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Review

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Paper Microfluidics for Point-of-Care Blood-Based Analysis and Diagnostics

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INTRODUCTION

Blood is the most intensely studied human biofluid and is used as the main indicator of the health status of an individual. Blood is a very complex fluid, containing a multiplicity of components, including cells and cell fragments (red and white blood cells, platelets), proteins, and many other organic molecules. Laboratory-based blood analysis (usually considered "gold standard") is a well-established procedure that provides a wealth of information about the concentrations of key blood components and enables medical personnel to establish diagnoses and treatments. It is typically performed in hospital or laboratory settings using a variety of methods and equipment,[1,2] with the emphasis on accuracy and measurement automation. To date, options for point-of-care blood analysis are limited and very low-cost options even more so. One analytical platform that contains the promise to fill this need utilizes paper-based microfluidic devices. Paper-based microfluidics has opened the door to point-of-care (POC) or point-of-use (POU) testing for various medical conditions (e.g., pregnancy testing, virus assays, etc.), and this research area has exhibited significant growth in the past few years, primarily because of its simplicity, fundamental low cost, and versatility. This article provides a review of the convergence of these two aspects into an exciting new field of paper microfluidics for POC/POU diagnostics using *whole blood*. Several recent reviews[3−10] have covered some subsets of the field, usually oriented toward a "detection" aspect of a specific medical condition. In this article, we aim to provide a broader view by starting with a brief overview of the properties of blood, followed by methods and instrumentation for conventional and novel blood sampling, and then by a review of the state-of-art of the field (from 2010, the beginning of the field, to the present) that discusses several important applications. While much work is being carried out on the use of plasma and serum for diagnostics, this article focuses on whole blood since it is the most likely format for POC/POU diagnostics, with the exception of those cases where a built-in plasma separation membrane is integrated into the platform.

Brief History of Blood. Prior to the discovery[11] of the heart pumping function and blood circulation by William Harvey (1578−1657), little accurate information was known about blood and its functionality within the human body. The ancient theory[12] of Hippocrates (∼460−370 BC) premised that human health was controlled by four "humors", blood, phlegm, black bile, and yellow bile, and being ill indicated the imbalance between those humors. On the basis of this theory, the treatment of illnesses by removing an amount of the excessive humor was developed,[12] including bloodletting, catharsis, diuresis, etc. After blood was declared to be the most dominant humor[13] by Galen (129−200 AD), bloodletting was widely adopted and continued to be used in disease treatment for nearly two thousand years until discredited in the late 19th century.[12] For diagnostic purposes, urine was considered the most important specimen from ancient times.[14] The examination of urine by visual inspection (uroscopy) was the oldest known diagnostic test using body fluids. It was known[14] to be first performed before 400 BC,

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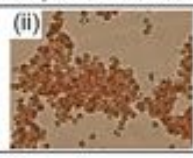
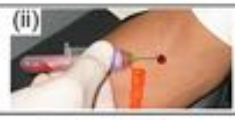
A Clumping Mechanism	Erythrocyte Aggregation	Hemagglutination	Coagulation
Cause	Natural phenomenon when blood under low shear forces or in stasis	Reaction between antigens in RBCs and matching antibodies in plasma	Activation of coagulation cascade
Clump Morphology	RBCs only; face-to-face stacking	RBCs only; no specific orientation	RBCs, platelets & fibrin; no specific orientation
Microscopic Image			
Major Determinants	Plasma protein composition, RBC morphology, blood hematocrit	Presence of matching antibody and antigen	Concentrations of clotting factors (e.g. prothrombin, fibrinogen, etc.); platelet function
Effect on Blood Rheology	Increases resistance of microcirculatory and venous blood flow	Increases blood viscosity	Liquid → solid gel; viscosity doubles at high shear rate, 6-10X at low shear rate
B Method for Blood Sampling	**Finger Prick**	**Venipuncture**	**Vacuum-Based Painless Sampling (TAP)**
Collection Tool	Lancet & capillary tube	Needle & vacutainer	Micro-needle & microfluidic reservoir
Collection Images			
Sampling Volume	A few drops (~8-10 μL per drop)	Typically 25 mL	100 μL
Maximum # of Tests per Sampling	Up to 13	Typically 200-300 (up to 1,000)	Unknown
Sample-to-Result Time	Immediate	Up to a few business days	Depends on instrumentation
Test Accuracy	Good (due to inherent variation in blood droplets)	Excellent (considered "gold standard")	Good (FDA-cleared)
Detection Range	Good (due to limitation of miniaturized instrument)	Excellent (considered "gold standard")	Depends on instrumentation
Major Risks	Pain, minor bruising	Bruising, hematoma, infection, etc.	Unknown

Figure 1. Blood clumping mechanisms and blood sampling methods. (A) Summary of erythrocyte aggregation, hemagglutination, and coagulation: clump morphology of (i) erythrocyte aggregation[38] (reprinted with permission from Lim, L. The effect of red blood cell aggregation and how it can be reversed. Copyright 2010 MedicLights Research Inc.), (ii) hemagglutination[39] [reproduced from Jiang, S.-Y.; Ma, Z.; Ramachandran, S. Evolutionary history and stress regulation of the lectin superfamily in higher plants. *BMC Evol. Biol.* **2010**, *10*, 79 (ref 39). Copyright 2010 BioMed Central Ltd.], and (iii) coagulation[40] (reprinted with permission from Fuzis. Copyright 2010. CC BY-SA 4.0 License. https://commons.wikimedia.org/wiki/File:Red_Blood_Cells_In_Clot.png). (B) Comparison between finger prick, venipuncture, and vacuum-based painless collection for blood sampling: (i) finger prick[41] [Sabidó, M.; Benzaken, A. S.; Rodrigues, Ê. J.; Mayaud, P. *Emerg. Infect. Dis.* **2009**, *15*, pp 647−649. (ref 41) DOI: 10.3201/eid1504.081293. Reproduced from public domain.]; (ii) venipuncture[42] (reprinted with permission from Bobjgalindo. Copyright 2004. GNU Free Documentation License. https://commons.wikimedia.org/wiki/File:Venipuncture.JPG); (iii) TAP device[43] (reprinted with permission from Seventh Sense Biosystems. Copyright 2018).

gained immense popularity during early medieval times, and became nearly universally adopted in European medicine by 1300 AD.[14] However, starting with the invention of the microscope in the 17th century, diagnostics using blood advanced rapidly. Blood cells were observed for the first time[14] under a microscope by Kircher (1602−1680), and blood transfusion experiments[14] were started by Lower (1631−1691). In the late 18th century and the 19th century, scientific discoveries emerged which gave a clearer and more complete understanding of the role and mechanisms of blood:[14] Hewson's (1739−1774) discovery of "coagulable lymph" (now known as fibrinogen) in plasma as the cause of coagulation, Dobson's discovery of sugar as the cause of sweetness in diabetic plasma in 1776, Denis's description of the separation of plasma proteins into albumins and globulins by salt precipitation in 1841, Vierordt's development of an accurate counting method of blood cells (now known as hemocytometry) in 1852, and Osler's descriptions of hemoglobin estimation in 1892 and of blood culture and blood agglutination in 1898. In the 20th century, blood banking and clinical chemistry of blood became established after several major events:[15] Landsteiner's discovery of human

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blood types in 1900 and the establishment of ABO grouping system in 1909, Conway and Cook's development of the first clinical laboratory method to measure blood ammonia in 1939, and Borgstrom's development of the clotting time test using whole blood in 1945.

Key Blood Components and Their Functions. Blood, the circulating portion of the extracellular fluid, is primarily responsible[16,17] for transporting nutrients and wastes from one part of the body to another, regulating body temperature, proper osmotic pressure, and pH of body fluids (~7.4), carrying the cells for defense against the invasion of pathogens, and protecting against blood loss in case of an injury to the vessel wall. Blood represents ~7% of an adult's total body weight. Whole blood consists of ~45% (v/v) cellular components: >99% of erythrocyte (red blood cells or RBCs) and <1% of combined leukocytes (white blood cells or WBCs) and platelets.[16,17] Plasma is the 55% (v/v) liquid component of blood. It contains 92% (w/w) of water, 7% (w/w) of dissolved proteins (e.g., albumin, globulins, fibrinogen, etc.), and 1% of combined dissolved organic molecules (e.g., amino acids, glucose, lipids, etc.), dissolved gases (e.g., O_2 and CO_2), ions (e.g., Na^+, K^+, Cl^-, Ca^{2+}, etc.), trace elements (e.g., copper, zinc, fluoride, iodine, etc.), and vitamins (e.g., A, B-complex, C, D, etc.).[16,17] Blood serum is obtained by removing the clotting proteins from plasma. All blood cells are derived from a single cell type called pluripotent hematopoietic stem cells[16] (blood stem cells), which can be found in bone marrow and peripheral blood. RBCs are primarily responsible for transporting O_2 from lungs to tissues and transporting CO_2 from tissues to lungs with the help of hemoglobin (Hb) within the cells. WBCs are primarily responsible for the body's immune responses to defend against foreign organisms, such as parasites, bacteria, and viruses. Platelets, the cellular fragments of megakaryocytes,[16] are primarily responsible for hemostasis during which insoluble blood clots are formed at the damaged vessel walls and eventually stops the bleeding. The packed volume percentage of RBCs, also known as hematocrit (Hct), is determined by centrifuging a small volume of blood drawn in a capillary tube. The heaviest RBCs precipitate to the bottom of the tube, followed by a "buffy coat" of lighter WBCs and platelets, and the plasma at the top. The normal range[18] of hematocrit is 40−54% for adult men and 36−48% for adult women.

Whole blood viscosity is heavily influenced by three RBC "clumping" mechanisms summarized in Figure 1A: erythrocyte aggregation, hemagglutination, and coagulation. Erythrocyte aggregation is the reversible and shear-dependent (2-D or 3-D) aggregation of RBCs that occurs in aqueous solutions with large plasma proteins or polymers.[19] The resulting face-to-face stacked RBC cluster ("rouleaux") is easily dispersed at high shear forces and reforms at low shear forces or at stasis.[19] Erythrocyte aggregation is primarily influenced by the composition of plasma proteins, the surface properties of RBCs, and the magnitude of shear force being experienced.[20] Aggregation is found to increase the resistance of microcirculatory blood flow as well as the venous blood flow *in vivo*.[21] Hemagglutination is the agglutination of RBCs occurring when the antigens on the RBC surface come in contact with the matching antibodies in plasma and thus trigger the amorphous clumping of RBCs.[22,23] Hemagglutination elevates blood viscosity and impedes the flow of RBCs due to the presence of cell clusters in blood. Blood coagulation is part of hemostasis that forms blood clots and grows fibrous tissues into the clot to close a ruptured site in blood vessels.[24,25] Coagulation can begin within the blood or with trauma to the vessel wall and surrounding tissues. The overall process is highly regulated by platelets and clotting factors.[24,25] The final product of coagulation is the blood clot that primarily consists of RBCs, platelets, and insoluble fibrin fibers that connect them together. The effect of coagulation on blood viscosity can be quite significant since the blood changes from a viscoelastic liquid to a viscoelastic solid (gel) during coagulation. Compared to the blood viscosity when coagulation is not activated, the peak viscosity of blood at coagulation gel point[26] can be 2× higher under high shear rate conditions and 6−10× higher at low shear rate.

Whole blood tests assist in a physician's decision during disease diagnosis and therapy monitoring by providing important information on patients' internal organ functions, cardiovascular risk factors, blood sugar levels, immune system wellness, and hormonal balance.[27] These tests primarily include complete blood count (CBC), metabolic panel, lipoprotein panel, blood coagulation test, and blood enzyme test. The CBC, one of the most routinely conducted laboratory tests in medicine, includes RBC count, hemoglobin, hematocrit, RBC indices, reticulocyte count, WBC count and differential, and platelet count.[28] The metabolic panel is typically conducted to assess patients' muscles, bones, and internal organs by measuring glucose, calcium, electrolytes, albumin, total protein, and biomarkers for kidney function (urea nitrogen, creatinine, etc.) and liver function (alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, etc.).[27] The lipoprotein panel, which includes total cholesterol, high-density and low-density lipoprotein cholesterol (known as "good" and "bad" cholesterol), and triglycerides, is typically conducted to evaluate the risk of cardiovascular disease and its management.[29] The blood coagulation test typically includes activated partial thromboplastin time (aPTT), prothrombin time (PT), and thrombin time (TT) to evaluate the intrinsic, extrinsic, and common pathways of the coagulation cascade,[30] as well as the platelet function test to evaluate the platelet phase of hemostasis.[31] The blood enzyme test typically measures cardiac enzymes including troponin T, creatine kinase, and myoglobin to diagnose heart attack or damage on heart muscles.[27]

Commercial POC Blood Analyzers. As opposed to centralized laboratory testing, point-of-care (POC) testing refers to the diagnostic testing that is carried out in a nonlaboratory, patient-centered environment (e.g., in-home, doctor's office) that does not require sample transportation or long turn-around time.[32,33] The market of POC testing has grown tremendously since its first introduction four decades ago.[33] The global POC testing market was valued at ~15.4 billion USD in 2015, with North America being the largest market (>43% share) and Asia Pacific being the most attractive regional market.[34] Besides the assessment of blood glucose and blood gas, POC diagnostic devices can also perform a wide variety of diagnostic tests including electrolytes, clinical chemistry, immunology, hematology, coagulation, lipids, cardiac markers, etc.[32,33] The utilization of those devices can be found in inpatient care (e.g., operating room, intensive care unit, emergency admission, etc.), outpatient care (e.g., house visits, nursing care, etc.), and patient self-monitoring (e.g., glucose monitoring and anticoagulant monitoring).[32] The accuracy and sensitivity of POC diagnostic devices are quite important, especially because therapeutic decisions (e.g.,

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insulin dose, anticoagulant dose) may be made by physicians based on those results. It was reported[35] that 53 out of 98 (54%) evaluated POC glucose meters met the requirement[36] of ISO 15197:2003 (≥95% of the meter results fall within ±15 mg/dL of the laboratory results at concentrations <75 mg/dL and within ±20% at concentrations ≥75 mg/dL) and 31 out of 98 (32%) evaluated POC glucose meters met the requirement[37] of ISO 15197:2013 (≥95% of the meter results fall within ±15 mg/dL of the laboratory results at concentrations <100 mg/dL and within ±15% at concentrations ≥100 mg/dL). Regarding POC coagulometers, it was reported[44] that the precision and accuracy of those devices are generally comparable to the laboratory instruments and thus are considered sufficient for clinical testing.

Conventional and Novel Blood Sampling Methods. Blood sampling methods and techniques are important for reliable and accurate clinical blood tests. Finger prick and venipuncture are two common sampling methods for blood tests, with finger prick mainly used for POC testing and venipuncture for laboratory testing. Finger prick sampling typically requires a lancet and a capillary collection tube, and a few droplets of capillary blood (~8−10 μL per droplet) are collected at the punctured site on a fingertip. It was reported[45] that the maximum number of tests that can be performed with one-time finger prick sampling is 13 when using an i-STAT POC blood analyzer from Abbott Point of Care Inc. Since POC blood analyzers are primarily used for finger prick samples, the test-to-result time is quite rapid (usually a few seconds to a few minutes), and the detection range is wide enough for a quick diagnosis or initial screening. The inherent variation of successive blood droplets can be an issue because their compositions can vary significantly and thus reduce the accuracy of the blood test. It was reported[46] that the coefficient of variation (CV) of hemoglobin values measured by a POC hemoglobinometer is up to 5× higher using finger prick blood than using venous blood. The authors also concluded[46] that multiple finger prick drops should be used (~4−8 drops, ≤80 μL) in the hemoglobinometer to reduce fluctuations of test results. Laboratory testing using venous blood has been the gold standard of blood tests due to its particularly wide component detection range and very-high test accuracy, due to the use of sophisticated laboratory instruments and the consistent test results produced by venous blood. Venipuncture sampling requires a syringe and a few vacutainers with sealed vacuum and anticoagulant. Typically, ~25 mL of venous blood is collected in one sampling. It was reported[45] that 200−300 routine blood tests can be performed in a clinical laboratory using venous blood, and the number of tests can rise to ~1000 if including nonroutine tests. However, the test-to-result time is usually from a few hours to a few days, which is much longer compared to POC blood testing with finger prick blood. Also, the associated risks[47] of venipuncture are much higher than finger prick, including bruising (reported[48] to occur in 12.3% of venipunctures), hematoma, nerve damage, infection, excessive bleeding, etc.

Recently, significant attention is being devoted to the development of various techniques for minimizing the blood sample volume needed for testing and the pain and discomfort associated with drawing blood. Vacuum-based blood extraction is one approach being pursued. For example, the touch activated phlebotomy TAP device[49] from Seventh Sense Biosystems is an FDA-cleared, single-use blood sampling device that utilizes a circular array of stainless-steel microneedles and prestored vacuum suction to collect capillary blood and transport and store the blood sample in microfluidic components containing anticoagulant. After adhering the device on a desired sampling location, the skin is punctured upon pushing an actuation button, and 100 μL of capillary blood is collected into the device within a few minutes. After collection, the device can be sent to a testing location to perform necessary blood tests. The Genteel Lancing Device[50] from Genteel LLC is a novel vacuum-based lancet that is FDA-cleared to be used in patient self-testing at home for blood glucose monitoring with minimal pain and discomfort. Due to its apparently very small sampling volume, applications beyond glucose testing are not currently available. Figure 1B shows a brief comparison between the above-mentioned three blood sampling methods: finger prick, venipuncture, and vacuum-based painless blood collection.

Emergence of Paper-Based Blood Tests. Paper-based diagnostic devices have gained increasing popularity due to their ability to conduct rapid and low-cost diagnostic tests in a nonlaboratory and patient-friendly environment including in a physician's office, in an ambulance, at home, and in a remote site.[51] Paper is a very attractive material for bioanalytical tests because it is abundant, cheap, biocompatible, and naturally hydrophilic.[52] Fluid flow in paper does not require external force but can rely on capillary action, which is caused by the intermolecular force between the fluid and the porous cellulose matrix.[53] Paper-based diagnostic devices are simple to use, disposable, low cost, and environmentally friendly.[54] The disposable nature of paper and paper-derived materials reduces the risk of cross contamination, and the low cost of these materials allow broader application and more frequent testing. The first immunoassay was reported by Berson and Yalow in 1959, who later received the Nobel Prize in Medicine in 1977 for this contribution.[55] Lateral flow immunoassay (LFIA), which is one type of paper-based assay, went through significant technological improvement in the 1970s. The first commercial lateral flow immunoassay, the pregnancy test kit, was introduced to the consumer's market in the late 1980s[56] and has become a staple of consumer-oriented assays. The assay measured the level of a hormone that is produced in the body during pregnancy (hCG, human chorionic gonadotropin). In diagnostic applications, various biological samples have been reported[57] to be tested in LFIAs (e.g., sweat, saliva, serum, plasma, and whole blood) to qualitatively or quantitatively detect specific antigens and antibodies, as well as products of gene amplification. LFIAs are also reported[57] to be utilized in many other applications, including veterinary medicine, quality control, product safety, food production, and environmental health and safety to identify animal diseases, pathogens, chemicals, toxins, and water pollutants.

The introduction[58,59] of microfluidic paper-based analytical devices (μPADs) by the Whitesides group utilizing simple fabrication methods, multiple chemical reactions between analytes and stored reagents, and simple visual colorimetric detection opened new possibilities for very low cost but sufficiently accurate diagnostics tests that can be easily accessed by most of the global population. Paper-based diagnostic devices utilize lateral and vertical fluid flows in paper and paper-like materials to screen or diagnose various medical conditions relevant to human[60] and animal[61] health and to monitor environmental pollution[62] and food safety.[63] Initially, being mostly qualitative or semiquantitative, recently developed paper-based POC diagnostic tests are capable of

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Table 1. Summary of Whole Blood Tests Using Paper-Based Diagnostic Devices

application	principal	detection	paper type	POC	ref.
plasma separation	RBC agglutination	N/A	chromatography	yes	67
	RBC aggregation	N/A	filter	yes	69, 70
	physical separation	N/A	chromatography + plasma separation	yes	68
blood grouping	RBC agglutination	colorimetric (distance-based)	chromatography; Kleenex towel; filter	yes	73−79
hematocrit (Hct)	viscosity-based RBC flow in porous media	colorimetric (distance-based)	chromatography	yes	81
hemoglobin (Hb)	hemolysis	colorimetric (color-based)	chromatography	yes	84
	hemolysis + absorption	spectrophotometric	chromatography	yes	85
sickle cell disease	hemolysis + sickle Hb polymerization	colorimetric (distance-based)	chromatography	yes	87−90
	hemolysis + immunoassay	colorimetric (color-based)	cellulose + NC + fiber glass	yes	91, 92
blood coagulation	viscosity-based RBC flow in porous media	colorimetric (distance-based)	cellulose + NC + fiber glass	yes	107−111
	quantum dot emission	fluorescent	filter	yes	113
glucose	enzymatic reaction	electrochemical	chromatography; chromatography + plasma separation	yes	115−117
		SERS	NC	no	118
biomarkers	enzymatic reaction (liver function markers)	colorimetric (color-based)	chromatography + plasma separation	yes	121
	immunoassay (cancer markers)	chemiluminescent	chromatography + blotting	no	123
	immunoassay (C-reactive protein)	colorimetric (color-based)	cellulose + NC + fiber glass + plasma separation	yes	125
	aptamer binding (mucin-1)	SERS	filter	no	127
malaria	immunoassay (malaria and dengue fever)	colorimetric (color-based)	chromatography + plasma separation + blotting	yes	130
	DNA amplification (malaria only)	fluorescent	filter + fiber glass	no	131
therapy monitoring	potentiometric cell (Li level)	electrochemical	filter	yes	133
	enzymatic reaction (phenylalanine level)	colorimetric (color-based)	plasma separation + fiber glass	yes	135

performing quantitative analysis of single or multiple target analytes with the aid of digital image analysis or a conventional laboratory spectrometer.[64] With the emergence of smartphone-based sensing techniques that can perform automated image analysis of colorimetric signals and display the test result to the user, true quantitative POC diagnostics using paper-based devices may become a reality.

Despite being readily available and easy to extract, whole blood has not been the preferred test sample compared to blood plasma or serum for paper-based POC diagnostic devices using colorimetric or optical detection since the intense red color produced by hemoglobin within RBCs strongly interferes with the signal from the target analyte.[65] Conventionally, RBCs are removed from whole blood by centrifugation to address this problem, which undesirably increases the test complexity and the sample preparation time. With the recent development of high-performance plasma separation membranes, on-chip RBC filtration from whole blood has become a significant step toward simple and low-cost whole blood analysis, expanding the applications of paper-based POC diagnostics beyond blood grouping and hematology and into the quantitative detection of metabolites, biomarkers, and pathogens. In this article, the various applications of paper-based POC diagnostic devices in whole blood testing are reviewed. Therefore, the reported studies must use whole blood as test sample on paper-based POC devices, with or without on-chip plasma separation. On the basis of a literature search, the related applications that have been reported are plasma separation, blood grouping, hematology (hematocrit and hemoglobin), sickle cell disease, blood coagulation, biomarkers (liver function biomarkers, cancer biomarkers, C-reactive protein, and mucin-1), malaria (with or without simultaneous detection of dengue fever), and therapy monitoring (Li therapy and PKU therapy).

In addition to cellulose (conventional paper), quite a few paper-like materials are widely utilized in the diagnostic devices, including nitrocellulose (NC), polysulfone (PSF), polyvinylidene difluoride (PVDF), nylon, and fiber glass. Cellulose is most commonly used as the absorbent pad for sample dispensing and as the medium for colorimetric chemical reactions. NC is typically employed as the "analytical" membrane for bioaffinity reactions between matching antibody and antigen in LFIAs. Fiber glass is frequently used for plasma separation from whole blood and for conjugate pad to store and release conjugated antibodies in LFIAs. PSF membrane is mainly used for plasma separation from whole blood, and PVDF and nylon membrane are mainly used for protein blotting. Table 1 summarizes the current applications of paper-based POC diagnostic devices in whole blood testing, as well as the corresponding experimental principles, detection methods, utilized paper types, and qualification as POC. The utilization of laboratory instruments (e.g., various spectrometers) disqualifies the study from being considered POC at its current reported stage.

APPLICATIONS OF PAPER-BASED DEVICES IN WHOLE BLOOD DIAGNOSTICS

Plasma Separation. As indicated in the Introduction, blood plasma is the noncellular part of whole blood and liquid medium very rich in proteins and organic molecules. Plasma is one of the most convenient sources of circulating biomarkers and is widely used for detecting various diseases including cancer, Alzheimer's, and sepsis.[65] Plasma is often chosen as the

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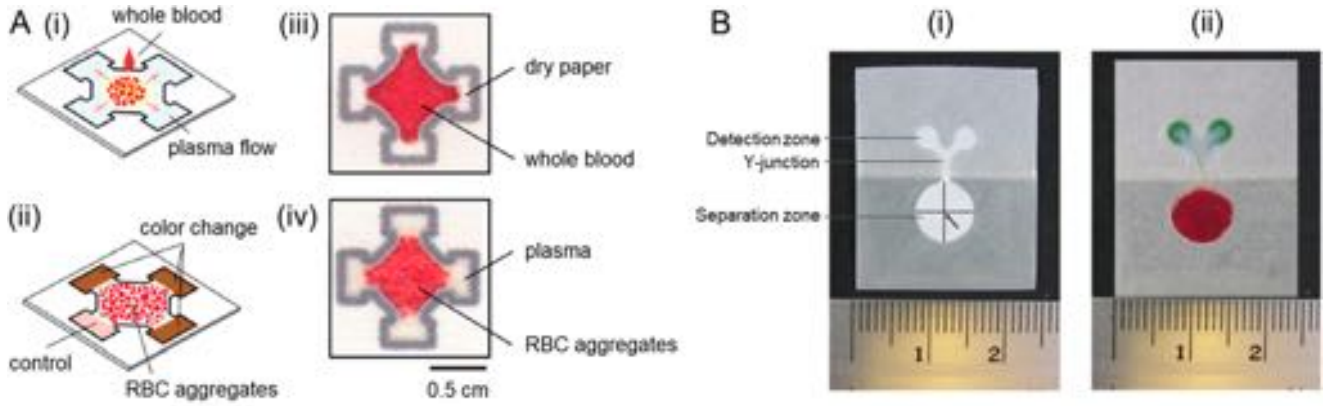

Figure 2. Plasma separation using paper-based devices. (A) Separation based on hemagglutination: (i, ii) illustrations of plasma separation on paper device; (iii, iv) photos of RBCs agglutinated in the center of paper device and plasma collected on the periphery. Reproduced from Yang, X.; Forouzan, O.; Brown, T. P.; Shevkoplyas, S. S. *Lab Chip* **2012**, *12*, 274−280 (ref 67), with the permission of The Royal Society of Chemistry. (B) Separation based on physical filtration: (i) photo of paper device with separation and detection zones indicated; (ii) photo of RBCs trapped in the separation zone and plasma flowed to the detection zone and reacted with the reagent of the protein colorimetric assay. Reproduced from Songjaroen, T.; Dungchai, W.; Chailapakul, O.; Henry, C. S.; Laiwattanapaisal, W. *Lab Chip* **2012**, *12*, 3392−3398 (ref 68), with the permission of The Royal Society of Chemistry.

preferred sample over whole blood for bench and POC testing due to the interference of hemoglobin from RBCs as well as the release of nucleic acids and other cellular components from blood cells.[65] Plasma is traditionally separated from whole blood using a centrifuge, which spins at high centrifugal force and precipitates cellular components in whole blood.[66] In paper-based POC devices, on-chip plasma separation is generally achieved through filtration with or without the assistance of other reagents.

Yang et al. reported[67] a single-step paper-based assay for hemagglutination-based plasma separation from whole blood and associated colorimetric detection of glucose concentration in the plasma. The device was fabricated by printing wax patterns on chromatography paper and then creating hydrophobic barriers by melting the wax. RBC agglutinating antibodies were spotted in the center of the device (separation zone), and the reagents for the colorimetric assay were spotted in the periphery of the device (readout zone). During the test, a droplet of whole blood was applied onto the separation zone. Once the RBCs were agglutinated, only plasma was wicked to the readout zone where it reacted with the reagents of the colorimetric assay. The fabrication and operation of this paper-based plasma separation approach is shown in Figure 2A. Songjaroen et al. developed[68] a single-step paper-based assay that was capable of plasma separation from whole blood and colorimetric detection of plasma protein concentration without agglutinating antibodies. The device was fabricated by the wax dipping method using an overlapped plasma separation membrane for the separation zone and chromatography paper for the detection zone. Upon the application of blood sample on the separation zone, both RBCs and WBCs were trapped and only plasma wicked onto the detection zone where colorimetric detection of proteins was performed, as illustrated in Figure 2B. The device was proven to be functional with 24−55% hematocrit without dilution and capable of separating plasma within 2 min after applying 15−22 μL of whole blood. The protein assay was shown to be comparable to the conventional method and exhibit good reproducibility for within-day (CV = 2.62%, n = 10) and between-day tests (CV = 5.84%, n = 30). Nilghaz and Shen developed[69] a salt-functionalized paper device for ultralow-cost single-step plasma separation from whole blood and colorimetric detection of glucose concentration in the plasma. The device was fabricated on filter paper using the wax printing method and was functionalized by applying 0.5 μL of 0.68 M saline solution. When whole blood was in contact with salt-impregnated paper, osmotic pressure generated around RBCs led to their deformation into deflated disks. This caused strong aggregation of RBCs that was sufficient to separate RBCs chromatographically from the plasma front. Kar et al. reported[70] a preliminary concept to separate plasma from whole blood with the aid of PBS solution. An H-shaped channel device (2 inputs, common channel, 2 outputs) was fabricated on filter paper. Whole blood and PBS solution were dispensed at the two inputs. During simultaneous capillary flow through the linear region of the device, the lighter molecules suspended in the bloodstream diffuse into the PBS stream. This results in RBC-rich and plasma-rich components being separately collected in each of the two outputs of the device.

Blood Grouping. Blood grouping, also known as blood typing, is a test that examines a person's blood type. Receiving blood that is not compatible with a person's own blood type can cause serious incompatibility reactions which include disseminated intravascular coagulation, shock, acute renal failure, and even death.[22] Blood grouping primarily consists of two parallel tests: ABO grouping and Rhesus grouping. ABO grouping determines if a person has type A, B, AB, or O blood. Rhesus grouping determines if a person has Rh positive (+) or Rh negative (−) blood. ABO blood group is determined by whether or not antigen A and/or antigen B is present in a person's RBCs.[22,71] A person's plasma contains antibodies that do not recognize the antigens in his/her own blood cells.[22,71] If a person with type A blood receives type B blood by transfusion, anti-B antibodies in his/her plasma will attack the newly received type B RBCs until they are destroyed. Note that transfusions use packed RBCs that are separated from the plasma of the donor. Individuals with type O blood are universal donors (absence of both antigen A and antigen B in RBCs) and people with type AB blood are universal receivers (absence of both anti-A and anti-B antibodies in plasma). Rhesus blood group is related to the presence of antigen D. Rh + type indicates antigen D is present on RBCs while Rh− type

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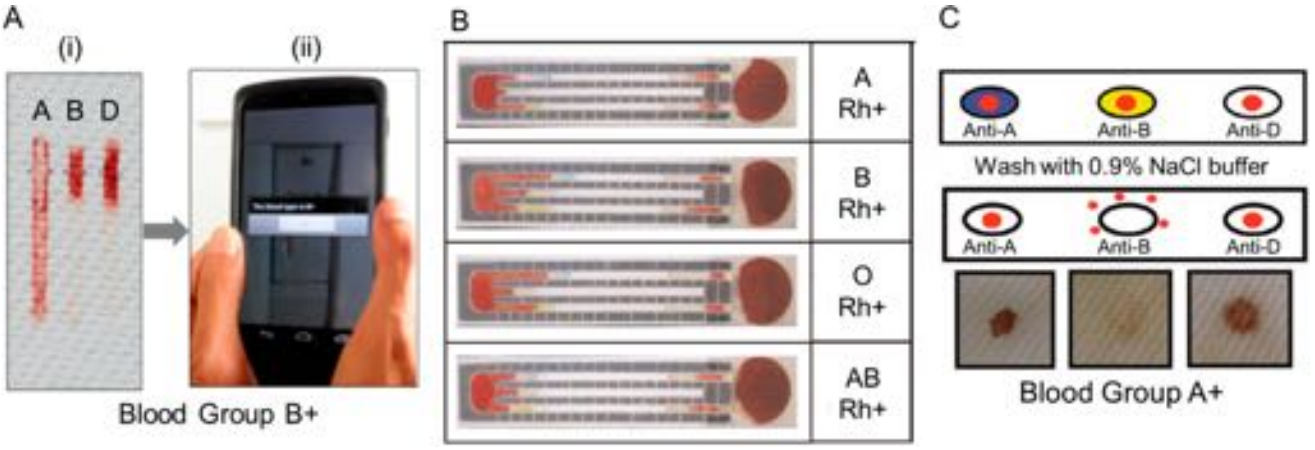

Figure 3. Blood grouping using paper-based devices. (A) Barcode-like paper device with designated phone app for blood grouping: (i) photo of paper device when applying B+ blood—blood with matching antibodies traveling a shorter distance and vice versa; (ii) reading the test result using a smartphone and app. Reproduced from Guan, L.; Tian, J.; Cao, R.; Li, M.; Cai, Z.; Shen, W. *Anal. Chem.* **2014**, *86*, 11362−11367 (ref 74). Copyright 2014 American Chemical Society. (B) Simultaneous forward and reverse blood grouping: left side, forward grouping (using diluted blood); right side, reverse grouping (using separated plasma). Reprinted from *Biosens. Bioelectron.*, Vol. *67*, Noiphung, J.; Talalak, K.; Hongwarittorrn, I.; Pupinyo, N.; Thirabowonkitphithan, P.; Laiwattanapaisal, W. A novel paper-based assay for the simultaneous determination of Rh typing and forward and reverse ABO blood groups, pp. 485−489 (ref 75). Copyright 2015, with permission from Elsevier. (C) Spot-based blood grouping when applying A+ blood—blood with matching antibodies left a dark spot and vice versa. Reproduced from Al-Tamimi, M.; Shen, W.; Zeineddine, R.; Tran, H.; Garnier, G. *Anal. Chem.* **2012**, *84*, 1661−1668 (ref 76). Copyright 2012 American Chemical Society.

indicates antigen D is absent. Similar to the mechanism of ABO blood group, individuals with Rh− blood cannot receive RBCs from a donor with Rh+ blood and vice versa. Since not all blood types are compatible, the blood grouping test is essential before a person can receive a blood transfusion or donate his/her blood.

Hemagglutination is the primary mechanism behind blood grouping tests that occurs when the antigens on RBC membranes come in contact with the matching antibodies, triggering the agglutination of RBCs.[22,23] RBCs in agglutinated blood clump together and exhibit higher viscosity than RBCs in nonagglutinated blood. This gives a visual indicator of the matching RBCs during forward grouping or matching plasma during reverse grouping, and the blood group of a person can be determined subsequently. During ABO grouping, both forward grouping and reverse grouping are conducted to ensure the test accuracy. Forward grouping, also called RBC grouping, is mixing blood samples with each of anti-A, anti-B, and anti-D antibodies, while reverse grouping, also called plasma (or serum) grouping, involves mixing plasma (or serum) samples with each of type A and type B RBCs.[72]

Khan et al. investigated[73] the transport properties of agglutinated and nonagglutinated blood in chromatography paper to develop paper-based blood grouping tests. Applying samples of premixed anticoagulated blood with various concentrations of matching antibodies resulted in different travel distances of blood samples on blotting paper. Blood samples with lower concentrations of antibodies traveled a longer distance and vice versa, and the blood sample with no antibodies traveled the longest distance. On the basis of this phenomenon, paper-based blood grouping was also explored using RBC antibodies immobilized on paper strips which would then react with antigens present in whole blood samples. Guan et al. expanded the idea that agglutinated RBCs and plasma flow at different speed in paper and developed[74] a barcode-like paper device for simultaneous forward ABO and Rhesus blood grouping using a cell phone. Three hydrophilic bar channels were printed on Kleenex paper towel and treated with anti-A, anti-B, and anti-D antibodies. After applying blood samples to the channels and allowing 30 s for reaction, blood type could be easily identified by evaluating RBC eluting length in each bar channel using PBS buffer solution, as shown in Figure 3A. A smartphone app was developed to recognize and scan the bar channels, calculate the eluting length, and report the blood grouping results to the users. Noiphung et al. also adopted[75] the approach of RBC agglutination using immobilized antibodies on a paper strip and reported a paper-based assay that performs simultaneous forward and reverse ABO and Rhesus grouping. The device had three gridded channels for forward grouping, two of which were also used for reverse grouping; see Figure 3B. The device was fabricated using chromatography paper and plasma separation membrane with combined wax printing and wax dipping methods. In forward grouping, blood sample diluted with 1:2 ratio was applied at the forward side of the device (chromatography paper) and flowed into the three channels prespotted with anti-A, anti-B, and anti-D antibodies in each channel. In reverse grouping, undiluted blood was applied at the reverse side of the device (plasma separation membrane) and plasma flowed into the two channels prespotted with A-type RBCs and B-type RBCs. Distances of RBC and plasma transport in the wax channels were measured, and the ratio of the two distances were calculated to determine the blood type. The device was capable of detecting blood type A, B, AB, O, and Rh+ with the accuracy of 92%, 85%, 89%, 93%, and 96%, respectively. In addition to flow-based blood grouping, spot-based blood grouping was also investigated. Al-Tamimi et al. developed[76] a paper-based assay that performed simultaneous forward ABO and Rhesus blood grouping by applying three droplets of blood on three separate locations on the Kleenex towel paper or filter paper where anti-A, anti-B, and anti-D antibodies were prespotted. After eluting blood spots in a thin film chromatography tank using saline solution, nonagglutinated RBCs were easily eluted by the solution, leaving a clear trace of RBCs in the wicking path, whereas agglutinated RBCs stayed fixed on the paper with no visible trace in the wicking path; see

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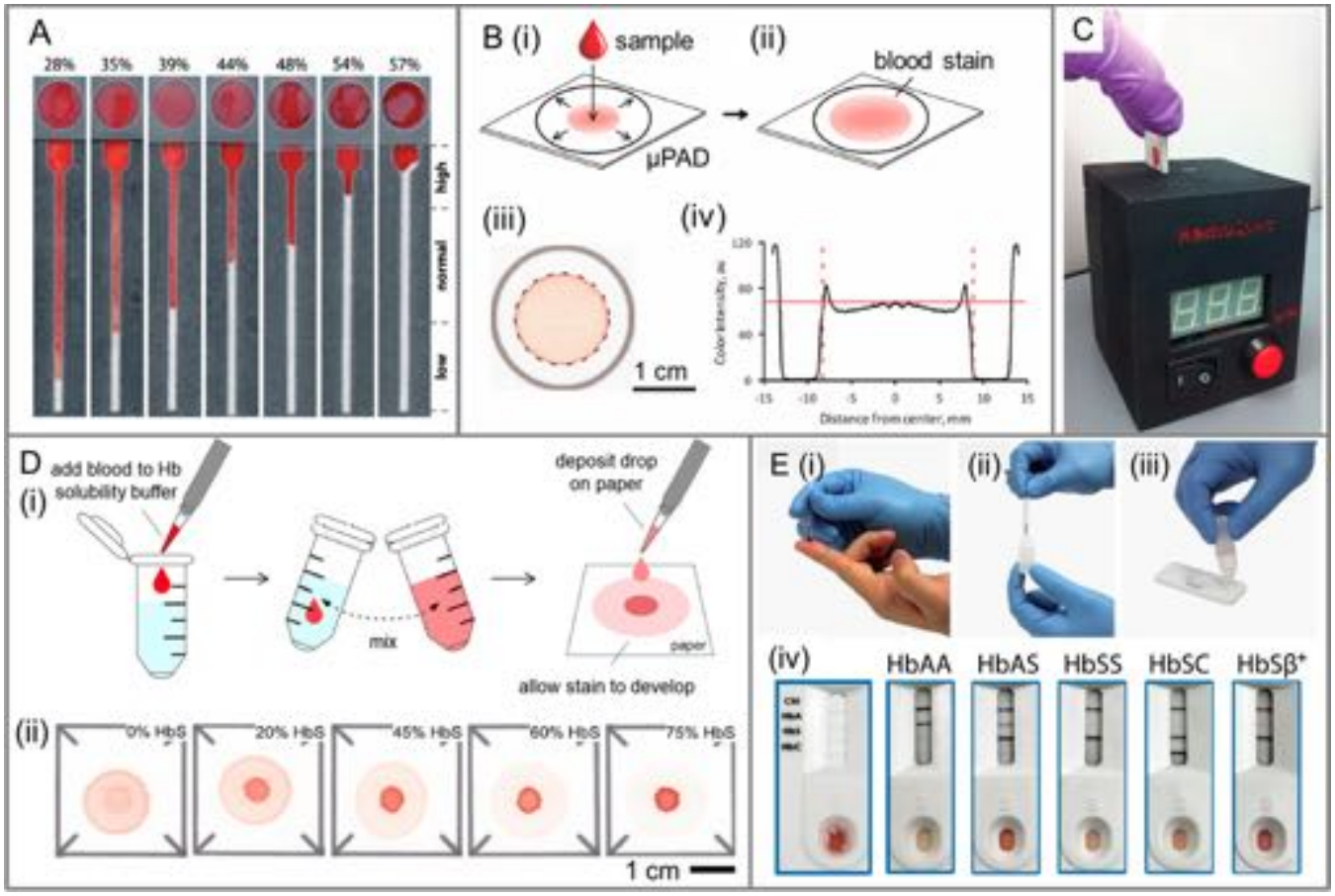

Figure 4. Hematology tests (hematocrit and hemoglobin) and sickle cell disease detection using paper-based devices. (A) Hematocrit measurement on a paper device; higher hematocrit resulted in shorter blood travel distance and vice versa. Reproduced from Berry, S. B.; Fernandes, S. C.; Rajaratnam, A.; DeChiara, N. S.; Mace, C. R. *Lab Chip* **2016**, *16*, 3689−3694 (ref 81), with the permission of The Royal Society of Chemistry. (B) Hemoglobin measurement on a paper device using digital image analysis: (i, ii) illustrations of test procedures; (iii) photo of a paper device after the formation of blood stain; (iv) plot of red color intensity vs distance from the center when digitally analyzing blood stain image from (iii). Xiaoxi Yang, Nathaniel Z. Piety, Seth M. Vignes, Melody S. Benton, Julie Kanter, Sergey S. Shevkoplyas. Simple Paper-Based Test for Measuring Blood Hemoglobin Concentration in Resource-Limited Settings. *Clinical Chemistry* Oct **2013**, *59* (10) 1506−1513 (ref 84); DOI: 10.1373/clinchem.2013.204701. Reproduced with permission from the American Association for Clinical Chemistry. (C) Hemoglobin measurement on a paper device using the spectrophotometric method; disposable paper device (housed in a 3D printed reusable frame) being inserted into a custom-made hand-held spectrometer. Reproduced from Bond, M.; Elguea, C.; Yan, J. S.; Pawlowski, M.; Williams, J.; Wahed, A.; Oden, M.; Tkaczyk, T. S.; Richards-Kortum, R. *Lab Chip* **2013**, *13*, 2381−2388 (ref 85), with the permission of The Royal Society of Chemistry. (D) Sickle hemoglobin detection on a paper device using digital image analysis: (i) illustrations of test procedures; (ii) photos of blood stains with various percentages of sickle hemoglobin (0, 20%, 45%, 60%, and 75%). Reproduced from A rapid paper-based test for quantifying sickle hemoglobin in blood samples from patients with sickle cell disease, Piety, N. Z.; Yang, X.; Lezzar, D.; George, A.; Shevkoplyas, S. S. *Am. J. Hematol.*, Vol. *90*, Issue 6 (ref 88). Copyright 2015 Wiley. (E) Sickle hemoglobin detection on a paper device using lateral flow immunoassay: (i−iii) photos of test procedures; (iv) test results showing the paper device capable of detecting HbAA, HbAS, HbSS, HbSC, and HbSβ^+ genotypes. Reproduced from Kanter, J.; Telen, M. J.; Hoppe, C.; Roberts, C. L.; Kim, J. S., Yang, X. Validation of a novel point of care testing device for sickle cell disease. *BMC Med.* **2015**, *13*, 225 (ref 91). Copyright 2015 BioMed Central Ltd.

Figure 3C. This assay demonstrated excellent reproducibility by being able to detect the same blood types when using 26 blood samples of various blood types on two different days. Li et al. reported[77] a paper-based blood grouping device that displayed the test results in text. Inspired by the novel "Harry Potter and the Chamber of Secrets", they printed symbols "A", "B", "O", "X", and "+" on paper with an inkjet printer using anti-A, anti-B, and anti-D antibody solutions as well as nonbioactive water-insoluble ink. The "A" and "B" symbols were printed with anti-A and anti-B antibodies, respectively; "|" symbol (part of "+") was printed with anti-D antibodies. "O" and "-" (part of "+") symbols were printed with water-insoluble ink, and "X" symbol which overwrites "O" was printed with an equal mixture of anti-A and anti-B antibodies. As a result, the paper device was able to display ABO and Rhesus blood groups in unambiguous written text without involving extra calculation or analysis steps.

Complementing the above application-driven approaches, researchers also investigated fundamentals of the interaction of agglutinating blood and the properties of cellulose-based materials. Su et al. reported[78] the effect of various paper properties (fiber composition, basis weight, density, and porosity) on the visualization of test results during blood grouping. Commercial (Kleenex) and experimental (blotting paper and filter paper) cellulose-based materials were tested for their ability to distinguish agglutinated RBCs from non-agglutinated RBCs. Ten μL of anti-A, anti-B, and anti-D antibody solutions were spotted on the paper followed by 3 μL

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of whole blood. After 30 s of reaction, the paper was eluded in saline solution, dried at room temperature for 10 min, and then placed into a scanner to capture the image of the blood pattern. Through image analysis with ImageJ, it was concluded that thin and porous papers were able to better distinguish between agglutinated and nonagglutinated RBCs. The separation between a positive test and a negative test increased with increased pore size and with reduced density and thickness. Li et al. studied[79] the mechanisms of RBC transport and immobilization on a paper-based blood grouping device using confocal microscopy. The morphologies of a free, nonagglutinated RBC spot and an immobilized, agglutinated RBC spot were compared on a paper device, as well as the chromatographic elution patterns of nonagglutinated RBCs and agglutinated RBCs. This work provides an insight into the flow behavior of agglutinated and nonagglutinated RBCs within the porous network of cellulose fibers.

Hematology and Sickle Cell Disease. Hematocrit (Hct) is the packed volume percentage of RBCs in the whole blood. Hct is typically determined[18] by centrifuging a small volume of blood drawn in a capillary tube or by counting the number of RBCs and multiplying it by mean cell volume (MCV) using an automated cell counter. The normal range[18] of hematocrit is 40−54% for adult men and 36−48% for adult women. Abnormal Hct values assist with a physician's decision on diagnosing anemia, infection, WBC disorder such as leukemia or lymphoma, vitamin or mineral deficiencies, recent or long-term blood loss, dehydration, polycythemia vera, and lung or heart disease.[80] Berry et al. reported[81] a simple and low-cost paper-based microfluidic device for Hct measurement using wax-patterned chromatography paper. The device consists of a top lamination sheet, a sample adding layer (made of chromatography paper) that prevents WBCs from entering the channel, a cutter-patterned double-sided adhesive, a wax-patterned microfluidic channel (made of chromatography paper) pretreated with 4.5 mM EDTA and 5 mM NaCl solutions for blood flow enhancement, and a bottom lamination sheet. Upon dispensing whole blood sample, WBCs were trapped in the sample adding layer, while RBCs and plasma traveled through it and onto the microfluidic channel (see illustration in Figure 4A). Blood with lower (higher) Hct blood traveled a longer (shorter) distance after a fixed period of time. The resulting device can detect Hct ranging from ∼30% to 55% in 30 min after sample application with high linearity between blood travel distance and Hct value within the detection range (R^2 = 0.991) and with good reproducibility (mean ± SD = 20.1 ± 3.2 mm and CV = 16%, when Hct = 42% and n = 20).

Hemoglobin (Hb) is the protein found in RBCs that is primarily responsible for delivering oxygen to cells and tissues. Each normal adult hemoglobin molecule consists of four subunits:[82] two identical α polypeptide chains and two identical β polypeptide chains. An adequate level of hemoglobin must be maintained in order to ensure sufficient oxygenation of tissues. The normal[18] hemoglobin levels for adult men and women are 14−18 and 12−16 g/dL, respectively. Values <7 or >21 g/dL are considered critical.[83] A decreased hemoglobin level is an indication of anemia, which can be caused by blood loss (hemorrhage), abnormal destruction of RBCs (hemolysis), or reduced RBC production due to bone marrow failure.[83] Traditionally, hemoglobin determination is conducted by an automated cell counter using a tube of well-mixed EDTA-anticoagulated blood that is filled to a predetermined level[18] (usually a few milliliters). Paper-based hemoglobin detection is mainly based on colorimetric detection followed by image analysis or spectrophotometric detection using a hand-held reader. Yang et al. developed[84] a simple paper-based test for the measurement of blood hemoglobin concentration using chromatography paper. The device was fabricated by printing a circular wax pattern on paper followed by heating, in which a 20 μL mixture of whole blood and Drabkin's reagent (for Hb colorimetric analysis) was applied and the subsequent blood stain was scanned and digitally analyzed, as shown in Figure 4B. The authors demonstrated high correlation (R^2 = 0.9598) between Hb values from 54 human subjects measured with paper devices and with a Medonic M-Series hematology analyzer. Bond et al. developed[85] a low-cost accurate spectrophotometric detection of blood hemoglobin concentration using chromatography paper (see Figure 4C). The paper device consists of a piece of chromatography paper prespotted with cell lysing agent and a 3D-printed reusable plastic frame to prevent the reader from touching biohazardous material. The reader was a custom-made, hand-held spectrophotometer that consists of two LEDs (528 and 656 nm), two plastic collimating lenses, one 50/50 beam splitter, one broadband photodiode, one microcontroller, and a custom-written program for obtaining and analyzing data. After applying a drop of whole blood, the paper device was vertically inserted into the battery-powered reader which displayed the test result. The authors demonstrated that this method was accurate to within 2 g/dL of results from the HemoCue analyzer for 95% of the samples and was insensitive to sample volume from 5 to 25 μL and that the absorbance difference between 528 and 656 nm was stable for the measurements taken up to 10 min after sample application.

The diagnostics of sickle cell disease is typically achieved by the detection of sickle hemoglobin (HbS) that exists within sickle RBCs. Despite the existence of HbS in the human race for thousands of years, not until 1910 were sickle cells first reported in circulating blood of an anemia patient.[82] Sickle cell disease is one type of anemia that is primarily inherited and caused by the production of HbS. As mentioned earlier, normal adult hemoglobin (HbA) has two identical α chains and two identical β chains. A single base-pair mutation of the gene that encodes the β chain replaces hydrophilic glutamic acid with hydrophobic valine within the hemoglobin molecule and subsequently forms HbS.[82] A deoxygenated form of HbS joins together and forms insoluble polymers, which in sufficient concentration leads to the distinctive sickle morphology of RBCs.[82] Individuals who inherit two normal copies of the gene encoding HbA have the genotype of HbAA. Individuals who inherited one copy of the gene encoding HbS and one copy of the gene encoding HbA (genotype HbAS) are considered to possess the sickle cell trait but are generally healthy.[86] Finally, individuals who inherit two copies of HbS-encoding gene (genotype HbSS) develop one of the most severe forms of sickle cell disease.[86] Other rarer forms of sickle cell disease are caused[87] by mutations leading to other types of abnormal hemoglobin, such as HbC or HbE.

Yang et al. proposed[87] a simple, rapid, and low-cost paper device for sickle cell disease diagnostics using wax-patterned chromatography paper. Whole blood was mixed with the standard hemoglobin solubility assay (SickleDex) at 1:20 volume ratio, and 20 μL of the blood mixture was applied onto the center of paper device after 5 min of incubation at room temperature. Upon contact with SickleDex, sickle hemoglobin

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(HbS) became polymerized and later entangled into the fibrous network of paper and formed a dark-red center spot, whereas normal hemoglobin remained soluble and wicked through the paper upon contact to form an almost uniform light-red circular pattern. Thus, the blood stain gave a visual indication that is able to differentiate between blood samples with or without sickle hemoglobin. After the full development of blood stain, the paper device was digitally scanned, and image analysis was performed to capture the change of red color intensity over the distance from the center of the blood stain. The authors successfully demonstrated the device's capability to distinguish between normal (HbAA), sickle cell trait (HbAS), and sickle cell disease (HbSS, HbSβ^+, HbSC) genotypes. Utilizing the same device, Piety et al. took one step forward and performed[88] sickle hemoglobin quantification using blood samples from 88 human subjects and demonstrated that the paper-based assay had high correlation (R^2 = 0.86) and strong agreement (standard deviation of difference = 7% HbS) with conventional electrophoresis measurement of % HbS; see Figure 4D. The test was proven to be accurate across 5.6−12.9 g/dL hemoglobin concentration with 10−97% of HbS and was insensitive to high levels of fetal hemoglobin (up to 80.6%). The same paper-based assay was also performed[89] among 226 postpartum women in a primary obstetric hospital in Cabinda, Angola, and was able to identify sickle trait carriers with 94% sensitivity and 97% specificity with only naked-eye evaluation of the test results. Furthermore, the assay was proven[90] to be capable of detecting HbS in blood samples among 159 newborn babies with a limit of detection of 2% HbS. The test identified the presence of HbS with 81.8% sensitivity and 83.3% specificity and diagnosed sickle cell disease from newborns with 100.0% sensitivity and 70.7% specificity. Kanter et al. developed[91] a paper-based device called Sickle SCAN for colorimetric diagnostics of sickle cell disease. The Sickle SCAN test is a sandwich-type lateral flow immunoassay that can qualitatively detect the presence of HbA, HbS, and HbC. The test strip consists of cellulose sample pad, fiber glass conjugate pad, cellulose wicking pad, and nitrocellulose analytical membrane with HbA, HbS, and HbC test lines and a control line. Five μL of capillary blood was mixed with hemoglobin solubility buffer, and after 20 s of incubation at room temperature, 5 drops of the sample mixture was dispensed onto the device. With the presence of HbA, HbS, and/or HbC, the corresponding test lines on the device appear and serve as disease indicators; see Figure 4E. In laboratory testing using venous blood, Sickle SCAN demonstrated 99% sensitivity and 99% specificity compared to known patient genotypes for the detection of HbSS, HbAS, HbSC, HbAC, and HbAA. In the bedside testing among 71 human subjects using capillary blood, the device was able to correctly detect the presence of HbA, HbS, and HbC with an overall accuracy of 99%. Bond et al. also developed[92] a paper-based sandwich-type lateral flow immunoassay for sickle cell disease diagnostics. The paper device consists of cellulose sample pad, fiber glass conjugate pad, and nitrocellulose analytical membrane with HbA and HbS test lines and a control line. Only 0.5−3 μL of undiluted whole blood sample was required to mix with hemoglobin solubility buffer, and the test time was no more than 10 min. This device was proven to be capable of detecting sickle cell disease with 90% sensitivity and 100% specificity with either naked-eye or computer-aided analysis.

Blood Coagulation. Hemostasis, meaning the prevention of blood loss, is achieved by four mechanisms when a blood vessel is damaged or ruptured:[24,25] (1) constriction of the vascular wall, (2) formation of a platelet plug, (3) formation of a blood clot due to coagulation, and (4) formation of fibrous tissues in the clot to permanently close the vascular wall. Blood coagulation, as part of hemostasis, occurs in three critical steps:[24,25] (1) formation of prothrombin activator by a complex cascade of chemical reactions, also known as coagulation cascade (triggered by the damaged or ruptured vascular wall or by the blood itself), (2) conversion of prothrombin into thrombin (catalyzed by prothrombin activator), and (3) conversion of fibrinogen into insoluble fibrin fibers (catalyzed by thrombin), which mesh platelets and RBCs together to eventually form a blood clot. The coagulation cascade can be triggered by either the intrinsic pathway (begins within the blood itself) or the extrinsic pathway (begins with trauma to vascular wall and surrounding tissues), or both, and then merge into a common pathway for final clot formation.[24,25] The entire process is highly regulated by platelets and 12 clotting factors designated by Roman numerals.[24,25] Blood coagulation tests aid in the diagnosis of hemophilia, the coagulopathy of liver disease, and disseminated intravascular coagulation, as well as the monitoring of anticoagulation therapy with heparin and warfarin.[93] Evaluation of blood coagulation can be achieved by four relatively simple tests:[94,95] the activated partial thromboplastin time (aPTT) to evaluate intrinsic and common pathways, prothrombin time (PT) to evaluate extrinsic pathway, and thrombin time (TT) and fibrinogen assays to evaluate common pathway. Traditionally, these tests have to be performed in a laboratory setting using large and expensive instrumentation, with a relatively long turn-around time. Thanks to the emergence of miniaturized POC coagulation analyzers,[95] taking the test in a physician's office or in the home and receiving the test result in a few minutes have become a reality. However, due to the high cost of the test reader unit ($600−700) and of the individual disposable strip ($5−6), POC coagulation analyzers are still an expensive proposition for many individuals.

Recent and current research[96] on blood coagulation analysis has focused on coagulation time measurements utilizing acoustics, optics, or the electrochemical method to detect the change in viscosity, transmittance, or electrical properties of whole blood or centrifuged plasma during the coagulation process. In the acoustics category, a microfluidic whole blood coagulation assay utilizing Rayleigh surface-acoustic wave (SAW) demonstrated[97] an increased SAW-induced clotting time with increasing dosage of various anticoagulants including heparin, argatroban, dabigatran, and rivaroxaban. Lamb wave-based microfluidic plasma coagulation assay was also reported[98] in which the acoustic streaming of fluorescent particles in citrated plasma was observed under the influence of Lamb waves. The test end point was when the particles ceased motion due to increasing viscosity of the plasma droplet. The PT values measured on a Lamb wave-based device had high correlation with those from a commercial instrument (R^2 = 0.9432). An acoustic resonator was also reported[99] to monitor the *in vitro* whole blood coagulation process and was able to quantitatively determine the coagulation time and the start/end of the fibrin generation. In the optics category, a lab-on-disc blood coagulation testing system with on-chip centrifugation was demonstrated[100] to be able to perform both PT and aPTT tests using spectrophotometric (transmission) measurement. High correlations (R > 0.96) between the results from

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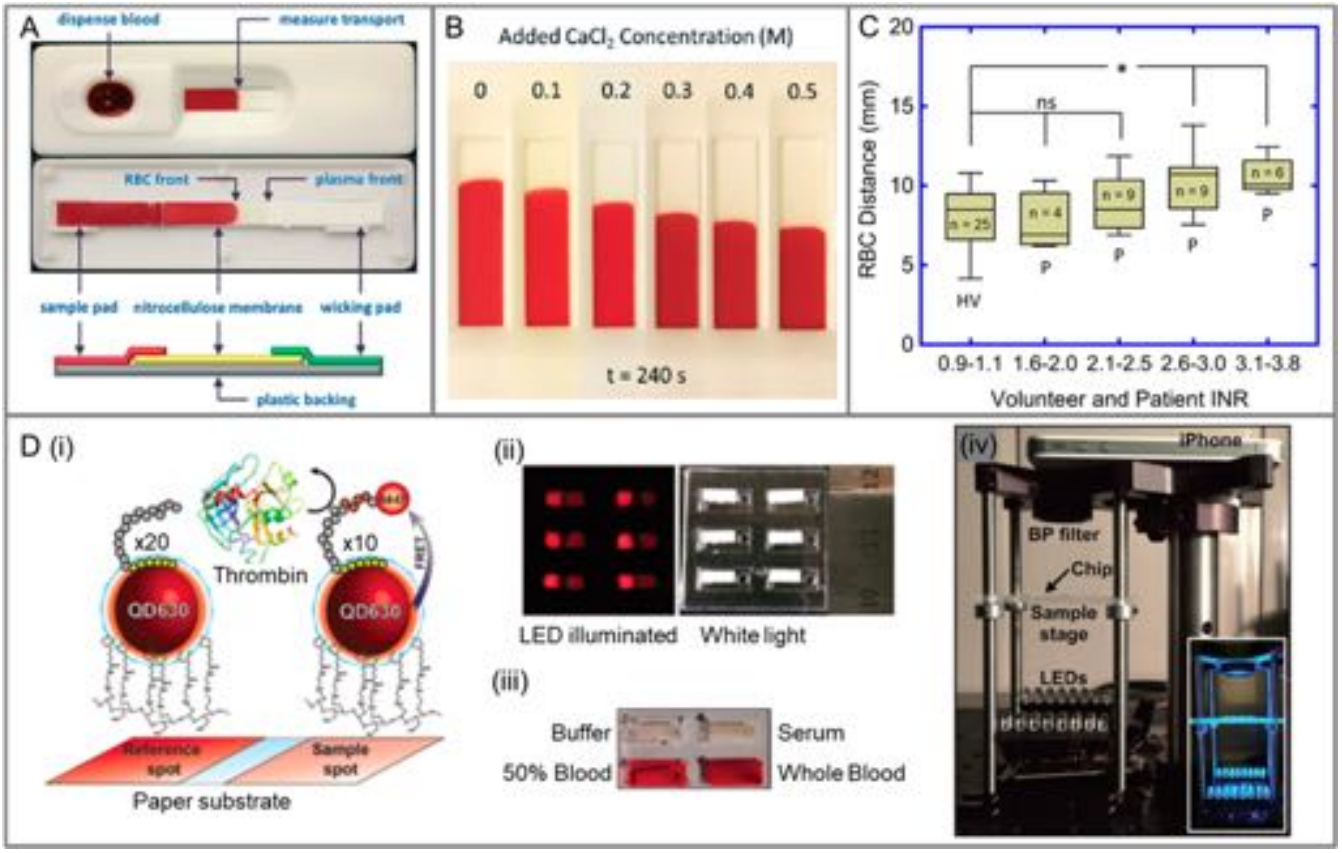

Figure 5. Whole blood coagulation analysis using paper-based devices. (A–C) Whole blood coagulation screening using the nrLFA device. (A) Photos of the nrLFA device (top: plastic cassette and nrLFA test strip) and schematic of nrLFA test strip (bottom: sample pad, analytical membrane, wicking pad, and plastic backing). Reproduced from Li, H.; Han, D.; Pauletti, G. M.; Steckl, A. J. *Lab Chip* **2014**, *14*, 4035–4041 (ref 107), with permission of The Royal Society of Chemistry. (B) RBCs in whole blood travel a shorter distance in the nrLFA with increasing coagulation ability induced by increasing concentration of $CaCl_2$ solution and vice versa. Reproduced from Li, H.; Han, D.; Pauletti, G. M.; Steckl, A. J. *Lab Chip* **2014**, *14*, 4035–4041 (ref 107), with permission of The Royal Society of Chemistry. (C) Human clinical trial result: increasing INR value (indicating decreasing coagulation ability) resulted in longer RBC travel distance in the nrLFA; significant difference ($p < 0.05$) revealed between healthy volunteer group (INR = 0.9–1.1) and patient groups with INR ≥ 2.6. Reprinted by permission from Springer Nature: Springer, *Biomed. Microdevices*, Point-of-care coagulation monitoring: first clinical experience using a paper-based lateral flow diagnostic device, Hegener, M. A.; Li, H.; Han, D.; Steckl, A. J.; Pauletti, G. M. (ref 109), Copyright 2017. (D) Thrombin detection from whole blood using paper-based device and smartphone camera: (i) illustrations of device schematic and interaction between thrombin and peptide-conjugated quantum dots (QD630); (ii) photos of reference and sample spots on paper device under LED- and white-light illumination; (iii) photo of device filled with various biological samples; (iv) photo of test setup for thrombin detection from blood and serum on paper device using LEDs and smartphone camera. Reproduced from Petryayeva, E.; Algar, W. R. *Analyst* **2015**, *140*, 4037–4045 (ref 113), with permission of The Royal Society of Chemistry.

the lab-on-disc device and from a clinical instrument were shown for PT and aPTT tests using clinically acquired whole blood samples. An optical transmission-based coagulation assay was reported[101] using whole blood samples without centrifugation and successfully obtained the PT results from 153 out of 167 (91.6%) blood samples. The measured PT values had high correlation with both the whole blood international normalized ratio (INR) (R = 0.985) and the plasma INR (R = 0.948) measured by a clinical instrument. Another transmittance-based microfluidic coagulation assay, which utilized a pinch valve for fluid motion, was demonstrated[102] to be capable of quantitatively measuring whole blood coagulation time as well as simultaneously evaluating the degree of erythrocyte aggregation within the blood sample. In the electrochemical category, two PDMS-based microfluidic devices were reported[103,104] to perform coagulation tests using whole blood by detecting the change in electrical impedance during coagulation, and one of the two devices was demonstrated to be able to perform aPTT tests[103] using whole blood samples. Also reported was a microfluidic dielectric sensor[105] with a 3D capacitive structure and a floating in-channel electrode measuring the dielectric spectrum of human whole blood during coagulation. A peak in the real part of the complex relative dielectric permittivity was observed at 1 MHz at 4–5 min when using blood samples, which corresponds to the $CaCl_2$-initiated coagulation of blood samples.

Upon the activation of the coagulation cascade, the viscosity of blood gradually increases, and the physical properties of blood change from a viscoelastic fluid to a viscoelastic solid after the formation of cross-linked fibrin clot.[106] On the basis of this mechanism, Li et al. developed[107] a simple, low-cost paper-based lateral flow device for blood coagulation screening based on the change of blood viscosity during coagulation. A device similar to existing lateral flow immunoassay test kits was developed, consisting of a paper-based test strip incorporating a fiber glass sample pad, a nitrocellulose analytical membrane, and a cellulose wicking pad. The assembled strip was inserted

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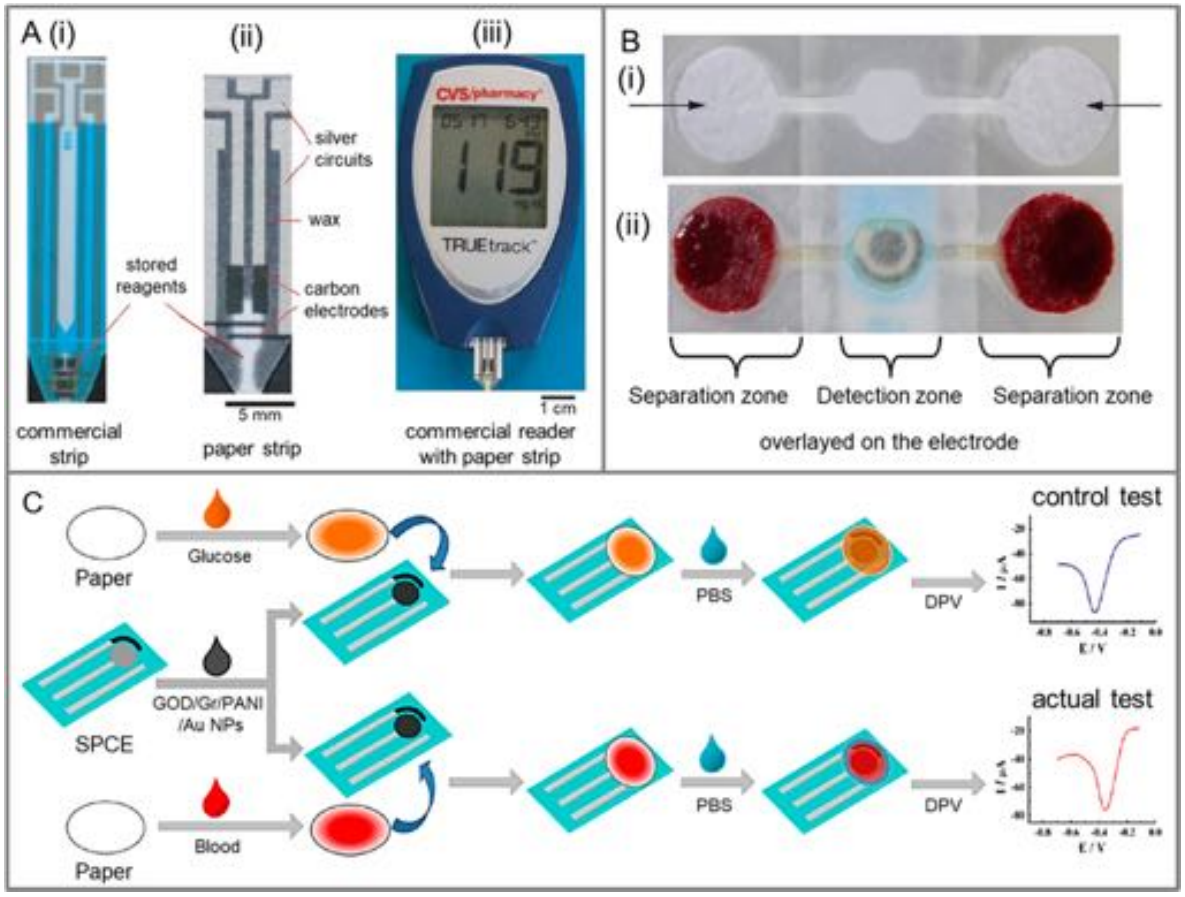

Figure 6. Quantitative glucose detection from whole blood using paper-based devices. (A) Glucose detection using novel wax- and silver-printed paper strip paired with a commercial glucometer: (i) commercial glucose test strip; (ii) novel paper strip with wax-defined fluid channels, silver circuits, and prestored reagents for glucose testing; (iii) glucose testing using a novel paper strip with a commercial glucometer. Reproduced from Nie, Z.; Deiss, F.; Liu, X.; Akbulut, O.; Whitesides, G. M. *Lab Chip* **2010**, *10*, 3163−3169 (ref 115), with permission of The Royal Society of Chemistry. (B) Glucose detection using disposable paper component and reusable electrode: (i) paper device consists of two plasma separation zones and one detection zone; (ii) whole blood samples applied to separation zones; plasma collected in detection zone and reacted with prestored reagents for glucose testing. Reprinted from *Anal. Chim. Acta*, Vol. *788*, Noiphung, J.; Songjaroen, T.; Dungchai, W.; Henry, C. S.; Chailapakul, O.; Laiwattanapaisal, W. Electrochemical detection of glucose from whole blood using paper-based microfluidic devices, pp. 39−45 (ref 116). Copyright 2013, with permission from Elsevier. (C) Glucose detection using a dry blood-impregnated disposable paper disc and screen-printed carbon electrode; enzymatic reaction activated when adding water to paper disc, generating a decrease in current signal. Reprinted from *Biosens. Bioelectron.*, Vol. *56*, Kong, F.-Y.; Gu, S.-X.; Li, W.-W.; Chen, T.-T.; Xu, Q.; Wang, W. A paper disk equipped with graphene/polyaniline/Au nanoparticles/glucose oxidase biocomposite modified screen-printed electrode: toward whole blood glucose determination, pp. 77−82 (ref 117). Copyright 2014, with permission from Elsevier.

into a plastic housing with openings for sample dispensing and flow observation; see Figure 5A. Because the device did not contain any stored reagents and did not operate on the basis of any bioaffinity reactions, it was named a *no-reaction* lateral flow assay (nrLFA) device. Instead of utilizing the antigen−antibody immunoreaction, the nrLFA takes advantage of the porous nature of nitrocellulose membranes to transport fluids of different viscosities at different speeds through capillary action. A mixture of anticoagulated (citrated) rabbit blood and increasing concentrations of reversal agent ($CaCl_2$) solution were utilized to mimic blood with increasing coagulation abilities and thus increasing viscosities. The nrLFA was able to distinguish the coagulation abilities of various samples; the sample with the strongest coagulation traveled the shortest distance after a predetermined time period and vice versa; see Figure 5B. The authors also reported that, in a comparison between the nrLFA and the CoaData 2000 Fibrintimer, a clinical instrument for clotting time measurement, comparable performance between two methods was obtained, with the nrLFA being slightly superior at detecting very weak or very strong coagulation. Due to the nrLFA's critical dependence on fluid flow in porous materials, the same group also investigated[108] the reproducibility of the nrLFA and related materials, as well the effect of hematocrit and sample volume on fluid travel in the device. Flow rate evaluations were conducted using MilliporeSigma nitrocellulose membranes (HF075, HF135, HF180) with various biofluids (whole blood, plasma, artificial sweat), and excellent flow reproducibility and high performance in distinguishing fluids of various viscosities were reported. Flow rate evaluation on the nrLFA (with HF075 membrane) demonstrated superior reproducibility of the device with a coefficient of variation CV = 2% for the RBC front and a CV = 5% for the plasma front during blood coagulation tests (n = 10). The effect of hematocrit percentage on blood travel distance on the nrLFA was investigated, and the minimum sample volume for reliable testing was determined to be ∼20 μL. A clinical trial of the nrLFA was conducted[109] for blood coagulation monitoring of patients on

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warfarin anticoagulation therapy. The international normalized ratio (INR) is a standard clinical parameter for routine monitoring of warfarin therapy. Lower INR indicates the patient's blood coagulates faster and vice versa. In their study, the nrLFA was able to statistically distinguish between healthy volunteers with INR = 0.9−1.1 and the patient group with INR ≥ 2.6, demonstrating its potential for clinical utility in therapy monitoring; see Figure 5C. The same device was also reported[110] to be capable of detecting different $CaCl_2$-induced coagulation for various animal species including rabbit, equine, bovine, and beagle, as well as different heparin-induced coagulation for rabbit and bovine. The results indicated that the nrLFA has the potential for veterinary applications as a blood coagulation screening tool for animals on anticoagulation therapy. Furthermore, a simple calibration method was recently reported[111] by the same group to quantify the effect of Hct value on RBC transport and correct for the dependence of RBC distance on Hct value in the nrLFA, while maintaining desired dependence of RBC distance on blood coagulation.

Thrombin (factor IIa), the activated form of prothrombin (factor II), is a critical clotting factor produced in the common pathway of the coagulation cascade. Thrombin assay analyzes the generation (due to procoagulants) and decay (due to anticoagulants) of thrombin, making it suitable for representing the dynamic coagulation balance *in vivo*, which cannot be achieved by *ex vivo* tests such as PT and aPTT.[112] Petryayeva and Algar reported[113] a quantum dot (QD) incorporated paper-in-PDMS-on-glass device for thrombin detection in serum and whole blood using a smartphone. After the pretreatment of filter paper to remove disulfate groups, QD630−peptide conjugates labeled with Alexa Fluor 647 were spotted directly on one end of the paper strip as sample spots with label-less reference spots at the other end; see Figure 5D. The resulting strip was enclosed within a PDMS-on-glass device with built-in sample reservoirs for buffer, serum, or whole blood. After sample application, the device was illuminated by blue LEDs and the photoluminescence intensity from QDs was captured using a smartphone camera. The device was proven to be capable of quantitatively detecting 15.1−484 U/mL thrombin in buffer, serum, and whole blood within 30 min after sample application, with a detection limit of 18 U/mL when using 12 μL of whole blood.

Glucose Monitoring. Self-monitoring of glucose level using finger prick blood was first[114] introduced in the 1980s and is now widely utilized. With very small sample volume, relatively inexpensive disposable test strips, and a portable electronic reader, patients' self-monitoring of glucose has been a critical part of effective diabetes management, especially for those on insulin medication.[114] The basic principle of the test utilizes the electrochemistry of glucose oxidase (GOD). While whole blood glucose POC testing is quite a mature technology, there has been continuing interest in developing more cost-effective and environmentally friendly approaches. Cellulose-based materials are a desirable alternative to conventional plastic materials in test strip fabrication for being ultralow cost, biodegradable, and easy-to-dispose.

Nie et al. reported[115] a simple paper-based analytical device designed to operate in conjunction with a commercial hand-held glucometer (i.e., TRUEtrack from CVS/Pharmacy) and was capable of rapid and quantitative detection of metabolites including glucose, cholesterol, lactate, and alcohol. The paper device, in the shape of a strip, consists of microfluidic channels fabricated by wax printing and carbon electrodes and silver wires fabricated by screen printing on one sheet of chromatography paper. The device required ∼1 μL of fluid sample (blood, plasma, or aqueous solution) to fully wet the channels, and the chemical reagents required for the tests were stored in the detection zone of the device. The paper strip worked in an identical manner compared to the commercial plastic test strip in glucose and alcohol testing; see Figure 6A. An additional preincubation process was needed for lactate and cholesterol testing due to their relatively long waiting period to complete the enzymatic reactions. When used with the commercial hand-held glucometer, the paper strip demonstrated excellent performance in whole blood glucose testing with 9.1% CV (4.1% CV for commercial test strip), as well as its potential to be used for lactate and cholesterol testing in plasma and alcohol testing in aqueous solution. Noiphung et al. developed[116] a paper-based device for electrochemical detection of glucose from whole blood utilizing disposable paper components and reusable electrode. The dumbbell-shaped paper device consists of one plasma separation zone (made of plasma separation membrane) at each of two ends and one detection zone (made of chromatography paper) in the middle, as shown in Figure 6B. The reusable Prussian blue screen-printed electrode was placed underneath the detection zone of the device and was connected to an electrochemical analyzer (Autolab PGSTAT101) using alligator clips. During the test, a minimum of 200 μL of undiluted blood was divided into two equal parts and applied onto two separation zones simultaneously, generating a homogeneous flow of separated plasma to the detection zone. The device was reported to have a linear range of up to ∼33 mM glucose concentration in whole blood (R^2 = 0.987) and CVs of 6.5%, 9.0%, and 8.0% when using blood samples with glucose concentrations of 3.4, 6.3, and 15.6 mM, respectively. Kong et al. developed[117] a paper-based whole blood glucose assay that utilized a disposable screen-printed carbon electrode (SPCE) equipped with a chromatography paper disk. During the tests, the paper disk was first impregnated with at least 2 μL of blood sample and allowed to dry in air. Once dried, the paper disk was laminated onto the electrode strip, which was pretreated with the graphene/polyaniline/gold nanoparticles (AuNPs)/GOD combination that covered the three-electrode configuration completely. Upon the addition of PBS solution on the paper disk, the current of flavin adenine dinucleotide in GOD started to decrease due to the enzyme−substrate reaction, and the current signal was measured using differential pulse voltammetry. The operation of the assay with both a control (glucose) solution and actual blood sample is illustrated in Figure 6C. The paper-based assay was demonstrated to cover the entire physiological range of glucose concentration in whole blood and was proven to be comparable to a conventional clinical method (using Roche automatic clinical chemistry analyzer). Torul et al. reported[118] a paper-based surface-enhanced Raman scattering (SERS) technique to evaluate blood glucose concentration using nitrocellulose membrane with wax-printed microfluidic channels. The area for SERS measurement was prepared by applying AuNPs on a nitrocellulose membrane, and a 20× diluted blood sample was applied within a hydrophilic channel defined by hydrophobic wax barriers. The blood cells and proteins were retained on the nitrocellulose membrane, whereas the glucose molecules traveled along the channel and reached the SERS measurement area. Increasing glucose concentration in whole blood was

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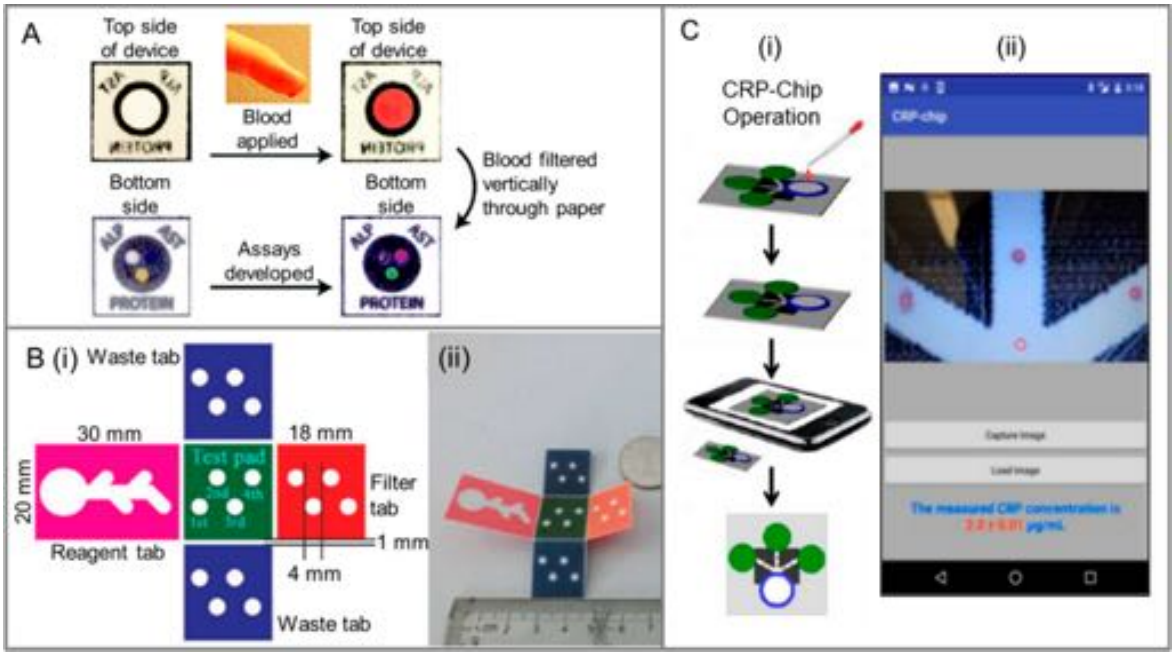

Figure 7. Detection of biomarkers from whole blood using paper-based devices. (A) Quantitative and qualitative detection of three liver function biomarkers (ALP, AST, and total serum protein) using a wax-pattern multilayer paper device. Reproduced from Vella, S. J.; Beattie, P.; Cademartiri, R.; Laromaine, A.; Martinez, A. W.; Phillips, S. T.; Mirica, K. A.; Whitesides, G. M. *Anal. Chem.* **2012**, *84*, 2883−2891 (ref 121). Copyright 2012 American Chemical Society. (B) Quantitative detection of four cancer biomarkers (r-fetoprotein, carcinoma antigen 153, carcinoma antigen 199, and carcinoembryonic antigen) using sandwich chemiluminescent immunoassay in an origami-like paper device: (i) device schematic: one reagent tab, one filter tab, one test pad (with four detection zones), and two waste tabs; (ii) device size: approximately the size of a quarter when folded. Reproduced from Ge, L.; Wang, S.; Song, X.; Ge, S.; Yu, J. *Lab Chip* **2012**, *12*, 3150−3158 (ref 123), with permission of The Royal Society of Chemistry. (C) Quantitative detection of C-reactive protein using paper-based immunoassay and designated phone app: (i) schematic of test procedures; (ii) reading test result using a smartphone and app. Reproduced from Dong, M.; Wu, J.; Ma, Z.; Peretz-Soroka, H.; Zhang, M.; Komenda, P.; Tangri, N.; Liu, Y.; Rigatto, C.; Lin, F. Rapid and Low-Cost CRP Measurement by Integrating a Paper-Based Microfluidic Immunoassay with Smartphone (CRP-Chip). *Sensors* **2017**, *17*, 684 (ref 125). Copyright 2017 MDPI.

reported to result in a monotonic decrease in the SERS signal intensity of the band at 1070 cm^{-1}. When using a reference blood sample with a certified glucose concentration of 6.17 mM, the paper-based SERS glucose assay produced a test result of 5.43 mM, ~88% recovery of glucose concentration in reference blood. The optimal volume of blood sample was found to be 5 μL for the highest SERS signal intensities.

Biomarker Detection. A biological marker, biomarker for short, is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention".[119] Biomarkers can be detected in body tissues, circulation (blood, lymph, etc.), and fluids (urine, stool, sputum, etc.).[120] Detecting circulating biomarkers in blood has been a powerful tool for diagnostics of various diseases. Due to the variability in biomarker signal caused by cellular components, pure plasma is more desired as test specimen than the highly complex whole blood.[65] However, the extra centrifugation and separation steps complicate the test procedures and lengthen the sample preparation time, which is not desirable in the pursuit of simple, rapid, and low-cost diagnostic devices. Therefore, detecting biomarkers directly from whole blood is a particularly challenging but promising method that can be widely utilized in resource-limited settings.

Vella et al. reported[121] a paper-based assay that simultaneously measures two enzymatic biomarkers, alkaline phosphate(ALP) and aspartate aminotransferase (AST), as well as total serum protein for liver function analysis using fingerstick blood. ALP and AST are primarily produced by liver cells and normally circulate in the bloodstream at very low concentrations. In the case of damaged or diseased liver, ALP and AST concentrations rise dramatically, making them target biomarkers for liver disease diagnostics.[121] The analytical device consists of four components: a top lamination sheath with a circular opening for sample dispensing, a circular plasma separation membrane that fits in the opening of the top sheath, a wax-patterned chromatography paper containing prespotted reagents, and a bottom lamination sheath. Plasma separation membrane was placed right on top of the three reaction zones so that the plasma can be transferred vertically onto the reactions zones after filtration, and the test result was read from the bottom side of the device where the presence of target analytes was detected by generating colorimetric signals that can be observed with the naked eye; see Figure 7A. The device was capable of both qualitative and quantitative detection of ALP, AST, and total serum protein, with detection limits of ~44 U/L, ~15 U/L, and ~7 g/L for AST, ALP, and the protein, respectively. The device was proven to be suitable for clinical use by showing the standard deviations during the calibration process as well as the absence of cross-reactivity using "spiked" samples.

An innovative "origami"-like paper device was first developed[122] by Liu and Crooks which utilized a 3D combination of vertical and lateral fluid flows in photolithography-patterned channels for performing parallel assays on a cleverly folded single sheet of paper. Using the same origami approach, Ge et al. reported[123] the simultaneous

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detection of four cancer biomarkers utilizing a sandwich chemiluminescent (CL) immunoassay. The device, which is made from wax-patterned chromatography and blotting paper, consists of one central test tab surrounded by four folding tabs including one reagent, one plasma filter, and two waste tabs, as shown in Figure 7B. The test tab contains four test zones with corresponding antibodies immobilized for the CL detection of four cancer biomarkers (r-fetoprotein, carcinoma antigen 153, carcinoma antigen 199, and carcinoembryonic antigen), and the reagent pad contains one reservoir and four microfluidic channels that provide H_2O_2 to four test zones on the test tab. During the test, the filter tab was folded onto the test tab and 6 μL of pretreated whole blood sample spiked with target antigens was applied on the filter tab. After sufficient plasma was transported onto the test tab, the filter tab was removed, and the bottom waste tab was folded underneath the test tab, followed by applying washing buffer to the four test zones. After washing, the used waste tab was removed, and AgNPs-luminol/Ab_2 solution was applied to the test zones, followed by another washing step with the top waste tab folded underneath the test tab. After disposing of the second waste tab, the reagent tab was folded onto the test tab and H_2O_2 was applied on the reagent tab to trigger CL reactions on the test pad. Using a benchtop luminescence spectrum analyzer, the device was reported to be capable of quantitatively detecting four cancer biomarkers simultaneously with high linearity (R > 0.99 for all four biomarkers) and excellent reproducibility (CVs ranging from 2.2% to 5.8% for the four biomarkers).

C-reactive protein (CRP) is a biomarker released by the liver in response to a variety of inflammatory cytokines.[124] The CRP level in blood increases rapidly in the event of trauma, inflammation, and infection and decreases just as rapidly when the condition is resolved.[124] Dong et al. developed[125] a paper-based immunoassay for the quantitative detection of CRP from finger-prick blood coupled with smartphone read out for the diagnosis of chronic heart and kidney disease. The device consists of a backing pad, a nitrocellulose membrane with wax-patterned test channels prespotted with test and control antibodies, three cellulose absorbent pads, a fiber glass conjugate pad with prespotted AuNP conjugated antibodies, and a plasma separation pad stacked on top of the conjugate pad. During the test, 20 μL of whole blood was dispensed onto the plasma separation pad, with the filtered plasma propagating onto the conjugate pad and then through three test channels in the NC membrane, as illustrated in Figure 7C. With the presence of CRP in plasma, control and test spots appeared in the channel, and the color intensity of the test spots was captured and analyzed using a smartphone. A PDMS microlens was attached onto the camera of a commercial smartphone, and the paper device was inserted into a 3D-printed plastic holder mounted to the phone with a built-in device holder and focus knob. The paper-based assay was reported to have high reproducibility and a linear detection range up to 2 μg/mL with a detection limit of 54 ng/mL. The material cost of a CRP triplicate test is reported to be <50 cents, and the result of the test can be obtained within 15 min.

Mucin-1 is a protein biomarker that is overexpressed in various cancers (e.g., breast, ovarian, prostate, pancreatic, etc.) and has an important role in cancer progression.[126] Mucin-1 promotes cancer growth and spread and its resistance to drugs and is critical for preserving "stemness" in cancer stem cells.[126] Hu et al. developed[127] a paper-based SERS test strip for quantitative analysis of mucin-1 in whole blood. The SERS strip was fabricated by the deposition of AuNPs on carbon dot-modified filter paper, followed by the sequential deposition of aptamer probe solution and CY-5 labeled reporter DNA solution. During the test, heparinized human blood spiked with mucin-1 was applied onto the SERS strip which was subsequently washed after a 1 h incubation, and then, the Raman signal on the strip was measured using a 633 nm laser. The paper-based SERS test strip was demonstrated to be capable of quantitatively detecting mucin-1 ranging from 0 to 1500 ng/mL in human whole blood samples, and the test results of mucin-1 concentrations among five healthy volunteers were consistent with the physiological range of normal adults.

Malaria Diagnosis. Malaria has been a primary cause of mortality and morbidity in tropical and subtropical counties, resulting in over one million deaths every year around the globe.[128] It is caused by protozoan parasites of the genus *Plasmodium*, which have a complicated life cycle in the mosquito vectors and vertebrate hosts.[129] Timely and accurate diagnosis is essential in effective treatment of malaria. In a laboratory, malaria diagnostics can be achieved by microscopic examination of thin and thick peripheral blood smears, the quantitative buffy coat method, various rapid diagnostic tests (e.g., OptiMAL, ICT, Para-HIT-f, ParaScreen, etc.), and polymerase chain reaction (PCR).[128] However, due to the requirement of laboratory equipment and skilled microscope operators or well-trained technicians, the above-mentioned diagnostic methods are not ideal for developing countries. A simple and low-cost paper-based diagnostic device can be an attractive solution for cheap and rapid malaria diagnosis without the need for laboratory equipment or trained healthcare professionals.

Deraney et al. developed[130] a paper-based immunoassay for multiplexed detection of malaria and dengue fever using a 3D 8-layer paper device. From top to bottom of the device, layers were designed for plasma separation, sample input, sample distribution, conjugate storage, incubation, capture/test read out, washing, and blotting. A plasma separation membrane, chromatography paper, a cellulose absorbent pad, and a Western blotting membrane were utilized for device fabrication. The cellulose layers were patterned by wax printing, and all layers were assembled together by adhesives and lamination sheets. Using lysed human blood spiked with 500 ng/mL single antigen or a combination of antigens, the device was capable of detecting all negative, all positive, and all possible combinations of malaria HRP2, malaria pLDH, and dengue NS1 type 2 by yielding a clean background for negative tests and an intense colorimetric signal for positive tests. It was also reported that no significant difference in performance was observed between multiplexed and single immunoassay structures. Xu et al. reported[131] a paper-based assay for multiplexed detection of malaria from finger-prick volume of whole blood within 45 min using the origami paper-folding technique. The device was designed to perform sequential steps of DNA extraction, loop-mediated isothermal amplification (LAMP), and multiplexed (array) fluorescent detection. The device consists of five wax-patterned filter paper panels (panels 1−5) in one row with fiber glass sample pad on panel 3, as shown in Figure 8. First, after dispensing whole blood on the device (panel 3), by folding and stacking panels 1, 2, and 3 in a predetermined sequence, the blood sample was lysed, and pure DNA was extracted. Second, by flipping panels 2 and 3 onto panels 4 and 5, the extracted DNA was transferred to

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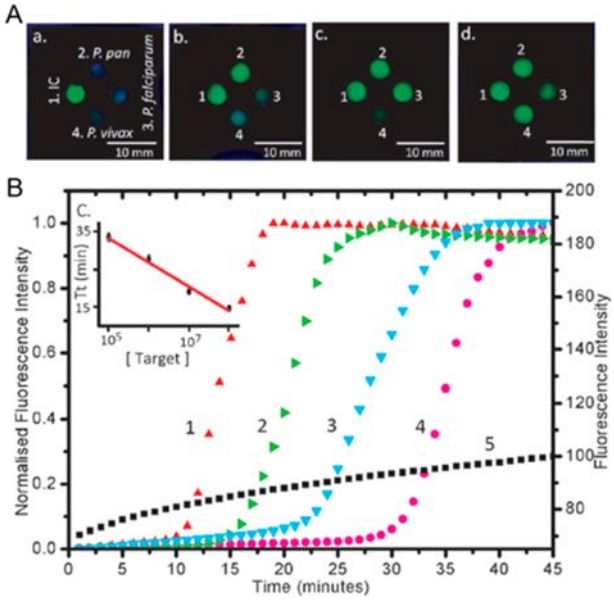

Figure 8. Quantitative detection of malaria from whole blood using a paper-based device. (A) Multiplexed detection of malaria (three species of *Plasmodium*) with on-chip DNA extraction and loop-mediated isothermal amplification in an origami-like paper device using fluorescence. (B) Plot of normalized fluorescence intensity vs time for four detection zones. Reproduced from A rapid paper-based test for quantifying sickle hemoglobin in blood samples from patients with sickle cell disease, Xu, G.; Nolder, D.; Reboud, J.; Oguike, M. C.; van Schalkwyk, D. A.; Sutherland, C. J.; Cooper, J. M. *Angew. Chem. Int. Ed.*, Vol. *55*, Issue 49 (ref 131). Copyright 2016 Wiley.

LAMP spots through elution, where DNA amplification and fluorescent detection were carried out. The fluorescent signal was obtained by shining a UV light with excitation wavelength of 365 nm. The device was demonstrated to have high specificity and good sensitivity for detecting three species of *Plasmodium* in human blood when compared to the gold-standard PCR.

Therapy Monitoring. Lithium (Li) is a commonly used substance in psychiatry, especially for the treatment of bipolar disorder.[132] Li has complicated mechanisms of action, and its effects include mood stability as well as neuroprotective and antisuicidal properties.[132] Patients undergoing Li therapy require regular monitoring of blood Li level as well as timely dosage adjustments in a very narrow range for safe and effective treatment.[132] Novell et al. developed[133] a paper-based potentiometric cell for POC Li level monitoring in whole blood using electrodes made of carbon nanotube (CNT) coated filter paper. For the potentiometric cell, both the Li^+ ion selective electrode (ISE) and the reference electrode consist of four major components: a top plastic mask with a circular opening, a circular Li^+ ion selective membrane (ISM) for Li^+ ISE or a reference membrane for reference electrode, a CNT-coated filter paper with a resistance of ~500 Ω sq^{-1}, and a bottom plastic mask. Paper ISE and reference electrode were assembled together with a piece of neoprene rubber in between forming a cavity of ~50 μL, and both electrodes were connected to a potentiometer by their conductive ends, as illustrated in Figure 9A. After dispensing blood or serum sample in the cavity, the fluid interacts with the Li^+ ISM and reference membrane and generates a potential difference between Li^+ ISE and reference electrode, which is subsequently detected by a potentiometer. The paper-based potentiometric cell was demonstrated to be capable of detecting Li level in clinically obtained human blood and serum from patients undergoing Li therapy, and in both cases, a recovery higher than 90% was obtained when compared to the atomic emission spectroscopy, a routine clinical tool for Li level measurement.

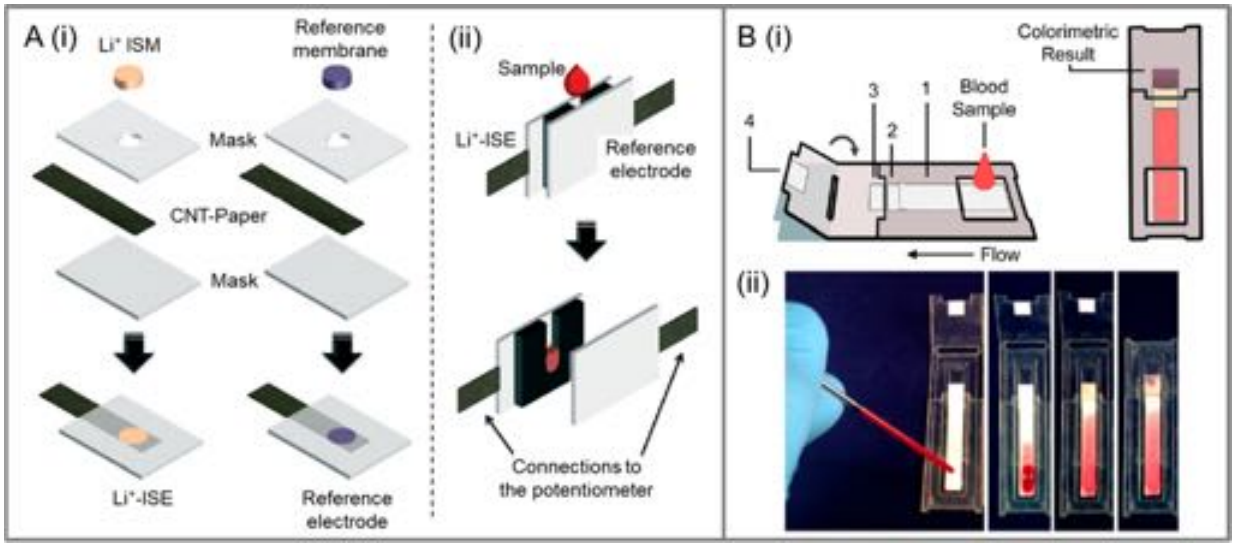

Figure 9. Therapy monitoring using whole blood in paper-based devices. (A) Lithium therapy monitoring using a potentiometric cell with electrodes made of carbon nanotube-coated filter paper: (i) illustration of fabrication procedures of potentiometric cell (Li^+ ion selective electrode and reference electrode); (ii) illustration of test procedures for quantitative detection of Li^+ ions. Reproduced from Novell, M.; Guinovart, T.; Blondeau, P.; Rius, F. X.; Andrade, F. J. *Lab Chip* **2014**, *14*, 1308−1314 (ref 133), with permission of The Royal Society of Chemistry. (B) Semiquantitative Phe detection using a paper-based lateral flow device in PKU therapy monitoring: (i) illustration of test principles: plasma separation and colorimetric reaction between Phe in plasma and prestored reagents; (ii) photos of test procedures. Reproduced from Robinson, R.; Wong, L.; Monnat, R.; Fu, E. Development of a Whole Blood Paper-Based Device for Phenylalanine Detection in the Context of PKU Therapy Monitoring. *Micromachines* **2016**, *7*, 28 (ref 135). Copyright 2016 MDPI.

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Phenylketonuria (PKU) is a genetic disorder that causes abnormal metabolism of the amino acid phenylalanine (Phe), which can result in growth failure, poor skin pigmentation, microcephaly, seizures, global developmental delay, and even severe intellectual impairment if left untreated during childhood.[134] PKU therapy is primarily based on a low Phe diet that reduces Phe concentration in a patient's body to prevent the progress of neurological and psychological degradation.[134] Robinson et al. developed[135] a paper-based device for semiquantitative Phe detection in whole blood for PKU therapy monitoring. The paper-based device consists of a plastic folding card for reagent transport and backing support, a separation membrane for extracting plasma from whole blood, and three pieces of fiber glass for enzymatic reaction and colorimetric detection, as shown in Figure 9B. After applying whole blood sample on the separation membrane, plasma was separated and flowed sequentially onto the pretreated fiber glass pads 1 and 2 for enzymatic reaction. Then, the third pretreated pad was folded onto pad 2 for colorimetric detection. The test requires 40 μL of blood sample, and the result is ready to be read ~8 min after sample application. The paper-based device was demonstrated to be able to semiquantitatively detect 1.1−16 mg/dL Phe in whole blood.

CONCLUSIONS, CHALLENGES, AND FUTURE PERSPECTIVES

Paper-based POC devices for whole blood analysis are most widely used for hematology (hematocrit and hemoglobin) and sickle cell disease testing since the blood itself, or to be specific, the RBCs within the blood, are the test subjects. The utilization of whole blood in non-RBC related analysis is much less common compared to plasma, serum, or urine because the existence of cellular components, especially RBCs, complicates the device structure and challenges test accuracy due to their intense red color. When using colorimetric and optical detections, RBCs are typically removed from blood samples by on-chip filtration using a plasma separation membrane. For electrochemical and SERS-based assays, whole blood samples can be directly analyzed without RBC removal. In some applications such as blood typing, hematocrit determination and coagulation analysis, an unconventional, simple, and accurate colorimetric detection is utilized where the travel distance of the color is measured after a certain time period instead of the change of color.

From a broader perspective, it is hoped that this Review exposes the wider scientific community to the challenges and opportunities of studying the flow of this most complex multiphase fluid through the porous medium of cellulose membranes. Looking forward, we can identify several aspects of paper-based whole blood diagnostic devices that should be taken as goals for improvements: sample volume, test time, limit of detection, quantification, and communication of information. Some approaches for pursuing these improvements in various aspects are discussed below.

Reducing Sample Volume to Single Digit (<10 μL). The sample volume can probably be reduced using various methods, some of which are discussed next. First, the width of the LFIA strip can be narrowed in the future to reduce the sample volume. This approach does have limitations because of edge effects (liquid flow along edge propagating at faster rate than in the center of the strip) which can become more pronounced as the strip becomes narrower. Therefore, a "sweet spot" should be found in which the sample volume is as close to the commercial blood analyzers as possible but still generates uniform, reliable, and reproducible fluid flow in the LFIA strip. The bed volume of the fiber glass[136] sample pad is typically ~1−2 μL/cm^2, and the bed volume of nitrocellulose membrane[137] is ~10 μL/cm^2. These numbers make it possible to reduce sample volume to a single digit by reducing the width and length of each component in the LFIA strip. Second, dispensing samples directly on nitrocellulose membrane without the sample pad and plastic cassette can probably result in the smallest sample volume required for a single LFIA test. Instead of being housed in a plastic cassette, the LFIA test strip can be laminated between two plastic sheets for strip protection and easy handling. Third, hydrophobic barriers can be fabricated on nitrocellulose membranes using lithography or wax printing to reduce the channel width of the LFIA strip while maintaining the original structural width of the strip. This method allows the continuous utilization of existing dimensions of the LFIA strips as well as the existing design of the accompanying plastic cassette.

Reducing Test Time to <1 min. Rapid, low-cost whole blood tests can be very beneficial in many locations and situations where a quick assessment of a patient's health condition is required, such as in emergency rooms and ambulances. While many paper-based diagnostic devices produce a result in a few minutes, some require a longer time (as long as 20−30 min). Several possibilities exist in order to accelerate the process. For example, for the nrLFA device for blood coagulation monitoring, one can investigate the use of membranes with faster flow rate and utilize information associated with the plasma front which moves faster than the RBC front.

Simultaneous Assays Using a Single Sample. Simultaneous detection of blood coagulation and hematocrit fraction should be pursued in the future to enhance the clinical utility of nrLFA for blood coagulation monitoring. Due to the sensitivity of RBC travel distance to blood viscosity, frequent corrections in RBC travel distance in the nrLFA device are needed on the basis of an individual's hematocrit value. The simultaneous detection can be potentially achieved by several approaches. For example, a dual-channel nrLFA device can be developed to perform blood coagulation analysis and hematocrit measurement in parallel. High concentration heparin solution can be prespotted in the hematocrit channel to ensure the blood sample is not clotted and the RBC travel distance in that channel is influenced by hematocrit only, not coagulation. Another approach can investigate hematocrit quantification using spectrophotometry or digital image analysis by measuring the red color intensity of the fluid path in the nrLFA. A higher intensity indicates a higher hematocrit fraction and vice versa. While a linear dependence of red color intensity on hematocrit would be advantageous because it would simplify the conversion of the measurement to a hematocrit fraction, a reliable nonlinear (but monotonic) dependence can also be utilized for accurate hematocrit measurement by using a correction algorithm.

Advanced Features Using Smartphone Capabilities. The concept of using smartphone capabilities for enhancing the diagnostic POC assays is being widely pursued.[74,125,138,139] For example, by using the smartphone camera and processing capability, automated readout, analysis, and communication of the assay results are possible. While this has very obvious benefits, it is not without its difficulties (effect of ambient

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lighting conditions, shake-free detection, fast data analysis). For the blood coagulation nrLFA device, a smartphone app can be developed to automatically capture and analyze RBC travel distance on the nrLFA in order to provide rapid and hassle-free blood coagulation results for patients self-testing at home. A third example is the use of near field communication capability of smartphones to provide the electric power needed for on-chip integration[140] of opto/electronic components with the diagnostic assay.

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The manuscript was written through contributions of both authors. Both authors have given approval to the final version of the manuscript.

Notes

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