CHAPTER 4: A SIMPLE AND LOW-COST PAPER-BASED DEVICE FOR SIMULTANEOUS DETERMINATION OF HEMATOCRIT AND HEMOGLOBIN LEVELS IN POINT-OF-CARE SETTINGS

4.1 Chapter overview

This chapter describes the development of a paper-based analytical device to determine the hematocrit and hemoglobin levels simultaneously. This device exploits the area of the stain formed by a spreading drop of 20 μL of whole blood on Whatman filter paper immobilized with sodium chloride and Ethylenediaminetetraacetic acid for hematocrit level, whereas for hemoglobin concentration, it utilizes the grey color intensity of a 20 μL droplet of a mixture of blood and deionized water. The performance of the device is verified by comparing with gold-standard results of the automated hematology analyzer, showing a high degree of correlation (R^2) of 0.9651 and 0.9701 for hematocrit and hemoglobin levels, respectively. The bias and standard deviation of the differences between the two measurements for hematocrit determination are 0.002 and 1.005, respectively, while the bias and standard deviation of differences between the two methods for hemoglobin determination are 0.065 and 0.398, respectively. This device is likely to provide a simple, fast, disposable, and inexpensive tool to determine the hematocrit and hemoglobin levels in resource-constraint settings.

4.2 Introduction

Microfluidics-based technologies have shifted the fluid manipulation paradigm, leading to the development of highly efficient miniaturized platforms for medical diagnostics. These platforms are simple, inexpensive, user-friendly, environment-friendly, and disposable (Khiabani et al. 2016; Shih et al. 2012; Wang et al. 2017; Yager et al. 2006; Zehnle et al. 2017). The most important feature of these devices is their application in resource-limited settings as point-of-care (POC) bio-medical devices (Sayad et al. 2018; Yager et al. 2006). Microfluidics-based technologies have several manifestations such as droplet-based microfluidics (Chen et al. 2022), digital microfluidics (Noviana et al. 2020), centrifugal

microfluidics (Hwu and Boisen 2018; Marc Madou et al. 2006), and paper-based microfluidics (Huang et al. 2018; Yamada, Suzuki, and Citterio 2017). Out of these, paper-based microfluidic devices have filled the growing niche for affordable, transportable, and user-friendly medical diagnostic tools (Berthier, Brakke, and Berthier 2016). Paper-based microfluidic devices utilize the capillary force to transport the fluid in the micro-channels. The low-cost and self-pumping ability of paper-based microfluidic devices have made them an attractive platform for POC medical devices (Noviana et al. 2020). Some notable applications of paper devices are hematocrit measurement (Berry et al. 2016), hemoglobin level estimation (Biswas et al. 2021; Yang et al. 2013), detection of albumin-to-creatinine ratio (ACR) (Nurrahmah et al. 2022), study of coffee ring effect of red blood cells (Cao et al. 2020), pH monitoring of sweat (Vaquer, Barón, and De La Rica 2022), determination of forward and reverse blood group typing (Ferrari et al. 2022), and early detection of cancer (Mousavizadegan, Roshani, and Hosseini 2021).

Hemoglobin is a protein contained in the red blood cells responsible for the transportation of oxygen to organs through the circulatory system. Therefore, the mass concentration of the hemoglobin is used as an indicator for measuring the oxygen-carrying capacity of the blood (Lee et al. 2020). The normal hemoglobin concentration range differs by race and age (Lee et al. 2020), however, it ranges from 14 to 18 g/dl for healthy men and 12 to 16 g/dl for healthy women (Yang et al. 2013). A drop in hemoglobin concentration, known as anemia, might indicate chronic blood loss, reduced RBCs production, hemolysis, dietary deficiency, cirrhosis, renal disease, increased heart rate and stroke volume (Asare 2008; Aspuru et al. 2011; McLean et al. 2009). In the chronic anemic patients having hemoglobin level less than 5 g/dl more severe complications such as angina, congestive heart failure, and heart attack have been observed (Weiskopf et al. 1998). Polycythemia Vera (Marchioli et al. 2013), congenital heart disease (Macnee 1994),

severe chronic obstructive pulmonary disease (VICARI AURELIO M. et al. 1998), and severe dehydration (Tefferi 2003) are some serious complications that might be indicated by increased hemoglobin levels. Smoking (Aitchison and Russell 1988) and hypoxia at high altitudes (Horstman, Weiskopf, and Jackson 1980) may also increase hemoglobin concentration. In clinical laboratories, the hemoglobin level is measured with automated hematology analyzers, spectrophotometers, blood gas analyzers, and stand-alone CO-oximeters (Myers and Browne 2007). However, these equipments are costly and not portable and hence cannot be readily available in resource-limited settings. Although some low-cost alternative techniques such as the "one-third of hematocrit" method (Carneiro et al. 2007) and WHO Hemoglobin color scale (HCS) test (Stott and Lewis 1995) have been developed, these methods have several drawbacks which restrict their use in the field and cannot be deployed in POC settings. Although several paper-based devices also have been developed for hemoglobin estimation (Biswas et al. 2021; Yang et al. 2013), most of them requires costly reagents for lysis of the RBCs. Our paper-based device do not require any special reagent, it needs only deionized water for the lysis.

In addition to hemoglobin, the hematocrit level is an important parameter that affects the hemodynamics throughout the circulatory system. Hematocrit, also known as a packed-cell volume (PCV), is the volume fraction of the red blood cells (RBCs) in a given volume of the whole blood sample. The normal range of hematocrit for healthy men is 40 to 54%, whereas it is 36 to 48% for healthy women (Billett 1990). A lower hematocrit level can indicate insufficient healthy red blood cells (anemia) (Kosiborod et al. 2003), infection, or a white blood cell disorder such as leukemia (Johansson and Harrison 2010) or lymphoma (Bachiashvili et al. 2022). However, a higher hematocrit level can indicate dehydration (Koç and Şahin 2022) and lung (Perez-Padilla et al. 1992) or heart disease (Mayer 1965). It may also be noted that some other important clinical parameters such as

erythrocyte sedimentation rate (ESR) and blood viscosity which are the non-specific identification of the disease and considered under routine check-up are also dependent on hematocrit level. Hence, these clinical parameters can also be simultaneously estimated if hematocrit level is known (Kumar et al. 2020).

In clinical laboratories, the hematocrit is measured with centrifugation (Goldenfarb et al. 1971) and automated analyzers (Fleisher et al. 1989). However, the centrifuges and automated analyzers are not readily available in resource-limited settings due to the high cost associated with their acquisition, maintenance, and routine uses. Several alternative techniques have been developed to address some of the restrictions imposed by the limited-resource settings. For example, centrifuges rely on simple mechanical power to determine the hematocrit (Brown et al. 2011) or separation of plasma depends on solute analysis by clinical chemistry (Wong et al. 2008). In contrast, batteries powered centrifuges have also been developed to diagnose sickle cell diseases in POC settings (Kumar et al. 2014). Although these approaches are less expensive than traditional equipment, they require manual labor, access to power sources, and some modified instrumentation and consumables for the analysis. In a nutshell, to the best of our knowledge, there is no simple, cheap, disposable as well as affordable device which can simultaneously determine hematocrit level and hemoglobin concentration from sample to results integration in a single go. Therefore, developing such a device is the need of the hour which can be achieved using paper-based microfluidics.

We develop a simple, inexpensive, easy-to-fabricate, and disposable microfluidic paper-based analytical device to simultaneously determine hemoglobin and hematocrit level. The developed device consists of two detection zones, one for hematocrit estimation and the other for hemoglobin estimation. A 20 µl unprocessed whole blood is used onto the hematocrit detection zone after immobilizing NaCl and EDTA solution, while a 20 µl

mixture of blood and DI water is used onto the hemoglobin detection zone. The device is allowed to dry for 30 minutes after injection of the sample, and the images are digitalized with HP printer scanner. The area of spread is calculated for Hematocrit, and the mean grey color intensity of the dry stain is recorded for hemoglobin estimation using ImageJ software.

4.3 Materials and methods

4.3.1 Materials and equipments

The commercially available printer (LaserJet MFP M233 dw, HP) was used to prepare μPADs using Whatman cellulose filter paper (Grade 1, Whatman TM, GE Healthcare, UK). The post-treatment of the resulting μPADs are performed using a digital hotplate. The area and color intensity of the stains on the μPAD were digitalized using an inbuilt scanner of the printer, and ImageJ software was used to calculate the area and color intensity of the stain. In the present study, all chemicals used were of analytical grade. NaCl was purchased from Fisher Scientific, whereas 0.5 M EDTA was purchased from Sigma-Aldrich. DI water was used for osmotic-hemolysis of the RBCs. DI water was used to prepare all solutions.

4.3.2 Design and fabrication of μPAD

We designed the μPAD pattern using Inkscape software. Each μPAD consists of two detection zones for hematocrit and hemoglobin estimation. The dimension of the μPADs are 54 mm × 34 mm with 10 mm diameter of each detection zone. The μPAD pattern is designed using freely available software Inkscape. The design (Fig. 4.1) is patterned on cellulose chromatography paper using the printer. The printed devices are placed on a heated plate at 180°C for 4-5 minutes to melt the toner particles across the entire thickness

of the PADs, generating the hydrophobic barrier. The backside of μPADs was sealed with transparent tapes to make the device leak-proof and provide mechanical strength.

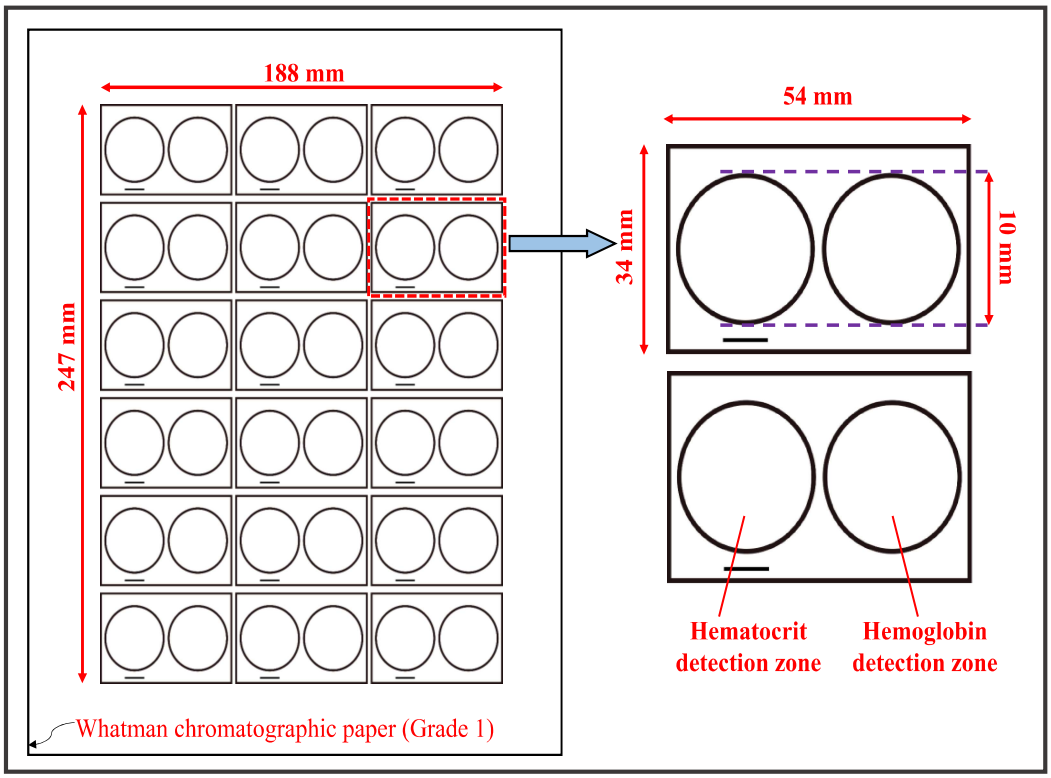

Fig. 4.1 Design of μPADs on cellulose chromatography paper

4.3.3 Blood sample collection and processing

We collected blood samples in vacutainer tubes (K_2EDTA) from the pathology laboratory of Sir Sunderlal hospital (Banaras Hindu University), Varanasi (India), after obtaining ethical approval from the Institutional Ethics Committee of Institute of Medical Sciences (Banaras Hindu University), Varanasi (India). We performed all experiments with blood samples having a wide range of hemoglobin (5 g/dl to 16 g/dl) and hematocrit (25 % to 50 %) levels. Before any experimental run, we shook all samples well to make a homogeneous suspension. The hematocrit and hemoglobin of each sample were estimated using an

Automated Hematology analyzer (KX-21 N, SYSMEX), which is assumed to be a gold standard result.

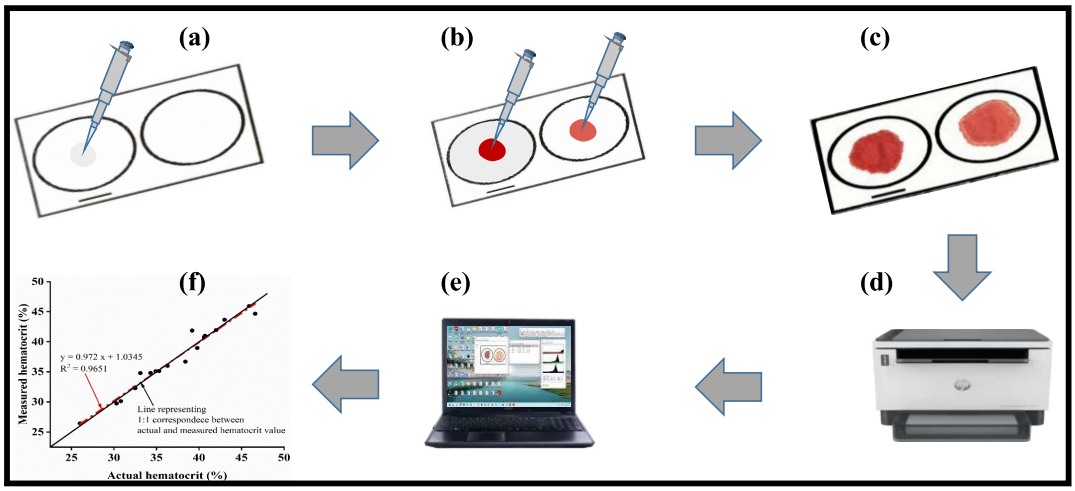

Fig. 4.2 (a) Immobilization of NaCl and EDTA on hematocrit detection zone and allow to dry for 10 minutes, (b) dispensing Whole blood on hematocrit detection zone and a mixture of blood and DI water on hemoglobin detection zone, (c) Radially spread of the sample on the µPAD; (d) digitalization of the sheets of chromatographic paper containing µPADs with the scanner, (e) Analyzing area of spread for Hematocrit and Grey color intensity for hemoglobin; (f) Comparison of the result for Hematocrit.

4.3.4 Experimental methodology

Blood samples are mixed in a ratio 1:2 (volume: volume) with DI water and allowed to incubate for 5 minutes at room temperature (20-25 ºC). The DI water has lysed the RBCs and released the hemoglobin in the supernatant. The EDTA solution and NaCl solution are dropped on hematocrit detection zone until they filled the circular area and allowed to dry for 10 minutes. Then, A 20 µl of unprocessed whole human blood, and 20 µl sample of mixture of blood and DI water are placed onto the center of Hematocrit and Hemoglobin detection zones, respectively. The chromatographic sheets containing µPADs are

digitalized with scanner after drying 30 minutes, and the resulting images are analyzed using ImageJ software. In this work, the mean area of the spread of the whole blood in the hematocrit zone is used for the determination of the hematocrit value, and grey color intensity is used for hemoglobin level estimation. The schematic of the experimental procedure is shown in Fig. 4.2.

4.3.5 Statistical analysis

Regression analysis has been performed to check the linearity of the data used for the calibration curve. The data obtained from the developed device and gold standard device have been directly compared by calculating regression coefficient. A Bland-Altman analysis has also been applied to investigate the agreement between the two devices. To visualize the comparison between the data from the gold standard data and data from our developed device, a chi-square (χ^2) hypothesis test has also been carried out in order to show an agreement between the gold standard value and the current device value.

4.4 Results and discussion

4.4.1 Hematocrit determination

Hematocrit is the volume fraction of red blood cells (RBCs) in a given sample of whole blood. The viscosity of the blood depends on hematocrit level. As the hematocrit level increases, the viscosity of the blood increases and consequently, the area of the stain decreases. Thus, the droplet with varying hematocrit levels generate a stain having a distinct area. The variation of the area of the stain with hematocrit level is shown in (Fig. 4.3(a)). We constructed a calibration curve by plotting the hematocrit value along the horizontal axis and the mean area of stain along the verticle axis (Fig. 4.3(b)). The mean

area of the stain shows a good correlation with the hematocrit level (Mean area of stain = -3.4687 × Hematocrit level + 347.47, $R^2 = 0.9777$).

The relative accuracy of the developed device is evaluated by comparing the hematocrit level measured with report obtained by gold-standard device from 19 different patients. The data points representing these measurements are distributed around the identity line (Fig. 4.3(c), red dashed line), indicating an excellent agreement between the hematocrit levels measured by the both devices. The linear least-square regression analysis (Fig. 4.3(c)), Black solid line) has showed a high degree of correlation between two measurements (y = 0.972 x + 1.0345, $R^2 = 0.9651$).

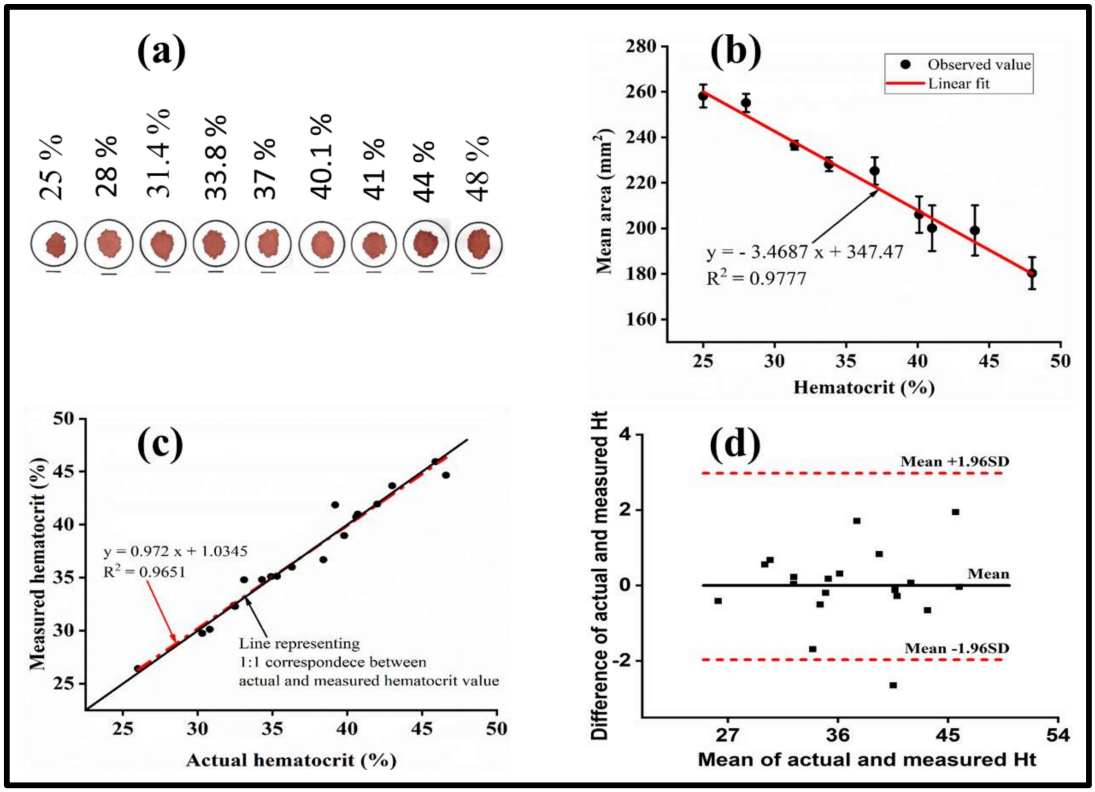

Fig. 4.3 Dependency of the mean area of the stain on hematocrit: (a) Variation of the area of the stain with hematocrit level, (b) Calibration curve was constructed by plotting the hematocrit level

along the horizontal axis and area of stain along the vertical axis. Comparison of hematocrit values measured with paper-based device and automated hematology analyzer (n = 19 samples): (c) The diagonal (black solid line) represents a 1:1 correspondence between actual and measured hematocrit values. The red dashed line represents linear regression analysis of the correlation between the hematocrit value measured by paper-based device and automated hematology analyzer, (d) Bland-Altman plot of the limit of agreement between the paper-based device and automated hematocrit analyzer. The solid black line is the mean of the difference between the hematocrit values measured by two methods (bias). Dashed red lines are the limits of agreements within 95% confidence interval.

Bland-Altman plot was also constructed to evaluate the agreement between the developed method and gold standard method (Fig. 4.3(d)). The mean and standard deviation (SD) of the difference between both methods are 0.002, and 1.006 repsectively. The limits of agreement between two methods are -1.975 and 2.98 %.

A chi-square (χ^2) hypothesis test was carried out to evaluate the agreement between the hematocrit values from gold standard device and the current device for 19 observations. The value of chi-square (χ^2) was found to be 0.49 for 0.005 level of significance and 18 degree of freedom, which was much lesser than (χ^2) from the table (28.869). Therefore, there is an excellent agreement between the hematocrit values from gold standard device and present device

The precision of the developed device was tested by evaluating repeatability and reproducibility (device-to-device and day-to-day performance). The repeatability was tested by measuring repeatedly (n = 3) the hematocrit level for a series of blood samples with hematocrit level 25%, 31%, 37%, 42%, and 47%. The repeatability was measured as the percentage of the relative standard deviation and found to be 4.4%, 6.2%, 5.1%, 8.3%, and 6.4 % respectively. The reproducibility of the device was evaluated as the percentage

of relative standard deviation. The device-to-device performance was evaluated by measuring repeatedly the hematocrit level for the blood sample with hematocrit level 40% in the devices fabricated on the different days, while day-to-day performance of the device was evaluated by measuring repeatedly the hemoglobin concentration for the blood sample with hematocrit level 40% in the devices on different days. The device to device and day-to-day performance were found to be 0.78%, and 0.36%, respectively. The limit of detection (LOD) and limit of quantification (LOQ, also known as sensitivity) of the assay was determined by standard curve method. The LOD and LOQ of the assay was found to be 2.2 and 6.7%, respectively.

4.4.2 Hemoglobin estimation

4.4.2.1 Extraction of the hemoglobin and optimization of the mixing ratio of the blood and DI water

Hemoglobin is a protein contained in RBCs, it is therefore required to extract the hemoglobin from the blood cells. Here, the mechanism of osmatic hemolysis has been utilized to extract the hemoglobin from the red blood cells (RBCs) using deionized (DI) water. DI water is devoid of ions; it is a hypertonic medium in nature, resulting in a net movement of the water into the RBCs via osmosis, causing all the cells to expand and, as a result, lose the integrity of their membranes, causing the hemolysis and release of the hemoglobin into the supernatant over time. For the complete lysis of the RBCs, an optimum mixing ratio of blood and DI water is required. Blood samples and DI water are mixed in different ratios (1:0.5, 1:1, 1:1.5, 1:2, 1:2.5, and 1:3) and incubated at room temperature for 5 minutes. The images of the RBC lysis at the different mixing ratios, captured by microscope (ZEISS Axio Vert.A1) with 10× resolution, are shown in Fig. 4.4. From Fig 4.4(d) it is observed that for the complete lysis of RBCs the mixing ratio of blood and DI

water is 1:2. Therefore, the optimum mixing ratio for the osmotic analysis of the RBCs is 1:2.

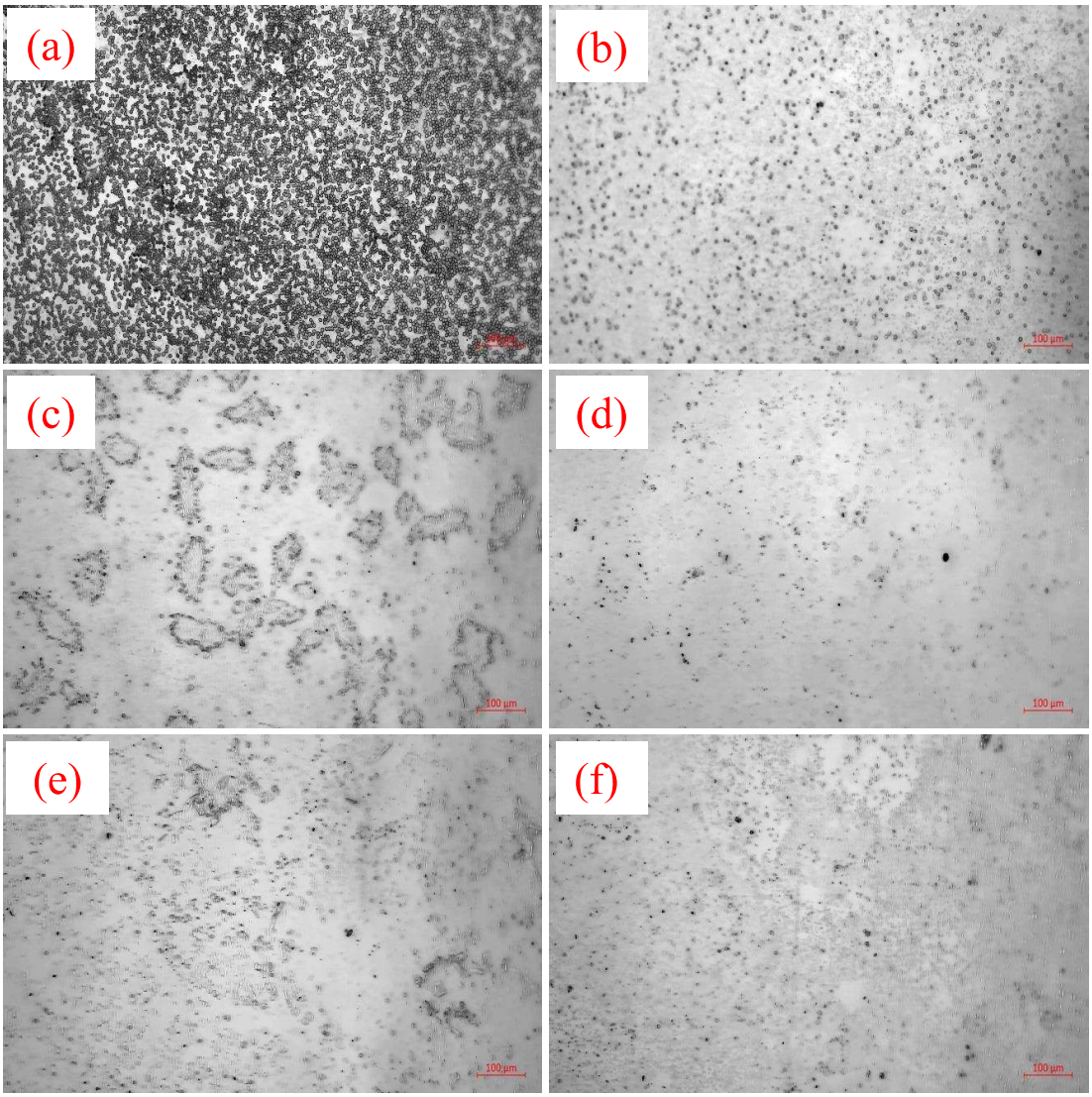

Fig. 4.4 The osmotic hemolysis of RBCs for the different ratios of blood and DI water: (a) 1:0.5, (b) 1:1, (c) 1:1.5, (d) 1:2, (e) 1:2.5, (f) 1:3

4.4.2.2 Operation of the paper-based Hb assay

Paper-based hemoglobin assay was performed by dispensing a 20 µL of the mixture onto the µPAD and allowing the blood-stain to develop. The droplet spreads radially from the center through the paper substrate towards the priphery of the µPAD due to wicking by

capillary action and forms a characteristic blood stain pattern. Droplets with significantly different Hemoglobin content produces stains with different color intensity. The variation in the color intensity of the blood stains with hemoglobin contents are shown in (Fig. 4.5(a)). A calibration curve was constructed by plotting mean grey color intensity along vertical axis and hemoglobin concentration along horizontal axis (Fig. 4.5(b)). The mean grey color intensity of each blood stain strongly correlates with its hemoglobin content (mean grey color intensity = 0.26472 × hemoglobin concentration + 72.079, $R^2 = 0.9975$).

The relative accuracy of the developed device was tested by comparing the hemoglobin concentration measured by Automated hematolyzer for blood sample from 31 different patients. The data points representing these measurements are distributed in vinicity of the diagonal line (Fig. 4.5(c)), red dashed line), which indicates a good agreement between the hemoglobin concentration estimated from the paper-based device and the actual hemoglobin concentration obtained from the gold standard device (Fig. 4.5(c)). The linear least-square regression analysis (Fig. 4.5(c)), Black solid line) shows a high degree of correlation btween two measurements ($y = 0.9797\ x + 0.1672$, $R^2 = 0.9701$).

Bland-Altman plot was also constructed to evaluate the agreement between the developed method and gold standard method (Fig. 4.5(d)). The mean and standard deviation (SD) of the differences between both methods were 0.065 g/dl and 0.398 g/dl respectively. The limits of agreement between two methods were - 0.715 g/dl and 0.846 g/dl.

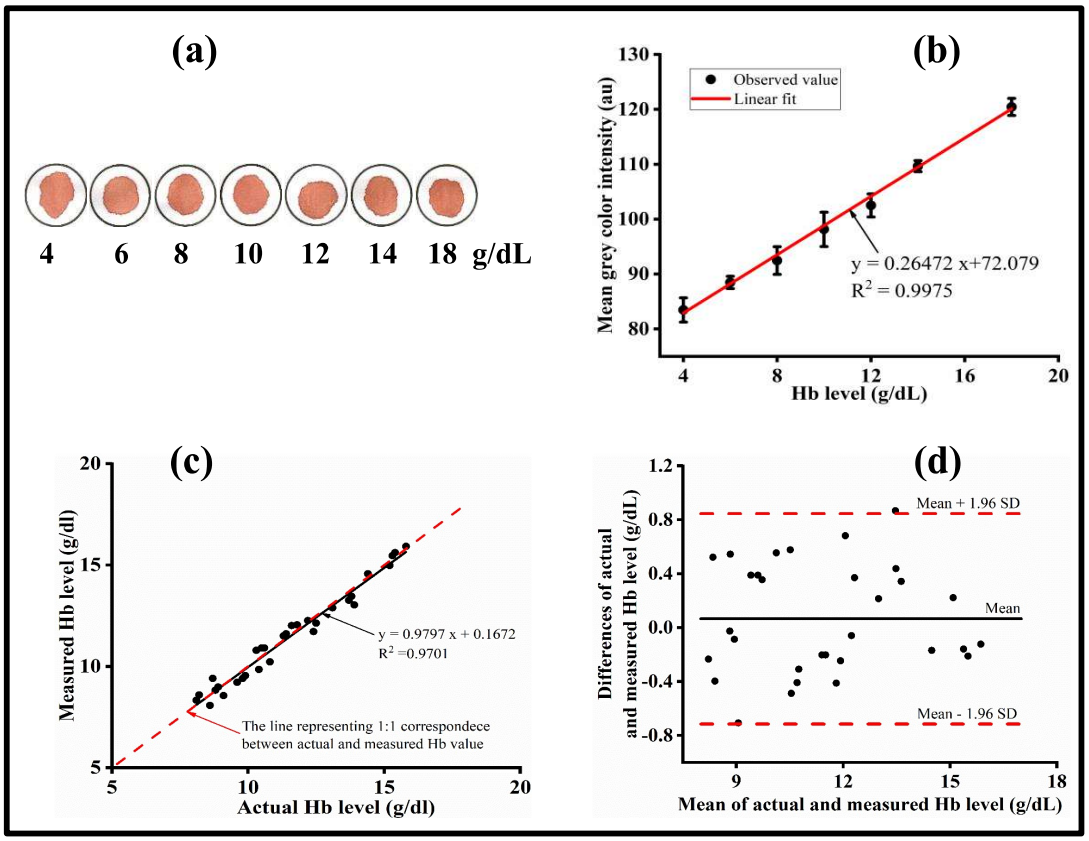

Fig. 4.5 Dependency of the mean color intensity of the stain on hemoglobin: (a) Variation of the color intensity of the stain with hemoglobin concentration, (b) Calibration curve was constructed by plotting the hemoglobin concentration along the horizontal axis and mean grey color intensity along the vertical axis. Comparison of hemoglobin values measured with paper-based device and automated hematology analyzer (n = 31 samples): (c) The diagonal (black solid line) represents a 1:1 correspondence between actual and measured hemoglobin values. The red dashed line represent linear regression analysis of the correlation between the hemoglobin value measured by paper-based device and automated hematology analyzer, (d) Bland-Altman plot of the limit of agreement between the paper-based device and automated hematology analyzer. The solid black line is the mean of the difference between the hemoglobin levels measured by two methods (bias). Dashed red lines are the limits of agreements within 95% confidence interval.

A chi-square (χ^2) hypothesis test was carried out to evaluate the agreement between the hematocrit values from gold standard device and the current device. For 31

observations, the and the level of significance (p-value) is set as 0.05. With this p-value and DOF, the value of chi-square ((χ^2)) is 0.48, which is much lesser than (χ^2) from the table (30.14). therefore, there is an excellent agreement between the hemoglobin values from gold standard device and present device.

The precision of the developed device was tested by evaluating repeatability and reproducibility (device-to-device and day-to-day performance). The repeatability was tested by measuring repeatedly (n = 3) the hemoglobin concentration for a series of blood samples 5, 10, 15, 20, 25 g/dl. The repeatability was measured as the percentage of the relative standard deviation and found to be 3.1%, 3.7%, 3.1%, 6.3%, and 7.4 % respectively. The reproducibility of the device was evaluated as the percentage of relative standard deviation. The device-to-device performance was evaluated by measuring repeatedly the hemoglobin concentration for the blood sample with hemoglobin concentration 10 g/dl in the devices fabricated on the different days, while day-to-day performance of the device was evaluated by measuring repeatedly the hemoglobin concentration for the blood sample with hemoglobin concentration 10 g/dl in the devices on different days. The device to device and day-to-day performance are found to be 0.45%, and 0.37%, respctively. The limit of detection (LOD) and limit of quantification (LOQ, also known as sensitivity) of the assay was determined by standard curve method. The LOD and LOQ of the assay was found to be 0.35 and 1 g/dl, respectively.

4.5 Conclusion

This chapter describes demonstrates a simple paper-based device for simultaneous determination of hematocrit and hemoglobin levels using static image analysis of the stain formed by a 20 μL droplet of the sample, without necessitating any sensitive reagent. Our

paper-based device simply exploits the area of the stain produced by the blood sample on paper for hematocrit estimation whereas it rely on the grey color intensity of the stain developed by the 20 μL droplet of a mixture of blood and DI water on the paper for hemoglobin estimation. We used scanner and laptop computer to digitalize and analyze the developed stains which makes our device independent of the ambient light because scanner itself provides consistent illumination. In summary, we developed and validated a paper-based device for assessment of the hematocrit and hemoglobin levels. This device represents a true potential toward point-of-care and point-of-need testing in resource-limited settings.