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REVIEW

Emerging point-of-care technologies for sickle cell disease screening and monitoring

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ABSTRACT

Introduction: Sickle Cell Disease (SCD) affects 100,000 Americans and more than 14 million people globally, mostly in economically disadvantaged populations, and requires early diagnosis after birth and constant monitoring throughout the life-span of the patient.

Areas covered: Early diagnosis of SCD still remains a challenge in preventing childhood mortality in the developing world due to requirements of skilled personnel and high-cost of currently available modalities. On the other hand, SCD monitoring presents insurmountable challenges due to heterogeneities among patient populations, as well as in the same individual longitudinally. Here, we describe emerging point-of-care micro/nano platform technologies for SCD screening and monitoring, and critically discuss current state of the art, potential challenges associated with these technologies, and future directions.

Expert commentary: Recently developed microtechnologies offer simple, rapid, and affordable screening of SCD and have the potential to facilitate universal screening in resource-limited settings and developing countries. On the other hand, monitoring of SCD is more complicated compared to diagnosis and requires comprehensive validation of efficacy. Early use of novel microdevices for patient monitoring might come in especially handy in new clinical trial designs of emerging therapies.

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1. Introduction

Sickle cell disease (SCD) is a genetically inherited debilitating illness, caused by a point mutation in the beta-globin gene that requires early diagnosis after birth and constant monitoring throughout the life span of the patient. Sickle cell anemia was first clinically described in the United States in 1910 [1], and the mutated heritable sickle hemoglobin (HbS) molecule was identified in 1949 [2]. It is estimated that 100,000 Americans and more than 14 million individuals worldwide [3] have SCD, disproportionally in economically disadvantaged populations. SCD is estimated to cost more than $1 billion per year in health-care costs in the United States, while the full economic burden of SCD is likely to be greater considering the additional contributions of productivity loss, uncompensated care, reduced quality of life, and premature mortality [4,5].

The underlying mutation of a single amino acid in the beta chain of HbS belies the complex, highly morbid, and sometimes life-threatening clinical phenotype of SCD [6,7]. The pathophysiology of SCD is a consequence of abnormal polymerization of HbS and its effects on red cell membrane properties, shape, and density, and subsequent critical changes in inflammatory cell and endothelial cell function. Observed pathophysiologic changes in SCD include alterations in adhesion amongst sickled red blood cells (RBCs) and activated white blood cells (WBCs) and endothelium, and abnormal numbers of circulating endothelial cells and hematopoietic precursor cells. The clinical consequences of SCD are anemia, painful crisis, widespread organ damage, and early mortality [4].

Neonatal diagnosis of SCD is critical for the management of the disease, since undiagnosed children are especially in great danger of early mortality due to infections and stroke. Early diagnosis of SCD still remains a critical challenge in preventing childhood mortality in resource limited, developing regions of the world, such as sub-Saharan Africa, due to requirements of skilled personnel and high cost of instrumentation and testing associated with conventional approaches. SCD diagnosis can be generally achieved through protein or molecular tests in the developed world due to its genetically inherited nature. However, monitoring of SCD patients presents insurmountable challenges due to heterogeneities among patients, as well as in the same individual from time to time, and the multisystem nature of the disease. Furthermore, neither conventional monitoring techniques nor conventional screening tools

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currently available are feasible for operation at the point of care (POC), impeding easy access to health care, as well as exacerbating patients' quality of life.

Micro/Nano platform technologies emerged in the last couple of decades [8,9], through advancements in fabrication techniques and versatile materials, offer unique advantages in overcoming the challenges associated with conventional SCD screening and monitoring tools. This review article describes prominent platform technologies for SCD screening and monitoring and critically discusses current state of the art, potential challenges associated with these technologies, and future directions.

2. Global scope of SCD

Even though the main birthplace of SCD is Africa, its geographical distribution is now spread worldwide due to migration. Today, SCD is most prevalent in regions of sub-Saharan Africa, The Americas, Saudi Arabia, India, and Mediterranean countries such as Turkey, Greece, Italy, and South East Asia [10,11]. SCD is highly prevalent in malaria endemic regions of the world [11]. The greatest burden of SCD still lies in Africa, where the number of newborns affected by SCD is estimated to be more than 200,000 annually [10,12,13]. In Africa, SCD is associated with high rate of childhood mortality, 50–90% of African children with SCD die early in their childhood [11,14]. In other words, approximately 1000 babies are born with SCD in Africa every day and more than half die before they are 5 years old [14]. The sickle cell carrier frequency across equatorial Africa is between 10% and 40%, which results in an SCD prevalence of at least 2% [11]. In some parts of Western and Central Africa, the prevalence of sickle cell trait is as high as 25%. SCD also has a high prevalence in central and western regions of India, where approximately 20% of children born with SCD die before the age of 2 [15]. Estimated number of people with sickle trait in North America is 2–3 million, whereas the number is 1–2 million in Brazil [10]. According to the data available, over 6000 annual births and 100,000–150,000 adults are affected by SCD in Latin America [16]. According to National Health Service in the United Kingdom, the number of people affected by SCD is estimated to be between 12,500 and 15,000, which makes SCD the most common inherited disease in the United Kingdom [17]. A percentage of 4.2 of the total population in Saudi Arabia is a carrier of sickle cell trait whereas 0.26% is affected by SCD [18]. In Jamaica, 10% of the total population carries some sort of genetic disorder related to SCD [19]. In the United States, SCD is the most common inherited blood disorder, and most of the people who suffer from SCD are of African descent. About 100,000 Americans are affected by SCD; it occurs in about 1 in 365 African-American births and 1 in every 16,300 Hispanic American births [11]. Sickle cell trait is estimated to occur in about 1 in 13 African-American births [11].

3. SCD pathophysiology

The pathophysiology of SCD is a consequence of abnormal deoxygenated HbS polymerization and its deleterious effects on RBC membrane, shape, density, deformability, and adhesion. The pathophysiology of SCD mainly consists of anemia, inflammation, hemolysis, vaso-occlusion, and consequent tissue ischemia, pain crisis, and organ damage. Though being the first discovered molecular disease, SCD has been known to be highly complex due to its heterogeneous characteristics in pathophysiology, making it hard to pinpoint the underlining biological mechanisms. Many facets of SCD pathophysiology have been investigated, including hemoglobin polymerization [20–22], cellular deformability [23–28], adhesion [25,29–34], hemodynamic changes [35,36], and clinical heterogeneity [6,7].

The original powerful observation that RBCs show abnormal adhesion to endothelial cells has since been deepened and expanded to describe a complex pathophysiology in which abnormal WBC adhesion also plays an important role. Therefore, along with RBC abnormalities, any approach to understanding SCD pathophysiology must also take into account endothelial, WBC, and platelet activation and adhesion, inflammation, and activation of coagulation [37–50]. Together, these heterotypic cellular and blood plasma abnormalities, arising ultimately from HbS polymerization, yield a clinical syndrome that is characterized by acute and chronic pain, cumulative organ damage, and early mortality [51,52].

3.1. Vaso-occlusion

Abnormal adherence to endothelium, by sickle RBCs and WBCs, as a possible root cause of vaso-occlusion and pain, was described in the 1980s, highlighting inflammation and abnormal cellular adhesion as key features of SCD [48,53–55]. A myriad of interconnecting abnormal interactions can be envisioned, amongst HbS-containing RBCs, activated WBCs, and activated endothelial cells in SCD (Figure 1). Key clinical and experimental studies in SCD literature, performed via flow chambers or *ex vivo* rat mesocecum [29,30,54,56], have shown that RBC adhesion and deformability, WBC adhesion and activation [57], and endothelial activation contribute to the pathogenesis of vaso-occlusion [33,56,58,59] and may correlate with disease severity [34,48,60,61]. Abnormal RBC adhesion to endothelium has associated with disease activity [34,48] and has diminished with treatment [34,62], with variable but elevated adhesion at clinical baseline. Associations with clinical status have shown using FACS analysis of membrane protein components [63–65]. However, few longitudinal measurements of adhesion at baseline and with therapy have been performed due to lack of convenient reproducible adhesion assays [30,34].

Abnormal monocyte, neutrophil, platelet, and endothelial cell activation and adhesion are present in SCD, and complementary models of vaso-occlusive crises (VOC) describe initial reticulocyte and neutrophil adhesion to an activated endothelium and/or subendothelial matrix (Laminin, LN; Fibronectin, FN; von Willebrand Factor, vWF), followed by dense (irreversibly sickled) red cell trapping and vaso-occlusion [33,66,67]. Further refinements in the model, based on *ex vivo* and *in vivo* experiments, is one in which the endothelium is activated by cytokines and white cells, primarily monocytes, which are themselves activated by sickle RBC-derived factors [40,68–70]. These factors combine to increase the adhesiveness of RBCs

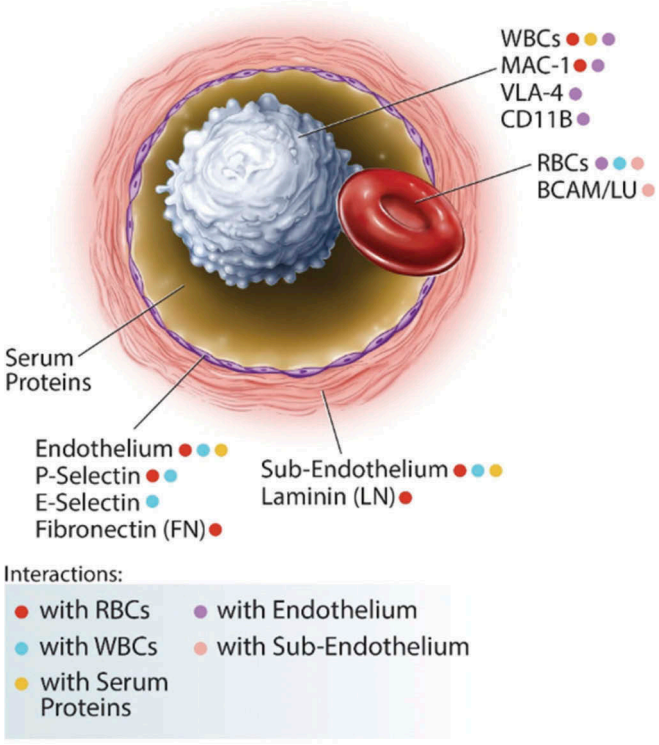

Figure 1. A subset of interactions between cellular and sub-cellular components in SCD. Abnormal interactions, amongst HbS-containing RBCs, soluble serum proteins (such as thrombospondin, TSP, and von Willebrand Factor, vWF), cytokine- and WBC- ($CD11b^+$ monocytes) activated endothelial cells (through integrins, integrin receptors, adhesion molecules, and selectins), subendothelial matrix components (including TSP, vWF, fibronectin, and laminin), and activated WBCs (via $MAC\text{-}1^+$, $LFA\text{-}1^+$, $VLA\text{-}4^+$ neutrophils), which themselves also directly adhere to the endothelium. (Illustration credit: Grace Gongaware, Cleveland Institute of Art.).

and white cells, primarily neutrophils and monocytes, to each other and to the endothelium and subendothelium, leading to vaso-occlusion. Soluble bridging factors (Thrombospondin, TSP; FN; vWF) are also important, although the interactions are not simply quantified [33,41,46,57,66,69,71–75]. Further, activated endothelial cells and hematopoietic precursor cells circulate at an unusually high level in SCD [40,48,76] and correlate with end-organ damage [77]. Some membrane/cellular interactions have been studied during VOC [48,76,78], or compellingly demonstrated in animal models [57,79], but broad clinically correlative studies are absent.

3.2. RBC adhesion and deformability

A healthy biconcave HbA-containing RBC deforms easily and passes through minuscule vessels and capillaries in the body [80–82]. Deoxygenated HbS polymerizes inside the red cell [83], altering its membrane, shape, and density [30,33,48,56,83–85]. These biophysical changes cause reduced deformability, increased stiffness, and abnormal adhesion of the HbS-containing RBC (SCD RBC) and may result in blockage of blood vessels [48,83,85,86] and reduced red cell half-life (hemolysis) [87,88].

Sympathetic tone and 'stress' signals, such as epinephrine, are modulators of SCD RBC adhesion and of abnormal vascular tone [89–93]. Importantly, intravascular heme arising from hemolysis impairs endothelial cell function and vascular tone, while triggering WBC activation, inflammation, and activation of coagulation [94–98]. In SCD, RBC membrane abnormalities include aberrant timing or abnormal persistence during maturation, and abnormal activation, by 'stress signals,' of surface molecules such as Very Late Antigen-4 (VLA-4), Cluster of Differentiation 36 (CD36), LW glycoprotein, and Basal Cell Adhesion Molecule/Lutheran (BCAM/LU) [74,99–106]. Cumulative oxidative damage, resulting in excessive phosphatidylserine externalization on the SCD RBC membrane, causes abnormal adhesion [107,108]. Anti-SCD RBC adhesion therapy has been validated preclinically, and, importantly, these targets are beginning to reach clinical trial, including VLA-4 blocking antibodies [109], and beta-adrenergic receptor blockade (via a US FDA-approved medication, propranalol [110]) targeting epinephrine-mediated red cell adhesion [92,99,106,111,112]. Small molecules (αVβ3 integrin) [113] and low molecular weight heparin (P-selectin) [59,114] were utilized to target RBC adhesion to an activated endothelium specifically, and an oral agent for this purpose is in phase I/II studies in humans (P-selectin) [58,115,116].

Studies showed that heme and plasma from SCD patients induce neutrophil extracellular traps (NETs) in murine models of SCD [97], resulting in capture of RBCs and platelets [117,118]. It is not known why hemolysis is more active in some patients [87], nor why hemolysis can exacerbate during severe painful crises [119–121]. SCD RBC deformability associates with hemolysis and adverse clinical outcomes [122], without definitive causality [123,124]. Adhesion to the endothelium may prolong delay time, and increase polymer formation and fragility as the RBC passes through the vasculature [51]. Furthermore, an association between hemolysis and increased SCD RBC adhesion to components of the endothelium/subendothelial surface has been shown recently [34].

3.3. WBC adhesion

Elevated numbers of activated WBCs (monocytes [40,69,125,126] and neutrophils [42,127,128]) in SCD patients have long been associated with adverse outcomes in SCD, such as stroke and even early mortality [52,69,98,129–132]. Moreover, increased rates of endothelial activation and inflammation in SCD induce abnormal leukocyte recruitment to the vessel wall [57,133]. The initiation and propagation of vaso-occlusive events subsequently takes place due to interactions between sickle RBCs and adherent leukocytes [39]. Using an SS mouse model and intra-vital microscopy, Turhan et al. showed that these interactions occurred in postcapillary venules and some of them indeed caused VOC *in vivo* [57]. On the other hand, in mice deficient of both E-selectin and P-selectin, vaso-occlusive events did not develop upon tumor necrosis factor α (TNF-α) induction [57]. Adherent leukocytes and RBC-leukocyte aggregates also distort the local microcirculation that increases the RBC transit time. This phenomenon renders RBCs more susceptible to sickling due to longer exposure to deoxygenation in the microvasculature which could lead to mediated RBC-leukocyte interactions [134].

Even though both P- and E-selectin are essential for WBC adhesion to the endothelium, E-selectin can further trigger secondary activation signals in the WBC. These signals result

in polarized activated $\alpha_M\beta2$ integrin (CD11b/CD18 or Mac-1) expression at the leading edge of the crawling neutrophil, and SCD RBC capture [135]. Surprisingly, inhibition of E-selectin abrogates these effects, whereas inhibition of P-selectin has only a partial effect, which was tested *in vivo* in VOC using the novel synthetic pan-selectin inhibitor (GMI-1070) with maximal activity against E-selectin [136,137]. Many studies in the literature suggest that blocking FcγRIII receptor activity on neutrophils by intravenous immunoglobulin infusions (IVIG) may interrupt Mac-1 activation and RBC capture by neutrophils. NET formation is also inhibited by FcγRIII blockade.

3.4. Endothelial dysfunction and inflammation

A growing body of evidence suggests that interplay between vascular dysfunction and high levels of inflammation remarkably contribute to the pathophysiology of SCD [40,42,138–144]. As an endothelial mediator, nitric oxide (NO) has been shown to correlate with the impaired endothelium functioning in sickle cell patients [145,146]. Elevated rates of hemolysis in SCD reduce the bioavailability of NO leading to vasoconstriction and further release of pro-inflammatory cytokines into plasma, which activates the endothelium [147]. Indeed, it has been shown that circulating endothelial cells are significantly increased in sickle cell patients regardless of their clinical status [76]. Subsequently, through NO-dependent activation pathways, the adhesion molecules such as VCAM-1, E-selectin, P-selectin, and ICAM-1 are overexpressed on the endothelial layer at significantly higher rates contributing to following vaso-occlusion and painful crises [148,149]. Other than NO, endothelium activation is also induced by the adhesion of activated platelets in SCD [150].

Furthermore, SCD can be associated with elevated counts of leukocytes, activated platelets, and pro-inflammatory cytokines, all of which are indicators of a marked chronic inflammatory state [151–154]. Activated monocytes and platelet monocyte aggregates in sickle cell patients trigger endothelial inflammatory response through the nuclear factor κB (NF-κB) pathway [155]. This interaction is mediated by several cytokines produced by monocytes including TNF-α and interleukin-1β (IL-1β) [40]. Moreover, invariant natural killer T cells (iNKT) in sickle cell patients overexpress chemokines CXCR3 and IFN-γ that has been shown to mediate pulmonary inflammation [156,157].

4. SCD screening at the POC

4.1. Ongoing challenges and unmet needs in the clinic

4.1.1. Developed world

POC screening for SCD in the developed world could allow more cost-effective identification of children at risk. Consistent and economic screening may improve care in these regions with less prevalent hemoglobin gene disorders and incomplete lab-based support. Even though a well-established universal screening program for SCD is in place in some resource-rich countries, such as the United States and United Kingdom, uniform newborn screening is not in place in many developed countries due to economic and technical challenges. Recent studies suggest that universal screening could prevent early childhood mortality in SCD, since unscreened patients in low-prevalence regions in developed countries are at greater risk for life-threatening complications during early childhood [158–161]. Moreover, prevalence of SCD is steadily increasing in European countries due to immigration [162–166], requiring additional health-care support and expanded screening programs. Screening platforms adapted for mobile phone use in the developing world could increase patient engagement in resource-rich settings, by giving patients their own mobile diagnostic. Finally, a cheaper, more-widely available platform could increase access for re-screening, as people reach reproductive age, to allow self-identification in those at risk for transmitting the HbS or HbC genes, i.e. those most at risk for having children with SCD. While this could not fully evaluate genetic risk in all patients, e.g. those with beta thalassemia trait would likely be missed, an accessible, affordable hemoglobin screen, although imperfect, could screen for those at greatest risk for transmitting SCD.

4.1.2. Low-resource settings

With its origins in sub-Saharan Africa, the Indian subcontinent, and the Arabian Peninsula, the sickle β-globin gene has spread throughout the world. It is estimated that more than three quarters of those homozygous for the hemoglobin S gene are born in Africa alone, with half the global burden borne by just three countries: Nigeria, India, and Democratic Republic of Congo [161]. In low-income countries, limited resources for diagnosis and treatment, aggravated by a dearth of government strategies to combat SCD, have led to poor patient outcomes. The World Health Organization (WHO) estimates that more than half of the children born with SCD in sub-Saharan Africa die before the age of 5 years [11,164,167]. This calls for the widespread implementation of affordable and evidence-based interventions that can be integrated into existing health systems to ensure their sustainability. Evidence from high- as well as low-income countries has shown that implementation of a range of interventions, including newborn screening, penicillin prophylaxis, pneumococcal vaccination, and parental education, significantly reduces morbidity and mortality [168–171]. However, in low-resource settings, diagnosis of SCD is hampered by the high cost of currently available laboratory methodologies, posing a major barrier to implementing life-saving interventions. Further, limited contact with health-care delivery systems requires that screening methodologies be timely and generate easily interpretable results to enable initiation of interventions at the POC. The deployment of low-cost, rapid, and accurate POC screening tools will be transformative in helping break the diagnostic barrier. These POC solutions lend themselves to integration into already existing public health programs such as primary immunization, a critical factor in ensuring sustainability in low-resource settings. Generation of easy-to-read results that requires only minimal training for health-care workers and adaptability to delivery via mobile phone platforms are great assets in employing POC techniques for widespread screening of SCD within public health systems with limited resources.

4.2. Conventional techniques for SCD diagnosis

The diagnosis of homozygous HbSS (sickle cell anemia, SCA) and heterozygous HbSA (sickle cell trait, SCT), HbSC disease, and HbS-β thalassemias is based on the varying percentages and combinations of HbS, HbA, HbF, HbC, and HbA2 present in RBCs. The most basic tests used to identify the presence of HbS are the sickling test and the sickle solubility test [172]. In sickling test, sodium metabisulfite is used to induce polymerization of HbS and consequent sickling of RBCs by reducing oxygen tension. Then, the diluted blood sample is observed under a microscope to observe the sickled RBCs. Although simple, this test cannot differentiate between HbSS, HbSA, HbSC, or HbS-β thalassemias.

The solubility test works by making HbS insoluble in a concentrated phosphate buffer solution. In this reduced state, HbS precipitates and forms tactoids that refract light creating a turbid solution. The result is compared to positive and negative control blood. An important issue is that these simple screening tests cannot be performed on newborns because of the predominance of HbF at birth. It takes several months after birth for newborns with HbSS (SCA) or HbSA (SCT) to produce significant amounts of HbS, which can be detected with these tests. If used at birth, the tests may produce false-negative results, if HbS is less than 10% of the total hemoglobin.

Additional tests are needed to confirm which form of SCD the patient has. There are four tests that are commonly used: hemoglobin electrophoresis, isoelectric focusing (IEF), high-performance liquid chromatography (HPLC), and DNA analysis [173,174]. The electrophoresis-based tests work based on the principle that different Hb types migrate with different velocities when placed in an electric field due to their different net charges. Following is a brief description of each of the aforementioned techniques.

4.2.1. Bench-top hemoglobin electrophoresis

Hemoglobin electrophoresis is a laboratory method that can be performed under alkaline or acidic conditions with a variety of sieving materials such as gel or paper [165]. Under alkaline conditions, hemoglobin types C, A2, S, F, and A have net negative charges and migrate toward the positively charged electrode. Various factors such as charges of the hemoglobin, the pore size of the medium, and the ionic concentration of the buffer solution determine how far each hemoglobin type migrates. The separation of hemoglobin types forms visible bands that can be used to identify various hemoglobin disorders. Hb electrophoresis is especially useful for the rapid screening of a small number of samples, and its results can be quantified using densitometry, which may suffer from inaccuracies at very low concentrations. Alkaline Hb electrophoresis displays lower resolution between HbS and HbF, particularly in neonates who have high HbF levels. Finally, electrophoresis carried out in a capillary tube is known as capillary zone electrophoresis [165]. This method allows for the use of higher voltages and shorter run times, which renders it especially advantageous for high-throughput screening.

4.2.2. IEF

IEF exploits the fact that the net charge of a protein varies with the pH of the surrounding medium. Utilizing this variation, proteins are separated based on their isoelectric points (pI), which can be defined as the point at which a protein possesses zero net charge. The technique uses an applied electrical field across a gel medium with a fixed pH gradient, in which each Hb type becomes immobilized once it reaches its pI. IEF exhibits higher resolution than Hb electrophoresis, thus it is capable of distinguishing between a larger number of Hb variants [165,166]. However, due to the larger number of bands that this higher resolution results in, IEF results are harder to interpret [164,165]. IEF is also more expensive and, as in hemoglobin electrophoresis, quantification is achieved using densitometry which can be inaccurate especially at low hemoglobin concentrations. Despite these challenges, IEF is considered to be the standard for newborn screening, since diagnosis is possible with very small sample volume or even an eluate from a dried blood spot [164].

4.2.3. HPLC

HPLC separates a fluid into its components based on molecular size and charge using cation exchange chromatography to identify the various hemoglobin types in a blood sample. HPLC utilizes absorbent materials such as granular silica or other polymers as a sieving medium. A pressure pump drives the fluid through the material, and a computer detects the separation. Unique aspects of this test are full automation and accurate quantification of the hemoglobin levels. These machines are relatively expensive and are not readily available in developing countries. In resource-rich countries like the United States, HPLC has largely replaced Hb electrophoresis and IEF as a primary screening test. This is because Hb electrophoresis and IEF are labor intensive, time consuming, and are not designed to quantify Hb levels. The ability to quantify Hb levels with HPLC makes it useful for monitoring patients who are on hydroxyurea (HU) or transfusion therapies [175].

4.2.4. DNA analysis

DNA-based assays can be used to detect the mutations in β globin that produce abnormal Hb [176]. However, it is generally more expensive than the previously described methods. An earlier popular method for the DNA-based assay utilizes the point mutation on the β-globin gene with restriction enzyme digestion and polymerase chain reaction (PCR). The point mutation that causes SCD changes the normal β-globin gene sequence, which removes the restriction site for the restriction enzyme DdeI. During the restriction enzyme digestion of the β-globin gene using DdeI, the gene is split into two fragments if the mutation is not present. However, if the mutation is present, the gene remains as a single large fragment. PCR is then used to amplify the fragments for identification using electrophoresis. Currently, the most robust testing strategy utilizes direct sequencing of β-globin combined with copy number variation analysis of the beta-globin locus.

4.3. Emerging POC technologies for SCD screening

The most recent technological trend for SCD screening focusses on adapting available diagnostic tools for feasible operation at the POC, especially in resource-challenged regions. The emerging technologies have veered toward overcoming concerns of cost, fabrication complexity, portability, as

well as the need for highly trained operators associated with conventional techniques. According to their operating principles and detection schemes, the emerging techniques in the past few years can be categorized into four groups: (1) paper-based hemoglobin solubility assays, (2) lateral flow immunoassays, (3) density-based separation, and (4) microengineered electrophoresis. The work reported in each of these categories is reviewed in this section and summarized in Table 1.

4.3.1. Paper-based hemoglobin solubility assay

This technique exploits the insolubility of HbS and the filtration properties of the paper substrate used in microfluidic paper-based analytical devices as a means to visually detect the presence of HbS. To perform the test, a drop of blood (20 μL) mixed with a hemoglobin solubility buffer in a 1:10 ratio is applied onto patterned chromatography paper. The difference in capillary action transport of polymerized HbS and other hemoglobin types results in different blood stain patterns (Figure 2(a)). These patterns are used to differentiate between HbAA, HbAS, and HbSS, and the test process can be completed within 20 min [177–180]. Initial validation results of this test in a resource-limited setting showed that HbS can be visually identified with 94.2% sensitivity and 97.7% specificity [180]. Furthermore, when combined with a custom image analysis algorithm, the relative color intensity of the center spot can be used to quantify Hb concentration in the sample.

This paper-based assay offers the advantages of ease of use, low cost ($0.77 per test), simple fabrication, and minimal sample processing as it requires only one step of mixing. Moreover, it utilizes the natural color of blood for detection without resorting to complex color change detection or labels. In addition, tests can be performed individually and no batching is required.

Despite the advantages of this technique, the results may be affected by clotting of the blood samples which would prevent the wicking of the blood through the paper substrate. In addition, the test relies on naked-eye detection which may render it prone to operator error, which can be overcome by pairing the test with an automated image processing algorithm. Moreover, the validation results show that this test cannot accurately distinguish between HbSC and HbAS even when used with automated image processing [179,180]. Finally, high levels of HbF, especially present in newborns, prevent the polymerization and precipitation of HbS, and consequently hinder the application of the test to newborn screening [171].

4.3.2. Lateral flow immunoassays

Kanter et al. reported the testing results of a lateral flow immunosassay, known as Sickle SCAN™, developed to detect the presence of HbA, HbS, and HbC with the unassisted eye [181]. The assay consists of a test strip, with polyclonal antibodies conjugated with colored nanoparticles immobilized on four different test lines (Figure 2(b)). Each line corresponds to one of the three hemoglobin types and the fourth line serves as a control to verify proper operation of the device. Once the test specimen consisting of 5 μL of blood in Hb solubility buffer at a 200:1 ratio is added into the device, the solution diffuses to the test zones where the Hb is captured forming antibody–antigen complexes. Consequently, the appearance of a blue line at any of the test lines signals the presence of the targeted hemoglobin types. A readout can be obtained from the device within 2 min. The limit of detection (LOD) varies between different hemoglobin types. The reported LOD values for HbA, HbS, and HbC are 40%, 1%, and 2%, respectively, with sensitivity and specificity of 99%.

A recent validation study for this lateral flow assay was reported by McGann et al. [182]. In this study, sensitivities of 98.3%, 99.5%, 100%, as well as specificity values of 94%, 92.5% and, 100%, were reported for HbA, HbS, and HbC, respectively, with Hb concentrations as low as 2%. This study also revealed that the presence of high concentrations of HbF did not interfere with the detection of HbS or HbC. Moreover, an evaluation of the shelf-life of the device was performed. The device has been proven to function properly even after storage at 37°C for 30 days. This technology is proposed as an initial assessment tool since it offers the advantages of ease of use, absence of auxiliary equipment, and short turnaround time. However, the assay relies on human visual interpretation, which may be critical to the test result especially in the case where faint HbA lines in patients with HbAS may be misinterpreted, despite the fact that the band intensities do not correlate with corresponding hemoglobin percentages [181]. Therefore, a weaker HbA line along with a stronger HbS line might be misinterpreted as HbSβ^+, instead of SCD trait. In addition, the assay is not suited for quantitative assessment for the concentrations of the different Hb types. Finally, the immobilization of the antibodies conjugated with nanoparticles increases the fabrication complexity of the assay adding to its cost and limiting its shelf-life especially at high-temperature environments without refrigeration or air conditioning.

Recently, a new lateral flow assay has been developed under the name HemoTypeSC™. The assay utilizes monoclonal antibodies specific to HbA, HbS, and HbC. The assay consists of (1) laminated fiberglass sample pads, (2) nitrocellulose membrane with antibodies deposited on four different locations, corresponding to each of the three hemoglobin types as well as a control, and (3) a cellulosic wick [183]. To perform the test, 1 μL of blood is diluted in a 1:1000 ratio in distilled water. Next, 15 μL of the diluted blood is applied to the sample pad and the strip is dipped in a sample vial containing red-colored colloidal gold nanoparticles rehydrated in 150 μL of assay buffer. The strip is allowed to wick the liquids for 10 min before it is taken out of the vial. Upon visual detection of the test strip, the absence of a red line on one or more of the four specific locations indicates the presence of the corresponding hemoglobin type. A result is obtained from this assay within 20 min.

This lateral flow assay was validated by testing 100 patients with the specific Hb types. Identification of HbA, HbS, HbC was reported to be achieved with 100% sensitivity and specificity. The LOD was estimated at 2.7% for HbA, 3.3% for HbS, and 1.3% for HbC. The estimated cost of the materials used for the fabrication of this assay was $0.25, while a more realistic cost estimation per test was not provided. As in the previous lateral flow assay, quantification of the results is not possible, and the

Table 1. Emerging POC technologies for SCD screening.

Technique	Advantages	Disadvantages	Cost per test	Equipment cost	Turnaround time	Sensitivity	Specificity	LOD	Refs.
Paper-based hemoglobin solubility	Simple fabrication, ease of use, uses natural color of blood, no batching	Failure to distinguish between HbSC and HbAS. Blood clotting interferes with the test. Interpretation of results is susceptible to human error	$0.7	$300–500 (for automated detection)	20 min	94.2%[a]	97.7%[a]	NA	[177–180]
Lateral flow immunoassay (Sickle SCAN)	Ease of use. Rapid results. Absence of auxiliary equipment	Complex fabrication. Interpretation of results is susceptible to human error. Hb quantification is not supported	>$5[b]	NA	2 min	99%[c] 98.3–100%[d]	99%[c] 92.5–100%[d]	HbA: 40%; HbS: 1%; HbC: 2% 1–2%	[181] [182]
Lateral flow immunoassay (HemoTypeSC)	Ease of use. Absence of auxiliary equipment	Complex fabrication. Interpretation of results is susceptible to human error. Hb quantification is not supported	$0.25[e]	NA	20 min	100%[f]	100%[f]	HbA: 2.7%; HbS: 3.3%; HbC: 1.3%	[183]
Density-based separation	Simple testing procedure. Rapid results	Need for bulky centrifuge limits applicability to POC setting. Batching required for centrifuge operation. Inability to distinguish between HbAA and HbAS. Inaccuracies due to high HbF levels, health and treatment conditions, and genetic factors affect RBC density	$0.5	$150–1600	10 min	90–91%[g]	88–97%[g]	2.8% (Dense cells)	[184]
Microengineered electrophoresis (HemeChip)	Low cost. Ease of use. Robustness. Rapid testing. Results comparable to standard electrophoresis tests. Possible integration with mobile devices. Works on the principles of clinical standard electrophoresis test	High HbF concentration present in newborns less than 4 weeks of age may affect test results	$0.9	~$500[h] (for automated detection)	<10 min	89–100%[i]	82–89%[i]	HbS: 10%; HbF: 10%; HbA:10%; HbC/A2: 3%	[185]

[a]For the detection of the HbS presence. [b]Based on personal communication. [c]For the detection of different Hb genotypes. [d]For the detection of the presence of different Hb types. [e]Estimated material cost only. [f]For the differentiation between different Hb phenotypes. [g]For the identification of HbSS and HbSC with two-phase and three-phase AMPS. [h]Initial assessment of automated reader cost. [i]For differentiating between adjacent bands corresponding to various Hb types.

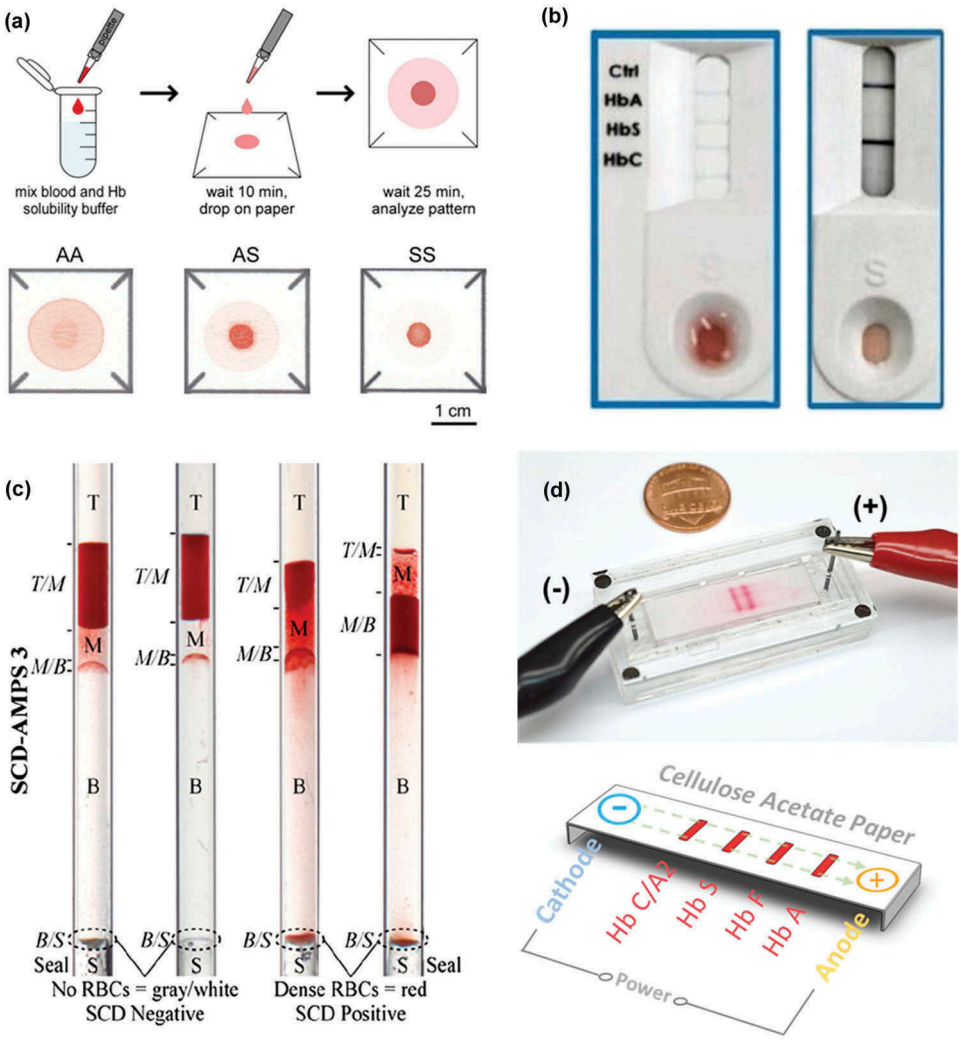

Figure 2. Illustrations of the principle of operation of the emerging technologies for SCD diagnosis. (a) Paper-based Hemoglobin solubility [180] (Figure is open access). A droplet of blood mixed with Hb solubility buffer is dropped on chromatography paper, and a blood stain is allowed to form. The stain on paper is analyzed and the color intensity profiles are used to determine the Hb type in the sample. (b) Sickle Scan™ lateral flow immunoassay [181] (Figure is open access). The test specimen consisting of a drop of blood mixed with Hb solubility buffer is dropped onto the sample loading zone. The solution then diffuses to the test zones where Hb is captured by color-conjugated antibodies. The type of Hb is determined by the appearance of a blue line at the different test zones along the test strip. (c) Density-based separation [184] (Figure is open access). The blood sample is mixed with aqueous polymeric solutions in capillary tubes. Upon centrifugation, the precipitation of a dense RBC layer at the bottom of the tube indicates SCD. (d) Microengineered electrophoresis (HemeChip). After loading the blood sample mixed with DI water into the chip, an applied electric field causes Hb separation. Due to the differences in mobility among Hb types, each type will travel a unique distance across the paper strip. Full color available online.

processes involved in its fabrication are complicated. The assay is able to distinguish between HbAA, HbAS, HbAC, HbSS, HbSC, and HbCC. However, identification of other Hb types such as HbF and HbA2 is not possible. In addition, HbSβ$^{+\text{-thal}}$ and HbSβ° gave results consistent with HbAS and HbSS, respectively.

4.3.3. Density-based separation

Density-based separation detects sickled RBCs via cell density measurements using aqueous multiphase systems (AMPS). Kumar et al. developed two- and three-phase AMPS capable of distinguishing dense SCD cells from normal cells with a sensitivity of 90% and a specificity of 97% for the two-phase system, whereas the detection with the three-phase system had a sensitivity of 91% and a specificity of 88%. The estimated LOD was 2.8% for dense cells and the cost per test is around $0.5 [184].

The test requires 5 µL of blood to be mixed with aqueous polymeric solutions. The mixture is loaded into capillary tubes and centrifuged for 10 min. SCD is detected by the precipitation of a dense RBC layer at the bottom of the tubes (Figure 2(c)). In addition, the combination of larger centrifugation time and the use of an optical reader enable the distinction between HbSS and HbSC. Further analysis of the sediment layer would also allow for the quantification of the fraction of dense cells. The density-based test is simple and rapid. However, the use of a centrifuge increases the cost of the test and its applicability at the POC. The turn-around time would also be affected since the samples need to be processed in batches. Density-based separation is also incapable of differentiating between HbAA and HbAS. It should also be noted that this test might not be suitable for detection of SCD in newborns since dense RBC cells are not present yet due to high levels of HbF in the first

4–6 months of life. Also, the test might not be accurate for patients with persistent high HbF levels, such as patients with the Arab-Indian haplotype. Furthermore, many health conditions, treatment processes, and prescribed medications, as well as genetic factors, influence the RBCs density and in turn limit the validity of the test.

4.3.4. Microengineered electrophoresis

Microengineered electrophoresis (HemeChip) has been recently developed to identify and quantify hemoglobin types including HbC/A2, S, F, and A, among others. As depicted in Figure 2(d), the HemeChip consists of a microfabricated polymethyl methacrylate (PMMA) chamber housing an electrophoresis cellulose acetate paper strip, which is used to separate hemoglobin types via an applied electric field [185]. A mobile image processing application has also been developed for automated and objective quantification of HemeChip results at the POC.

The test starts by mixing a blood sample (<5 μL) with pure or deionized (DI) water to lyse the cells and release Hb, and <1 μL of the mixture is stamped onto the paper substrate inside the chip. Next, an electric field is applied across integrated electrodes. The electric field causes hemoglobin separation with distinct bands, and due to differences in mobility among the different hemoglobin bands, each type will have a unique travel distance from the application point across the paper strip. Screening is achieved in under 10 min, and test results showed 90% sensitivity and 89% specificity in differentiating between HbC/A2 and HbS bands, 89% sensitivity and 82% specificity in differentiating between HbS and HbF bands, and 100% sensitivity and 86% specificity in differentiating between HbF and HbA bands. Large-scale field testing of HemeChip with newborns is pending for assessment of clinical sensitivity and specificity in determining healthy versus diseased states and high-risk infants. Since HemeChip works on the principles of current clinical standard electrophoresis method, once the large-scale field testing is done, the clinical sensitivity and specificity values of HemeChip are expected to be comparable to those of the standard electrophoresis test (93.1–99.9%) [172]. The assessment of the LOD for this technique was carried out using adult blood samples. The LOD for adult SCD and SCT were determined to be around 10% for HbS, HbF, and HbA, and 3% for HbC/A2.

HemeChip is low cost ($0.9 per chip), rapid, robust, and accurate. Moreover, hemoglobin detection and quantification results using the HemeChip were shown to be in strong agreement with the standard HPLC and laboratory-scale electrophoresis tests. This technology also offers the advantage of possible integration with mobile devices for more accurate analysis. A potential challenge when using this technology, as with any other newborn screening method, may originate from high percentages of HbF masking other Hb types (e.g. HbS and HbA). Additionally, HemeChip test setup currently utilizes a bench-top power supply. However, due to the low-power requirement for the test, this power supply can be replaced by portable rechargeable batteries for real-world applications.

5. SCD monitoring at the POC

5.1. Ongoing challenges and unmet clinical needs

More than 100,000 Americans and millions worldwide have SCD [11]. In the United States, SCD is estimated to cost >8 million dollars per patient over a 50-year life-span [4]. Life expectancy of SCD patients has increased significantly, thanks to the introduction of cost-effective interventions such as prophylactic penicillin, widespread vaccination, and HU. Nonetheless, high morbidity, from chronic complications and organ damage, and early mortality are still having a great impact on patients with SCD. To date, correlative studies in SCD have ranged amongst clinical reports, based on tests, interventions, and chart review [52,186–191], and, at the other extreme, SCD population-based genetic analyses of gene polymorphisms [192–195]. Despite significant advances in the understanding of the fundamental pathophysiology of SCD, we are still without markers that can reliably reflect the clinical course of patients in real time. SCD is unusually susceptible to an examination of cell membrane properties and cellular activation. RBC stiffness, RBC density, RBC & WBC adhesion, WBC repertoire and activation, and whole blood viscosity are excellent candidate biophysical surrogates for disease activity, and some of which (RBC density, RBC and WBC adhesion, and WBC repertoire and activation) are already targets in therapeutic trials.

Many important observations about membrane and cellular abnormalities, and their relationship to clinical complications in SCD, have been made since the 1980s [48,53–55]. However, most pathophysiologic studies have been undertaken in modest numbers of subjects at a single time point and single institution. Further, it has not been feasible for more than one analysis to be performed on a single patient sample, e.g. simultaneous evaluations of membrane properties, inflammatory cell activation, and circulating endothelial cell numbers. Often, promising 'cutting-edge' or biologically illuminating correlative tests are too expensive, complex, or difficult to 'export' to widespread use outside of a few specialized research centers. Widespread access to longitudinal examination of key pathophysiologic endpoints, such as intercellular adhesion of RBCs, WBCs, and endothelium (and subendothelium), if reproducible and feasible, could provide a critical additional dimension to clinical studies that could improve clinical care, guide clinical trial design, and decrease the physical and financial burden of SCD. Non-biased characterization of cellular biophysical properties should enable more precise targeting of disease modifying interventions in SCD. Most notably, reproducible and clinically feasible serial evaluation of RBC or WBC adhesion and WBC activation could guide intervention if, for instance, MAC-1 activation predominated in some clinical scenarios [196,197], P-selectin adhesion in others [115,137], and iNKT cell expansion in a third [156,157,198]. Finally, the burden of SCD is in resource-limited settings. Therefore, inexpensive and simple POC discriminants (and diagnostics) of disease activity could be extremely valuable tools worldwide [161,177].

More than half of patients living with SCD are treated with HU or are receiving regular transfusions to prevent severe and life-

threatening complications of SCD. HU is the only FDA-approved drug to treat SCD. Regular blood transfusions are commonly performed in approximately 10% of pediatric and up to 20% of adult patients with SCD. HU increases the overall HbF% and the percentage of red cells containing detectable HbF (F cells), in children and adults [199–201], thereby decreasing the tendency toward intracellular polymerization of HbS [199,202,203]. Importantly, the decision to initiate HU therapy, especially in children, must be thoughtful and therapy must be monitored and adjusted to achieve optimal results. HbF is an important biomarker for efficacy and adherence to treatment [202,204]. Greater treatment-related increases in HbF may predict a more robust response to treatment in children [205]. Patients require frequent (monthly or bi-monthly) blood testing and monitoring, once HU therapy is initiated. These safety labs comprise a CBC and reticulocyte count; the next month's dose is not dispensed until that day's blood counts are available. The dose needed for maximal clinical benefit, which may or may not be the maximum-tolerated dose (MTD) [206], is generally identified within 6–8 months of initiating HU therapy, but should be established and assigned only after the patient tolerates the dose for at least 8 weeks [52,58]. Because HbF response to HU is dose dependent, HbF levels, measured serially, help to establish MTD in individuals [202]. Clinical trials with escalation to MTD have reported higher percentage of HbF and Hb as well as mean corpuscular volume (MCV) [207]. Careful attention to patients' response to treatment and the resulting individualized therapy has the potential to improve clinical outcomes [208]. Increases in Hb and HbF associate with a clinical response to HU therapy, and are sustained, especially in children [199,209,210]. Close monitoring and follow-up are vital to ensure adherence to treatment and appropriateness of dose.

Healthy RBC transfusion (HbA) can be lifesaving and is proven to help prevent complications of SCD. Transfusion can prevent stroke in children at high risk and, post-stroke, may prevent recurrence [211]. Regular (monthly) blood transfusions are common therapy, utilized in approximately 10% of pediatric and 20% of adult patients with SCD. The primary objective of long-term transfusion programs is to maintain low proportions of HbS in the blood [212], often at <30% HbS percentage with a total Hb between 9 and 12 g/dL [213]. Ongoing monitoring of HbS and HbA levels during transfusion care informs therapeutic and technical decisions (about the length and frequency of transfusions) [214]. Accurate administration of blood transfusion limits excessive transfusion and reduces the risks associated with transfusion, such as alloimmunization, hemolytic reactions, and iron overload [215]. However, current methods to monitor Hb composition require that samples be sent to a lab, resulting in delays in patient feedback, provider decision-making, and treatment modification. Patient self-efficacy and provider monitoring and management would benefit from a POC test that delivers immediate results.

5.2. Currently available methods for SCD monitoring in clinical research

Conventional monitoring of SCD patients in the clinic relies on measurement of different blood components quantitatively and qualitatively, including amount of cell types and proteins to physical properties of cells via complete blood count (CBC) test and biochemical assays [216–219]. Even though other techniques, including flow cytometry, ektacytometry, and flow chambers, have been occasionally utilized in clinical research and in some clinical trials [220–223], they are not integrated into routine patient monitoring. Apart from these modalities, HPLC, which was described in detail in Section 4.2, is used for hemoglobin quantification to monitor patients undergoing HU therapy or transfusion therapy [224,225].

5.2.1. CBC

A typical CBC test includes WBC count (number of WBCs/μL), RBC count (number of RBCs/μL), hemoglobin (g/dL), hematocrit (percentage of RBCs in blood), MCV (average size of a single RBC), mean corpuscular hemoglobin (average amount of hemoglobin in a single RBC), red cell distribution width (RDW, variation in RBC size), reticulocyte count (amount of immature RBCs), and platelet count. Homozygous SS and heterozygous S/β° patients typically have lower RBC count, hemoglobin, and hematocrit due to the hemolytic anemia. On the other hand, WBC count, platelet count, and reticulocyte count are elevated, though they can fluctuate. Reticulocyte counts may alter depending on the degree of anemia, due to hemolysis or other causes (including sequestration), and bone marrow responsiveness to anemia. MCV has shown to rise in SCD patients treated with HU. Furthermore, RDW is elevated in SCD patients due to increased heterogeneity within RBC subpopulations. Even though CBC is widely used and provides valuable information on blood cell properties, it is insufficient to provide an integrated and complete evaluation of the patient's status.

5.2.2. Biochemical assays

Enzymatic and non-enzymatic biochemical markers in blood, measured via activity, reactivity, and immunosorbent assays, can be direct indicators of hepatic dysfunction, kidney damage, endothelial damage, inflammation, and intravascular hemolysis. In SCD patients, plasma levels of lactate dehydrogenase (LDH), total bilirubin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) are used as principal hemolytic markers. ALT, AST, and total bilirubin, along with alkaline phosphatase are also indicators of hepatic dysfunction. Serum cystatin c and creatinine levels are used as indicators for kidney function and elevated levels in SCD patients are associated with renal decline. Elevated c-reactive protein levels in blood plasma are associated with acute and chronic inflammation. Moreover, soluble adhesion molecules, including VCAM-1, ICAM-1, E-selectin, and P-selectin, are associated with increased cell adhesion and VOC and can be quantified via enzyme-linked immunosorbent assay (ELISA) [226,227]. Even though these soluble adhesion molecules utilized to better understand the SCD pathophysiology and targeted in some clinical trials [37], these markers need thorough validation before clinical use. Overall, biochemical assays can be important in measuring several protein and enzymatic markers that are associated with end-organ damage and vasculopathy; however, they are time- and labor-intensive.

5.2.3. Flow cytometry

Surface characteristics of blood cells are typically measured with conventional techniques, such as fluorescent-activated cell sorting (FACS), immunohistochemistry, or microscopic imaging methods. In FACS, cells of interest are isolated, extensively processed, incubated with a fluorescent-labeled antibody raised against a cellular protein (e.g. integrin, receptor, adhesion molecule), and sorted by optical recognition. Measurement by flow cytometry of aberrant surface molecule expression or activation has served as a surrogate for directly measuring abnormal adhesion in humans with SCD [63,196,228]. However, quantitative changes in surface molecule expression (e.g. BCAM/LU) do not always faithfully recapitulate qualitative changes [102,229]. Furthermore, FACS requires high-cost instrumentation, skilled personnel, and time- and labor-intensive operation, which limits its application in most clinical and research settings.

5.2.4. Ektacytometry

Ektacytometry has been widely utilized in deformability measurement of RBCs in SCD [197,198]. Ektacytometry involves a stationary inner cylinder and an outer cup, and between the two a narrow gap where blood fills in [230]. Varying levels of shear stress can be generated on blood when the outer cup is rotated at different speeds and osmotic gradient is varied from below physiological osmolality to above physiological levels. Elliptical diffraction patterns of sheared cells are obtained using a laser and a lens, and deformability is calculated from the dimensions of the elliptical diffraction pattern [230]. Despite its frequent use in SCD research studies, ektacytometry lacks physiologically relevant flow conditions that are present in the blood vessels. Further, it is not feasible as a POC analysis tool at the clinic due to high-cost and need for skilled personnel.

5.2.5. Flow chambers

Flow chambers are composed of a gasket, with inlet, outlet, and vacuum ports, assembled on a glass slide either functionalized with biomolecules or coated with endothelial cells [231]. Gasket thickness determines the height of the chamber and flow rates pumped into the chamber can be optimized to simulate physiological shear stress levels in the blood vessels. Flow chambers were widely utilized in the early SCD literature to mimic blood cell and vessel endothelium interactions. In particular, they were employed frequently in SCD research to mimic post-capillary venules, where vaso-occlusion occurs, and analyze abnormal adhesion of RBCs to endothelium and endothelium-associated proteins [29,54]. Even though flow chambers provide high-throughput analysis of cell interactions and ability to mimic physiologically relevant conditions, they require complicated equipment and skilled personnel for operation, which limits their wide-spread application in the clinic and as a research tool.

5.3. Emerging technologies for SCD monitoring in clinical research

Despite vast knowledge gained in identifying and targeting cellular abnormalities and interactions in SCD over the last 30 years, such expertise has not been translated into clinical care or trial design due to requirements for complicated custom-designed devices, trained technicians, specially collected patient blood samples, and extensive sample processing and manipulation. Groundbreaking studies on cellular adhesion and deformability in SCD relied on complex systems requiring skilled personnel, which include laser diffraction ektacytometry, FACS, and parallel flow chambers. These important proof-of-concept studies used techniques that required extensive preprocessing, separation of cellular populations, and washing of RBCs [29,30,56]. Further, preprocessing wash steps typically removed plasma proteins that contribute to cellular adhesion [25,232]. Complex and labor-intensive techniques to measure cellular adhesive interactions have not been used in longitudinal analyses or in large-scale clinical studies, applied at more than one center. These significant technological barriers have hindered the widespread evaluation of abnormal cellular characteristics, clinically and as a research tool.

Recent advances in micro and nano-fabrication technologies have yielded microfluidic platforms that can probe single cell behavior and tissue response simultaneously under precisely controlled biological, biophysical, and flow conditions, mimicking physiological systems at baseline and with disease [27,233–244]. These technologies have been used to model SCD vasculature [83,240,244], and are likely to yield important insights about SCD pathophysiology. Furthermore, such advanced modeling of *in vivo* microvasculature conditions might produce simple, reliable, and rapid platform technologies for personalized medicine applications, from therapeutics to monitoring.

5.3.1. Endothelialized microchannels

Endothelial cells play an essential role in the pathophysiological manifestations of SCD [33,55,245]. Activation of the endothelial layer mediates cell adhesion and aggregation by further inducing inflammatory response and subsequent complications. In that sense, several researchers have recently directed their focus toward fabricating endothelialized parallel flow chambers and microfluidic devices to better recapitulate the biophysical and hemodynamic properties of the microvasculature environment [246,247]. For example, utilizing Human Umbilical Vein Endothelial Cells (HUVEC)-coated flow chamber assays, Zennadi et al. [111] demonstrated that epinephrine-mediated sickle RBC adhesion to the endothelium through LW-αvβ3 interactions. In addition, epinephrine-stimulated RBCs were shown to activate peripheral blood mononuclear cells (PBMC) and promote PBMC adhesion to the endothelium increasing the risk of vaso-occlusive events. Matsui et al. [59] reported elevated sickle RBC adhesion to thrombin-treated HUVECs in a parallel flow chamber under normoxic conditions. The rolling adhesion of cells showed P-selectin pathway dependency while firm adhesion required additional pathways. Unfractionated heparin was shown to block the P-selectin dependent pathways by diminishing the thrombin-enhanced rolling adhesion of cells [59]. Stimulation of endothelial cells *in vitro* through IL-1β was also shown to enhance sickle RBC adhesion to the endothelium in a time-dependent manner [68]. During the early stages of the activation, RBC adhesion to the HUVEC monolayer exhibited a

negligible dependency on integrin α4β1 on the RBC surface. However, 9 h following the IL-β1 stimulation, the adhesion was both α4β1 and VCAM-I mediated. Even though these studies provided valuable insight into cellular adhesive interactions in SCD, they relied on extensive sample preprocessing, removing soluble adhesion molecules and other activation factors in plasma that are critical *in vivo*.

Recently, Tsai et al. developed a microfluidic platform with an inner surface coated by a confluent monolayer of endothelial cells for modeling vascular occlusion and thrombosis in hematologic diseases, such as SCD (Figure 3(a)) [244]. Comparison of the flow behavior of whole blood samples from two patient populations, receiving (HU^+) and not-receiving (HU^-) HU treatment, revealed that HU^+ blood achieved higher average velocities within the channels. Moreover, HU^- samples caused greater levels of obstruction indicating the efficacy of the drug in SCD. Furthermore, interaction of TNF-α activated Human Lung Microvascular Endothelial Cells with TNF-α activated leukocytes resulted in a significant increase in the obstruction of flow as well as a decrease in flow rate. Despite their advantages in high-fidelity mimicking of microvasculature, endothelium-based models are not yet widely applicable to large numbers of patients, because of the requirement for labor-intensive and technically challenging tools, and a need for a constant supply of cultured endothelial cells.

5.3.2. Sickle blood rheology in microfluidic channels

The rheology of blood is dominated by RBC deformability, which is affected by changes in local flow conditions, such as high shear rate induced by vessel size [248]. In SCD, hemoglobin polymerization triggered by deoxygenation and subsequent decrease in cell deformability dramatically affects blood rheology (e.g. increase in viscosity), most specifically during microcirculation when a single RBC occupies the vessel. Moreover, low deformability dramatically decreases the shear thinning capability of blood, which is imperative to prevent any occlusion in postcapillary venules. The flowing suspension of soft cells (RBCs) that change their morphology and rheology relatively quickly upon deoxygenation is a key source for the

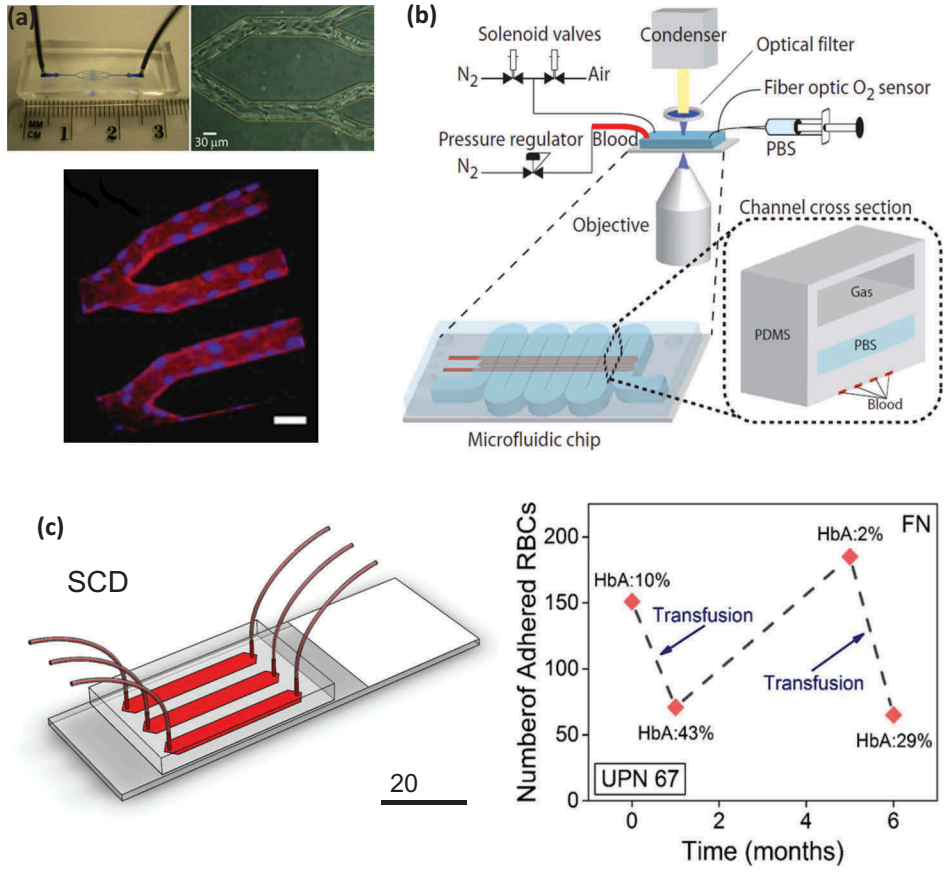

Figure 3. Emerging POC technologies for SCD monitoring. (a) An endothelialized microfluidic platform to model microvascular occlusion *in vitro* [247]. Brightfield microscopy shows the immobilized endothelial cells 48 hours after the cells are seeded in the microfluidic device. The cultured cells round up the rectangular cross-section mimicking *in vivo* blood vessel geometry and size scale. The endothelialized microfluidic platform can recapitulate altered blood flow and occlusive events in physiologically relevant flow conditions. The influence of hydroxyurea treatment of sickle cell patients on cell adhesion and subsequent microvascular occlusion is also observed using fluorescent microscopy. Reprinted with permission from J. Vis. Exp. (b) A microfluidic platform to probe blood rheology of SCD patient blood samples in physiologically relevant flow and oxygen tension conditions [243]. Blood flow conductance can be measured in microfluidic channels at the bottom layer, whereas the channel at the top layer are filled with N_2 to deoxygenate the blood through gas diffusion across the separating PDMS membrane. Reprinted with permission from AAAS. (c) SCD Biochip as a functional RBC adhesion assay to monitor SCD [34]. Adhesion of RBCs to endothelium and sub-endothelium associated proteins in physiologically relevant size scale and flow conditions are analyzed and associated with clinical course of SCD patients. SCD Biochip provides rapid, fully enclosed, standardized, and pre-processing-free analysis of RBC adhesion in whole SCD patient blood samples, enabling longitudinal studies.

vaso-occlusion heterogeneity in SCD. Two fundamental time scales in vaso-occlusion are the polymerization time (τ_p) and kinetic time (τ_k). The polymerization time scale is a function of HbS concentration whereas the kinetic time scale is a function of the pressure gradient, vessel diameter, and the effective viscosity of blood. The ratio of these two time scales governs the vaso-occlusion phenomenon [242].

Microfluidic systems provide several advantages in studying rheological properties of blood in SCD, including physiologically relevant size scale and flow conditions. In the SCD literature, microfluidic channels have been utilized to probe both individual sickle RBC rheology (occurs at a length scale of 10 μm) [240], and whole blood rheology along with vaso-occlusion dynamics (occurs at a length scale of 100 μm) [243]. Du et al. [240] developed a microfluidic system incorporating micropillar arrays forcing single RBCs to squeeze and deform through microgaps. Moreover, microfluidic channels were deoxygenated by gas diffusion through a PDMS membrane separating another layer of microchannels injected with N_2, allowing analysis of RBC deformability at hypoxic conditions.

To study the effect of deoxygenation on kinematic rheology and vaso-occlusion process, Higgins et al. [242] and Wood et al. [243] utilized a polydimethylsiloxane microfluidic channel, fabricated via photolithography and soft lithography. Blood in the microfluidic channels was deoxygenated by gas diffusion through a gas membrane separating a second layer of microchannels (Figure 3(b)) [242,243]. Decreased rate of conductance upon deoxygenation was analyzed in SCD patient blood samples and associated with clinical course of the corresponding patients [243]. Blood samples from benign SCD patients showed no drop of conductance upon deoxygenation, whereas blood samples from severe SCD patients showed large and rapid change in conductance following deoxygenation. Furthermore, the effect of HbF fraction on the rate of conductance was analyzed and it was shown that conductance rate of HU-treated patients, with increased HbF levels, was comparable to untreated severe samples.

5.3.3. RBC adhesion in microphysiological flow

Despite the remarkable insights about abnormal RBC adhesion in SCD that have been made, there remain gaps in knowledge about these complex adhesive interactions. Because of their technically challenging and labor-intensive nature, currently available techniques do not allow rapid measurement of diverse cellular adhesive events in the clinic simultaneously, e.g. abnormal RBC adhesion to fibronectin, laminin, and P-selectin. Further, there is no established 'atlas' of abnormal adhesive events, examined longitudinally and in a standardized manner in a large heterogeneous population of SCD patients (HbSS and HbSC, children, and adults) under a range of clinical circumstances and with and without treatment. Neither the topography of adhesive events for an individual patient, nor for the SCD population as a whole is known. Better knowledge of the nature and scope of abnormal adhesive events is critical to the goals of establishing associations with clinical outcomes and successfully identifying therapeutic targets in clinical trials.

Recently, a versatile microfluidic platform (SCD Biochip, Figure 3(c)) mimicking the dimensions of the postcapillary venule was developed for evaluation of RBC adhesion and deformability. Whole blood samples were flowed through microchannels functionalized with endothelium-associated proteins (FN and LN) at physiological shear stress levels (1–5 dyne/cm^2). The SCD Biochip allows rapid, standardized, and preprocessing-free analysis of whole blood samples, incorporating effects of both cellular and plasma components [27]. No work prior to SCD Biochip has analyzed cellular adhesion directly using whole blood (taken from patients being seen in clinic) at the micro-vasculature scale.

In SCD Biochip, among blood samples with different hemoglobin phenotypes, the number of adhered RBCs was significantly higher in HbSS blood samples compared to HbSC/Sβ^+-thalassemia blood samples or HbAA blood samples, both for FN and LN [34,249]. It was shown that HbS-containing RBCs were heterogeneous in deformability, adhesion strength, and in the number of adhesion sites [27,28]. Based on morphological and biophysical characteristics (i.e. deformation in flow), adhered HbS-containing RBCs were categorized as deformable or non-deformable. While deformable RBCs formed a single attachment site, non-deformable RBCs displayed multiple attachment points.

Moreover, RBC adhesion to FN- and LN-coated microchannels in SCD patient blood samples was analyzed, focusing on markers of hemolysis, including an elevated reticulocyte count and an elevated serum LDH [95,250,251] level, since hemolysis may play a critical role in VOC and organ damage in SCD [252]. Non-deformable HbS-containing RBCs adhered more strongly to FN under flow conditions, which may be the result of their multiple attachment sites. Deformable and non-deformable HbS-containing RBCs at a physiologically relevant shear stress (1 dyne/cm^2) [27] were quantified, and shown to have a significant association between adhered non-deformable RBCs (%) and serum LDH levels in adult SCD subjects. It is plausible that adherent non-deformable cells are causally associated with hemolysis, due to prolonged delay time in the vasculature [51,253]. These findings from the SCD Biochip support an association between deformability of adhered RBCs and hemolysis. In addition, RBC adhesion to FN or LN was significantly higher in subjects with low HbF (<8%) compared with those from subjects with high HbF (≥8%). This is in agreement with the known beneficial effect of fetal hemoglobin in SCD [254–256].

Using SCD Biochip, samples from over 100 SCD patients, obtained primarily during outpatient clinic visits, were examined. Furthermore, a small number of these individuals were examined longitudinally using SCD Biochip, performed ≥1 month apart [34]. For example, in UPN 67 (Unique Patient Identifier 67), monitored over 6 months, RBC adhesion to FN decreased after two episodes of transfusion (Figure 3(c)). Overall, these results obtained via SCD Biochip support the idea that changes in RBC adhesion can reflect clinical state and treatment response in SCD. However, these results need to be validated and expanded by increasing the number of subjects, expanding the longitudinal scope, and broadening the range of adhesive interactions that are studied.

6. Expert commentary

WHO has declared SCD as a public health priority [164,167,257]. More recently, American Society of Hematology, in partnership with other organizations, launched the 'Sickle Cell Disease Coalition' and called for action to improve the patient care in SCD [11]. Estimated 50–80% of the babies born with SCD in Africa die before the age of five due to lack of diagnosis followed by basic treatment and care [14,167]. Current laboratory-based screening platforms, although widely available in developed countries, are not feasible for operation at the POC in the resource-limited settings and in the developing world due to high infrastructure and operational costs, as well as the need for skilled operators. Moreover, typical turnaround time for screening test results in low-resource environment is too long (2–6 weeks). In such settings, it is critical to screen the baby, obtain the results, and inform the parents before they leave the testing center, otherwise, tested newborns may be lost to follow-up before the test results are available. Therefore, there is a need for simple, rapid, and mobile analyses of hemoglobin types in newborn blood with which to screen for hemoglobinopathies while the baby is still on-site. WHO estimates that more than 70% of SCD-related deaths are preventable with simple, cost-efficient interventions, such as early POC newborn screening followed by treatment and care [258]. Recently developed novel microtechnologies offer simple, rapid, and affordable screening of SCD and have the potential to facilitate universal screening in developing countries. Smart utilization and wide-spread application of these technologies may revolutionize SCD screening in resource-limited settings and dramatically decrease early mortality due to SCD.

Efficacy in both HU and transfusions is reflected in changes in Hb composition. Close monitoring of HbF can reflect adherence to treatment and appropriateness of HU dose. Current monitoring relies on laboratory tests causing significant delays in patient feedback and treatment optimization. A rapid POC test that quantifies HbS, HbF, and HbA, performed during a scheduled HU monitoring visit, would allow for personalized and precise dose adjustment. Importantly, communication of results immediately and directly to the patient (without the wait for lab results) may improve adherence to, and clinical efficacy of, HU therapy. Furthermore, it is known that real-time assessment of HbS and HbA levels prior to and during transfusions can improve the accuracy of treatment. There is no rapid real-time POC monitoring test for HbS and HbA levels in blood. A rapid POC test that quantifies HbS and HbA would help clinical management, monitor therapeutic response, improve clinical efficacy and safety, and may ultimately reduce the cost of care while improving patient satisfaction.

In SCD monitoring, current analyses performed at the clinic to evaluate the course of the disease only provide single-faceted information and largely lack complex cellular/tissue level response, which have restricted our ability to integrate complex biophysical phenomena into our understanding of SCD. Further, the relative contribution of red cell versus white cell interactions as well as endothelium response and rheological changes in individual patients with SCD, at baseline, with clinical disease activity, and following therapeutic interventions has not been studied in a sizable SCD population, using reproducible, unbiased, and standardized evaluations. Development of novel microtechnologies for measuring the impact of interventions on cell/tissue biophysical properties, such as RBC adhesion and deformability, may accelerate the development or adoption of new pharmaceutical and treatment approaches. Clinically feasible measurements are especially important as abnormal adhesive interactions emerge as therapeutic targets [116,136,137,259,260]. Indeed, we do not yet have the tools to tell us whether there is a difference amongst patients in degree or type of aberrant cellular adhesion; that is, though some patients show exaggerated RBC adhesion when compared with other patients, who show exaggerated WBC adhesion. Absent a clinical adhesion assay, current and future studies of anti-adhesion strategies in SCD, antibody- or drug-based, may underestimate the response of patients who have predominant abnormalities in one targeted pathway more than another, e.g. differential sensitivity to agents that target MAC-1 activation, BCAM/LU phosphorylation, or selectin binding. Alternatively, overall cellular adhesion of all cell types, and therefore sensitivity to a range of targeted agents, may increase in a patient who has striking abnormalities in a single cell type.

7. Five-year view and future perspectives

POC technologies for disease screening and patient monitoring are attracting increasing attention with each passing year due to improvements in their cost-effectiveness, speed, and user-friendly operation. Such technologies not only enable a better access to health care by millions of underserved people but also improve patients' quality of life by allowing quick result turnaround at home or at the clinical site.

7.1. Diagnosis

Some of the screening platforms for SCD described in this review are already in field trials in sub-Saharan Africa, whereas others have been validated in local hospitals in the United States. Therefore, it is plausible for these technologies to be in market in the next couple of years, especially in developing regions of the world including Africa, India, and Southeast Asia, where the need is more urgent and the demand is the greatest. High HbF levels have shown to be problematic for all screening platforms, decreasing sensitivity and specificity of the test. However, HbF levels continually decrease in babies over the course of the first 6 months after birth, and at 6 weeks, HbF level decreases to be around 60%. This timing is especially opportune for POC screening as this time frame aligns well with typical immunization schedules for children in Africa. When these devices are proved to be effective, reliable, and cost-efficient in the developing world, there is a great potential for their use in developed countries: (1) where the infrastructure for universal screening is not in place, as in most European Countries, and (2) where these cost-efficient diagnostic alternatives can replace bulky, complicated, and costly traditional techniques, as in the United States.

7.2. Monitoring

On the monitoring front, which is much more complicated in comparison to diagnosis and requires thorough validation of efficacy, early use of the novel microdevices might come in especially handy in the design of clinical trials for emerging therapies, most of which are performed in developed countries. Since all these advanced microtechnologies are based on mimicking human microvasculature features and biophysical properties, measurements and evaluations performed in these devices reflect cellular/tissue level responses, such as cell adhesion and vaso-occlusion. Therefore, such platforms can be utilized in therapeutic clinical trials targeting biophysical interactions in human vasculature. These platforms may be used for selective patient enrollment in trials and evaluating impact of therapeutics in enrolled patient blood samples. However, the long-term goal for all of these microtechnologies are patient monitoring at the POC, whether it can be home or clinic, either as a complimentary to other blood tests or a standalone monitoring modality. Successful development of such novel monitoring technologies can also provide a glimpse of hope for SCD patients in the developing world, where insufficient number of health-care providers and infrastructure impedes proper patient tracking and follow-ups.

Key Issues

- SCD affects 100,000 Americans and more than 14 million people globally, disproportionally in economically disadvantaged populations.
- SCD requires early diagnosis after birth and constant monitoring throughout the life-span of the patient.
- Currently available screening and monitoring tools are not feasible for operation at the point of care (POC), which increase the economic burden with hospital visits and costly tests, as well as exacerbate patients' quality of life.
- The emerging POC technologies offer cost-efficient, rapid, and reliable screening of SCD, which is essential to facilitate universal screening programs in developing countries.
- The emerging microfluidic platforms mimicking and modeling biophysical conditions of SCD microvasculature can reflect cellular/tissue level responses, providing unique capabilities for efficient, reliable, and convenient monitoring of SCD patients at clinical baseline, with disease, and under treatment.

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Y. Alapan, U. Gurkan, J. Little and R. Ung have a financial interest in Hemex Health (licensor of the HemeChip Technology) including licensed intellectual property, stock ownership, and consulting. A patent application pertaining to HemeChip and SCD Biochip technologies described this paper has been filed: Patent Cooperation Treaty Application (PCT/US2015/042907): "Biochips to Diagnose Hemoglobin Disorders and Monitor Blood Cells". The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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