Characterizing osmotic lysis kinetics under microfluidic hydrodynamic focusing for erythrocyte fragility studies

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The biomechanics of erythrocytes, determined by the membrane integrity and cytoskeletal structure, provides critical information on diseases such as diabetes mellitus, myocardial infarction, hypertension, and sickle cell anemia. Here we demonstrate a simple microfluidic tool for examining erythrocyte fragility based on characterizing osmotic lysis kinetics. Hydrodynamic focusing is used for generating rapid dilution of the buffer and producing lysis of erythrocytes during their flow. The lysis kinetics are tracked by monitoring the release of intracellular contents from cells via recording the light intensity of erythrocytes at various locations in the channel. Such release profile reflects sensitively the changes in erythrocyte fragility induced by chemical, heating, and glucose treatment. Our tool provides a simple approach for probing red blood cell fragility in both basic research and clinical settings.

Introduction

The erythrocyte (i.e. red blood cell or RBC) membrane is composed of roughly equal amounts of lipids and proteins. Erythrocyte shape, stability and deformability strongly depend on the membrane–cytoskeleton interactions. Erythrocyte deformability, representing the ability to undergo reversible shape changes in response to an external stress on the cell, is a very important property that is highly relevant to capillary passage and oxygen supply to tissues. Parameters that affect erythrocyte deformability include the integrity and organization of cytoskeletal proteins, surface to volume ratio of the cell, and the hemoglobin concentration and physicochemical properties. For example, in the case of insufficient cytoskeletal protein synthesis, enzyme activity for neutralizing oxidants decreases. The oxidative damage results in abnormal hemoglobin concentration and changed elastoviscous behavior of erythrocytes. Furthermore, change in the surface area to volume ratio due to aging makes the erythrocyte flow rheologically unfavorable. Decrease in the erythrocyte deformability has been linked to many diseases including diabetes mellitus, hypertension, acute myocardial infarction and sickle cell anemia with different underlying mechanisms. For example due to diabetes mellitus, the change in the ratio between the phospholipids and cholesterol causes a decrease in the erythrocyte deformability. Abnormal erythrocyte phospholipid account leads to cell rigidity in hypertension patients, specifically due to a decrease of phospholipids and phosphatidyl ethanolamine and increase of sphingomyelin. For acute myocardial infarction, the decreased erythrocyte deformability is caused by an increase in the lactate concentration of the extracellular environment. In sickle cell anemia, hemoglobin is polymerized under hypoxic conditions by replacing glutamate with valine at the sixth position of the β-globin chain, which results in decreased cell deformability. As a result, erythrocyte deformability quantification is interesting for disease diagnosis, monitoring, and staging.

Current techniques for measuring erythrocyte deformability include osmotic fragility tests, filtration, ektacytometry, rheoscopy, micropipette aspiration and atomic force microscopy (AFM). Osmotic fragility tests expose red cells to a series of hypotonic saline solutions for a long period of time (e.g. 24 h) and measure the degree of hemolysis based on optical density for these samples. The percent lysis is then plotted against the saline concentration and the curve is compared to the control. The susceptibility of RBCs to osmotic lysis is primarily determined by their surface area to volume ratio. Based on measuring the ensemble properties of large cell populations, filtration records passage time of erythrocytes through the membrane with micropores. In the ektacytometry method, laser diffraction analysis is utilized to examine the erythrocyte deformability under varying stress. In contrast, the other three methods examine the deformability at a single cell level. Rheoscopes analyze images of erythrocytes that deform due to shear stress produced by two rotating plates. Micropipette aspiration studies the cell behavior under suction stress to estimate the area rigidity modulus. AFM assesses the elasticity of an erythrocyte by deforming the cell surface with a cantilever tip and then records the laser deflection.

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In this report, we developed a microfluidic method for rapid study of erythrocyte biomechanics based on analyzing osmotic lysis kinetics under hydrodynamic focusing. Although the manipulation and analysis of erythrocytes on a microfluidic platform has been well practised in the field, the link between the osmotic lysis kinetics and the red cell biomechanics has not been explored previously. In our method, we use hydrodynamic focusing to create an interface between pure water and isotonic buffer where ion mixing and dilution occur. Osmotic lysis of RBCs can be observed by a change in the optical intensity of RBCs along the flow path. The low-Reynolds-number hydrodynamic focusing in the microfluidic device generates reproducible RBC lysis data. Combined with results on the ion concentration modeled by COMSOL, our approach provides a simple way to study RBC lysis kinetics with complete information. We show that the lysis kinetics data generated by this method are highly reflective of cell fragility varied by chemical, heating, and glucose treatment. Although both based on examination of RBCs under osmotic stress, our approach and the conventional osmotic fragility test are different, with our test focusing on obtaining the kinetics and the conventional assay focusing on the equilibrium measurements.

Materials and methods

Sample preparation

Whole blood samples from healthy humans (purchased from Bioreclamation, Westbury, NY) were collected into K3 EDTA vacutainer tubes. Then the whole blood was washed three times with filtered PBS buffer by centrifuging at 1200 g for 10 min before carefully removing the plasma supernatant and buffy coat. The remaining pellet containing red blood cells were suspended in PBS and kept at 4 °C until needed for experiments. For preparation of chemically treated erythrocytes, the washed erythrocytes were suspended and incubated at 25 °C for 30 min in either glutaraldehyde (Sigma, St. Louis, MO) of four different concentrations (0, 0.3, 0.5 and 1 mM) or hydrogen peroxide (Electron Microscopy Sciences, Hatfield, PA) of four different concentrations (0, 0.3, 0.5 and 1 mM). For heating treatment, the washed erythrocytes were suspended in a PBS solution and incubated at 25 °C for 30 min in either glutaraldehyde (Sigma, St. Louis, MO) of four different concentrations (0, 0.3, 0.5 and 1 mM) or hydrogen peroxide (Electron Microscopy Sciences, Hatfield, PA) of four different concentrations (0, 0.3, 0.5 and 1 mM). For heating treatment, the washed erythrocytes were suspended in a PBS solution and incubated at 25 °C for 30 min. For glucose treatment, the red blood cells were suspended in various glucose (Sigma) concentrations (5 and 15 mM) in PBS of 40% hematocrit and incubated in a shaking incubator at 37 °C for 24 h. After treatment, the erythrocytes were washed with PBS and resuspended in a PBS solution until required.

Microfluidic device fabrication

Microfluidic devices were fabricated based on PDMS using a standard soft lithography method. Microscale patterns were first created using computer-aided design software (FreeHand MX, Macromedia, San Francisco, CA) before being printed out on a high resolution (5080 dpi) transparency. SU-8 2025 negative photoresist (~23 μm in the thickness) (Microchem Corp., Newton, MA) was spun on a 3 inch silicon wafer before pre-baking. Then the photoresist was exposed to UV light before development to form the master. PDMS prepolymer mixture consisting of monomer (A) and curing agent (B) (General Electric Silicones RTV 615, MG Chemicals, Toronto, Ontario, Canada) was poured on the master and heated at 80 °C for 1 h to cure before it was peeled off and punched to form inlet and outlet. The PDMS chip and a pre-cleaned glass slide were bonded together immediately after both surfaces were oxidized in a plasma cleaner (Harrick Plasma, Ithaca, NY). The whole device was baked at 80 °C for an additional 30 min to attain strong bonding.

Microfluidic chip operation

As shown in Fig. 1, erythrocyte suspension was flowed into the device from the central inlet under hydrodynamic focusing by two flows of pure water from two side inlets. All solutions were filtered by 0.2 μm filter before use to prevent channel clogging. The flow rates from the central inlet and side inlets were varied to obtain different focusing effects. Erythrocyte lysis was recorded at different positions of the channel using a CCD camera (ORCA-285, Hamamatsu, Bridgewater, NJ) on an inverted fluorescence microscope (IX-71, Olympus, Melville, NY) equipped with a 10× dry objective at a frame rate of 16 frames per second. We adjusted focusing (by moving the objective along the Z direction) at each location in order to obtain sharp images of the cells.

Image analysis

A MATLAB program was written and used to analyze the image series which recorded erythrocyte lysis. Specifically, the channel segment covered by the CCD camera frame was about 0.9 mm long. Once steady flow of RBCs was established, we took image series at 13 locations that were evenly distributed along the horizontal channel with 150 frames captured at each location. The MATLAB program recognized all erythrocytes within the frames and calculated the average light intensity of cells at each location. The averaged light intensity of the erythrocytes at...
different positions were then plotted against the distance from the channel intersection \((x = 0)\) to generate the lysis profile. Every experiment was conducted in triplicate. In order to quantify the percent release, we set the light intensity of cells that were intact (when the side streams were also PBS buffer) to be 100\% and that of completely lysed cells (exposed to pure water for 30 min) to be 0.

**Modeling**

The flow in the microfluidic device was modeled using COMSOL Multiphysics 4.1. The Navier--Stokes and the continuity equations were coupled with the diffusion equation to solve the problem computationally. The boundary conditions were determined by setting specific inlet velocities and the pressure to 0 at the outlet. We assumed a no-slip condition at the walls and incompressible fluid.

**Results and discussion**

Hydrodynamic focusing has been widely used in microfluidics to generate steady and rapid mixing of molecules across the interface of parallel streams.\(^{42–45}\) We use hydrodynamic focusing here to create continuous lysis for flowing RBCs. As shown in Fig. 1, erythrocytes suspended in PBS buffer flow into the central inlet of a cross channel, the RBC-carrying stream is hydrodynamically focused by two streams of pure water from the two side inlets. The scheme creates steady-state concentration profiles for the ions along three dimensions of the horizontal channel. RBC osmotic lysis starts in the downstream of the channel intersection where the osmolarity is low due to water dilution. The lysis continues to release intracellular contents of RBCs (mostly hemoglobin) while they flow downstream along the length of the channel. Such release of intracellular materials is reflected by the change in the light intensity of cells, as shown in the inset images of Fig. 1. We take images of flowing RBCs at various locations along the channel length and quantify the RBC lysis/release at these locations by averaging the light intensity of a number of cells. In our experiment, the concentrations and velocity in the microfluidic device can be easily modeled by software package such as COMSOL. Thus this simple device and design allow us to characterize RBC lysis/hemoglobin release in relation to the time and osmolarity (determined by ion concentrations).

Fig. 2 shows ion concentration distribution in the microfluidic channel in the vicinity of the intersection under various hydrodynamic focusing conditions modeled by COMSOL (ignoring the effects from flowing RBCs). In the modeling, we follow the most abundant salt NaCl (137 mM in PBS buffer) and assume that other ionic species follow very similar trends. In the left panel of Fig. 2, we increased the flow rates of the two side water streams (from 0.3 to 1.8 \(\mu\)l min\(^{-1}\) in each stream) while keeping the central PBS flow at 0.1 \(\mu\)l min\(^{-1}\). The increase in the side stream flow rate under constant central stream flow rate creates more rapid mixing and dilution in a given channel length. As shown in the left panel of Fig. 2, the decay of NaCl concentration to its 15\% occurs at 12.5 mm downstream from the intersection under 0.1–0.6 \(\mu\)l min\(^{-1}\) (referring to the central stream of 0.1 \(\mu\)l min\(^{-1}\) and the side streams of 0.3 \(\mu\)l min\(^{-1}\) each) and at 2.0 mm under 0.1–3.6 \(\mu\)l min\(^{-1}\). In contrast, in the right panel, we increased the flow rates of the central stream and the side streams proportionally from 0.1–1.2 to 0.4–4.8 \(\mu\)l min\(^{-1}\). The dilution along the channel length occurs at the closest distance under 0.1–1.2 \(\mu\)l min\(^{-1}\) (decay to 15\% when \(x = 4.9\) mm) and at the farthest under 0.4–4.8 \(\mu\)l min\(^{-1}\) (decay to 15\% when \(x = 18.6\) mm).

The results generated by our experiments are in the form of plots between the percent release of hemoglobin from RBCs due to osmotic lysis and the distance \(x\) along the channel length (by setting the left side of the intersection as \(x = 0\)). The percent release was calculated based on the average optical intensity of cells at a specific location along the channel length, in reference to both intact RBCs and RBC ghosts. As seen in Fig. 3, the percent release–distance curves are fairly reproducible with the same type of RBCs, yet change under different focusing conditions.

It is necessary to probe the origin of the change in the curves under various focusing conditions because such investigation yields insights into what parameter(s) affects kinetics of osmotic lysis in our experiments. Under hydrodynamic focusing, the osmotic lysis is potentially affected by two parameters: the concentration of the salts (which determines the osmolarity and varies along the channel length) and the exposure time of RBCs to hypotonic buffer (the time required for cells to move from the origin at \(x = 0\) to a given downstream location). Both parameters change with the focusing conditions. In Fig. 4, we show the relationship between the NaCl concentration and the exposure time with data generated by COMSOL modeling. Fig. 4a shows the relationship between the two when the side flows increase and the central stream is constant. The concentration gets lowered more rapidly when the side flows are set at higher values. In contrast, when the central and side flows are varied proportionally (Fig. 4b), the relationship between the exposure time and the concentration is essentially unchanged.

In Fig. 5, we plot the percent release of RBCs against the salt concentration and the exposure time respectively. Fig. 5a and 5b (with the flows varying from 0.1–0.6 to 0.1–3.6 \(\mu\)l min\(^{-1}\)) show that there is not a consistent correlation between the concentration and the percent release \((R^2 = 0.061\) in Fig. 5a\) but there is a strong correlation between the exposure time and percent release \((R^2 = 0.818\) in Fig. 5b\). Thus we conclude that the exposure time of RBCs to the diluted buffer quantitatively affects the percent
release in the most substantial way. The lysis starts once RBCs are in hypoosmotic buffer, with the actual salt concentration (or osmolarity) exerting little influence on release kinetics. Fig. 5c and 5d show that there are strong correlations when the percent release is plotted against either the salt concentration or the exposure time, when the flow rates of the central and side streams were increased proportionally. This is due to the fact that the exposure time and the salt concentration were well correlated under these focusing conditions, as shown in Fig. 4b.

In order to study cells of different biomechanics, we observe the cell lysis profile along the channel length using fixed focusing conditions. In Fig. 6, we set the central carrier stream at 0.1 mL min⁻¹ and the side flows at 0.6 mL min⁻¹ each and examine the lysis kinetics of untreated RBCs and RBCs treated by chemicals including glutaraldehyde and hydrogen peroxide. Glutaraldehyde reacts with various functional groups of amino acids, such as amino, imino, hydroxyl and thio groups, and such treatment leads to cross-linking of proteins that destroys the membrane integrity and alters the membrane structure.⁴⁶–⁴⁹ Hydrogen peroxide induces oxidative damage to hemoglobin and thus alters the composition of RBC cytoplasm, specifically by introducing free radicals across the membrane.⁵⁰ Both treatments lead to hardened cell membrane and thus a decrease in RBC deformability.⁵¹ The low deformability in turn suppresses hemolysis and increases its stability under osmotic lysis condition. As seen in Fig. 6a, various concentrations of glutaraldehyde (0.3–1 mM) affect the releasing to different degrees. Even treatment with a very low concentration of 0.3 mM leads to a substantial change in the release profile of RBCs. In general, higher glutaraldehyde concentration leads to less release at a certain channel location. At the high concentration of 1 mM, nearly no releasing happens, indicating a very high degree of erythrocyte hardening. Similar to glutaraldehyde, hydrogen peroxide also produces less release of cellular contents, but to a less significant degree compared to glutaraldehyde of the same concentration. At the concentration of 1 mM hydrogen peroxide, there is ~40% of content release at the end of the channel, in comparison to less than 5% with 1 mM glutaraldehyde. Results from both chemical treatments suggest that the release profiles of RBCs under hydrodynamic focusing reflect delicate changes in the cell biomechanics.

Heating has been proved to change RBC biomechanics by denaturing spectrin in the cytoskeleton.⁵²,⁵³ Fig. 7 shows incubating erythrocytes at 49 °C (at which the change in spectrin is substantial) affects the release profile, depending on the heating duration. Heating for 10 min at 49 °C does not introduce substantial change in the release profile. However, heating of 30 min produces decreased release especially at the more downstream channel locations.

We also investigate the utility of our method for clinically relevant studies. Diabetes mellitus and hyperglycemia are known to cause increased RBC fragility.⁵⁴,⁵⁵ A number of mechanisms including membrane lipid peroxidation,⁵⁶ glycosylation of structural and functional proteins,⁵⁷ and production of oxygen free
radicals\textsuperscript{58} have been associated with the phenomenon. In Fig. 8, we show that the change induced by glucose incubation in the RBC fragility can be detected by our method. We set the hydrodynamic focusing at 0.1–0.6 $\text{ml min}^{-1}$ and examine the lysis kinetics of untreated RBCs and RBCs treated by glucose of 5 and 15 mM at 37 °C for 24 h (normal blood glucose level is 4–6 mM). The data indicate that the treatment with glucose increases the fragility and produces faster lysis kinetics. Treatment with 15 mM glucose (i.e. hyperglycemia) yields higher lysis rate than the one with 5 mM glucose treatment and the untreated cells are associated with the lowest lysis rate. Our experiment reveals the potential of this method for detecting pathological changes.

It needs to be noted that while cell biomechanics and deformability have been mostly linked to the physical properties of the cell membrane and cytoskeleton, the treatment described above also changed the properties of intracellular hemoglobin and might have affected its release. Thus our data not only

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**Fig. 5** The percent release of RBCs plotted against the NaCl concentration (a and c) and the exposure time (b and d). In (a) and (b) the side flows increase while the central stream is constant, while in (c) and (d) the central and side flows are varied proportionally.

**Fig. 6** The release profiles (the percent release plotted against the distance $x$) of untreated RBCs and RBCs treated by glutaraldehyde (a) and hydrogen peroxide (b), respectively. 0.1–1.2 $\mu\text{l min}^{-1}$ is used as the hydrodynamic focusing condition. RBCs are treated with either chemical of various concentrations at 25 °C for 30 min.

**Fig. 7** The release profiles of untreated RBCs and RBCs heated at 49 °C for 10 min and 30 min. 0.1–1.2 $\mu\text{l min}^{-1}$ is used as the hydrodynamic focusing condition.
reflected the mechanical changes in the membrane or the cytoskeleton, but also contribute from the change in the properties of hemoglobin under the treatment. The accuracy of the data may be affected by the cell movement in the direction of the channel depth. For a particular cell model, the channel design and the flow conditions require optimization for maximal differentiation of cells of various fragilities.

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References