A biophysical marker of severity in sickle cell disease

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Abstract

The search for predictive biomarkers of disease has largely focused on molecular indicators; however, mechanical and biophysical markers, which can integrate multiple pathways across length scales, may provide a more global picture of the underlying pathophysiology. Sickle cell disease, the first disease to have its molecular origins decoded, affects millions of people worldwide and has been studied intensively at the molecular, cellular, tissue and organismal level for a century since its initial description. However, there are still few, if any, markers that allow us to characterize the severity of this disease. Because the complications of sickle cell disease are largely due to vaso-occlusive events, we hypothesized that a physical metric characterizing the vaso-occlusive process could serve as a marker of disease severity. Here we use a simple microfluidic device to characterize the dynamics of jamming in physiologically relevant conditions, using the rate of change of the resistance to flow following a sudden deoxygenation event. Our studies show that this single biophysical parameter could be used to distinguish between patients with divergent clinical outcomes, unlike existing laboratory tests. Our assay provides a biophysical marker of disease severity that could be used to guide timing of clinical interventions, to monitor the progression of the disease, and to measure the efficacy of drug response, transfusion, and novel small molecules in an in vitro setting.

Introduction

Biomarkers for diseases are critical to guide timing of clinical interventions, to monitor treatment efficacy, and to speed the development of new therapies. The search for such markers has yielded many new genetic, epigenetic, and proteomic indicators [1–5], but the need for new markers continues to outpace their validation and use [6]. This problem is
particularly acute for some diseases, such as sickle cell disease, where no molecular markers have been found to correlate reliably with the clinical picture [7–10]. Markers that provide integrative systemic readouts are complementary to molecular indicators; for example, biophysical markers are already used clinically and range from long-standing measurements such as blood pressure to newer imaging modalities such as Doppler ultrasound and cerebral flow magnetic resonance imaging [11–13]. Because these biophysical metrics integrate multiple length scales, molecular players, and pathways, they have the potential to correlate better with disease state than their molecular counterparts. Even at the cellular scale, recent studies suggest new possibilities for biophysical markers such as cell motility or cell deformability as an important indicator of metastatic potential in cancer [14, 15] and other diseases [16, 17].

Sickle cell disease, which affects more than 13 million people worldwide [18] and costs more than US$1.1 billion per year in the United States alone [19], is an ideal candidate for a biophysical marker. The molecular origin of the disease is a mutation in the β-globin gene that results in a variant hemoglobin molecule (hemoglobin S, or HbS) that polymerizes into long chains and extended gels upon deoxygenation [20, 21]. Sickle cells (erythrocytes or RBCs containing HbS) stiffen when polymerization occurs, causing changes in sickle cell blood flow, which can lead to clinical vaso-occlusive crisis events [22]. Although the molecular origins of sickle cell disease have been known for more than 60 years [23], the factors and processes that link these molecular and cellular events to clinical outcomes in patients remain elusive [24–26]. This search for mechanisms is particularly challenging because pathogenesis of vaso-occlusion involves a number of processes across multiple length and time scales, including polymerization and melting of hemoglobin, stiffening and morphological change of RBCs, and increasing apparent viscosity of blood. Each of these processes has been examined separately in various in vitro systems [20, 21, 27–30]. However, studies that integrate all of these processes in physiologic regimes are rare, even though their integrated effects are likely decisive in determining clinical outcomes [28, 31].

In the absence of objective biomarkers of disease severity [7–10], it is difficult to predict the frequency of painful and damaging vaso-occlusive crises and overall severity of disease from one patient to the next. Common laboratory tests measure HbS fraction and fetal hemoglobin (HbF) fraction, neither of which is predictive of clinical outcome for individual patients [9, 32]. Thus we have no method to predict which patients will require urgent or frequent treatment [8, 10], and it is very difficult to assess efficacy of different treatments. This lack of clear predictors also confounds the development of new therapeutics because we have no reliable in vitro methods to test which molecules will work in vivo [22]. Because the morbidity and mortality are caused by vaso-occlusions [33], biophysical markers might provide a link to clinical phenotype. For example, in vivo cerebral blood flow velocity measurements have shown value in guiding transfusion therapy for pediatric sickle cell patients [34–36], suggesting that it may be possible to translate the extensive research on biophysical aspects of sickle cell disease [37] into in vitro biophysical markers.

Recent studies have shown that it is possible to halt the flow of sickle cell blood in a capillary-sized microchannel under constant pressure simply by lowering the oxygen concentration, thereby simulating the most basic features of a vaso-occlusive event [38, 39]. Here we show that a measurement of the dynamics of the integrated physical vaso-occlusive process in vitro serves as a biophysical marker of pathophysiology in a physiologically relevant range of hemoglobin concentration, hemoglobin composition, RBC volume fraction, and local oxygen concentration [28]. This measurement characterizes the dynamics of blood rheological properties during in vitro vaso-occlusion (Scheme 1, Movie S1). Our experiments evaluate this measurement as a biophysical marker of disease severity in patients by benchmarking it against currently available clinical laboratory tests for sickle
cell disease, and as an *in vitro* assessment of the efficacy of existing and experimental treatments.

**Results**

**Quantifying Blood Conductance Dynamics**

We developed a microfluidic system to measure how the flow of sickle cell blood changes after deoxygenation. The system includes a microfluidic device with a capillary-sized channel that is diffusively coupled to a gas reservoir (Scheme 1). This system allows control over many parameters important to vaso-occlusion, including channel size, blood pressure, and oxygen concentration. Whole EDTA-anticoagulated human blood is driven through the channel under constant pressure, while gas with prescribed oxygen concentration is driven through the gas reservoir. RBCs are imaged in the Soret band [40] allowing determination of hemoglobin oxygenation state based on a change in optical absorption (Fig. S1). As sickle cells become deoxygenated, hemoglobin polymerizes, leading to cell stiffening and sickling and an increase in the blood’s resistance to flow with a resulting drop in flow velocity despite being subject to a constant driving pressure head, i.e. there is a drop in the blood conductance defined as velocity per unit pressure drop and inversely proportional to the effective viscosity of the suspension. By measuring the changes in flow velocity for a given constant applied pressure head, we can quantify the kinetics of the change in conductance for a blood sample following deoxygenation, as shown in Figure 1 for a blood sample from a sickle cell patient (Movie 1 shows a full deoxygenation-reoxygenation cycle with vaso-occlusion and recovery). When the oxygen concentration in the gas reservoir is reduced rapidly (≈20 s, Fig. 1a) from 21% to 0%, it causes hemoglobin desaturation (see Fig. S2 for oxygen measurements in blood channel), cell deoxygenation, stiffening and the overall blood flow velocity decrease though the pressure drop across the device remains unchanged (Fig. 1b, right y-axis). In contrast, blood samples from individuals with either normal hemoglobin A or sickle cell trait show no response (see Fig. S3), and the steady-state velocity at 21% oxygen depends linearly on the pressure drop, consistent with Poiseuille’s Law[41], with the maximum shear rates in the middle of the channel in the range 100–400/s, similar to shear rates found in the *in vivo* microcirculation [42]. For sickle cell blood deoxygenation is followed by a velocity that also varies linearly with applied pressure, i.e. the rate of decrease in conductance is independent of pressure as shown in Fig. 1c. We thus use the rates of conductance decrease in blood samples from different sickle cell patients as measures of disease severity.

**Conductance Dynamics Correlate with Clinical Outcomes**

If the rate of conductance decrease captures critical aspects of *in vivo* sickle cell pathophysiology, then we would expect blood samples from the most severely affected patients to show a more rapid reduction in conductance than samples from the least severely affected patients. To test this hypothesis, we used simple criteria to distinguish “benign” and “severe” blood samples. A “benign” sample is one collected from a sickle cell patient who had not been treated with hydroxyurea, had not received any transfusions, and had no unscheduled medical visits for a sickle cell disease-related indication in the 12 months before or 2 months after the date of sample collection (see Methods); a “benign” blood sample shows little or no drop in conductance following deoxygenation (Fig. S4). “Severe” samples were those from sickle cell patients who had either received a transfusion or made an unscheduled hospital visit for a sickle cell disease-related indication in the 12 months before or 2 months after the date of sample collection; Figure 1 shows a large and rapid conductance change following deoxygenation for a “severe” blood sample. Tests were carried out with 23 severe blood samples and 6 benign blood samples, each from a unique patient; all showed similar oxygenated conductances after adjustment to a hematocrit of

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25%. (See Methods for details on conductance calculation). In Figure 2a, we show that
deoxygenation tests indicate that the rate of conductance decrease correlates strongly with
benign and severe classification for this set of blood samples, averaged over at least 3
deoxygenation experiments. Figure S5 shows de-oxygenation trials for 4 additional severe
blood samples, and Table S1 gives raw data for all samples. Blood samples from patients
with more benign disease showed a significantly (p < 0.01) slower decrease in the
conductance following deoxygenation. The conductance of the median severe sample fell
more than twice as fast as that of the median benign sample. We also calculated the receiver
operating characteristic (ROC), which characterizes the sensitivity and specificity for a
binary test as a function of the discrimination threshold, and the area under the curve (AUC),
which quantifies the ability of the test to discriminate between states [6, 43]. In contrast to a
random prediction (Fig. 2b, dashed line), which has no information content, our
measurement (Fig. 2b, solid line) demonstrates strong diagnostic potential with an area AUC
of 0.85 (95% confidence level = 0.65 – 0.95).

We explored the possibility that this effect could be explained by other blood parameters,
and we found no systematic difference between the benign and severe groups for HbS
fraction (Fig. 2c), unadjusted hematocrit (HCT, Fig. S6a), mean hemoglobin concentration
(MCHC, Fig. S6b), sample storage time (Fig. S6c) or steady-state oxygenated conductance
(Fig. S6d). We also considered the role of white blood cells (WBCs) and found no
correlation between sample severity and WBC count (Fig. S6e). We expect that most WBCs
are not viable at the time of testing and therefore that WBCs are not responsible for the
differences in rheodynamics. We further explored this hypothesis by depleting WBCs from a
severe sample and showing that the rheodynamics were not significantly altered (Fig. S6f),
i.e. none of these can serve as a predictive biomarker for disease severity.

Another potential explanation of our results is that the benign group has a higher fraction of
HbF, which inhibits HbS polymerization [44]. In order to compare HbF fractions between
the benign and severe samples, we first subdivided the severe samples into those from
patients who had been or had not been treated with hydroxyurea (“treated severe” or
“untreated severe”), a drug that works in part by increasing HbF expression [45, 44]. As
shown in Fig. 2d, benign blood samples had higher HbF fractions than untreated severe
samples. However, the HbF fractions for treated severe samples were comparable to those of
the benign samples. Despite elevated HbF fraction, the hydroxyurea-treated patients had a
more severe clinical course, and their blood samples showed rates of conductance decrease
comparable to those of untreated severe samples (Fig. 2e). While increased HbF fraction
may account for some of the difference in rates of conductance decrease between benign
samples and untreated severe samples, HbF fraction cannot account for the difference
between the benign samples and the treated severe samples. Further, if HbF fraction alone
determined the rate of conductance decrease, then one would not expect the untreated and
treated severe samples to show such similar rates of conductance decrease. We also assessed
the role of cell volume by comparing the mean cell volumes (MCV) in the groups.
Hydroxyurea is known to raise MCV, and we find a slight elevation in MCV in the
hydroxyurea-treated group as expected (see Fig. S6g). We find no significant different
between the benign and untreated severe groups, however, making it unlikely that
differences in RBC volume are responsible for the differences in rheodynamics.

Simulated Transfusion Reduces Rates of Conductance Change

We also tested whether a simulated transfusion of normal (hemoglobin A, or HbA) blood to
lower HbS fraction would lead to slower declines in conductance analogous to the way that
it improves patient clinical status [45]. We mixed ABO- and RhD-compatible HbA blood
with blood samples from sickle cell patients. Figure 2f shows how the flow dynamics of 4
severe samples changed following a simulated transfusion that reduced the HbS fraction by
about one third. As expected, lower HbS fraction is associated with slower conductance decreases for each sample. The magnitude of the effect is quite variable between patients. Neither the initial HbS fraction nor the fractional decrease predicts how much slower the conductance would fall.

**Small Molecule Treatment Modulates Rate of Conductance Change**

If the rate of conductance decrease correlates with clinical outcome, small molecules with demonstrated clinical potential should reduce the rate of conductance decrease. Recent work has shown that a small molecule that increases hemoglobin oxygen affinity (5-hydroxymethyl furfural, or 5HMF) can significantly improve phenotypes in a mouse model of sickle cell disease [46]. We used our system to assess the *in vitro* effect of this experimental therapy on the rates of conductance decrease in patient blood samples. Figure 3 compares a sample that was treated with either 0 mM (Fig. 3a) or 10 mM 5HMF (Fig. 3b). The results are dramatic, with a large and rapid conductance decrease in response to deoxygenation for the untreated sample but very little conductance change for the treated sample. Treatment with 5HMF causes a 5-fold slower conductance decrease (Fig. 3c). This marked rheodynamic response is associated with a slower change in the optical absorption measurement (Fig. 3b) as the treated sample unloads oxygen much more slowly than the untreated sample.

**Discussion**

Our microfluidic system enables us to make a novel measurement of the speed of the rheological response of patient blood samples to deoxygenation by integrating the molecular, cellular, and rheological processes that likely occur during *in vivo* vaso-occlusion, in sharp contrast with previous approaches that have typically focused on just one aspect of the vaso-occlusive process, such as HbS polymerization, RBC sickling, or blood rheology. We have shown that the rate of change in the rheological properties of blood samples, characterized by the rate of change in conductance during an *in vitro* vaso-occlusive event, is strongly correlated with overall patient disease severity for the set of patients in our study. Our finding in this patient cohort is statistically robust (p < 0.01) with > 90% statistical power (for p = 0.05). From a diagnostic perspective, our biophysical marker, with an AUC = 0.85, compares well against other biomarkers in development, such as a micro-NMR based cancer diagnostic (AUC = 0.44 – 0.88) and urinary biomarkers for kidney injury (AUC = 0.73 – 0.91) [47, 48]. This diagnostic efficiency contrasts sharply with existing laboratory tests such as HbS and HbF fractions which are much more weakly correlated with patient outcomes [10, 49]. Because we find no benign samples with fast rheodynamics, this novel measurement may be most useful for identifying patients at increased risk of complications. Although the sudden and seemingly unpredictable onset of vaso-occlusive crises suggests that it may never be possible to predict the exact timing of individual occlusive events, our measurement may provide the basis for an objective and accurate tool to identify those patients who have not responded as intended to hydroxyurea or transfusion or who may benefit most from initiation such treatment.

Our study highlights the potential diagnostic power of a biophysical marker to integrate multiple interdependent pathophysiological processes, in contrast to previous studies at the molecular or cellular level [20, 21, 27–30]. Furthermore, we see that a previously unappreciated difference between patients is found in the dynamics of blood rheological properties, reflecting the pathophysiological transition of a soft suspension that flows steadily to a jammed one when the cells stiffen in response to deoxygenation. Thus, it should not be surprising that steady-state values, such as hematocrit, mean corpuscular volume, mean hemoglobin concentration, WBC counts, and oxygenated conductance do not correlate with clinical outcome, while the dynamics of flow do correlate with the occlusive events.
ultimately responsible for disease severity. Indeed, more generally, stasis and hyperviscosity lead to thrombosis in vivo and thus imply a lower limit on steady-state velocity in the microcirculation [50]. It is therefore not surprising that the key determinant of vaso-occlusion is the rate at which flow approaches this threshold. In our measurements, the patients most at risk are those whose blood shows a rapid conductance change, indicating a high probability that it would slow sufficiently quickly in the microcirculation to produce a vaso-occlusion. Understanding the occurrence of these extreme events requires a dynamical assay such as that characterized here.

In summary, the strong correlation between blood rheodynamics and the clinical outcomes in our study population provides a valuable tool for scientific discovery, drug development, and possibly for patient monitoring and clinical decision-making in sickle cell disease. For example, although transfusion of HbA blood to lower the HbS fraction is the most common treatment for sickle cell disease in the US and provides short-term improvement [45], it is not clear what hematocrit and HbS fraction targets are optimal for each patient; efficacy in one cohort can lead to complications in another [8]. Our quantitative measurements of the effect of simulated transfusion on rates of conductance change (Fig 2f) may provide additional guidance.

Our approach may provide a better way to assess the efficacy of experimental pharmaceuticals in humans. Animal models of sickle cell disease are limited by significant phenotypic differences from human patients and the complexity of experimental protocols [7]. In contrast, our in vitro system can be used to identify potential drugs and quantify their efficacy much more easily. As an example, a reduced rate of conductance change following treatment of a blood sample with 5HMF (Fig. 3), is consistent with the known effects of this small molecule on increasing hemoglobin oxygen affinity, and thus slowing the kinetics of deoxygenation and subsequent polymerization of HbS [46]. However, these increases in hemoglobin oxygen affinity are large, and it is possible that a much milder increase in hemoglobin oxygen affinity may be sufficient to alter flow dynamics in vitro and possibly in vivo.

Our results also help focus the question of why hydroxyurea treatment has such variable efficacy [44, 51]. Hydroxyurea is currently the only approved pharmaceutical treatment for sickle cell disease, and it works in part by increasing the HbF fraction [10, 44, 52]. However, many patients treated with hydroxyurea show large increases in HbF fraction without significant clinical benefits. HbF is known to slow HbS polymerization and should therefore change the kinetics of RBC stiffening and blood conductance change [44]. Surprisingly, we find no consistent effect of total HbF fraction in a blood sample on the rate of conductance decrease, which is consistent with previous studies showing that the fraction of HbF in treated patients is not a useful predictor of clinical outcomes in individual patients [32]. One existing hypothesis to explain this result is that the uniformity of the HbF distribution across all RBCs may be more important than the total fraction of HbF [8, 44, 53]. This hypothesis is consistent with our findings, and our work focuses the question on analyzing the effects of HbF distribution on rate of conductance decrease and its correlation with patient clinical outcomes.

More generally, although our study is limited to a small cohort of patients and uses simple criteria for the “severity” of the disease, our results are robust and motivate further work to predict clinical course in larger populations over long periods of time. Our work also provides an impetus for the development of integrative markers of disease that link multiple molecular and cellular pathways across diverse length and time scales as tools to complement the growing biomolecular armamentarium in the quest to predict clinical outcomes and reveal the mechanisms underlying for human disease.
Methods

Blood Specimens

Blood specimens were collected during the normal course of patient care at two tertiary care hospitals and used in experiments 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 14 days. Hematocrit was determined using a Siemens ADVIA 2120 automated analyzer. Hemoglobin fractions were determined using cellulose agar electrophoresis and confirmed by high performance liquid chromatography (HPLC) with a Tosoh G7 column. Blood samples used in simulated transfusion experiments were tested to exclude the presence of any RBC alloantibodies.

For all measurements, hematocrit was adjusted to 25% by fractionating RBCs and plasma in a centrifuge at 3500 rpm for 10min. Separated plasma was then removed or added to adjust total hematocrit. For transfusion studies, hematocrit-adjusted sickle blood was mixed with type-matched HbA blood to achieve the desired sickle cell fraction. In order to treat with 5-hydroxymethyl furfural (5HMF, Sigma), we first dissolved 1M of drug in DMSO. Blood samples were treated with appropriate dose and incubated at 37°C for 1hr. After incubation, samples were transferred to 4°C until ready for use.

Patient blood samples were classified as “benign” if all of the following criteria were met during the 12 months prior to sample collection and the 2 months following: no patient hospital admissions or emergency room visits for sickle cell disease-related complications, no blood transfusions for sickle cell disease-related indications, and no hydroxyurea use. Patient blood samples were classified as “severe” if the patient had had at least one hospital admission or emergency room visit for a sickle cell disease-related complication or a blood transfusion for a sickle cell disease-related indication in the 12 months prior to sample collection or the 2 months following.

Device Fabrication

The device comprises 3 layers: the blood capillary, the gas reservoir, and a reservoir for hydration (Scheme 1). Each of these layers is separated from the adjacent layers by a 100 µm thick polydimethylsiloxane (PDMS) membrane, which allows for gas transport between the layers (Scheme 1). The blood capillary is 7 cm long with cross section 15 µm by 10 µm. The hydration layer, which is required to prevent dehydration of the blood through the membrane [54], is 30 cm long with cross section 1.5 mm by 100 µm. The gas reservoir completely overlaps the hydration layer but is 150 µm tall. The layers were fabricated in PDMS using soft lithography. The blood layer master was made by spinning SU-8 2015 (MicroChem Corp.) onto a silicon wafer at 3500 rpm for 30 s and then photocrosslinking the SU-8 through a 50,000 dpi transparency mask. The hydration and gas layer molds were made by spinning SU-8 2050 at 1500 rpm and 1000 rpm, respectively, for 30 s. The PDMS layers were made and assembled using thermal bonding. For the hydration layer and membrane, we mixed PDMS with a 20:1 elastomer:curing agent ratio and spun this onto the mold at 500 rpm for 30s. The gas layer was cast at 1 cm thickness using a 5:1 elastomer:curing agent ratio and bonded to the hydration layer at 80°C for 2h. The blood layer and corresponding membrane was made by mixing PDMS with a 5:1 elastomer:curing agent ratio and spin casting using the same conditions as for the hydration layer. The gas and hydration reservoirs were then removed from the hydration layer mold and bonded to the blood layer at 80°C overnight. The final structure was then plasma bonded to a glass microscope slide.
Device Operation

The experimental setup is shown in Scheme 1. Gas was flowed through the top chamber of the device (Scheme 1) under constant pressure (1 psi) from precision pressure regulators (PRG200-25, Omega). We switched between 0% oxygen (N\textsubscript{2}) and 21% oxygen (21% O\textsubscript{2}, 5% CO\textsubscript{2}, balance N\textsubscript{2}) using solenoid valves downstream of the regulators. We flowed 1X phosphate buffered saline (PBS) in the hydration layer at 1 ml/hr using a syringe pump. This aqueous layer prevented dehydration of the blood. Whole blood (EDTA-anti-coagulated) from human patients was flowed through the capillary under constant pressure, provided by compressed N\textsubscript{2}, using a digital pressure regulator (PCD, Alicat Scientific).

Oxygen Measurement

Oxygen concentration of the gas phase was measured using a fiber optic sensor (FOXY, Ocean Optics) connected to the outlet of the gas reservoir. We also measured the oxygen saturation of the RBCs directly using an optical technique. An optical filter (Brightline 434 +/- 17 nm, Semrock) was placed between the light source and the device (Scheme 1). The transmission band of this filter is centered on an absorption peak for deoxy-hemoglobin (Fig. S1). When the hemoglobin is oxygenated, the absorption peak shifts (20 nm) out of the transmission band of the filter (Fig. S1), causing deoxygenated cells to absorb more light and appear dark, while oxygenated cells remain transparent (Fig. S1 inset). A measurement of the RBC optical density through the filter revealed the oxygenation state of the cells (Fig. S1). For calibration of the measurement, oxygen concentration in the blood channel was measured directly using an oxygen-sensitive luminescence probe (Fig. S2). Tris(4,7-diphenyl-1,10-phenanthroline)ruthenium(II) dichloride complex \[55\] was dissolved in PBS and flowed through the blood channel under the same conditions as the blood measurement. Luminescence was measured under illumination at 488 nm.

Blood Conductance and Dynamics

Blood rheology was measured by capturing high speed videos in real time and performing video tracking of individual RBCs offline. Using this method we were able to compute instantaneous blood velocities over very short time intervals and track changes in blood velocity as a function of RBC oxygenation state. Video processing methods were described in detail previously \[39\]. Briefly, cells in each video frame were identified computationally based on morphologic criteria. Cell locations in subsequent frames were linked to form trajectories using heuristics and machine learning techniques. We defined the velocity at each point in time as the median cell velocity calculated over a 32-frame video captured at higher than 200fps. We excluded any noisy videos where the inter-quartile range of calculated velocities was greater than the larger of the median velocity or 40 um/s. All rheology data is reported as device conductance, which was calculated by dividing the median blood velocity by the applied pressure bias. Stretches of constant oxygen and pressure were identified automatically, and the beginning and ending of conductance transients were identified by examining a 5-point moving average of the conductance. Once the time range was defined for a conductance transient, rates of conductance change were computed and fit to a linear function using the robust least-squares method available in the MATLAB curve fitting toolbox (MathWorks). Average rates of conductance change for each sample were defined by taking the mean of calculated transients from at least 3 independent deoxygenation cycles. Nonparametric analysis to compare groups of samples was performed using the Mann-Whitney non-parametric test in the MATLAB statistics toolbox \[56\]. Power analysis was performed using bootstrapping methods (resampling with replacement from our data set) along with numerically simulated trials (N=100,000) \[57\]. Experiments flowing whole blood in microfluidic channels are prone to fouling, delamination, and other artifacts. We assessed the integrity of each deoxygenation/
reoxxygenation cycle by measuring the conductance before and after. Any significant (>20%) change likely reflects device compromise, and the results of such transients were excluded.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Measuring rate of conductance decrease after deoxygenation

Time course of measurement showing oxygen concentration (a) in the gas reservoir and corresponding median velocity (b, left y-scale and circles) of RBCs by video tracking as well as applied pressure bias (right y-scale and dot-dash line). Corresponding channel conductance (c) is measured by dividing instantaneous velocity by the pressure. We see that the rate of change of the conductance as the blood flow stops is effectively constant and independent of both the velocity and pressure. All conductance values are scaled by the mean HbA blood conductance ($C^*_{\text{HbA}} \sim 1.7 \mu\text{m/s/mmHg}$ (0.013 $\mu\text{m/s/Pa}$)), and time is scaled by the time scale for hemoglobin deoxygenation ($\tau_{\text{deox}} \sim 10\text{s}$).
Figure 2. Rate of conductance decrease correlates with patient clinical course

(a) Box plot comparing rates of conductance decrease for benign and severe samples. Receiver operating characteristics (b) for rate of conductance decrease (solid line) and a theoretical random prediction (dashed line). The areas under the ROC curves are 0.85 and 0.5 respectively. (c) Box plot comparing HbS fraction for benign and severe samples. HbF fractions (d) and rates of conductance change (e) for benign and severe samples further subdivided by hydroxyurea use. Rates of conductance decrease (f) for patient blood samples before and after addition of ABOand RhD-compatible HbA blood, simulating a blood transfusion. For each sample, data is normalized to unmodified sample. *p < 0.01 as determined by Mann-Whitney non-parametric analysis [56]. Bar heights in (f) represent means, and error bars represent standard deviations of at least 5 deoxygenation cycles. In box plots (a,b-e) red line is the median, blue box shows interquartile range (IQR), and dashed lines show extent of data within 1.5 times IQR. Rates of conductance decrease are scaled by mean HbA blood conductance ($C_{\text{HbA}}^* \sim 1.7 \, \mu \text{m/s/mmHg (0.013 } \mu \text{m/s/Pa)}$) divided by the time scale for hemoglobin deoxygenation ($\tau_{\text{deoxygenation}} \sim 10s$).
Figure 3. Rate of conductance decrease is modulated by a small molecule
Oxygen data as measured in the gas reservoir (top graph) and conductance data (bottom graph) are shown for an untreated severe sample (a) and the same sample treated (b) with 10mM 5-hydroxymethyl furfural (5HMF). Oxygen data (top graph) are shown as measured in the gas reservoir (dashed line) and in the blood channel (open circles, as measured by RBC intensity). Rates of conductance decrease (open circles) are quantified in (c). *p<0.05 as determined by Mann-Whitney non-parametric analysis [56]. Bar heights in (c) represent means, and error bars represent standard deviations of at least 5 independent deoxygenation cycles. All conductance values are scaled by the mean HbA blood conductance (C*HbA ~ 1.7 µm/s/mmHg (0.013 µm/s/Pa)), and time is scaled by the time scale for hemoglobin deoxygenation (τ_deox ~ 10s). Rates of conductance decrease are scaled by mean HbA blood conductance divided by the time scale for hemoglobin deoxygenation (C*HbA / τ_deox).

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Scheme 1. Microfluidic device for studying sickle cell blood flow conductance
The device comprises 3 layers (inset): artificial capillary for blood flow, hydration layer with PBS, and gas reservoir. Blood is flowed under constant pressure bias, controlled by a digital pressure regulator. Two solenoid valves control the gas in the top chamber. A fiber optic probe is used to measure the oxygen concentration in the gas reservoir. The device is illuminated through an optical filter whose transmission band (434±17 nm) is centered on an absorption peak for deoxy-hemoglobin (Fig. S1). The absorption peak of oxy-hemoglobin shifts making deoxygenated RBCs appear dark and oxygenated RBCs transparent. Qualitative measurements of hemoglobin oxygen saturation are made using the intensity of transmitted light.