

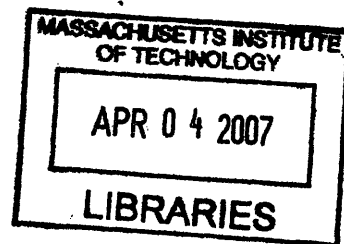
Mathematical and Mechanical Modeling of Vaso-occlusion in Sickle Cell Disease

by

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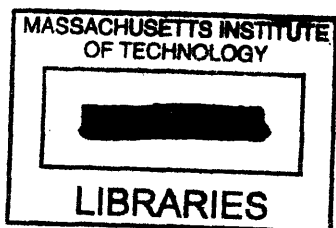
Submitted to the Harvard-MIT Division of Health Sciences and Technology in Partial
Fulfillment of the Requirements for the Degree of

Master of Health Sciences and Technology

at the

Massachusetts Institute of Technology

February 2007



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ABSTRACT

Vaso-occlusive crises cause most of the morbidity and mortality associated with sickle cell disease. The proximal causes of these occlusive events are not well understood. The risks and consequences of vaso-occlusion however are clear. Ten percent of sickle cell disease patients will have a stroke by the age of 20. Two thirds of sickle cell disease patients require more than one hospitalization per year for treatment of pain crises.

The flow behavior of blood samples from sickle cell patients was studied in an artificial microfluidic environment. This microfluidic environment allowed modulation of the hydrostatic pressure causing flow, the ambient oxygen concentration, and the vascular channel geometry. A range of blood samples was evaluated by selecting specimens with various hematocrits and concentrations of sickle hemoglobin.

Velocity profiles were calculated following sudden changes in oxygen concentration. From these profiles, it was possible to create a phase space of vaso-occlusion in the artificial microfluidic environment. This phase space characterizes the environmental conditions in which sickle cell blood will stop flowing within a given interval of time. This work is a first step in characterizing the inter-relationships between some of the control parameters governing vaso-occlusion: pressure, oxygen concentration, channel size, hematocrit, and sickle hemoglobin concentration. This artificial device enables a quantification of the effect of a clinical therapy, red blood cell exchange, as performed on an actual sickle cell patient. Additionally, three sample small molecules known to alter rates of sickle hemoglobin polymerization were evaluated for their ability to perturb the tendency of sickle cell blood to stop flowing. These results suggest a possible application of this technique to the diagnosis and monitoring of sickle cell patients as well as to the investigation of new regimens of existing treatments and altogether novel therapies.

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Introduction

Sickle cell disease was identified more than 50 years ago by Linus Pauling as the first “molecular disease.”¹ There are approximately 50,000 individuals with sickle cell anemia in the United States requiring frequent hospitalizations for acute events.² Sickle cell disease was responsible for an average of 75,000 acute hospitalizations between the years of 1989 and 1993, costing \$475 million annually.³ Vaso-occlusive events caused a large number of these hospitalizations. Efforts in the past half century to understand the mechanisms of disease and to find effective treatments provide overwhelming evidence that a useful understanding of the sickle cell disease process requires the integration of experiments and models at multiple levels of scale: microscopic hemoglobin polymerization, mesoscopic cellular sickling, and macroscopic vascular occlusion. Only by capturing and integrating processes at each level of scale can we hope to find meaningful and effective treatments.

¹ Pauling, L., H. A. Itano, et al. (1949). "Sickle cell anemia a molecular disease." *Science* 110(2865): 543-8.

² Selekmán, J., *Update: new guidelines for the treatment of infants with sickle cell disease. Agency for Health Care Policy and Research. Pediatr Nurs*, 1993. 19(6): p. 600-5.

³ Davis, H., R.M. Moore, Jr., and P.J. Gergen, *Cost of hospitalizations associated with sickle cell disease in the United States. Public Health Rep*, 1997. 112(1): p. 40-3.

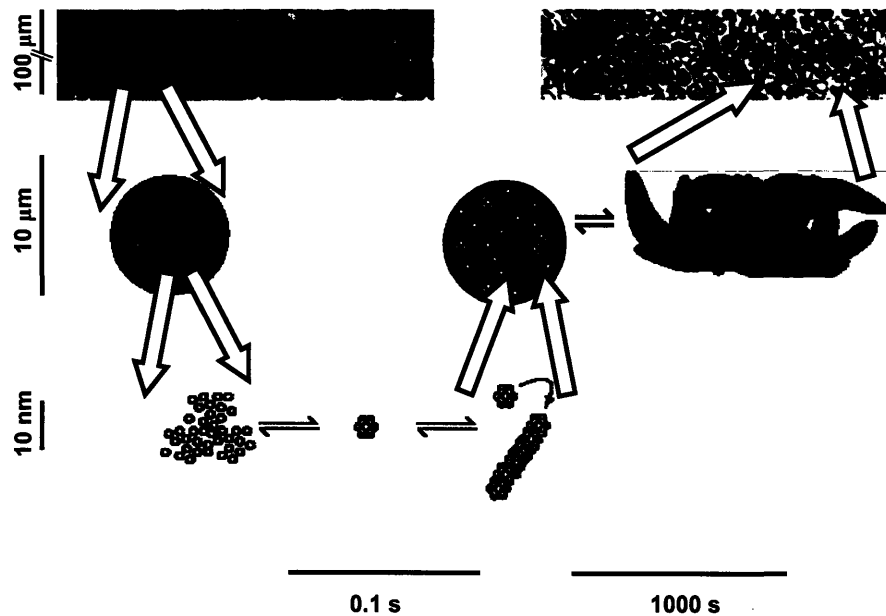


Figure 1: Multiscale schematic of the collective processes of vaso-occlusion: polymerization of hemoglobin S occurring at the 10-nm length scale, cell sickling at the 10-μm length scale, and vessel jamming at up to 100-μm, while the time scales for the different processes range from a fraction of a second for polymerization to a few minutes before a deoxygenation event leads to jamming of the artificial capillary by deformed red blood cells.

We undertook a multi-scale study of vaso-occlusion in sickle cell disease. By manipulating oxygen concentrations, we controlled microscopic hemoglobin polymerization. By selecting hemoglobin S concentrations, we controlled mesoscopic cellular sickling. By varying channel diameter and red blood cell concentrations, we controlled rates of occlusion. We hypothesize that control parameters for vaso-occlusion are vessel diameter, oxygen concentration, pressure gradient, red blood cell concentration, and hemoglobin S concentration. We performed experiments varying these parameters to study integrated processes with length scales varying over more than five orders of magnitude and time scales varying over more than four orders of magnitude (see Table 1).

	Hemoglobin S	Cell Sickling	Occlusion
Length (m)	10^{-9}	10^{-6}	10^{-4}
Time (s)		10^{-1}	10^3

Table 1: Length and time scales in sickle cell vaso-occlusion. Length scales vary from several nanometers for a single molecule of hemoglobin to several microns for a hemoglobin S polymer and a red blood cell to almost a millimeter for the maximal diameter of a flow channel. Time scales vary from less than one tenth of a second for hemoglobin polymerization to more than ten minutes for stoppage of flow.

Much is known about the molecular details of sickle hemoglobin polymerization.⁴

Much is also known about the clinical heterogeneity of sickle cell disease.⁵ There are many unanswered questions about the processes linking the pathologic polymerization of sickle hemoglobin with the devastating clinical effects of sickle cell disease. There have been many successful efforts to characterize parts of this integrated process, including hemoglobin polymerization rates⁶ and sickled cell deformability.^{7,8} The diversity of behaviors at each level also argues in favor of an integrative approach. The heterogeneity at the level of organism phenotype is qualitatively matched by the heterogeneity of hemoglobin S polymers which often contain a single polymer domain whose shape and therefore rheology are very difficult to model.⁹ By analyzing the process at an integrated level, we can average some stochastic effects, such as those of individual polymer domains, and assess their collective

⁴ Eaton, W. A. and J. Hofrichter (1990). "Sickle cell hemoglobin polymerization." *Adv Protein Chem* **40**: 63-279.

⁵ Bunn, H. F. (1997). "Pathogenesis and treatment of sickle cell disease." *N Engl J Med* **337**(11): 762-9.

⁶ Mozzarelli, A., J. Hofrichter, et al. (1987). "Delay time of hemoglobin S polymerization prevents most cells from sickling in vivo." *Science* **237**(4814): 500-6.

⁷ Gregersen, M. I., C. A. Bryant, et al. (1967). "Flow Characteristics of Human Erythrocytes through Polycarbonate Sieves." *Science* **157**(3790): 825-827.

⁸ Alexy, T., E. Pais, et al. (2006). "Rheologic behavior of sickle and normal red blood cell mixtures in sickle plasma: implications for transfusion therapy." *Transfusion* **46**(6): 912-8.

⁹ Ferrone, F. A. (2004). "Polymerization and sickle cell disease: a molecular view." *Microcirculation* **11**(2): 115-28.

action. It is this collective action leading to states of no blood flow which is medically and scientifically most important.

Vaso-occlusive events, or “crises,” are known to be responsible for the majority of morbidity associated with the disease, but little is certain about the proximal causes or the circumstances in which they occur. What are the necessary and sufficient conditions for a vaso-occlusive crisis? Hemoglobin S polymerization is certainly a common precursor event for all the effects of the disease, but is it a proximal cause or a distal enabling event? Are there particular circumstances in which hemoglobin polymerization leads to altered blood flow and imminent vaso-occlusion? Or does each cycle of polymerization and melting create a pro-inflammatory state which makes vaso-occlusion more and more likely?

A vaso-occlusive crisis can be modeled as two collective processes occurring in series. First, the individual hemoglobin S molecules cooperate to form polymers. Second, the individual sickled cells cooperate to occlude vessels. These two levels of cooperativity make discrete modeling very difficult.

With the help of collaborators Sangeeta Bhatia and David Eddington at MIT, we devised a microfluidic device which could recreate a polymerization-induced occlusion, and we characterized the conditions in which these occlusions occurred. We hope our results increase our understanding of the process of vaso-occlusion and bring us closer to the goal of more effective treatments.

Materials and Methods

Blood Specimens

Blood specimens were collected during the normal course of patient care at Brigham and Women’s Hospital in accordance with a research protocol approved by the Partners

Healthcare Institutional Review Board. Blood samples were collected in 5 ml EDTA vacutainers and stored at 4°C for up to 60 days. Hematocrit was determined using a Bayer Advia 2120 automated analyzer. Hemoglobin concentrations and isoform percentages were determined using cellulose agar electrophoresis and confirmed by high performance liquid chromatography (HPLC) using a Tosoh G7 column.

Fabrication of Microfluidic Devices

The microfluidic network was fabricated in poly(dimethylsiloxane) (PDMS) using previously described soft lithography techniques.¹⁰ The specific design and manufacturing process were developed under the supervision and guidance of David Eddington and Sangeeta Bhatia and are described elsewhere.^{11,12}

Experimental Setup

The assembled microfluidic device was mounted on an inverted microscope (Nikon TE-3000) and the blood and gas sources were connected as shown in Figure 2. The microfluidic channels begin 4 mm wide, then split into equal cross section areas until the smallest dimension (7, 15, 30, or 60 μm) which then traverses 4 cm until the channels recombine sequentially at the outlet. The blood velocity was monitored most often in the 250 μm channels which were fed by 4 60- μm , 8 30- μm , 16 15- μm , or 16 7- μm channels depending on the device studied. Two rotometers controlled the gas mixture fed through the oxygen channels. The gas mixture was switched between 0% and 10% oxygen to initiate

¹⁰ Duffy, D., J. McDonald, et al. (1998). "Rapid prototyping of microfluidic systems in poly(dimethylsiloxane)." *Analytical Chemistry* **70**(23): 4974-4984.

¹¹ Eddington, D. T., J.P. Puccinelli, and D.J. Beebe (2006). "Thermal aging and reduced hydrophobic recovery of polydimethylsiloxane." *Sensors and Actuators B-Chemical* **114**(1): 170-172.

¹² Higgins, J.M., D.T. Eddington, S. Bhatia, and L. Mahadevan (2007). "Sickle cell occlusive crisis and rescue in a microfluidic device." *Manuscript submitted for publication.*

occlusion or flow. The outlet gas concentration was monitored with a fluorescent oxygen probe (FOXY Fiber Optic Oxygen Sensor, Ocean Optics, Dunedin, FL) to monitor the gas concentrations within the gas microchannels. Gravity-driven flow was used to inject blood into the vascular network and resulted in flow rates up to $500 \mu\text{m}/\text{second}$.

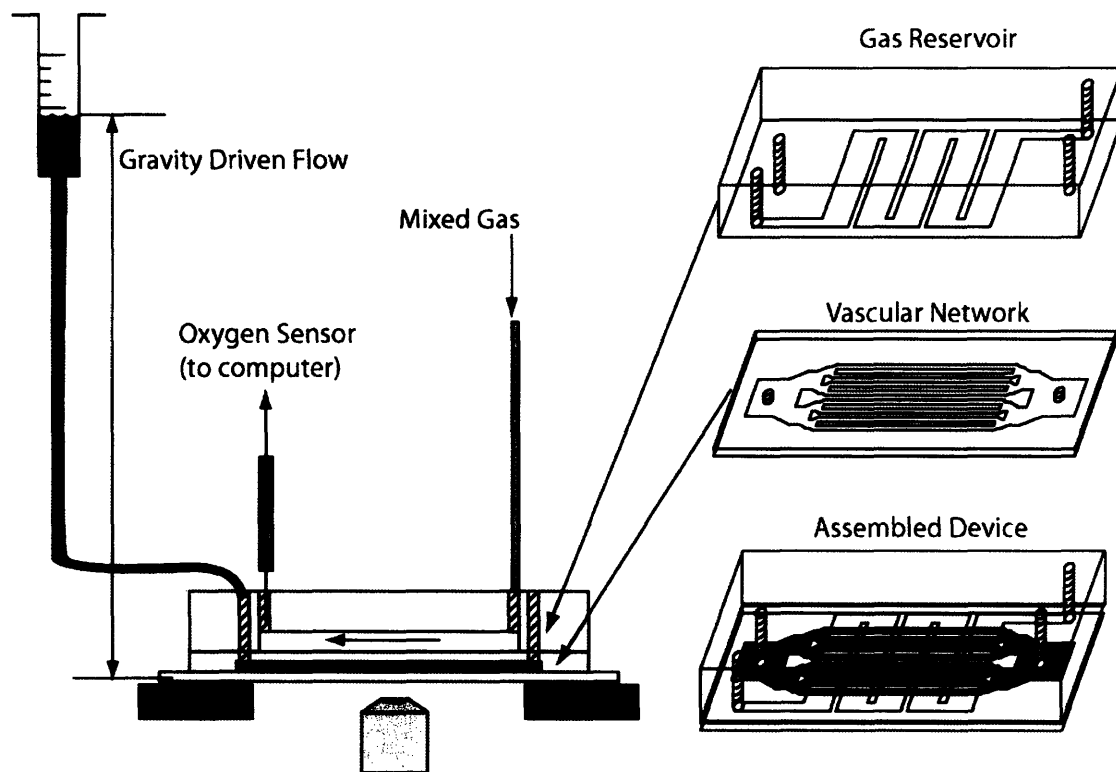


Figure 2: Fabrication and schematic of the device. The widest cross section in the vascular network on the left and right of the device is $4\text{-mm} \times 12\text{-}\mu\text{m}$. The vascular network then bifurcates, maintaining a roughly equal cross-sectional area. An open 5 mL syringe was connected to the device and raised and lowered to increase or decrease the flow rates through the device. The gas channels were connected to two rotometers regulating the ratio of 0% and 10% oxygen in the gas mixture which was fed into the device. The outlet of the gas network had an oxygen sensor to validate the oxygen concentration in the microchannels.

Oxygen Diffusion into Microchannels

The oxygen concentration within the vascular network was quantified by bonding the microfluidic network to a glass slide coated with a ruthenium complex (FOXY-SGS-M, Ocean Optics, Dunedin, FL), which fluoresces under 460 nm excitation and is quenched by

oxygen. The intensity of the fluorescence can be correlated to the oxygen concentration through the Stern-Vollmer equation.¹³ Water was used during these studies to simplify data collection, however the results should be similar to blood filled channels. See Figure 3.

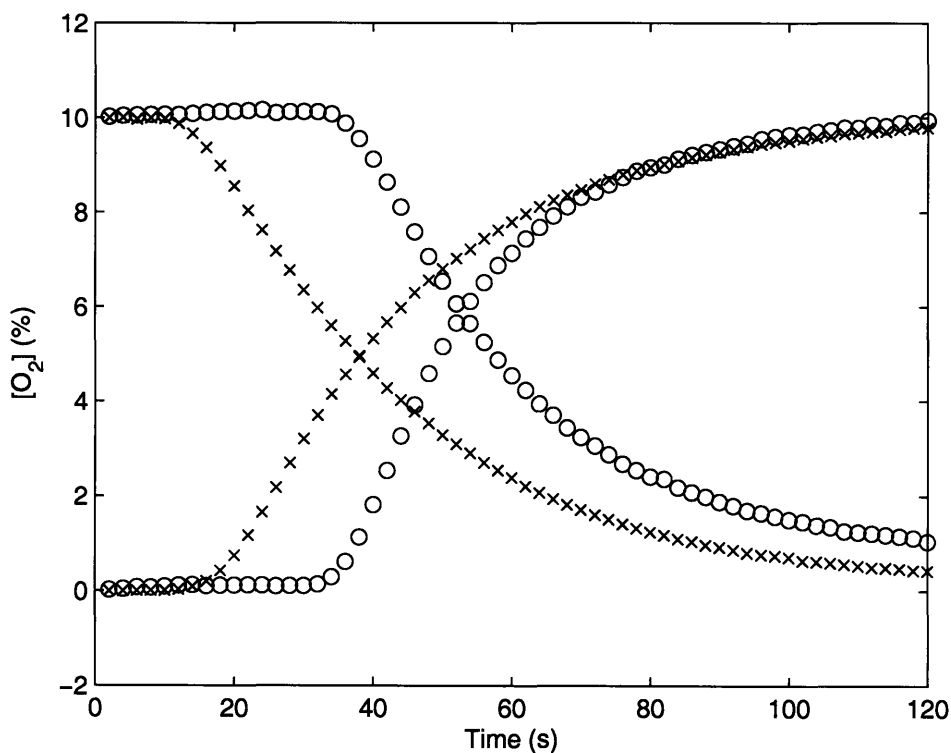


Figure 3: Oxygen concentration profiles following gas mixture change. A ruthenium-coated microscope slide was attached to the bottom of the microfluidic device. The X's represent measurements underneath the gas inlet (near the blood outlet) of the device. The O's represent measurements underneath the gas outlet (near the blood inlet) of the device. The red markers correspond to measurements made after increasing oxygen from 0% to 10% at time 0. The blue markers represent measurements made after decreasing the oxygen from 10% to 0% at time 0. These concentration profiles represent upper bounds (O's) and lower bounds (X's) on the concentrations in the fluid channels where data was collected because they represent concentrations at positions farther up and down the gas stream.

Data Collection and Analysis

Assays were performed at room temperature. Videos were captured with a PixeLink PL-A781 high-speed video camera (PixeLINK, Ottawa, Ontario). Videos were processed

¹³ Evans, R. C. and P. Douglas (2006). "Controlling the color space response of colorimetric luminescent oxygen sensors." *Anal Chem* 78(16): 5645-52.

and analyzed using MATLAB, the MATLAB Image Processing Toolbox, and the SIMULINK Video and Image Processing Blockset (The MathWorks, Natick Massachusetts).

Results

In Vitro Vaso-occlusion

Our first goal was to recreate a vaso-occlusion in the microfluidic device. An *in vivo* vaso-occlusive crisis occurs when a sufficient density of cells containing a sufficient concentration of sickle hemoglobin encounters regions of sufficiently low oxygen tension. The sickle hemoglobin then polymerizes, and these cells become less deformable, providing more resistance to flow until they slow down and eventually lead to thrombosis. We wanted to find out if we could stop steady blood flow in the device by changing the oxygen concentration. The velocity profiles in Figure 4 demonstrate this ability. By removing oxygen from the local environment, we were able to cause the blood flow in the channels to cease. Figure 4 also demonstrates our ability to reverse this stoppage of flow in the microfluidic device by reintroducing oxygen to the local environment. This ability to initiate and then to reverse an occlusion by manipulating oxygen concentrations was reproducible at varying pressure differences, oxygen concentration gradients, channel sizes, hematocrits, and hemoglobin S concentrations.

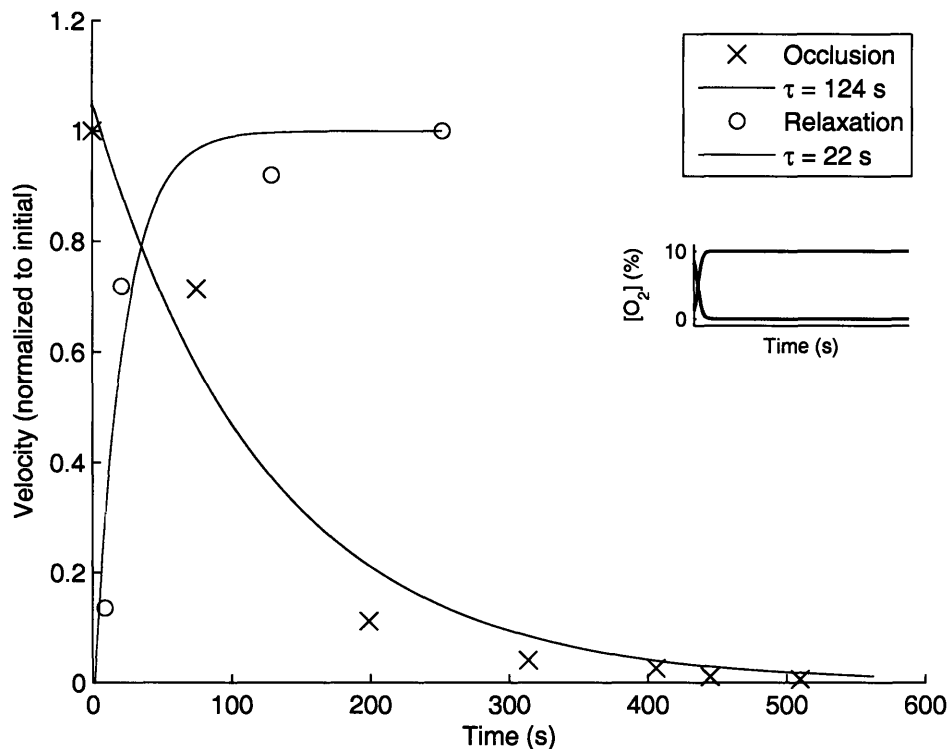


Figure 4: Velocity profiles for an occlusion and relaxation assay for a device with a minimal width of 30 μm and a blood sample with 92% hemoglobin S. Data points represent measured velocities normalized to the maximum within each assay. Lines represent least-squares exponential fits. The occlusion measurements had a time scale of about 124 seconds, while the corresponding time scale fit to the relaxation profile was about 22 seconds. We note that the velocity of the red blood cells actually does vanish on occlusion.

Furthermore, this behavior was not seen when control blood samples were used.

Figure 5 shows velocity profiles for patient specimens from normal and heterozygous individuals. Blood from normal individuals was collected and handled in the same way as that from the sickle cell patients and showed no significant reduction in velocity following deoxygenation. Additionally, samples from patients heterozygous for the sickle hemoglobin mutation were also assayed. Each red blood cell from a heterozygous or “sickle cell trait” patient contains roughly 1/3 sickle hemoglobin and 2/3 normal hemoglobin. Following deoxygenation in our device, these samples showed a slight initial reduction in flow rate with

no further significant deceleration, and no occlusion more than 20 minutes following deoxygenation.

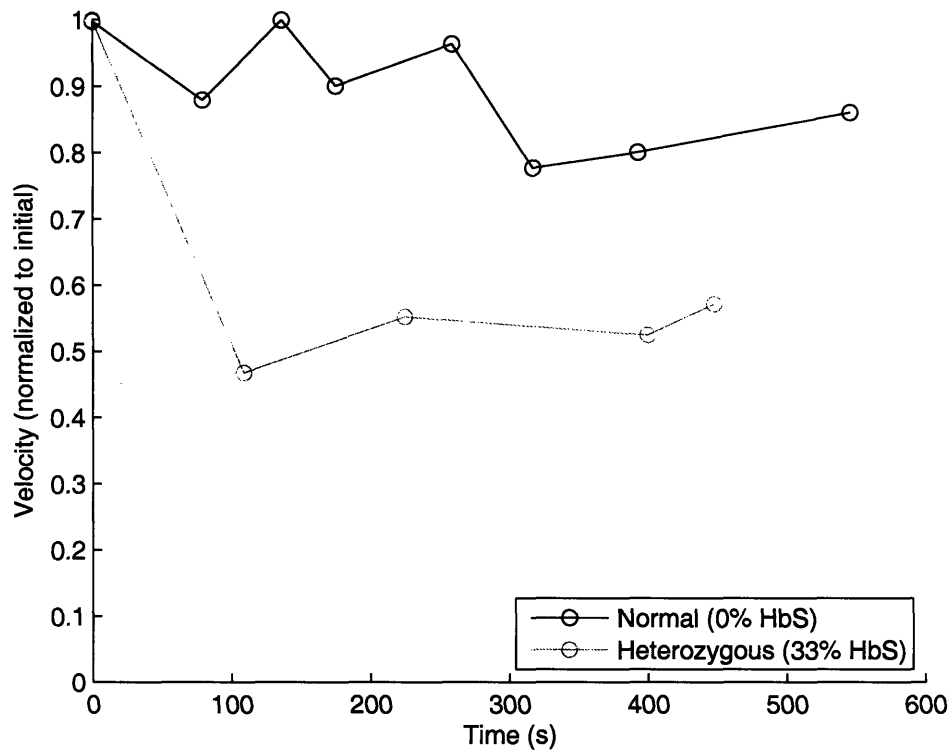


Figure 5: Velocity profiles for control specimens at 0% oxygen. Experiments were carried out in devices with minimal width of 15 μm . We see that there is no capillary occlusion in normal blood (no HbS) or blood from a patient with the heterozygous form, i.e. sickle-cell trait (33% HbS).

Phase Space of Vaso-occlusion

This ability to trigger and to reverse an occlusion immediately led to several questions about the nature of this dynamical process. How would the velocity profile change if the hydrostatic pressure were increased? Were there pressures above which occlusion would never occur? How would the velocity profiles change if the oxygen concentration were not reduced completely to zero? What was the maximum oxygen

concentration which would still lead to occlusion? How would the velocity profiles after deoxygenation change if we increased the size of the channels in the microfluidic device? What was the maximum channel size that would allow occlusion following deoxygenation? We attempted to answer some of these questions by measuring the phase space of vaso-occlusion. The phase space shown in Figure 6 depicts the inter-relationships between some of these control parameters. Which combinations of parameter values lead to occlusion in a specified period of time?

We performed over 100 different occlusion and rescue assays, capturing more than 1000 videos with more than 100,000 total frames. Given a device with a particular minimal diameter (7, 15, 30, or 60 μm), we flowed a patient blood specimen with a known hemoglobin S concentration and a known red blood cell concentration. We modulated the pressure difference by changing the height of the pressure head and modulated the gas concentration in the fluid channel by adjusting the gas mixture flowing through the adjacent gas channels. Videos were captured at intervals and processed to determine velocity as a function of time.

Figure 6 shows an isosurface in this phase space. Each point on a particular isosurface represents a state in the three dimensional space with coordinates specifying minimal radius, pressure difference, and oxygen concentration. Each state on this surface leads to the same time to occlusion. This phase space describes the behavior of patients whose samples contained hemoglobin S concentrations of at least 65% (mean 86%, standard deviation 6.7%). For unaffected individuals with normal hemoglobin (AA), isosurfaces for a any time to occlusion are shifted toward the origin, because the time to occlusion for any set of parameter values approaches infinity. In other words, with no hemoglobin S, the minimal

radius or the pressure difference would have to approach zero before the blood would stop flowing. This effect was depicted earlier in Figure 5.

Pressures shown in the phase space in Figure 6 were normalized both for hematocrit and for the slightly variable resistance of each individual microfluidic device. Pressures were increased or decreased due to the different resistance provided by blood samples with different hematocrits. The hematocrit normalization was calculated according to previously determined relationships between hematocrit and effective viscosity.¹⁴ In practice, this adjustment represented changes of less than 15% relative to the actual pressure.

Pressures were also normalized for the variable resistance provided by each individual microfluidic device. The resistance of each device was assumed to depend on both the specific vascular channel network topology and the number and quality of minor artifacts and defects typically acquired by each device during production. Device resistance was calculated prior to each occlusion assay as defined by Poiseuille's Law in terms of the known dimension, number, and arrangement of the smallest channels, the applied pressure difference, and the initial flow rate.

Normalized pressure therefore represents an estimate of the pressure that would be needed to generate the flow rate measured if the sample had a hematocrit of 25% and the device had a standard topology without defects as shown in Figure 2.

¹⁴ Lipowsky, H. H., S. Usami, et al. (1980). "In vivo measurements of apparent viscosity and microvessel hematocrit in the mesentery of the cat." *Microvasc Res* **19**(3): 297-319.

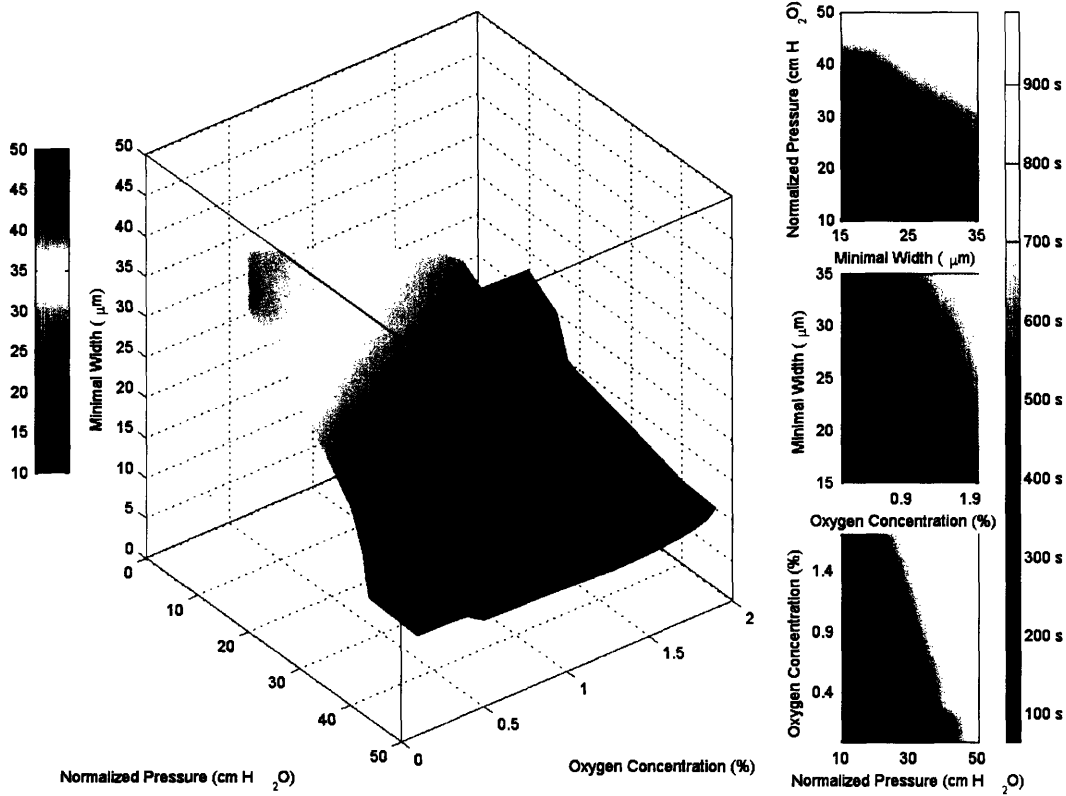


Figure 6: Phase space of vaso-occlusion. The colored surface represents a fitted hypersurface in 4-dimensional space: width, pressure, oxygen concentration, and occlusion time. The isosurface was computed from 43 data points using Delaunay triangulation (See the MATLAB *griddata3* function documentation, The MathWorks, Inc., Natick, Mass.). All points on the hypersurface correspond to triples of height, pressure, and oxygen concentration where the fitted time to occlusion was 500 seconds. The color of each point on the surface characterizes the minimal width in the device, and is redundant with the point's vertical (width) coordinate. Pressures were normalized for hematocrit and for individual device resistance. The filled contour plots represent slices through the fitted volume at specific planes (top: oxygen concentration = 0.5%, middle: normalized pressure = 20 cm H₂O, bottom: minimal width = 25 μm). This phase space describes the behavior of patient samples with hemoglobin S concentrations of at least 65% (mean 86%, standard deviation 6.7%).

The stochasticity in the vaso-occlusive event leads to large variations about the mean time for

jamming. We characterize the deviations from the mean time to occlusion by $X = \frac{1}{n} \sum \frac{|t_{fit} - t_{actual}|}{t_{actual}}$

We find that X is 46%; i.e., vaso-occlusion is highly heterogeneous temporally.

An effective treatment for a sickle cell patient would correspond to an intervention that caused these isosurfaces to move toward the origin. For example, the 500-second isosurface before treatment would include points with width, pressure, and oxygen combination coordinates much greater than those of that same isosurface following a successful treatment. The phase space of a treated sickle cell patient would therefore become more similar to that of a normal or sickle trait individual. Lower pressure, lower oxygen tension, or narrower channels would be required to cause an occlusion within a particular time interval.

Conversely, an increasing risk of occlusion would correspond to the movement of these isosurfaces away from the origin. A fixed combination of minimal radius, pressure difference, and oxygen concentration would then fall on an isosurface with a shorter and shorter time to occlusion. As the time since treatment increased for a given patient, greater pressures, greater oxygen tensions, and wider channels would be required to delay occlusion for a fixed period of time.

Crisis and Rescue Asymmetry

Our experiments also led to the identification of an asymmetry or hysteresis in the crisis-rescue process. As shown in Figure 4, the time scales of the occlusion and relaxation velocity profiles are not the same, with relaxation occurring over a few-fold shorter time scale than occlusion. The occlusion and relaxation assays depicted in the figure were performed in a device with a minimal diameter of 30 μm and a patient blood sample with a hemoglobin S concentration of 92%. The least squares exponential fit of the occlusion measurements had a time scale of about 124 seconds, while the corresponding time scale fit to the relaxation profile was about 22 seconds. This asymmetry between the time scales of

occlusion and relaxation held for all minimal device diameters tested as well as for all hemoglobin S concentrations above the 65% threshold set before experimenting. Since we have cast the vaso-occlusive event as a composite of hemoglobin polymerization and cellular jamming, this asymmetry may simply represent different time scales intrinsic to one or both of these sub-processes. There is reason to suspect that under some circumstances, the rates of hemoglobin gelation (polymerization) and melting (de-polymerization) will be different.¹⁵ This asymmetry at the chemical level has been postulated to be the result of multiple concentration-dependent mechanisms for polymer melting. Perhaps our experimental conditions (10% oxygen) were sufficient to enable the much faster “surface-melting” in addition to “end-melting.” It is also possible that there is an asymmetry intrinsic to the hydrodynamic process of cellular jamming and unjamming. We can speculate that the jamming of a channel requires the collective and simultaneous change in rheology and conformation of multiple cells. In contrast, the unjamming of these cells in the presence of a static pressure drop may require rheologic changes only for individual cells. Our results may reflect a combination of these effects as well as different phenomena altogether.

Effects of Small Molecule Inhibitors

Much of the research into sickle cell disease over the past few decades has involved attempts to identify small molecules which might inhibit sickle hemoglobin polymerization and lead to an amelioration of clinical symptoms. We hypothesized that our device could serve as a testing environment for such inhibitors. We therefore evaluated the effects of three small molecules on the flow properties of sickle cell blood in the device. We assayed two solid molecules, phenylalanine and pyridoxal (a 2,3 DPG analog), both of which had

¹⁵ Ferrone, "Polymerization and sickle cell disease: a molecular view," 115.

been shown previously to slow the rate of hemoglobin S polymerization at least modestly.¹⁶ Phenylalanine acts by binding covalently to hemoglobin, while pyridoxal acts non-covalently. Neither of these two soluble molecules had a significant impact on occlusion velocity profiles, as shown in Figure 7. We hypothesize that our experimental conditions generated concentrations of deoxygenated hemoglobin S far in excess of the mildly elevated solubilities achieved by these two molecules. Perhaps less severe experimental conditions would allow demonstration of the effect of these moderate increases in solubility on velocity profiles.

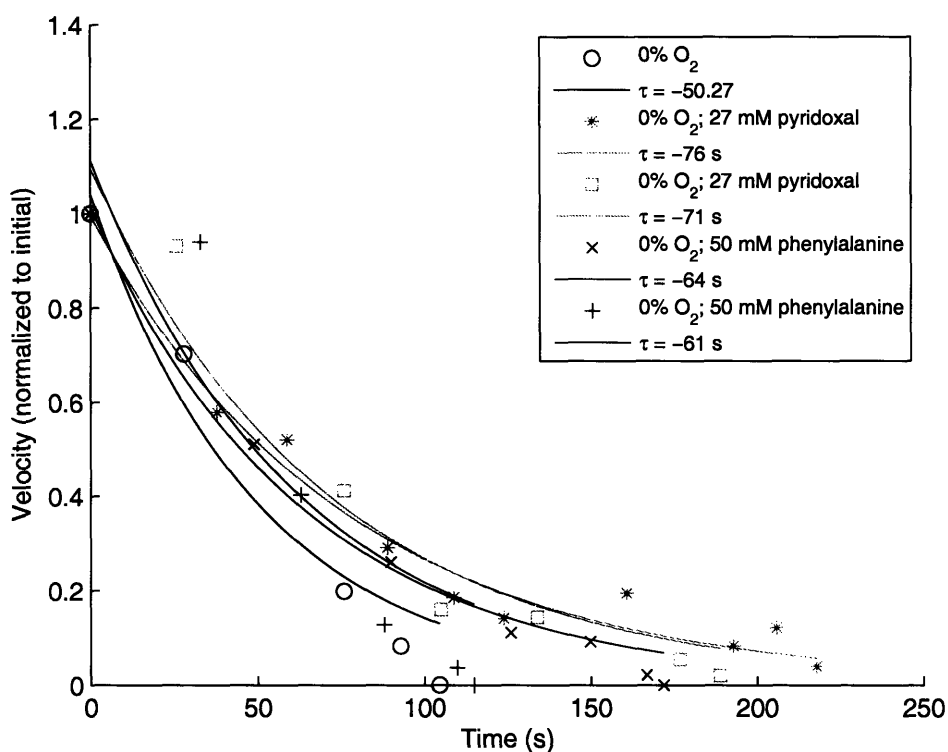


Figure 7: Velocity profiles for occlusion with and without addition of phenylalanine or pyridoxal, a 2,3 DPG analog when the ambient oxygen concentration is suddenly dropped to 0% at time $t=0$. Experiments were conducted in a device with a minimal width of $30\ \mu\text{m}$ and a blood sample with hemoglobin S concentration of 85.5%. We see that there is little observable change in the dynamics of occlusion due to the presence of these small molecule drugs.

¹⁶ Chang, H., S. M. Ewert, et al. (1983). "Comparative evaluation of fifteen anti-sickling agents." *Blood* 61(4): 693-704.

We also investigated the impact of very small concentrations of carbon monoxide (CO). CO is known to bind hemoglobin at least 200 times more tightly than oxygen and to occupy the same binding site.¹⁷ The hemoglobin monomers therefore retain their oxygenated conformation and do not polymerize. Figure 8 shows velocity profiles for occlusion assays with and without carbon monoxide. These assays were carried out in a device with a minimal diameter of 15 μm and with a blood sample containing 85.5% hemoglobin S. Our device clearly demonstrates the established inhibitory effect of CO on polymerization and further demonstrates that this inhibition leads to significant increases in flow, with no occlusion detectable after at least 20 minutes.

We also attempted rescue assays at 0% oxygen with 0.01% carbon monoxide and 99.99% nitrogen and did not see any evidence of polymer melting after at least 10 minutes. Given the similarity in chemical interactions between CO and hemoglobin and between oxygen and hemoglobin, one might expect the introduction of even this low level of CO to generate the same type of rescue as we had seen for the introduction of oxygen such as in Figure 4. We saw no rescue. While this result was surprising, it is possible that the very low concentration of CO restricted the melting process to the ends of the polymers and that there was no surface-based melting with its associated dramatic increase in depolymerization rate.¹⁸ Nevertheless, the results of our experiments with CO suggest that this device may be useful as a test environment for the identification of novel (and safer) inhibitors of sickle hemoglobin polymerization.

¹⁷ Mozzarelli, Hofrichter, et al. "Delay time of hemoglobin S polymerization prevents most cells from sickling in vivo." 500.

¹⁸ Ferrone, "Polymerization and sickle cell disease: a molecular view," 115.

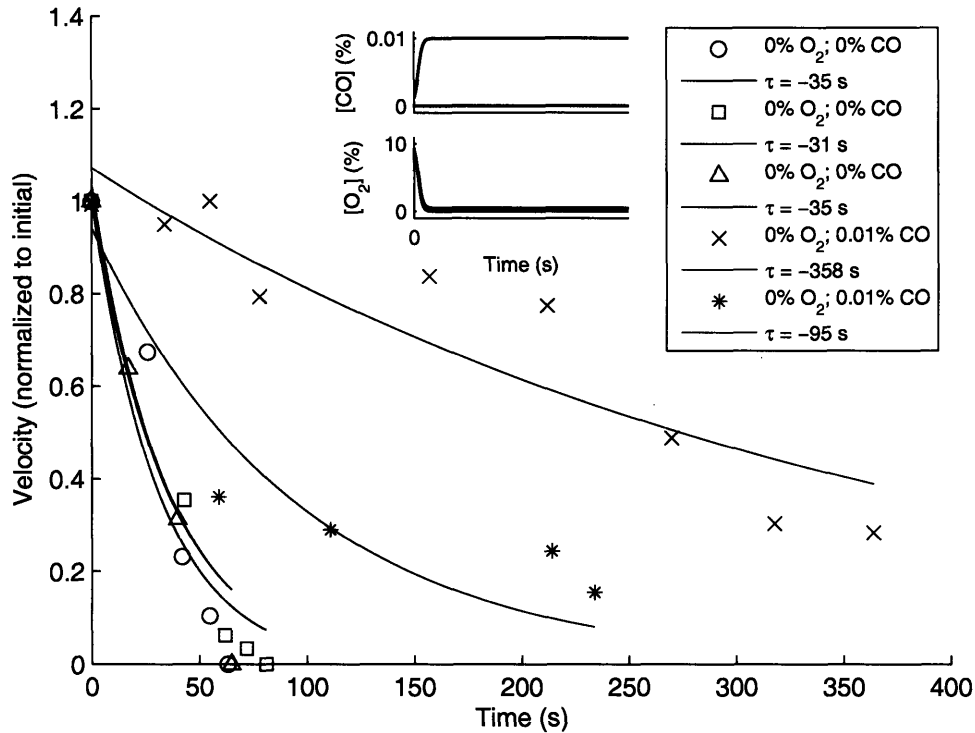


Figure 8: Velocity profiles for occlusion with and without carbon monoxide. All assays were carried out in a device with a minimal diameter of 15 μm and a patient blood sample with 85.5% hemoglobin S. The blue markers correspond to three different occlusion assays with no oxygen or carbon monoxide. The purple markers correspond to assays with 0.01% carbon monoxide and 0% oxygen.

Effects of Red Blood Cell Exchange

While small molecule inhibitors are not currently a part of established clinical practice guidelines for the management of sickle cell anemia, effective transfusion-based treatments are available. We wished to assess the clinical relevance of the behavior of blood samples in our device by comparing measurements of a patient sample before and after therapeutic red blood cell exchange (or erythrocytapheresis). In this procedure, the sickle cell patient's blood is withdrawn and centrifuged. The layer containing red blood cells is removed, and the remaining components are retransfused along with donor red blood cells

containing no hemoglobin S. This treatment approach allows the removal of pathologic cells, the introduction of therapeutic cells, while avoiding any significant volume challenges to the patients. We obtained blood samples before and after red blood cell exchange from a patient admitted to Brigham and Women's Hospital for prophylactic red cell exchange. Occlusion profiles are shown in Figure 9. The effect of this transfusion-based procedure is clearly demonstrated on the velocity profiles of the blood flowing in the microfluidic device.

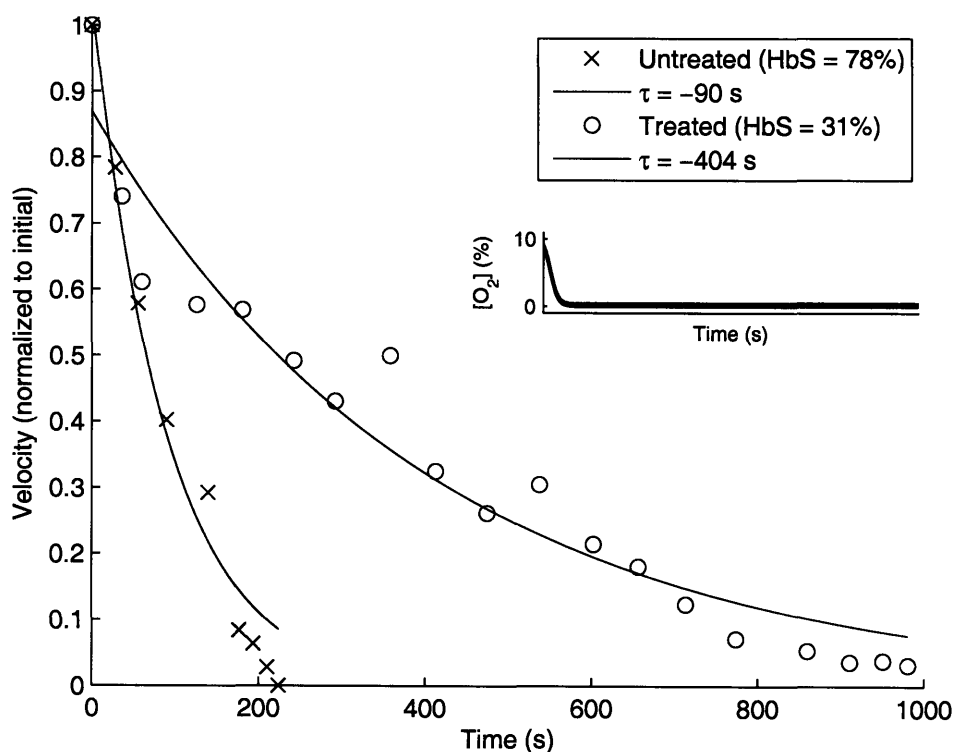


Figure 9: Velocity profiles for occlusion of a patient blood sample before and after therapeutic red blood cell exchange as measured in a device with a minimal diameter of 30 μm and ambient oxygen concentration that is suddenly reduced to 0%. Velocities are normalized to the maximum within each assay. The blue data points represent the behavior of the patient's sample prior to treatment (78% hemoglobin S). The red data points represent behavior of a sample obtained following treatment (31% hemoglobin S). The lines represent least-squares exponential fits. Note that the velocity of the untreated specimen vanishes after a finite time, while that of the treated specimen never vanishes.

Mathematical Models

Mathematical Model 1: In Vitro Vaso-occlusion

By Poiseuille's Law, the flow rate (volume/time) of blood in a channel depends on the ratio of the pressure drop and the resistance of the system.

$$Q = \frac{\Delta P}{R}$$

The resistance has contributions from the geometry and the fluid material properties, which are separable in the case of a simple, static geometry and an ideal Newtonian fluid:

$$R \propto \frac{L}{r^4} \mu$$

In the case of blood cells adhering to channel walls, the fluid properties may influence the geometry, and the reverse is possible as well. The total resistance is some arbitrary function of contributions from the physical geometry and contributions from the fluid material properties.

$$R_{total} = f(R_{geometry}, R_{blood})$$

In our experimental device, the only significant change in geometry between experiments was the radius of the smallest channel in the device.

$$R_{geometry} = f(r)$$

We hypothesize that the contribution of the blood specimen to the resistance is an increasing function of the total concentration of red blood cells (hematocrit) as well as the concentration of sickled red blood cells.

$$R_{blood} = f([RBC_{total}], [RBC_{sickled}])$$

The concentration of sickled red blood cells is determined by the concentration of intracellular hemoglobin S and the partial pressure of oxygen.

$$RBC_{sickled} = f([RBC_{total}], [HbS], [O_2])$$

After selecting an experimental device with a particular minimal radius and a patient specimen with a particular hemoglobin S concentration and hematocrit, these relations imply the ability to control occlusion. Our experimental device allows us to modulate oxygen concentration. We therefore control the concentration of sickled red blood cells, which in turn controls the rheology of the blood. Together with the resistance component from the geometry, we are able to manipulate all the independent variables determining flow rate.

$$Q = f([RBC_{total}], [HbS], [O_2], r)$$

See Figure 10 for a schematized view.

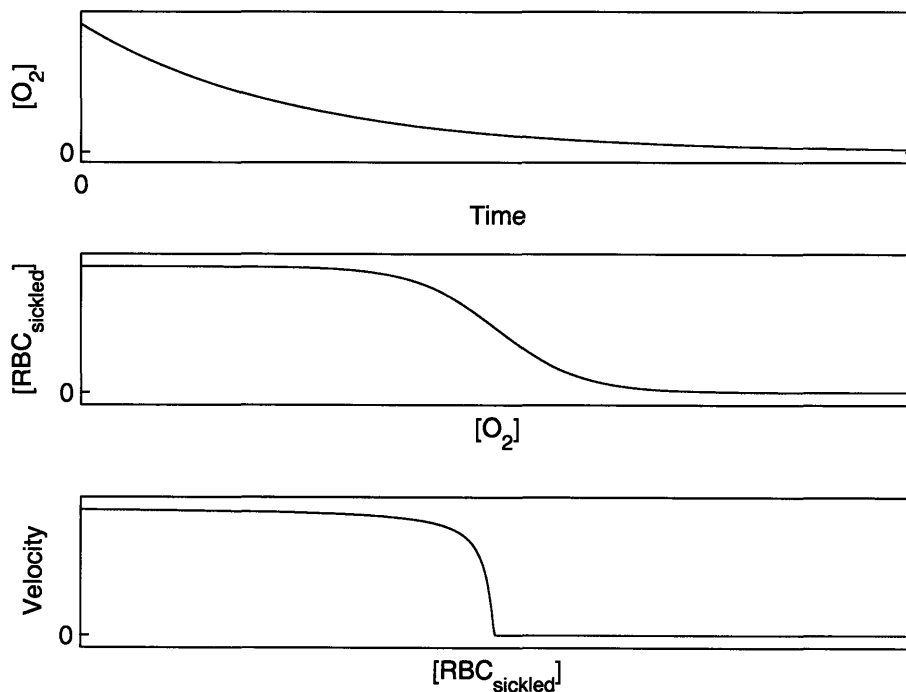


Figure 10: Simplified qualitative model of vaso-occlusion. As the concentration of oxygen decreases with time, the sickle cell concentration increases leading to a diverging viscosity of the blood which stops flowing.

Mathematical Model 2: Rate of In Vitro Vaso-occlusion

The steady state velocity in a channel is given by the flow rate equation above divided by the cross-section area. The minimal cross-section of all devices had a height of about 12 μm . The width of this minimal cross-section varied among 7, 15, 30, and 60 μm .

$$v = \frac{Q}{A} = \frac{\Delta P}{RA}$$

Substituting expressions from above in Mathematical Model 1 gives the following.

$$v = \frac{Q}{RA} = \frac{\Delta P}{f(R_{\text{geometry}}, R_{\text{blood}})A}$$

During an occlusion assay, the oxygen concentration was constant at its final value, and the pressure was also held constant. The increase in flow resistance that leads to occlusion must be some combination of increases in the resistance associated with the geometry of the device and the resistance associated with the blood specimen. Previous studies have examined the effect of polymerization on effective viscosity.¹⁹ Any change in geometry would be expected to occur as a result of sickled cells adhering to the wall of the channel and changing the patent cross-sectional area.

One might hypothesize that immediately following a drop in oxygen concentration, there has not been time for cells to adhere and that all of the increased resistance to flow is based on an effective viscosity change. This speculation is supported by the observation that sickle cell rheological properties change before observable alterations in physical conformation.²⁰

$$v = \frac{Q}{RA} = \frac{\Delta P}{f(R_{\text{geometry}}, R_{\text{blood}})A} = \frac{\Delta P}{f([RBC_{\text{total}}], [RBC_{\text{sickled}}])A} \propto \frac{\Delta P}{\mu_{\text{effective}}A}$$

¹⁹ Alexy et al. "Rheologic behavior of sickle and normal red blood cell mixtures in sickle plasma: implications for transfusion therapy," 912.

²⁰ Berger, S. A. and W. S. King (1980). "The flow of sickle-cell blood in the capillaries." *Biophys J* 29(1): 119-48.

Under this assumption, the change in velocity is governed by the change in effective viscosity.

$$\frac{dv}{dt} \propto \frac{d}{dt} \left(\frac{1}{\mu_{\text{effective}}} \right) = \frac{-1}{(\mu_{\text{effective}})^2} \frac{d\mu_{\text{effective}}}{dt}$$

At hemoglobin S concentrations typical of sickle cell disease patients and at 0% oxygen, the time scale of polymerization is very fast (much less than one second) relative to the time scale we see for velocity change (tens of seconds). An instantaneous increase in effective viscosity would, in theory, cause an exponential decrease in velocity to the new steady state with a time scale determined by the magnitude of the change in viscosity. Previous research estimates a 10-fold increase in viscosity following complete deoxygenation.²¹ If the reduction in flow is due only or mostly to an increase in fluid resistance, the velocity profiles should not depend on the radius of the smallest channels in the device other than as these profiles are related to radius by Poiseuille's Law.

If we consider the effect of adherent sickled cells as causing a reduction in the cross-section without dramatically effecting viscosity, we might expect a different relationship between velocity and time following complete deoxygenation.

$$v = \frac{Q}{RA} = \frac{\Delta P}{f(R_{\text{geometry}}, R_{\text{blood}})A} = \frac{\Delta P}{f(r)A}$$

In an idealized circular channel, a reduction in radius will have a different impact on change in velocity:

$$\frac{dv}{dt} \propto \frac{d}{dt} \left(\frac{1}{(r(t))^2} \right) = \frac{-2}{(r(t))^3} \frac{dr}{dt}$$

²¹ Ballas, S. K. and N. Mohandas (2004). "Sickle red cell microrheology and sickle blood rheology." *Microcirculation* 11(2): 209-25

Prior research has identified a nonlinear relationship for normal blood between flow and pressure at low flow velocities.²² Resistance increases as flow decreases.

Interestingly, our results appear to show a difference in the character of the velocity profiles for assays carried out under complete deoxygenation with pressure low enough to ensure complete occlusion within a few minutes. In the 7 μm channels, which can be blocked by an individual sickled cell, parameterized velocity profiles have generally less curvature than the velocity profiles for larger channels. In these small channels, significant increases in rigidity leading to a change of “geometry” would cause complete occlusion. This speculative analysis may therefore support a conclusion that the processes governing the velocity profiles are most likely operating through viscosity instead of geometry.

The 7 μm channels showed an average velocity profile with a negative mean curvature (-0.0818). The standard deviation of curvatures for these profiles was 0.0527, making this mean curvature slightly and significantly negative. For the 15 μm and 30 μm channels, the means and standard deviations were 0.0356 (0.0251) and 0.1330 (0.0432). These preliminary results may reflect the greater cooperativity that is required to occlude a channel with a cross-section larger than that of a red blood cell.

Since we measured flow rates in downstream 250 μm channels which emptied 16, 8, or 4 channels of minimal diameter, depending on the device, these changes may be due also to the averaging of flow rates across more and more channels. Also possibly relevant is the reduction in shear-thinning seen in the smallest blood vessels following sickling. The shear thinning seen in normal blood in very small capillaries depends on the deformability of red blood cells. As red blood cell deformability decreases following sickling, this shear-thinning effect will be decreased, and its reduction would only be relevant to the very small channels.

²² Lipowsky, H. H. (2005). "Microvascular rheology and hemodynamics." *Microcirculation* 12(1): 5-15.

Discussion

In summary, we report an integrative, multi-scale analysis of vaso-occlusion in sickle cell disease using a microfluidic device. Our experiments initiate and reverse occlusion by manipulating channel diameter, gas concentration, pressure gradient, hemoglobin concentration, and red blood cell concentration. These interventions control processes occurring with length scales varying over five orders of magnitude and time scales varying over four orders of magnitude. We believe that this integrative approach will increase our understanding of pathophysiologic processes like sickle cell vaso-occlusion and its associated devastating medical consequences.

Some Complications with the In Vitro Model

We demonstrate qualitative control of vaso-occlusion with this experimental device. The underlying processes may be too complicated to allow specific quantification based only on this initial study. There seemed to be a preconditioning effect on the behavior of the experimental setup. In extreme cases, specimens which had undergone repeated or prolonged cycles of sickling would become permanently occluded despite atmospheric oxygen concentrations and significant increases in applied pressure. The experimental process of jamming, while qualitatively controllable, was not precisely quantitatively predictable. Repeated vaso-occlusion assays using the same specimen in the same microfluidic device often yielded slightly different results. We speculate that this preconditioning was due to a number of factors including device aging, device failure, and local hematocrit changes.

Some of this variation was clearly the result of experimental device aging, as effective resistance to flow at full oxygenation increased following successive cycles of occlusion and resolution. Previous occlusive episodes may have altered the surface of the PDMS channels via protein deposition, pressure damage due to mechanical deformation, or other effects. There is also the possibility of small device failures. Undetectable delamination of the bilayer device would allow shunting of blood flow, decreasing that observed in the video frame. Alternatively, delamination events could also allow leakage of gas into the fluid channels, creating a gas bubble downstream of the video frame and reducing flow. We identified some such delamination events during the course of our experiments and discarded data in that case, but it is possible that some of these events escaped our detection.

Additionally, since each vaso-occlusive event required at least a local increase in hematocrit to the packing threshold, subsequent vaso-occlusive cycles, though initiated only after steady-state had resumed, may have had different initial fluid resistance due to increases in hematocrit or increases in irreversibly sickled cells or other changes to the fluid mechanical properties of the test specimen. We note that initial hematocrit within the range tested did not seem to alter the qualitative effect of vaso-occlusion, as might be expected from previous work measuring the incremental effect on viscosity of increasing red blood cell volume fraction.²³

Some Limitations of the In Vitro Device

This experimental device has a number of obvious limitations and departures from physiology. First, there were no endothelial cells in our setup. We believe that our results are nevertheless meaningful because they allow us to isolate the endothelial-independent

²³ Lipowsky and Usami, "In vivo measurements of apparent viscosity and microvessel hematocrit in the mesentery of the cat," 297.

effects, and we have clearly demonstrated recreation of occlusion despite the absence of endothelium-red blood cell interactions. Additionally, the device clearly distinguishes normal and sickle cell blood as well as treated and pre-treated specimens. Previous work has identified a vasodilatory response by endothelium to increasing viscosity.²⁴ While our device obviously cannot recapitulate this response, our results are still likely relevant to the flow dynamics occurring on time scales shorter than those of the endothelial response. Future work could involve modifying this device by decorating the PDMS walls with endothelial adhesion molecules or other mediators suspected of being involved in disease pathogenesis. Also, this device could help assess the involvement of endothelium by allowing the comparison of specimens from individuals with similar blood cell metrics but different clinical histories. Whether or not vaso-occlusion events are proximally caused by endothelial involvement and regardless of whether polymerization events precede crises by seconds or days or months, based on our current understanding, the prevention of polymerization will prevent crisis. If this device can help identify new ways to prevent polymerization, then it will clearly be clinically relevant.

A second difference between our device and *in vivo* physiology is the geometry of the networks. The experimental device is regular and not tortuous like the human microvasculature. Increasing tortuosity of channel networks would probably increase the likelihood of occlusion. We observed anecdotally that the order of jamming in channels appeared to be reproducible, with the smallest-diameter channels jamming in roughly the same order following a relaxation phase, but this assertion requires further experimentation. The soft lithographic manufacturing process allows for much more complicated geometries which would serve as a natural future extension of this work.

²⁴ Lipowsky, "Microvascular rheology and hemodynamics," 5.

Phase Space of Vaso-occlusion

We report the characterization of a phase space of vaso-occlusion in sickle cell disease (Figure 6). We believe that these results represent an important resource for a deeper understanding of this multi-scale process as well as for increased understanding of a pathophysiologic process responsible for the majority of morbidity and mortality in sickle cell disease. Specifically, this work allows us to begin to address several questions of great importance scientifically and medically.

Does this phase space imply that polymerization alone can cause vaso-occlusion *in vivo*? Based on our results, hemoglobin polymerization alone is sufficient to cause occlusion in capillary-sized channels under certain conditions of hematocrit, hemoglobin S, pressure, and oxygen tension. The hematocrit and hemoglobin S values were physiologic by design. Were the values of pressure and oxygen similar enough to those *in vivo*? Assessing the pressure drops achieved in our artificial device relative to those *in vivo* is difficult, partially because it is hard to measure these pressure gradients *in vivo*.²⁵ While our device had a larger overall resistance to flow than a typical capillary bed of comparable size,²⁶ the pressures we used were accordingly higher than physiologic. Some reported *in vivo* capillary flow velocities are a few times greater than the flow velocities seen in our device. Our device therefore probably relied on somewhat slower flow velocities than those seen in some capillary beds *in vivo*. Previous experiments determining transit times of red blood cells through capillary beds found extreme transit times a few times smaller than those required for occlusion in

²⁵ Lipowsky, "Microvascular rheology and hemodynamics," 5.

²⁶ Cima, L. G., D. E. Discher, et al. (1994). "A hydrodynamic interpretation of crisis in sickle cell anemia." *Microvasc Res* 47(1): 41-54.

our device.²⁷ There is limited data available on the *in vivo* conditions immediately prior to vaso-occlusive crisis, but one could argue that increasing the flow velocities to correspond to those *in vivo* would reduce the likelihood of an occlusion in our experimental device, as would decreasing the maximum time to correspond to those measured previously. Whether this reduced likelihood corresponds to the risk of vaso-occlusive crisis *in vivo* is hard to determine.

While the clinical effects of vaso-occlusion are devastating, given the certainty of the polymerization process upon deoxygenation, it is surprising that this event occurs as infrequently as it does. The average red blood cell undergoes relative deoxygenation about once per minute – each time it makes a circuit through the systemic circulation. Patients with the worst vaso-occlusive disease probably do not experience crisis events more than on the order of days or weeks. Therefore the vast majority of circuits through the systemic circulation must not result in vaso-occlusion, despite ongoing polymerization. Vaso-occlusion is therefore a very extreme event and is likely associated with extremes of both flow velocity and transit time and may require only a very small subpopulation of cells with these extreme characteristics. The uncertainty associated with this event *in vivo* therefore makes it difficult to assess the direct clinical relevance of the absolute pressures and oxygen concentrations shown in the phase space of vaso-occlusion derived from these experiments. What is less certain is the value of this phase space as a foundation for understanding the relationships between control parameter values and for assessing relative effects of existing and novel interventions.

²⁷ MacNee, W., B. A. Martin, et al. (1989). "Regional pulmonary transit times in humans." *J Appl Physiol* 66(2): 844-50.

Diagnostic and Monitoring Device

We report a demonstration of an *in vitro* model of vaso-occlusion in sickle cell disease with potentially important implications for diagnosis, monitoring, and treatment of sickle cell disease. As demonstrated by the velocity profiles in Figure 9, this microfluidic device can clearly distinguish between vaso-occlusive tendencies of a patient before and after treatment. Sickle cell patients may benefit from routine monitoring with such a device. Results of this monitoring may help determine which individuals should undergo red blood cell exchange procedures. Until now, the indications for red blood cell exchange have been vague and subjective. A device such as this one might help identify patients at risk for imminent pain crises or more serious complications before these crises or complications occur. Conversely, the velocity profiles obtained with this device may also potentially identify patients not at increased risk of vaso-occlusion, thereby avoiding the potential complications including infection and alloimmunization associated with unnecessary red blood cell exchange procedures.

This device might also be used to optimize the hematocrit and percent-hemoglobin S targets for each patient undergoing red blood cell exchange. These targets are currently based mostly on clinical experience and anecdote.²⁸ Measurements in this device would provide an objective quantification of treatment and allow the comparison of more intense of less intense treatment. It is interesting to note that while the hemoglobin fraction (~33%) of the average sickle cell trait patient is the same as the target hemoglobin fraction for a red blood cell exchange recipient, the behavior of these two different specimens in our device as shown in Figure 5 and Figure 9 is dramatically different. This effect clearly demonstrates that a non-uniform distribution of hemoglobin S across a population of red blood cells is likely to

²⁸ Alexy, et al. "Rheologic behavior of sickle and normal red blood cell mixtures in sickle plasma: implications for transfusion therapy," 912.

be much worse clinically than a uniform distribution of the same amount of hemoglobin S across the entire population. While this result would have been suspected based on the dependence of hemoglobin S gelation rates on its intracellular concentration,²⁹ these figures provide objective evidence.

There have been several published efforts investigating the value of red blood cell exchange or transfusion in clinical scenarios where the risk of vaso-occlusive complications seems to be higher: surgery,³⁰ pregnancy,³¹ renal failure. These studies have all yielded equivocal results. One could imagine stratifying patients in these categories based on an evaluation of their occlusive tendencies in this type of device before the decision to exchange or transfuse. Perhaps there is a subgroup of these patients who would benefit from such prophylaxis and who would be identifiable based on velocity profile evaluation.

A further potential application of this device is in the optimization of existing drug regimens. Hydroxyurea is an effective treatment for a subset of sickle cell patients but is potentially myelosuppressive and toxic. Evaluation of a patient's specimen following treatment with different doses might help clarify the risk/benefit ratio of higher doses.³² Hydroxyurea acts in part by causing an increase in hemoglobin F production and its inhibition of polymer formation. While there is evidence for additional therapeutic mechanisms for hydroxyurea, current practice involves assessing hydroxyurea efficacy by measuring levels of hemoglobin F. Increasing global levels of hemoglobin F are likely to represent increased concentration only in the small subpopulation of cells already containing hemoglobin F. This impact may be negligible in a fashion analogous to that discussed above

²⁹ Ferrone, "Polymerization and sickle cell disease: a molecular view," 115.

³⁰ Vichinsky, E. P., C. M. Haberkern, et al. (1995). "A comparison of conservative and aggressive transfusion regimens in the perioperative management of sickle cell disease. The Preoperative Transfusion in Sickle Cell Disease Study Group." *N Engl J Med* **333**(4): 206-13.

³¹ Koshy, M., L. Burd, et al. (1988). "Prophylactic red-cell transfusions in pregnant patients with sickle cell disease. A randomized cooperative study." *N Engl J Med* **319**(22): 1447-52.

³² Ballas and Mohandas, "Sickle red cell microrheology and sickle blood rheology," 209.

for sickle trait versus red cell exchanged samples. In other words, the cells containing some hemoglobin F may already be unlikely to polymerize and sickle at a low level of hydroxyurea treatment. Increasing the dose of hydroxyurea may lead to further increases in local concentrations of hemoglobin F in the cells already producing this protein, but this increased dose is unlikely to have any effect on tendency to occlude while putting the patient at increased risk of toxic side effects.³³

Pharmaceutical Research Device

This microfluidic device may also be helpful in identifying small molecule inhibitors effective in reducing or eliminating risk of vaso-occlusion with acceptable side effects. Figure 7 and Figure 8 suggest its value in assessing the clinical effect of anti-sickling inhibitors. In the clinical context, the degree and rate of polymerization is significant only as far as it causes vaso-occlusion. Prior efforts to identify such inhibitors were unsuccessful due either to lack of agent potency at achievable concentrations or unacceptable toxicity.³⁴ Efficacy was determined generally based only on an evaluation of the agent's effect on rates of hemoglobin polymerization. Perhaps these agents deserve re-evaluation in terms of their impact on the integrated process of vaso-occlusion. Perhaps an agent which causes only a modest decrease in rates of polymerization will be sufficient to prevent actual occlusion, depending on the time dependence of its effect. Similarly, perhaps agents which require toxic levels to inhibit hemoglobin polymerization fully can be shown in this type of device to prevent occlusion through partial inhibition of polymerization at lower concentrations. Prior reports have suggested that even disrupting the inter-fiber polymer bonds may be

³³ Nathan, D. G. (2002). "Search for improved therapy of sickle cell anemia." *J Pediatr Hematol Oncol* **24**(9): 700-3.

³⁴ Nathan, "Search for improved therapy of sickle cell anemia," 700.

sufficient to prevent cell sickling.³⁵ Agents with this sort of effect likely would have been overlooked using previous methods of evaluation but would be likely to be identified in this type of device.

Our integrative and multiscale experiments yield scientific and medical insights into a multiscale collective process. By manipulating channel diameter, gas concentration, pressure gradient, hemoglobin concentration, and red blood cell concentration, thereby controlling processes occurring with length scales varying over five orders of magnitude and time scales varying over four orders of magnitude we can initiate and reverse an *in vitro* sickle cell vaso-occlusion. This work may provide the foundation for improvements in diagnosis, disease monitoring, and treatment discovery.

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³⁵ Cohen, A. E. and L. Mahadevan (2003). "Kinks, rings, and rackets in filamentous structures." Proc Natl Acad Sci U S A **100**(21): 12141-6.