavior of individual glutaraldehyde- and diamide-exposed cells, we have used concentrations that in other stress fields have shown drastic decreases in deformation.

The non-dimensional strain rate ($\acute{Y}$), averaged for each cell type, tells us that the rate of change of deformation during entrance into the constriction is also not reduced by diamide at either concentrations. However, the glutaraldehyde-exposed cells did show a marked decrease in effective cellular strain rate. This result also demonstrates that the increased shear modulus of the spectrin–actin network does not play a significant role in RBC deformation in this type of flow.

Recoiling phenomena, in which the cell relaxes and lengthens inside the constriction, was more common for diamide-exposed RBCs. This "recoiling" may be in some ways similar to an earlier described vesicle phenomenon known as "vacillating-breathing" where the cell deforms periodically as it changes orientation (Misbah, 2006). However, we note that because the "recoiling" behavior does not occur outside the constriction in the region of lower shear, it is unlikely that this is a periodic behavior. The glutaraldehyde-exposed cells did not exhibit this recoiling behavior, while cells exposed to the highest concentration of diamide were most likely to recoil. It is likely that the diamide-treated cells have a higher variation in elastic energy when undergoing deformation due to the stiffened membrane skeleton. The resulting shape memory response would be stronger for these cells, possibly producing this "recoiling" effect via a folding mode (Fischer, 2004). This result indicates that while diamide does not play a significant role in deformation or tumbling behavior, it still alters the cells.

Glutaraldehyde-exposed cells showed a substantial increase in tumbling when compared to other cell types. This result is supported by tumbling models that rely on the viscosity contrast between the cell and fluid to predict tumbling behavior. In addition, it is known that tumbling cells increase the effective viscosity of the solution (Chien, 1987). This fact, taken together with our results, offers an explanation for the observations by Noji et al. (1991) who found that glutaraldehyde-exposed cells mixed with healthy cells had an apparent viscosity much larger than diamide-exposed cells mixed with healthy cells at the same ratio.

When evaluating the mechanics of blood cells in pathology or in medical therapeutics, such as drug delivery and blood substitution, our results indicate that it is important to use pressure-driven flows. Further work needs to be done to determine which models best describe RBC behavior in flow, taking into account the entire cell including the cytoplasm and other contributors to cell viscosity. We believe our work is relevant for understanding cardiovascular disease, as many correlations have been drawn between RBC deformation and these diseases, as well as other disorders, such as hereditary spherocytosis, which directly affect the deformation of RBCs (Mokken et al., 1992). The microfluidic channels we used are in the size range of arterioles, which have large amounts of vascular smooth muscle, that control vasodilation *in vivo*. Recent research has demonstrated a link between deformation of RBCs and adenosine triphosphate release by the red blood cells (Sprague et al., 1998; Price et al., 2004; Wan et al., 2008), which is believed to trigger vasodilation of the smooth muscle cells (Bogle et al., 1991). Therefore, cellular deformation dynamics and their relation to local chemistry in the arterioles play an important role in physiology and should be carefully considered.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mvr.2010.03.008.

References

Aarts, P.A., et al., 1984. Red-blood-cell deformability influences platelets–vessel wall interaction in flowing blood. Blood 64, 1228–1233.
Abkarian, M., et al., 2002. Tank treading and unbinding of deformable vesicles in shear flow: determination of the lift force. Phys. Rev. Lett. 88, 068103.
Ajmani, R.S., 1997. Hypertension and hemorheology. Clin. Hemorheol. Microcirc. 17, 397–420.
Bogle, R.G., et al., 1991. Bradykinin and ATP stimulate L-arginine uptake and nitric oxide release in vascular endothelial cells. Biochem. Biophys. Res. Commun. 180, 926–932.
Chien, S., 1987. Red-cell deformability and its relevance to blood-flow. Annu. Rev. Physiol. 49, 177–192.
Driessen, G.K., et al., 1982. Capillary resistance to flow of hardened (diamide treated) red-blood-cells (rbc). Pflug. Arch. Eur. J. Phys. 392, 261–267.
Driessen, G.K., et al., 1984. Flow behaviour of rigid red blood cells in the microcirculation. Int. J. Microcirc. Clin. Exp. 3, 197–210.
Duffy, D.C., et al., 1998. Rapid prototyping of microfluidic systems in poly(dimethylsiloxane). Anal. Chem. 70, 4974–4984.
Eike, J.H., Palmer, A.F., 2004. Effect of glutaraldehyde concentration on the physical properties of polymerized hemoglobin-based oxygen carriers. Biotechnol. Prog. 20, 1225–1232.
Engelhardt, H., Sackmann, E., 1988. On the measurement of shear elastic-moduli and viscosities of erythrocyte plasma-membranes by transient deformation in high-frequency electric-fields. Biophys. J. 54, 495–508.
Evans, E.A., Hochmuth, R.M., 1976. Membrane viscoelasticity. Biophys. J. 16, 1–11.
Faris, A., Spence, D.M., 2008. Measuring the simultaneous effects of hypoxia and deformation on ATP release from erythrocytes. Analyst 133, 678–682.
Feo, C., Mohandras, N., 1978. Role of ATP depletion on red cell shape and deformability. In: Bessis, M., Shohet, S.B., Mohandras, N. (Eds.), Red Cell Rheology. Springer-Verlag, New York.
Fischer, T.M., et al., 1978. Selective alteration of erythrocyte deformability by Sh-reagents—evidence for an involvement of spectrin in membrane shear elasticity. Biochim. Biophys. Acta 510, 270–282.
Fischer, T.M., 2004. Shape memory of human red blood cells. Biophys. J. 86, 3304–3313.
Giger, U., et al., 1995. Naturally-occurring human anti-band-3 autoantibodies accelerate clearance of erythrocytes in guinea-pigs. Blood 85, 1920–1928.
Gov, N.S., Safran, S.A., 2005. Red blood cell membrane fluctuations and shape controlled by ATP-induced cytoskeletal defects. Biophys. J. 88, 1859–1874.
Hochmuth, R.M., Waugh, R.E., 1987. Erythrocyte-membrane elasticity and viscosity. Annu. Rev. Physiol. 49, 209–219.
Hochmuth, R.M., et al., 1979. Red cell extensional recovery and the determination of membrane viscosity. Biophys. J. 26, 101–114.
Johnson, G.J., et al., 1980. Decreased survival in vivo of diamide-incubated dog erythrocytes—a model of oxidant-induced hemolysis. J. Clin. Invest. 66, 955–961.
Kantsler, V., Steinberg, V., 2006. Transition to tumbling and two regimes of tumbling motion of a vesicle in shear flow. Phys. Rev. Lett. 96, 036001.
Kon, K., et al., 1987. Erythrocyte deformation in shear-flow—influences of internal viscosity, membrane stiffness, and hematocrit. Blood 69, 727–734.
Marchesi, V.T., 2008. The relevance of research on red cell membranes to the understanding of complex human disease: a personal perspective. Annu. Rev. Pathol. 3, 1–9.
Marczak, A., Jozwiak, Z., 2008. The interaction of DNR and glutaraldehyde with cell membrane proteins leads to morphological changes in erythrocytes. Cancer Lett. 260, 118–126.
Misbah, C., 2006. Vacillating breathing and tumbling of vesicles under shear flow. Phys. Rev. Lett. 96, 028104.
Mokken, F.C., et al., 1992. The clinical importance of erythrocyte deformability, a hemorheological parameter. Ann. Hematol. 64, 113–122.
Morel, F.M.M., et al., 1971. Quantitation of human red blood cell fixation by glutaraldehyde. J. Cell Biol. 48, 91–100.

Noji, S., et al., 1991. An EPR study on erythrocyte deformability. Prog. Biophys. Mol. Biol. 55, 85–105.
Ogura, E., et al., 1991. Measurement of human red-blood-cell deformability using a single micropore on a thin Si_3N_4 film. IEEET Bio-Med. Eng. 38, 721–726.
Olla, P., 1997. The lift on a tank-treading ellipsoidal cell in a shear flow. J. Phys. Ii 7, 1533–1540.
Price, A.K., et al., 2004. Deformation-induced release of ATP from erythrocytes in a poly (dimethylsiloxane)-based microchip with channels that mimic resistance vessels. Anal. Chem. 76, 4849–4855.
Schmid-Schonbein, H., Gaehtgens, P., 1981. What is red-cell deformability. Scand. J. Clin. Lab. Investig. 41, 13–26.
Shelby, J.P., et al., 2003. A microfluidic model for single-cell capillary obstruction by *Plasmodium falciparum* infected erythrocytes. Proc. Natl. Acad. Sci. U.S.A. 100, 14618–14622.
Simchon, S., et al., 1985. Influence of red blood cell deformability on regional blood flow distribution. Fed. Proc. 44, 1005.
Simchon, S., et al., 1987. Influence of reduced red-cell deformability on regional blood-flow. Am. J. Physiol. 253, H898–H903.
Skotheim, J.M., Secomb, T.W., 2007. Red blood cells and other nonspherical capsules in shear flow: oscillatory dynamics and the tank-treading-to-tumbling transition. Phys. Rev. Lett. 98, 078301.
Sou, K., et al., 2005. Circulation kinetics and organ distribution of Hb-vesicles developed as a red blood cell substitute. J. Pharmacol. Exp. Ther. 312, 702–709.
Sprague, R.S., et al., 1998. Deformation-induced ATP release from red blood cells requires CFTR activity. Am. J. Physiol. 275, H1726–H1732.
Stone, H.A., 2007. Introduction to fluid dynamics for microfluidic flows. In: Lee, H., Westervelt, R.M., Ham, D. (Eds.), CMOS Biotechnology. Springer US, pp. 5–30.
Szwarocka, A., et al., 2001. The combined effect of IDA and glutaraldehyde on the properties of human erythrocytes. Int. J. Pharm. 220, 43–51.
Teitel, P., 1981. Polymicroviscometry of spectrin cross-linked red-blood-cells—theoretical and clinical implications. Scand. J. Clin. Lab. Investig. 41, 235–237.
Vennemann, P., et al., 2007. In vivo whole-field blood velocity measurement techniques. Exp. Fluids 42, 495–511.
Wan, J., et al., 2008. Dynamics of shear-induced ATP release from red blood cells. Proc. Natl. Acad. Sci. U.S.A. 105, 16432–16437.