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Density-based Separation in Multiphase Systems Provides a Simple Method to Identify Sickle Cell Disease

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Abstract

Although effective low-cost interventions exist, child mortality attributable to sickle cell disease (SCD) remains high in low-resource areas due, in large part, to the lack of accessible diagnostic methods. The presence of dense ($\rho > 1.120$ g/cm^3) cells is characteristic of SCD. The fluid, self-assembling step-gradients in density created by aqueous multiphase systems (AMPSs) identifies SCD by detecting dense cells. AMPS separate different forms of red blood cells by density in a microhematocrit centrifuge, and provide a visual means to distinguish individuals with SCD from those with normal hemoglobin, or with non-disease, sickle-cell trait, in under 12 minutes. Visual evaluation of a simple two-phase system identified the two main subclasses of SCD (Hb SS and Hb SC) with a sensitivity of 90% (73-98%) and a specificity of 97% (86-100%). A three-phase system identified these two types of SCD with a sensitivity of 91% (78-98%) and a specificity of 88% (74-98%). This system could also distinguish between Hb SS and Hb SC. This test demonstrates the first separation of cells by density with AMPS, and the usefulness of AMPSs in point-of-care diagnostic hematology.

Significance Statement

Red blood cells with a high density ($\rho > 1.120$ g/cm^3) are characteristic of sickle cell disease. This paper demonstrates a density-based separation of red blood cells in a system of aqueous multiphase polymers that enables a visual test that identifies sickle cell disease, starting from samples of whole blood, in less than 12 minutes. This low-cost, simple test could provide a means to enable diagnosis of sickle cell disease in low-resource settings, and enable life-saving interventions for children with the disease. The method itself provides a demonstration of the use of a biophysical indicator (here, density) rather than a biochemical marker (e.g., proteins separated by gel electrophoresis) as a means to do point-of-care hematology.

Main Text

Over 300,000 children (approximately 1% of births) are born with sickle cell disease (SCD) in Africa each year (1, 2). SCD is a genetic disorder caused by an array of genotypes (e.g., homozygous sickle cell disease—Hb SS, and hemoglobin SC disease—Hb SC) that lead to the sickling of erythrocytes and associated pathologies (**Table 1**). Severity and specific symptoms vary between the subtypes of SCD; the most prevalent form (Hb SS, ~ 75%)(3) is associated with the most severe effects.

Children with SCD suffer high mortality due to acute vaso-occlusive crises and increased risk of bacteremia (4, 5). Although inexpensive interventions exist to limit infection (e.g., penicillin prophylaxis, vaccinations) and reduce childhood mortality, over 50% of children < 5 years of age die in low-resource areas (4, 6, 7). Much of this mortality could be avoided, but to implement simple interventions, a diagnosis is necessary (8, 9). Standard diagnostic procedures, such as hemoglobin electrophoresis (HE) or high performance liquid chromatography (HPLC), which are used in well-equipped facilities to detect SCD, are either unfeasible in low-resource settings—where the disease is highly prevalent—or do not differentiate between SCD and the non-disease, sickle cell trait (Hb AS). This unmet need has motivated the recent development of creative diagnostic methods for SCD using blood stains on paper devices (10) and hemolysis in solutions of sucrose (11).

The distribution of density of red blood cells provides a biophysical indicator that is closely related to SCD pathophysiology (12, 13); the dehydration that leads to the formation of sickle cells increases the rate of the polymerization of hemoglobin S. Dehydration increases the density of the cell by reducing the volume and increasing the ratio of dense protein to less dense water in the cell. Although many of the cells with the highest density are also sickled, some may

Table 1. Genetic disorders classified as SCD.

Genotype	Description	Estimated Annual Births Worldide[a]
Hb SS	Homozygosity for Hb S	217,331
Hb SC	Heterozygosity for Hb S and Hb C	54,736
Hb $S\beta^{+/0}$	Heterogygous for Hb S with concurrent beta thalassemia (0 or +)	11,074
Hb SE	Heterozygosity for Hb S and Hb E	NA
Hb SD	Heterozygosity for Hb S and Hb D Punjab	NA
Hb SO	Heterozygosity for Hb S and Hb O Arab	NA

[a] estimates based on literature (3, 14)

also have irregular shapes variously described as a holly leaf or wreath shape (15–17). For simplicity, we refer to the entire class of high density cells characteristic of SCD as "dense, SCD cells."

This paper describes two rapid tests for the most prevalent forms of SCD (Hb SS and Hb SC) based on a sensitive but convenient measurement of the density (ρ, g/cm^3) of red blood cells using aqueous multiphase systems (AMPSs)—mixtures of polymers in water that form immiscible phases (18). AMPSs provide a method of separating particles by density (18); the discrimination between particles of different density using an AMPS can be high ($\Delta\rho < 0.001$ g/cm^3) (18). Each phase of an AMPS is separated by an interface that defines a step in density. AMPSs are thermodynamically stable and re-form if disturbed by stirring or shaking. AMPSs can be designed to be biocompatible, and have been used to separate mammalian cells by surface interactions (19, 20). This work presents the first use of AMPS to separate cells based on their density.

We designed two AMPSs to separate erythrocytes into multiple bins of density; the presence or absence of erythrocytes in the bins distinguishes individuals with the most prevalent forms of SCD from individuals with either normal hemoglobin (Hb AA) or sickle-cell trait (Hb AS). The simpler test, SCD-AMPS-2, uses two phases; the higher resolution test, SCD-AMPS-3, uses three phases. We evaluated our tests both visually and digitally in a population of 59 subjects (33 negative—Hb AA or Hb AS, 26 positive—Hb SS or Hb SC). Both tests identified SCD positive samples with a sensitivity > 90% and a specificity > 88%. Details of the design and contents of both SCD-AMPS are provided in *Materials and Methods*.

SCD refers to a large number of genetic variations. Establishing whether the presence of dense cells at an interface of an AMPS is diagnostic of SCD requires testing many genetic

variations of SCD, as well as concurrent conditions, that may affect the density of cells. **Table S1** lists several conditions that could affect the number of dense cells present in SCD. Homozygous sickle cell disease (Hb SS) and sickle cell with concurrent beta-0-thalassemia (Hb $S\beta^0$) are the most severe forms, whereas Hb SC and sickle cell with concurrent beta-+-thalassemia (Hb $S\beta^+$) are generally milder forms of the disease. In all cases, sickling of cells occurs, but the total amount of dense cells present may differ.

Hemoglobin C disease (Hb CC)—much more rare and geographically isolated than SCD (1, 14, 21, 22)—also increases the mass density of erythrocytes (23), and constitutes a potentially confounding interpretation. The distribution of densities of erythrocytes, however, may provide discrimination between SCD and Hb CC. In Hb CC, the entire distribution of densities of erythrocytes shifts to a slightly higher density; reticulocytes in Hb CC are more dense than those in Hb AA, but the high-density erythrocytes in Hb CC may be less dense than the densest cells in Hb SS (12). In sickle-cell disease, erythrocytes that are not sickled remain at normal densities, and the erythrocytes exist in two populations: a high-density, often sickled, population (~10% of cells) and a lower-density, predominantly normocytic, population, which also comprises reticulocytes and the youngest erythrocytes (24, 25).

For a proof-of-principle, we chose a population of subjects that represented the most prevalent forms of SCD (Hb SS and Hb SC) and those that represented typical negatives (Hb AA and Hb AS).

Dense cells are characteristic of SCD. Dense cells present in SCD have a mass density ($\rho \sim 1.12$ g/cm^3) that is higher than the most dense erythrocytes in healthy individuals ($\rho_{max} \sim$

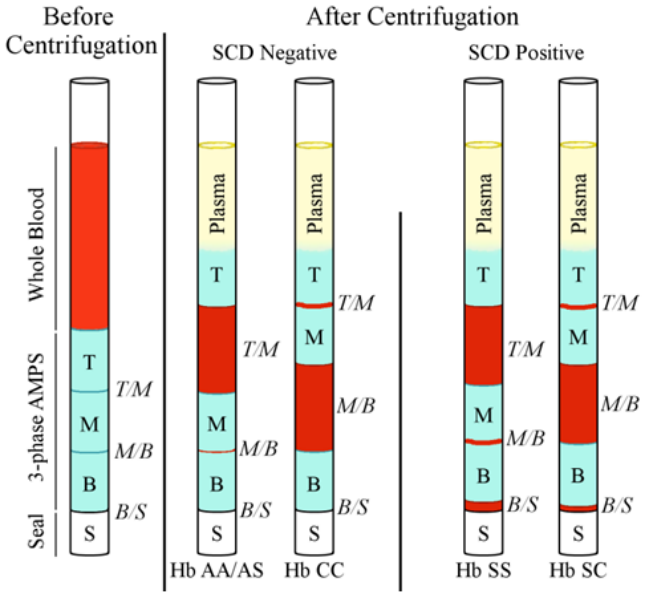
1.10 g/cm^3)[1] (12, 24–26); the difference in density between these cells makes SCD a candidate for a density-based diagnostic test.

Although the density distribution of erythrocytes in SCD has been studied extensively, commonly used methods of separating cells by density are not suitable for use in field settings (see Supporting Information). Methods that separate multiple populations require tediously layered gradient systems that are destroyed by agitation or mixing, and simple systems can only separate a single population. Centrifugation through AMPSs allows the separation of multiple populations in a thermodynamically stable (and, thus, simple to use) system. AMPS agitated by vigorous shaking readily reform quickly (minutes) under centrifugation, or more slowly (hours) under gravity (18).

The interfaces in AMPS provide bins of density to separate cells. The number of sub-populations of erythrocytes of interest determines the number of phases that should be used (see Supporting Information). An AMPS with three fluid phases provides enough interfaces—two liquid-liquid interfaces and a liquid-container interface—to separate the three populations of erythrocytes required in a test for SCD that can distinguish the major subtypes (Hb SS and Hb SC) of the disease (**Figure 1**). An AMPS with two phases provides two well-defined interfaces: one liquid-liquid interface between the phases, and one liquid-container interface. These interfaces are sufficient to separate dense, SCD cells from cells in the normal range of densities of erythrocytes, and to provide a test for SCD. Like the three-phase system depicted in **Figure 1**, the presence of red cells at the bottom of the tube indicates a positive test for SCD. The simplicity of interpreting only two phases comes at the cost of the ability to distinguish subtypes of SCD.

[1] Specific values of density vary in the literature and may be dependent on the media (e.g., Percoll, stractan or phthalate esters). The values cited here were found to be consistent in AMPS.

Figure 1. Schematic representations for the four most important outcomes of a density-based rapid test for sickle-cell disease. Upon centrifugation, erythrocytes move through the top (T), middle (M), and bottom (B) phases of the AMPS and collect at some combination of the three lower interfaces (two liquid/liquid interfaces, *T/M* and *M/B*, and one liquid/container interface, *B/S*). The distribution of cells between these interfaces will depend on the genotype; non-sickle hemoglobins (Hb AA, Hb AS, and Hb CC) are distinct from sickle hemoglobins (Hb SS and Hb SC). In all cases, the presence of red at *B/S* indicates sickle cell disease. Three interfaces allow further discrimination; a majority of erythrocytes at *M/B* indicates Hb SC or Hb CC.

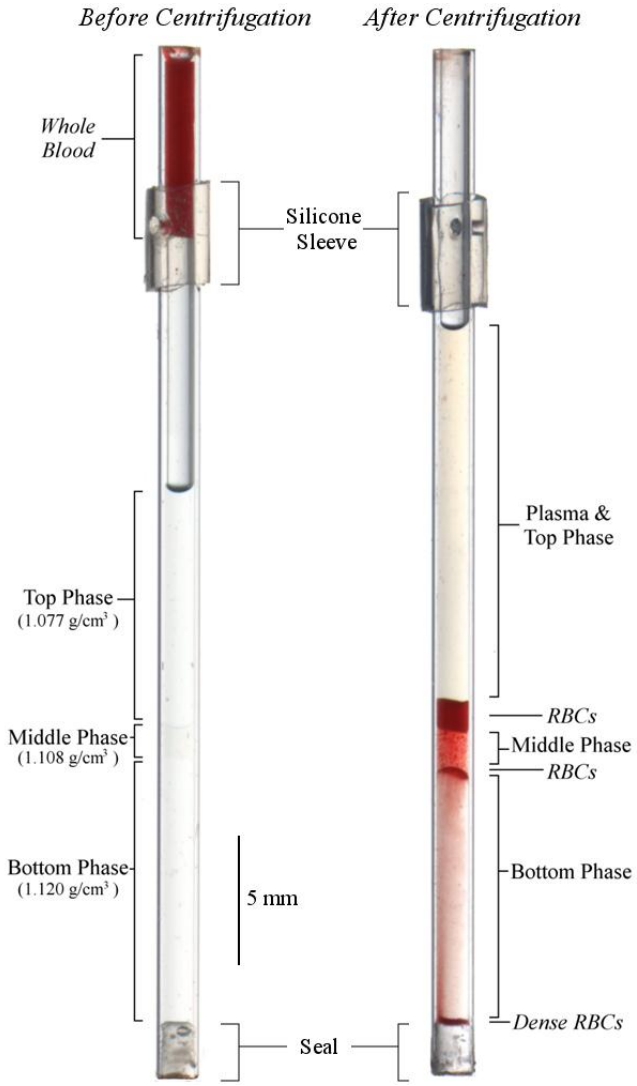


A point-of-care test imposes cost and time constraints on the assay design. To demonstrate the potential use of AMPS in point-of-care (POC) settings, we designed tests to use ~5 µL of blood (a volume easily obtained from a finger stick). This blood was added to a plastic capillary that had been preloaded with 14 µL of the SCD-AMPS-3 or SCD-AMPS-2 (**Figure 2**). We sealed the capillaries with either a white, vinyl-based sealant (Critoseal, Leica)—for ease of visual detection—or epoxy—for clarity when imaging tubes by transmission-mode in a scanner. Volumes were dictated by the capacity of the capillaries (see Supporting Information). The total cost of reagents and materials per test is ~$0.20 at this scale; when fabrication costs and packaging materials are accounted for, the cost per test is ~$0.50 (**Table S2**).

In rural settings, patients may travel for a day to seek medical care, and follow-up is challenging. Tests that can be coupled to actionable information and counseling must be rapid (ideally under 30 minutes). To meet this condition, we used a microhematocrit centrifuge (CritSpin, Iris Sample Processing) to centrifuge samples at 13,000 *g*; this centrifuge can perform 12 tests at a time and, with a simple, DC-to-DC converter can be powered by a car battery. We could distinguish blood of individuals with SCD from normal blood after six minutes; additional time in centrifugation enhanced the signal (**Figure S1**). We chose to use ten minutes for our test to ensure a strong positive response, while keeping the test rapid. The rest of the procedure, including the finger-stick, sample loading, and test interpretation, took less than two minutes. By comparison, the gold standard for analysis of SCD, hemoglobin electrophoresis, requires more than three hours to prepare samples, run electrophoresis, stain, and wash. Laboratories often batch samples to run on a single gel and report results several days later.

In addition, ease-of-use is a critical component of a POC test (27). Capillary action provides a simple mechanism to load blood. Designing a test to diagnose SCD from a

Figure 2. Example of an SCD-AMPS-3 rapid test loaded before and after centrifugation of blood from a sample without sickle cell disease. Blood wicks just past the hole in the side of the tube that is then covered with the silicone sleeve. After centrifugation, the cells separate from the plasma and pack between the phases of the AMPS. The test shown would be classified SCD positive because there is red below the bottom phase and above the seal.



finger-stick required a method to load blood into a capillary pre-loaded with SCD-AMPS and sealed on one end. Without modification, capillaries with closed ends will not wick additional fluid due to air trapped between the fluid and the sealed end. We used a "hole-in-tube" method to load blood into the capillary; puncturing a small hole in the side of the polycarbonate capillary at a specific distance from one end allowed a standard volume of blood (~5 µL) to wick into the tube (**Figure S2**).

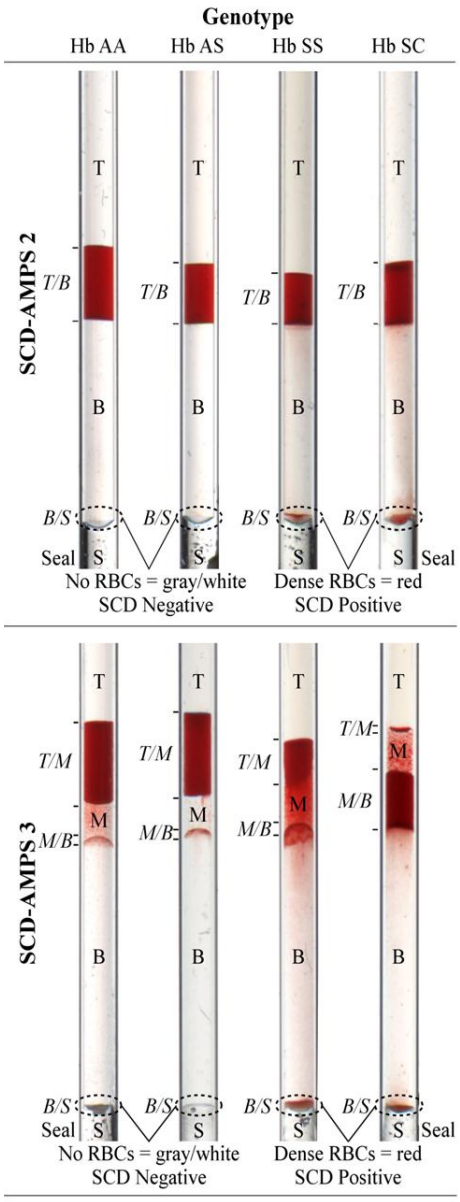
Results

Centrifugation of blood through the SCD-AMPS provides a visual separation of sickled cells. Over the course of ten minutes of centrifugation at 13,000 *g*, blood moved out of the loading zone and through the AMPS, and formed separated layers at the interfaces (**Figure 2**). In most cases, the boundary between the top phase and the plasma was not distinguishable. The total packed volume between each interface provides an estimate of the hematocrit (**Figure S3**): this estimate also provides a simple, if crude, method to identify severe anemia concurrently (28). We could distinguish samples from individuals with SCD from normal samples with a diagnostic accuracy of over 90%; a layer of red cells sat below the bottom phase of both SCD-AMPSs, and packed against the seal of the capillary (**Figure 3**); this layer was dominated by sickled and dehydrated cells (**Figure S6**). By comparing the volume of the packed cells at the seal, to the volume of cells at the other interfaces, we could estimate the percentage of dense cells (**Table S3**).

In the SCD-AMPS-3, most erythrocytes within the normal density range sat at the upper liquid/liquid interface. The densest normal erythrocytes (~1–5% of erythrocytes) collected at the lower liquid/liquid interface. In both SCD-AMPS-2 and SCD-AMPS-3, blood from individuals with sickle-cell trait appeared the same as that from normal individuals; this method

Figure 3. Representative examples of positive and negative tests in the SCD-AMPS-3 (ρ_{top} = 1.077 g/cm^3, ρ_{mid} = 1.108 g/cm^3, ρ_{bot} = 1.120 g/cm^3) and the SCD-AMPS-2 (ρ_{top} = 1.078 g/cm^3, ρ_{bot} = 1.129 g/cm^3), show a clear distinction between subjects with SCD (Hb SS and Hb SC) and those without SCD (Hb AA and Hb AS). In non-SCD blood in the SCD-AMPS-2 system, all cells pack at the liquid interface (*T/B*). In the SCD-AMPS-3 system, most cells have normal morphologies and densities (normocytes) and pack at the upper liquid interface (*T/M*), the densest normal shaped cells (dense normocytes) collect at the lower liquid interface (*M/B*), and some aggregated platelets are present at the bottom of the tube (gray) (*B/S*). Erythrocytes from a subject with SCD display greater heterogeneity at high densities. Dense, SCD cells form a layer below the bottom phase of both SCD-AMPS on top of the sealant (*B/S*). In the SCD-AMPS-3 test, the distribution of cells between the liquid interfaces (*T/M* and *M/B*) differentiates Hb SS from Hb SC. The difference in the total packed volume of the cells demonstrates the difference in the hematocrit between the normal subject and the anemic subject with SCD.

Figure 3 (cont.)



does not differentiate between Hb AS and Hb AA genotypes. Although a small amount of sickled cells may exist in blood from an individual with Hb AS, the amount is below our estimated limit of detection of 2.8% dense cells (**Figure S7**).

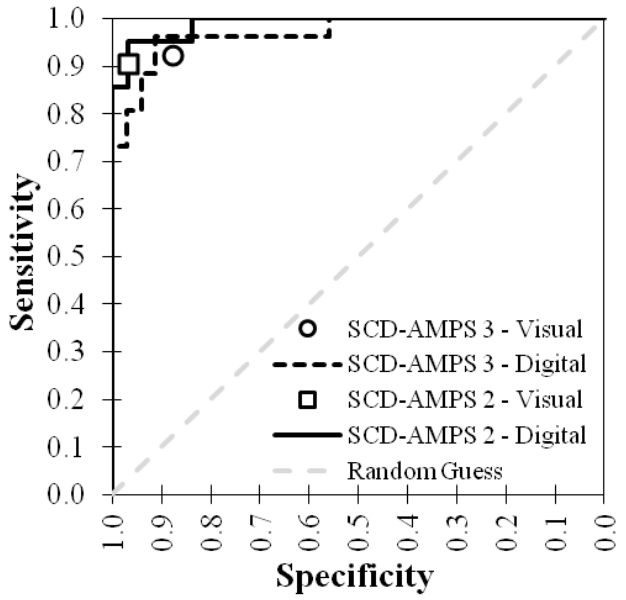
The presence of cells with a high density correlates with the presence of SCD. Samples that had a visible red band at the bottom of the AMPS correlated strongly with the presence of SCD in those with Hb SS or Hb SC. Conversely, samples negative for SCD rarely had red cells visibly present at the bottom of the AMPS (**Table S4**). The SCD-AMPS-2 had a true positive rate (sensitivity) of 90% with a Jeffreys 95% confidence interval (C.I.) of 73%–98% and a true negative rate (specificity) of 97% (C.I. = 86%–100%). The SCD-AMPS-3 had a sensitivity of 91% (C.I. = 78%–98%), and a specificity of 88% (C.I. = 74%–98%). Admittedly, visual inspection allows room for bias in a diagnostic test. To reduce biased evaluation, each test was evaluated independently by at least two people (see Supporting Information for more details). Samples negative for SCD included both Hb AA (n = 26 for SCD-AMPS-3, n = 24 for SCD-AMPS-2) and Hb AS (n = 7). Samples positive for SCD included Hb SS (n = 20 for SCD-AMPS-3, n = 15 for SCD-AMPS-2) and Hb SC (n = 6). The formulation of SCD-AMPS-2 was finalized after testing had begun on SCD-AMPS-3 and, thus, the former system was tested on fewer samples.

In about half of all tests where a clear red band was not present at the bottom of the tube, visual inspection showed a thin layer of white, yellow or pink material. Evaluation of these layers by microscopy revealed that platelets had clumped together to form large aggregates (**Figure S5**). In some cases, these aggregates appeared to have captured a small number of both red and white blood cells. In samples without SCD, we believe these aggregates may occasionally capture enough red blood cells to create a false positive.

We also evaluated scanned images of the capillaries digitally. Varying the thresholds for the intensity of the red color at the bottom of the tube produced a receiver operating characteristic (ROC) curve (**Figure 4**) (see Supporting Information). Our sensitivity and specificity from visual evaluation was near the ROC curve for both tests; this finding suggests two interrelated procedures: i) with proper training, the visual reading of the tests by health workers could match (and be checked by) the performance of the digital analysis; or ii) with higher-technology phones, digital analysis could replace training of readers. The fact that these two methods of reading give similar results suggest that reading the test will not be a major source of error.

The least dense red blood cells in Hb SC have a slightly higher density than the least dense red blood cells in Hb SS (23). In SCD-AMPS-3, blood from most individuals with Hb SC had distributions of red cells distinct from those with Hb SS. In half the cases, a thick band of red cells formed at the lower liquid interface whose height was comparable to or greater than that of the band of red cells at the upper interface (**Figure 3**). In some cases, an hour-glass shape of red cells connected the packed cells at the two interfaces (**Figure S8**). By contrast, blood from samples with Hb SS had two distinct bands of red with a clear majority of red cells at the upper liquid interface. These differences in the distribution of cells allowed us to distinguish visually between Hb SC (n = 6) and Hb SS (n = 20), the two most prevalent forms of SCD with a sensitivity of 67% (C.I. = 29%–92%) and a specificity of 100% (C.I. = 88%–100%). Centrifugation for an additional 10 minutes increased the sensitivity to 83% (C.I. = 44%–98%). In **Figure 3**, the red and pink, in the lower phases, indicated either that the dense cells had not all reached their equilibrium position, or that some cells had the same density as one of these phases. Additional centrifugation time (a total of 30 minutes) allowed these cells to sediment

Figure 4. The receiver operating characteristic (ROC) curve (solid line) for the digital evaluation of the presence of a red layer at the bottom of the SCD-AMPS demonstrates good diagnostic performance. Both curves are far from the gray line that indicates no ability to detect an event. Visual evaluation of the rapid tests matched the sensitivity and specificity of the digital analysis.



further and increased the thickness of the layer of red at the bottom of the tube (**Figure S1 & S4**), but required more time.

Discussion

An AMPS-based test for SCD is appropriate for use at the POC. The World Health Organization (WHO) recommends that POC devices be ASSURED: affordable, sensitive, specific, user-friendly, reliable, equipment-free, and deliverable to those in need (27). AMPS-based tests for SCD have the characteristics of sensitivity, affordability, and ease-of-use required to have an impact at the POC.

The "hole-in-tube" method allows blood to fill the tube automatically. Unlike tests that require lysing and incubating blood in a solution (e.g., Sickledex), whole blood is tested directly. Minimal handling of the sample reduces errors and risks to health workers performing the test. We estimate the cost of materials and manufacturing per test to be $0.50 (see Supporting Information). The centrifuge (CritSpin, Iris Sample Processing) we use in this study costs approximately $1,600, but we have verified that our system performs similarly on a basic centrifuge (SpinCrit, www.spincritcentrifuge.com) that costs $150, is portable, and runs on four AA batteries; this centrifuge can perform six tests at a time and standard AA batteries allow three separate 10 minutes spins per charge.

Although SCD-AMPS do not distinguish between Hb AS and Hb AA, they can distinguish between Hb AS and both Hb SS and Hb SC. SCD-AMPS-3 has the added ability to distinguish Hb SC from Hb SS, albeit with a lower sensitivity than that with which the test identifies SCD—the sensitivity to identify Hb SC may be improved with a slightly different density of the middle phase. In addition to providing diagnostic information, these tests measure a biophysical indicator that may help identify patients more likely to experience certain

complication of the disease; the fraction of erythrocytes that have a high density correlates with certain clinical manifestations of SCD (e.g., skin ulcers, priapism, and renal disfunction) (29). Monitoring the distribution of the density of cells could also provides a way to assess sickle crises (30).

The density of cells can be used as a diagnostic marker. The use of density as a tool for POC diagnostics began with the spun hematocrit, in which the difference in density between cells and plasma enables the measurement of the volume ratio of packed cells in blood. This measure has been helpful in the diagnosis of anemia (28). Modifications on the spun hematocrit concept have led to systems for measuring white blood cell counts and other parameters (31). The company Zyomyx has developed a technique to tag CD4+ cells with proprietary, high-density beads so that these cells can be separated from whole blood and quantified (32).

We have demonstrated, for the first time, that the self-assembling step-gradients in density formed by AMPS can separate cells by density, and provide a new method to identify SCD. The presence of dense cells correlates with the presence of SCD (12); AMPSs may provide a simple tool to identify dense cells in low-resource settings. Further testing will be required to verify the performance of the SCD-AMPS on larger population with more genetic variation and concurrent conditions, but this work demonstrates that density has the potential to be a sensitive and specific biophysical marker for diagnosing SCD.

One limitation of density-based methods is that density is a colligative property; density depends on the sum of the elements (solutes, proteins) contained in a cell rather than on a specific biochemical marker. As discussed earlier, factors that can affect the density of red blood cells, such as genetic variations and iron deficiency anemia (**Table S1**), must be carefully assessed to develop a POC diagnostic based on density. Testing on a larger population that

might include patients with these and other concomitant conditions would determine the generality of density as a diagnostic for SCD.

The densities of SCD-AMPS described here should discriminate between Hb CC and SCD; if, however, dense cells in Hb CC do provide false positives, an additional low-density band to isolate reticulocytes could discriminate Hb CC from Hb SC and Hb SS (23).

Density-based tests, also, will not be sensitive when dense cells are not present. At birth, children with SCD have predominantly Hb F, and generally begin to express appreciable amounts of Hb S between six months and one year of age. In some cases, high levels of Hb F may persist in children until they are two years old. The presence of dense, SCD cells relies on some amount of dehydration or sickling,of cells and, hence, on the presence of large amounts of Hb S. Screening young children with a high proportion under two years old, might have lower sensitivity than the performance described in this study. Only one of our Hb SS samples came from a child under one year old, and it appeared negative on both digital and visual inspection. Developing an accessible and affordable screening test for newborns remains a critical, unmet challenge. Rapid diagnostics that rely on the presence of sickled cells, such as ours, might play an important role in reducing child mortality if screenings are done near a child's first birthday; such diagnostic efforts could be carried out simultaneously with vaccination campaigns (e.g. measles) that target children between nine months and one year old.

The percentage of dense, SCD cells in the blood varies among individuals. Factors that can affect the amount of dense cells present in the blood include hydration and the occurrence of a recent vaso-occlusive crisis (30). Under most conditions, however, the blood of individuals with SCD has 13% (S.D. 8%) dense cells (29). This estimate of the amount of dense cells present includes a large population and modulators that can reduce the amount of dense cells

present, such as high levels of Hb F and concurrent alpha-thalassemia. In these individuals, sickled cells comprise a smaller proportion of erythrocytes and clinical symptoms are normally milder than others with SCD (33, 34).

An AMPS-based test for SCD can fill a gap in global health. With progress against infectious diseases around the world, the burden of morbidity and mortality due to hemoglobinopathies, such as SCD, will rise (3). Standard diagnostic techniques in well-equipped laboratories, such as HE and HPLC, are too expensive and require more infrastructure than is available in many countries with high burdens of SCD, especially in rural areas (35).

Sickling tests employing a deoxygenating agent and microscopy are subject to error from preparation and interpretation (36). Solubility tests are among the most used, microscope-free methods for screening for SCD in low-resource settings (37). In these tests a solution lyses and deoxygenates the blood; the polymerization of deoxyhemoglobin S causes the hemoglobin to form a nematic liquid crystal and makes the solution turbid (38, 39). Both sickling and solubility tests can detect the presence of Hb S, but cannot distinguish between Hb SS and Hb AS without the use of additional, expensive equipment (e.g., a turbidimeter); this distinction is non-trivial, as the former is a life-threatening condition, and the latter is, largely, benign.

In response to the outstanding need for rapid tests for SCD appropriate for the POC, a Request for Applications by the NIH in 2013 solicited the creation of tests for SCD with a sensitivity of at least 60% (RFA-HL-14-010). The AMPS-based tests described here have a sensitivity of over 87% in the patient samples used for this initial validation study.

Compared to currently available techniques, the density-based tests using AMPS combine four desirable properties: i) fieldability—they are amenable to use at the POC, ii) performance—they can distinguish Hb SC and Hb SS from Hb AA or Hb AS, iii) biophysical

information—by quantifying the percentage of dense, SCD cells, and iv) low cost. The AMPS-based method also compares favorably in ease-of-use to other technologies that have been developed recently to diagnose SCD (**Table S4**).

The high sensitivity and specificity of SCD-AMPS in identifying SCD in tests in a laboratory described here demonstrates that a density-based approach may provide a valuable screening tool. Further testing on different genotypes and concurrent conditions will determine whether such a test is appropriate as a POC diagnostic for SCD. Even if the performance is reduced, the densities could be adjusted to provide a higher sensitivity test (with lower specificity) to be used as a screening method; such a test would allow interventions where the cost-to-treat is low.

By combining simplicity and rapidity to measure a biophysical parameter (i.e., density), the density-based test using AMPSs could play an important role in diagnosing SCD at the point-of-care. Measuring the fraction of dense cells at the bottom of an SCD-AMPS could also have use beyond the diagnosis of SCD, and possibly aid in the management of the disease, but such uses will require clinical validation. More generally, density-based diagnostics illustrate how biophysical markers, such as density, and simple separation methods, such as centrifugation through AMPS, can combine to provide low-cost health solutions; AMPS-based separations of blood should enable hematology at the point-of-care.

Materials and Methods

Desiging SCD-AMPS. When designing an AMPS, we first seek systems whose phases are separated by the same differences in density as our target sub-populations of erythrocytes. We then use other additives to tune the overall density to the necessary levels.

An AMPS formed by mixing 7.0% (w/v) poly(ethylene glycol) (PEG) with a molecular weight (MW) of ~20 kD and 10.3% (w/v) Ficoll with a MW of ~400 kD provided phases with densities separated by the values required for a two-phase system useful in the diagnosis of SCD. An AMPS comprising 3% (w/v) PEG with a MW of ~20 kD, 10% (w/v) dextran with a MW of ~500 kD, and 5% (w/v) polymer of partially hydrolyzed poly(vinyl acetate) (containing 75% -OH and 25% $-OCOCH_3$ groups) with a MW of ~3 kD, provided phases separated by differences in density of the values required for our three-phase assay.

We added NaCl to make the system isotonic (as measured by vapor pressure osmometry) with blood. The pH of each system was adjusted to 7.40 ± 0.02 by the addition of concentrated (1-10 M) NaOH or HCl. We used a low-osmolality, high-density additive (Nycodenz, Accurate Chemical), to increase the density of each system to the proper range, and measured the density of each phase using an oscillatory U-tube densitometer. SCD-AMPS-2 contained 9.1% (w/v) Nycodenz and had phases with densities ρ_{top} = 1.078 g/cm^3 and ρ_{bot} = 1.129 g/cm^3. SCD-AMPS-3 contained 8.7% (w/v) Nycodenz and had phases with densities ρ_{top} = 1.077 g/cm^3, ρ_{mid} = 1.108 g/cm^3, and ρ_{bot} = 1.120 g/cm^3.

Supporting Information. The Supporting Information includes detailed descriptions of all chemicals and materials used, methods for obtaining blood samples, the preparation and characterization of the AMPS, estimations of the cost per rapid test (**Table S2**), analysis of test performance with different centrifugation times (**Figure S1 & S4**), experimental and rapid test design (**Figure S2**), estimation of hematocrit and fraction of dense cells (**Figure S3 & Table S3**), analysis of separated fractions of cells (**Figure S5 & S6**), comparison of methods to identify SCD (**Table S4**), estimation of the limit of detection using cells treated with Nystatin as a model

for dense, sickled cells (**Figure S7**), and detailed results on clinical samples (**Table S4 & Figure S8**).

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