Chapter 4 Quantification of Creatinine on Paper-based Devices from Whole Blood using RGB Sensor

4.1 Overview

Creatinine, an important biomarker for kidney function tests often performed from blood serum using gold standard Jaffe reaction. Conventional protocol is associated with a spectrophotometric measurement; not suited for point-of-care applications. Here, we report the development of a single step quantification of creatinine from whole blood using a paper-based microfluidic device. Our platform uses a combination of LF1 membrane and Whatman filter grade 1, fabricated using wax dipping technique; allows on-chip separation of blood plasma through LF1 membrane, while Whatman grade 1 component of the device contains the embedded reagents for Jaffé reaction. This combination of two different grades of paper allows a single step quantification of creatinine as the separated blood plasma traverses to the reaction zone through capillary imbibition. For on-spot quantification, the colorimetric signals were measured using an RGB sensor integrated within a 3D box; thereby virtually making the detection instrument free and suited for point-of-care settings. The limit of detection (LOD) of our device is 0.219 mg/dL which is well below within the normal physiological limits. Furthermore, we have validated the performance of our device with 35 clinical samples, delineating excellent agreement with the gold standard measurements.

4.2 Introduction

Exploration of different microfluidic platforms for pathological testing of physiological fluids has garnered significant attention in recent decades. This is mainly due to its potential

deployment in point-of-care (POC) settings, especially in resource-constrained settings for decentralized diagnostics at affordable pricing. POC settings eliminate the requirements for sophisticated infrastructure and allow to conduct diagnostic tests at patients' site with minimal interventions [124].

Amongst several microfluidic platforms, the micro paper-based analytical device (μPAD) stands out as a suitable alternative for POC settings due to its underlying capillarity-driven wicking of fluids lead to pump-free transport. Moreover, other advantages over like ease of fabrication, flexibility, low cost, excellent reagent storage capacity, quick detection, involvement of minimally skilled personnel, and biodegradability enhance the potential of these devices for several applications e.g., environmental testing [125], milk adulteration [126], and medical diagnostics [127] , to create portable and low-cost devices for onsite testing.

In porous-paper surface liquid transports via capillary imbibition and flow rate of liquid on the surface has been controlled by modifying channel geometries and engraving hydrophobic barriers over the paper substrate. To create the hydrophobic barrier over the hydrophilic paper substrates, different methods have already been utilized such as photolithography [120], wax patterning [28], wax dipping [128], inkjet printing [129], laser treatment [130], plotting [131], and flexographic printing [132]. Even after the modification of paper substrate the flexible nature of solid porous paper matrix allows the further integration of versatile detection methods including colorimetric [133], electrochemical [134], fluorescence [135] and distance based [136]. Among all the abovementioned methods colorimetric based detection methods are quite simple and easy to deploy at POC settings. The colorimetric methods render prominent visible color changes during the reaction of the analyte to be detected with the specific reagent which can be easily interpreted after the processing of images captured using simple portable tools such as smartphone, camera, scanner etc.

Over the past decade, United States has witnessed ~ 30% rise in the prevalence of chronic kidney disease (CKD), attributed due to the increasing life expectancy and the upsurge in lifestyle-related diseases [137]. Unfortunately, in lower- and middle-income countries (LMICs), there is a lack of longitudinal studies and limited statistics available to understand the prevalence of CKD. Increase in the prevalence of diabetes and hypertension often increases the chances of kidney dysfunctions. Creatinine is one of the most common biomarkers, has been utilized for the understanding of several renal disorders or seen as an early indication of kidney malfunction. Off late, different μPADs have been reported to measure proteins from blood-plasma or other biofluid such as urine by using colorimetric detection techniques [102], [138], [139]. Among several proteins present in plasma, creatinine has a crucial role since its detection is highly important for identifying renal diseases. The accepted healthy reference range of plasma creatinine in human body is 0.5–1.0 mg/dL for females and 0.7–1.2 mg/dL for males [140]. The gold standard colorimetric approach, known as the Jaffe reaction, was originally proposed by Max Jaffe in 1886. In the mechanism of Jaffe' reaction formation of a yellowish-orange colored product occurs when creatinine has a reaction with picric acid in an alkaline environment, as described by Lelis et al [141].

Talalak et al. [142] devised an economical enzymatic paper-based analytical device (enz-PAD) to quantify the content of creatinine in human urine. Tsang et al. [143] developed a portable microfluidic 3D paper-based device for creatinine detection from whole human blood utilizing small pore size filter paper. However, they have used wax printing method for device fabrication process which is costly and utilized CMOS camera for image capturing. The technique involves complexities and requires separate software for image processing. It has been observed that above mentioned methods suffer from either one or more of the following shortcomings: (1) requirement of blood-plasma separation in a separate device (2) involvement of costly fabrication technique for blood-plasma separation in the same μPAD (3) usage of

computationally well-equipped gadgets (i.e., smartphones) for image processing purpose, thus inhibiting it's usage in resource limited settings. Herein, we combine two different paper substrates of varying characteristics to ensure on-chip plasma separation from a drop of whole blood and thereafter allows the separated plasma to react with the embedded reagents. In sharp contrast to the reported techniques which discuss the use of smartphone and digital camera, we employ RGB sensor for color intensity measurement leading to the measurement of creatinine from whole blood sample-in answer-out format.

Here, we discuss the development of a microfluidic paper-based device which contains a combination of glass fiber LF1 filter paper and Whatman Grade 1 filter paper interface that has been fabricated using cost-effective wax dipping technique. The smaller pore size of LF1 membrane does not allow the larger size cells to pass through it's porous network leading to effective separation of plasma from whole blood. Whatman Grade 1 filter paper interface which was attached next to LF1 membrane, contains the picric acid reagent for facilitating Jaffé reaction leading to the development of a colorimetric assay. After the reaction, the device is placed inside image analysis platform (housed within a 3D box) which has been developed in-house with an integrated RGB color sensor to measure the color intensity of the reaction zone. It is important to note that the limit of detection is 0.219 mg/dL, which meets well within the physiological standards. Furthermore, our developed platform was validated with 35 clinical samples in laboratory settings against the gold standard measurements. Our validations study shows that out 35 clinical samples, 5 samples shows > 10% deviations while the rest of the samples delineates excellent agreement with the gold standard measurements (<10%); thus suited for quick screening (in less than 5 minutes) in resource limited settings.

4.3 Experimental Methodology

4.3.1 Materials and Method

Whatman (grade 1) filter paper, LF1 glass membrane, creatinine, picric acid, and NaOH were procured from Sigma-Aldrich (Bangalore, India). NaOH solutions were prepared in DI water. To prepare the alkaline picrate solution, picric acid and NaOH were mixed in 5:1 ratio. Standard creatinine concentration was prepared in DI water to produce calibration curve. All the reagents in the present study were of analytical grade and utilized without purification. The microstructure of fabricated device was analysed using Scanning Electron Microscopy (SEM, CARL ZEISS MICROSCOPY LTD, Oxford Instruments Nanoanalysis). To measure the surface roughness of filter paper Atomic Force Microscopy (AFM, NT-MDT Service & Logistics Ltd) was used. Wax (www.merckmillipore.com), iron sheet and permanent magnet were purchased from local market.

4.3.2 Device fabrication process

In the present scope of work wax dipping method was used to fabricate the μPAD. The first step during the fabrication process was to draw the device dimensions using AutoCAD software. After that Wire EDM (Express cut Series-Ex 4032C) was used to cut the channel dimension from an iron sheet. In the next step, LF1 and Whatman Grade 1 filter paper were cut in 12×20 mm dimension and placed horizontally on a clean glass slide overlapping 1 mm from edges as depicted in ***Figure 4.1****(a).* The prepared iron channel was placed on top of the paper surface by keeping blood inlet zone on LF1 side and a strong permanent magnet was placed at the bottom of the glass slide. Now the whole assembly was held vertically and placed inside a hot molten wax (120 ºC) for 1 sec and left at ambient for drying. The complete process of device fabrication was depicted in schematic ***Figure 4.1***. The final fabricated device has a clear hydrophobic wax boundary and a combination of LF1 membrane and Whatman (grade 1) filter

paper for quick quantification of plasma creatinine from finger-pricked blood samples. The same iron mold can further be used many times for device fabrication with dimensional stability.

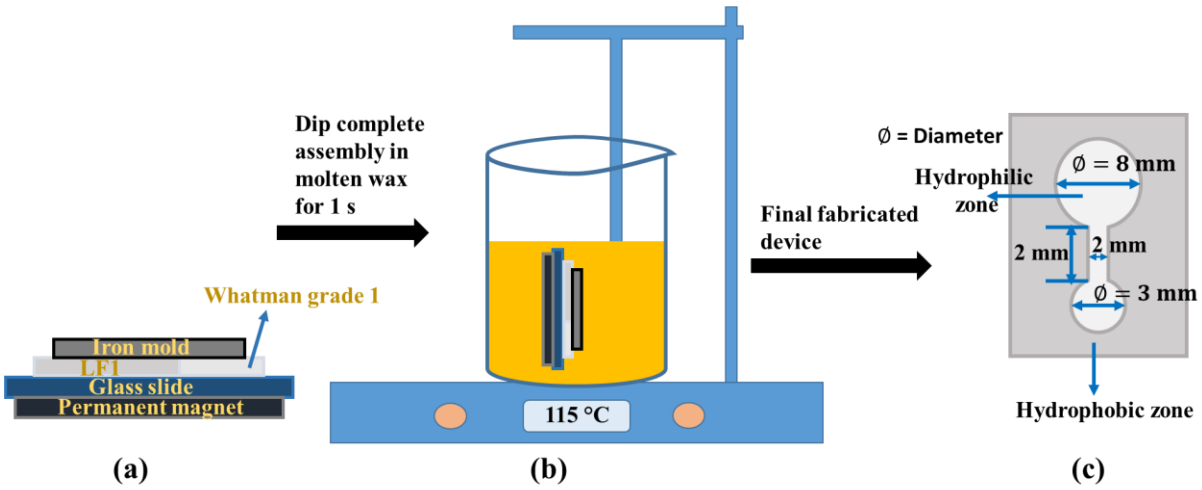

Figure 4.1 Schematic representation of wax dipping method (a) side view of the device assembly prior to dipping inside molten wax container. (b) Dip the whole assembly inside melted wax at 120 °C for 1 s. (c) Finally fabricated paper device with precise wax hydrophobic boundary and a combination of LF1 and Whatman filter paper.

4.3.3 Creatinine estimation method

In this study porous paper substrate has been used to measure the creatinine concentration from blood-plasma sample using the traditional Jaffé reaction. 10 % w/v aqueous NaOH and picric acid were mixed in 5:1 ratio to prepare the alkaline picrate reagent. The fabricated µPAD was placed on a horizontal surface and 10 µL of blood sample was pipetted smoothly at the sample inlet zone (made of LF1). At the same time 1 µL of alkaline picrate reagent were injected in the detection zone, which uniformly disperse in detection zone in 4-5 sec. The time taken by

plasma to separate and travel to the detection is one min. As the plasma reaches the reaction zone the creatinine reacts with alkaline picrate reagent and changes color from yellow to yellowish orange. After 3 minutes of the reaction µPAD was cut using scissor placed inside the in-house prepared 3D box to obtain the image intensity of the detection zone using a RGB sensor. The color intensity of the obtained images was compared with the plasma creatinine concentration to develop a correlation between image intensity and creatinine concentration. The overall process of clinical assay regarding creatinine estimation is illustrated in ***Figure 4.2***.

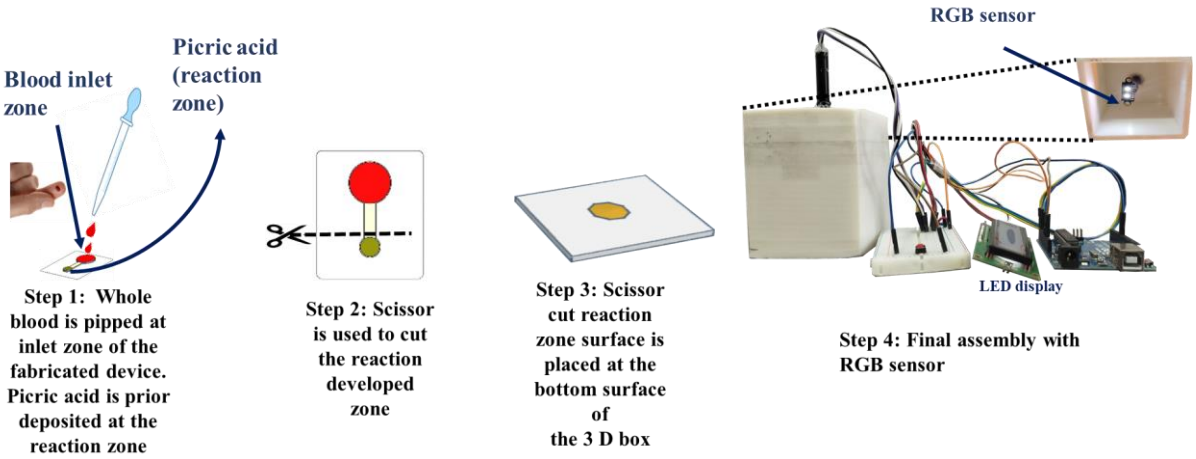

Figure 4.2 The schematic representation of overall colorimetric assay.

4.3.4 Image analysis through RGB sensor

RGB analysis using smartphones is highly device-dependent. A video can be captured through a mobile app to pick the best image for detecting the analyte with the highest precision; however, such a facility needs highly sophisticated machine-learning tools. Thus, the cost of the miniaturized device while using a smartphone app increases drastically. Furthermore, the

smartphone-based creatinine estimation process is complex and computationally intensive. It involves the following steps: image acquisition, spatial filtering for image enhancement, image analysis, region of interest (ROI) segmentation, background correction, pre-processing, feature extraction, and color intensity calculation.

On the other hand, image intensities can be accurately analyzed through RGB sensors without sophisticated computational facilities. Hence, under a low-cost paradigm, the RGB sensor is the best tool for image analysis. In this paper, an indigenous RGB color detector-based hardware module has been developed to measure real-time creatinine concentration. The developed prototype has several components: RGB color sensors, Arduino Uno microcontroller, alphanumeric LCD with I2C converter, micro-SD card reader, potentiometer, push buttons, and relevant passive components. In our experiments, we incorporate Arduino Uno based on the ATmega328P microcontroller. The significant design steps in our prototype are interfacing the RGB sensor with the Arduino controller, writing the color sensor code for the Arduino, data storage, and processing and displaying RGB components to estimate creatinine concentration using blood plasma samples. We can start the serial monitor at the baud rate of 9600. To verify the effectiveness of available RGB sensors, we performed several experiments using TCS34725, TCS3200, and TCS230 to match with the known creatinine concentrations. The TCS3200 has an array of photodiodes with four different filters, i.e., red, blue, green, and clear, having 16 photodiodes for each component. The sensor has a current-to-frequency converter that converts the photodiode recordings into a square wave with a frequency proportional to the light intensity of the chosen color. This frequency is further fed to a microcontroller to estimate RGB components. The TCS3200 supports a 5 V DC power supply and 32 KB flash memory, of which 2 KB is used by the bootloader, 2 KB SRAM, and 16 MHz clock Speed. The power consumption of the Arduino Uno is roughly 1.04 Watts. The

TCS230 color sensor is a light-to-digital converter that uses a photodiode to sense the intensity of light.

From extensive experiments, we observe that the TCS34725 RGB sensor provides a more accurate estimation of the corresponding creatinine values due to the effective RGB and clear light-sensing elements present in TCS34725, providing an incredible 3,800,000:1 dynamic range. The TCS34725 sensor uses an infrared (IR) blocking filter to minimize the IR spectral component of the incoming light. It allows color measurements to be made accurately close to the true color. One can power the breakout safely with 3-5VDC and level shifting for the I2C pins using 3.3V or 5V logic. We incorporated the following formula: Greyscale = 0.299×R + 0.587×G + 0.114×B, where grey scale intensity has been estimated from red (R), green (G), and blue (B) intensities considering their appropriate weights. The obtained grey scale value is further used to evaluate the creatinine concertation using the following expression: Creatinine concentration (mg/dL) = (greyscale intensity – 196.2) / 2.421. This intensity value is aligned with known creatinine concentrations through calibration. By utilizing the calibration curve, the proposed prototype predicts the concentration of an unknown sample. The overall functionality of the application is outlined in ***Figure 4.2*** (step 4).

4.4 Result and discussion

4.4.1 Reproducibility of fabricated channel

The study conducted by Jafery et al.[120] examines the impact of hydrophobic boundaries on the process of imbibition dynamics in paper channels. It has been found that in paper channels without hydrophobic boundaries, the flow velocity remains constant regardless of the channel width. However, when hydrophobic boundaries are included, the flow speed becomes dependent on the width of the channel. The surface tension force opposes the flow direction at

hydrophobic borders, resulting in a decrease in the flow velocity. This phenomenon becomes more pronounced as the channel width decreases, typically about 1 mm. To minimize the hydrophobic boundary resistance in this study, a 2 mm wide channel is fabricated to support the fluid flow.

In wax printed channels during the heating process uneven spreading of wax occurs in both lateral and vertical direction. Apart from this it is not possible to combine two different porosities filter membrane with dimensional accuracy in wax printing process. However, in our technique through wax dipping method a strong magnetic force is applied between mold and paper surface to create a zero gap between glass-paper and iron mold-paper interfaces. This results in the formation of a uniform hydrophobic wax barrier on the paper surface and consequently equal spreading of wax occurs at front and back side of the paper substrate.

To ensure reproducibility of these wax dipped fabricated channels five different iron molds of having inlet diameters of 5, 6, 7, 8, and 9 mm were prepared, and five sets of paper channels were fabricated with each mold. ***Figure 4.3*** *(c)* depicts a strong linear correlation ($y = 0.993\times - 0.1951$, $R^2 = 0.99$) between the mold inlet diameter and final fabricated channel inlet diameter. By using this linearized relation, we can fabricate the channels with the finest precision in the order of magnitude of 300 μm.

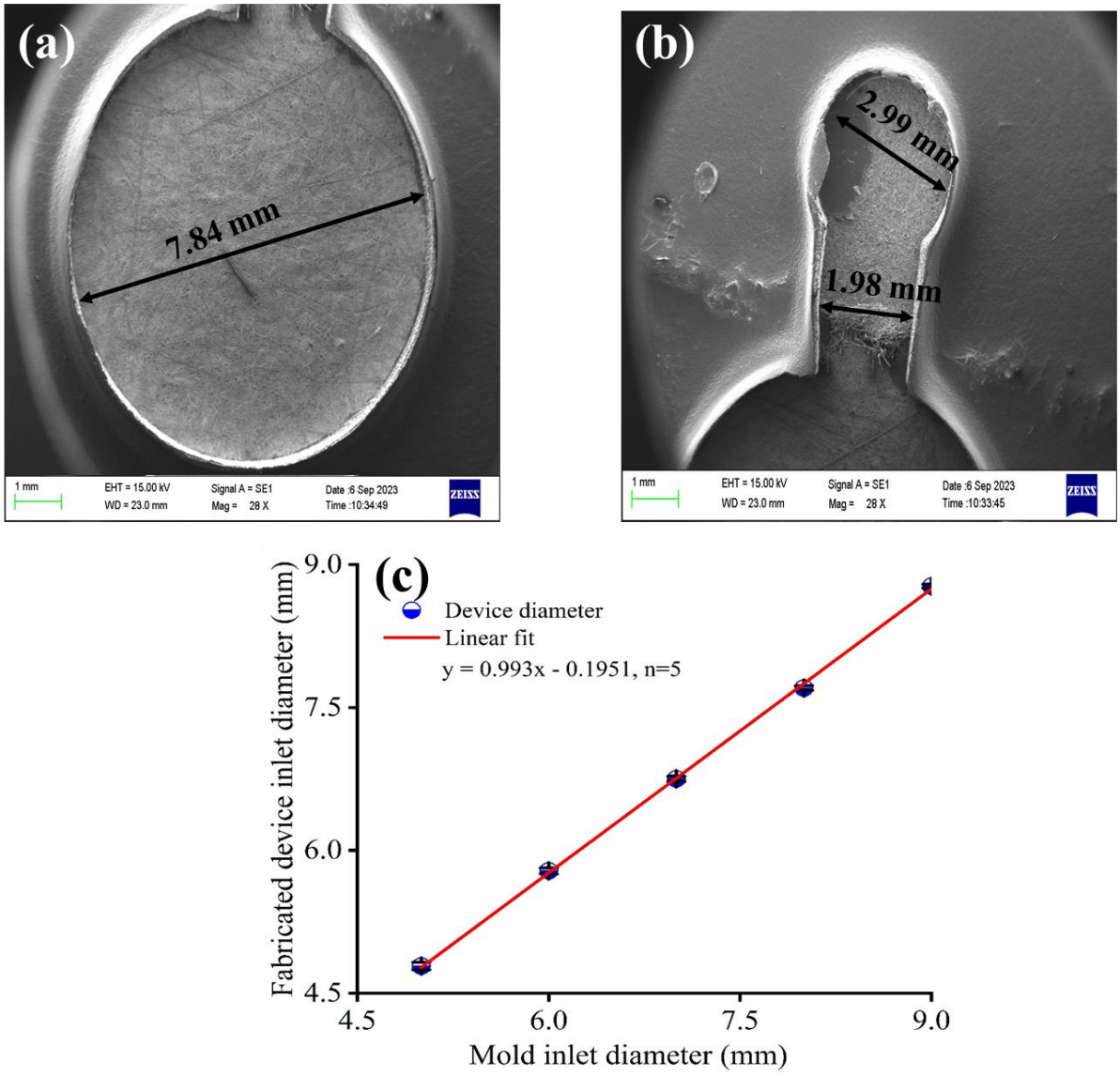

Figure 4.3 (a) FESEM image of sample inlet zone made of LF1 membrane (b) FESEM image of reaction zone made of Whatman grade 1 filter paper (c) a linear correlation between fabricated channel diameter and mold inlet diameter and error bar shows standard deviation for five measurements.

4.4.2 Blood plasma filtration mechanism through porous paper substrate

The mechanism of liquid flow through porous paper substrate is different from that of hollow microfluidic channels. Porous paper substrate is a combination of randomly arranged cellulose fibers and void spaces. The SEM images of LF1 and Whatman Grade 1 filter paper illustrated in ***Figure 4.6*** (a), (c), and (d) clearly depict the random distribution of fibers over the paper substrate. However, the particle retention size of Whatman Grade 1 filter paper and LF1 membrane exhibits notable variations. Specifically, Whatman Grade 1 has a particle retention capacity of 11 µm, whereas LF1 has a capacity of 2 µm. Separation of plasma occurs through the entrapment of RBC particles within the designated pores of the LF1 membrane and the separated plasma further travels through Whatman Grade 1 filter paper to the reaction zone as depicted in Fig ***Figure 4.4***.

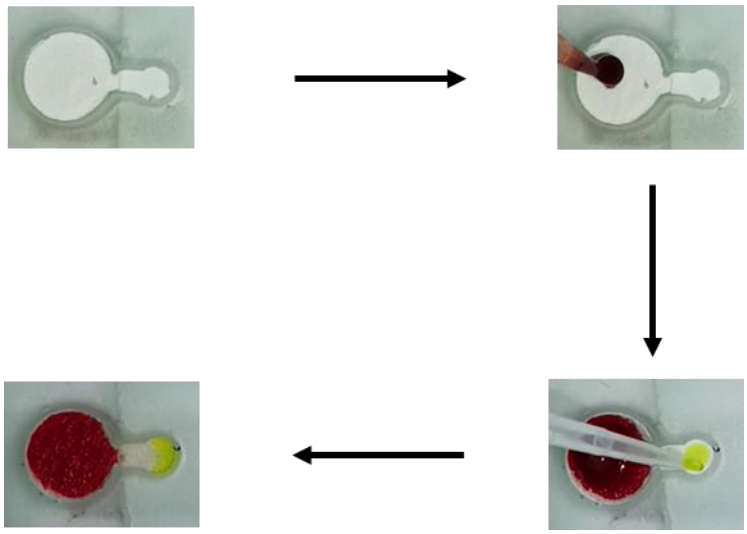

Figure 4.4 Blood-plasma separation and reaction mechanism on fabricated paper-based device.

Figure 4.5 depicts duration taken by plasma for reaching the reaction zone over the fabricated µPAD for different blood samples having varying haematocrit levels. The optimum value

obtained for highest Hct (55 %) blood sample is less than one min which is significantly lesser than traditional available methods confirming high throughputs of the present device even in comparison with other existing microfluidic devices.

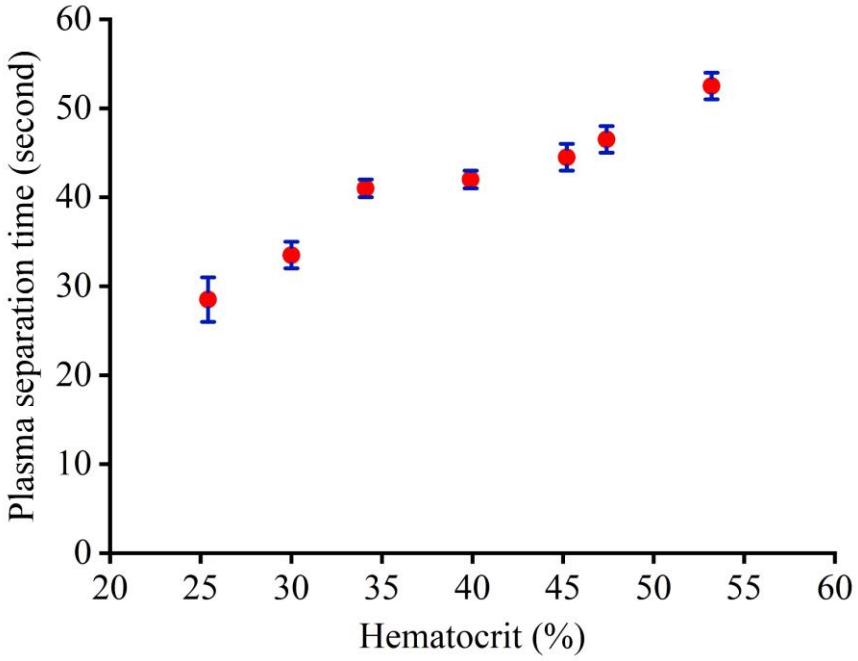

Figure 4.5 Graph between plasma separation time vs different hematocrit blood sample.

Figure ***4.4*** illustrates the fabricated μPAD to separate the RBCs, WBCs, and platelets from plasma through filter membrane and in the downstream direction separated plasma react with pre-deposited picric acid reagent to yield yellowish orange complex according to well-known Jaffe' reaction.

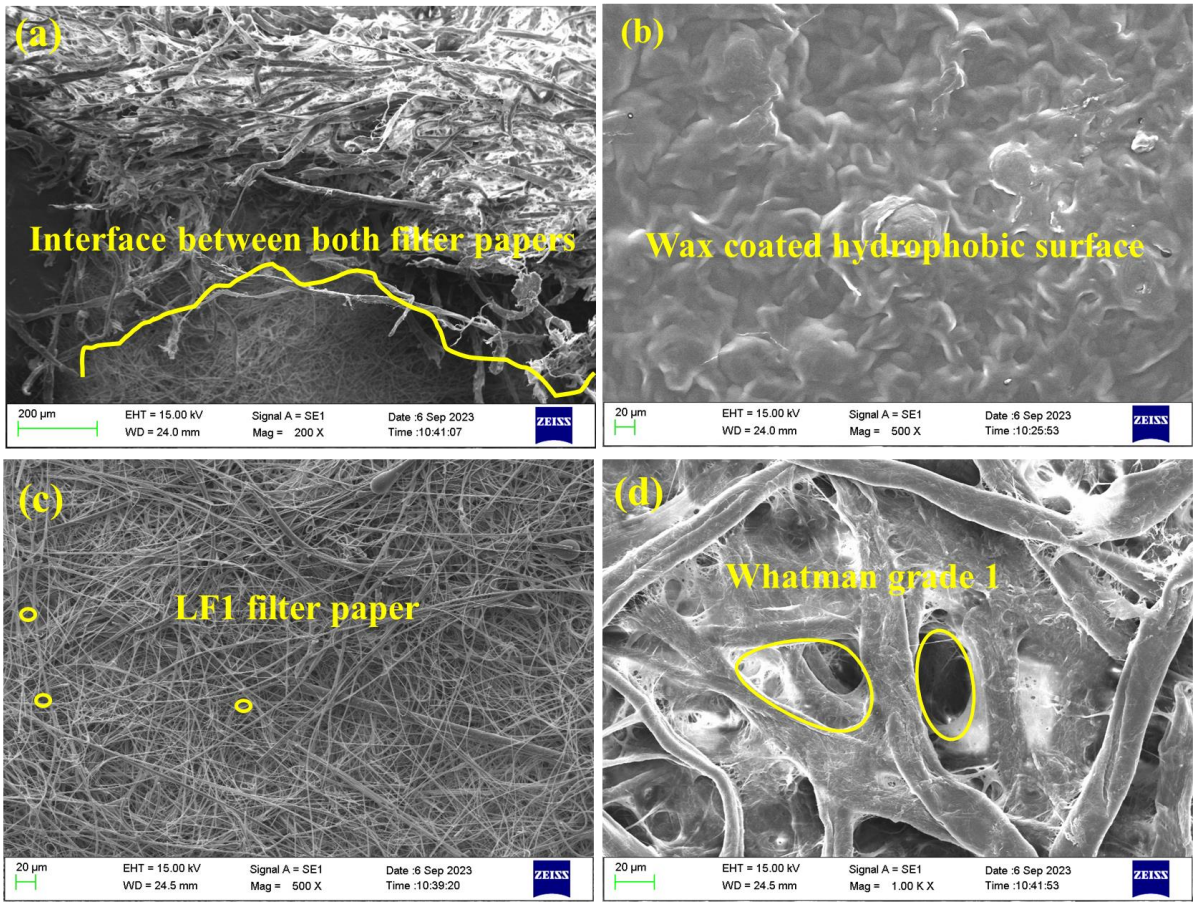

Figure 4.6 (a) depicts a scanning electron microscope (SEM) image of interface of Glass fiber LF1 and Whatman filter paper (b) wax coated hydrophobic surface (c) LF1 filter membrane image at 20 µm scale (d) Whatman grade 1 filter paper at 20 µm scale.

4.4.3 Advantage of combination of Whatman grade 1 and LF1 membrane

LF1 membrane exhibits remarkable efficiency in swiftly separating plasma from whole blood within a mere minute, all without the need for external force application. This characteristic makes it an appealing choice for rapid blood processing in various diagnostic applications. However, its application in colorimetric assays poses challenges due to the non-uniform distribution of reagents within the small micro-porous structure of the LF1 membrane. The colorimetric assay, reliant on the consistent and even distribution of reagents, encounters

limitations when implemented with the LF1 membrane. The intricate network of micro-pores within the membrane leads to uneven dispersion of the reagent, impacting the accuracy and reliability of the colorimetric measurements. Consequently, the LF1 membrane becomes less suitable for applications that demand precise and uniform distribution of reagents, such as in colorimetric assays. Furthermore, the potential use of LF1 membrane in fabricating a complete µPAD raises additional concerns. Integrating LF1 into the entire µPAD structure could compromise the mechanical strength of the device. The inherently delicate nature of the LF1 membrane, coupled with the demands of creating a robust and reliable diagnostic platform, poses challenges in maintaining the structural integrity of the final device.

The 3D projection of LF1 and G1 are shown in ***Figure 4.7*** (a & b). As revealed from the images, the particles embedded into the micropore channel of LF1 and adhered to the surface inhibiting the flow the solution. Conversely, ***Figure 4.7*** b illustrates the W1 paper surface with a uniform spread of analytes. This has been attributed to high mobility of the solution onto the substrate surface.

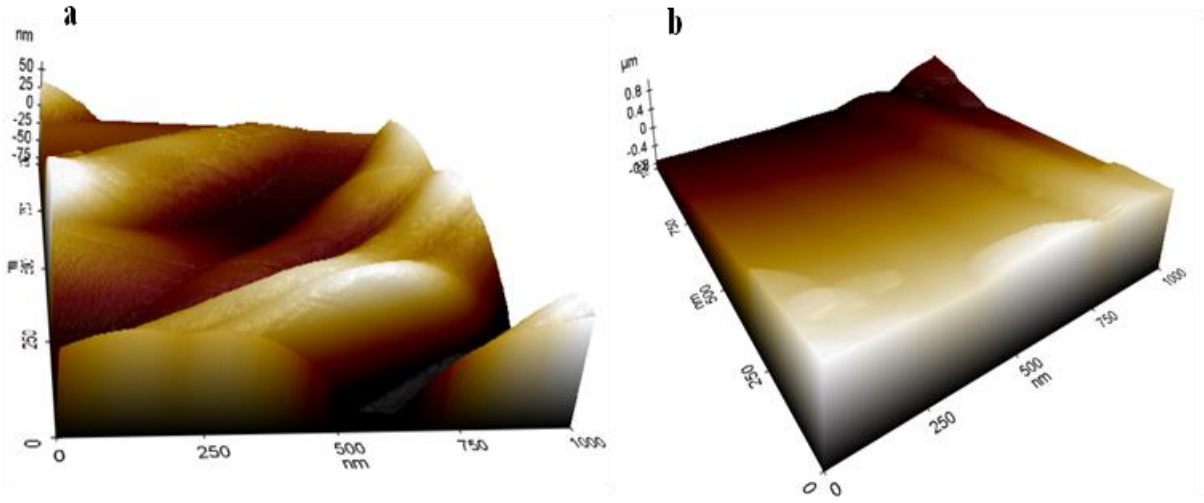

Figure 4.7 (a) 3D projection of LF1 filter paper and (b) Whatman grade 1 filter paper.

Additionally, the surface height histograms, depicted in ***Figure 4.8*** *(a-b),* align with the cantilever deflection observed during cyclic up and down movements. These histograms hold practical value in assessing surface topographical characteristics, particularly the Z-range. They facilitate quantification of height distribution, offering insights into the spread of measured heights. ***Figure 4.8*** *a* illustrates analyte accumulation on LF1 paper, while ***Figure 4.8*** demonstrates uniform analyte distribution across the W1 surface.

Moreover, the non-uniform distribution of reagents within the LF1 membrane becomes a critical issue when scaled up to fabricate the entire µPAD. This non-uniform distribution can introduce errors, particularly in measurements involving substances like creatinine, where precision is paramount. The inconsistencies in reagent distribution across the microfluidic channels could lead to inaccuracies in the analytical results, limiting the reliability of the miniaturized platform for diagnostic purposes.

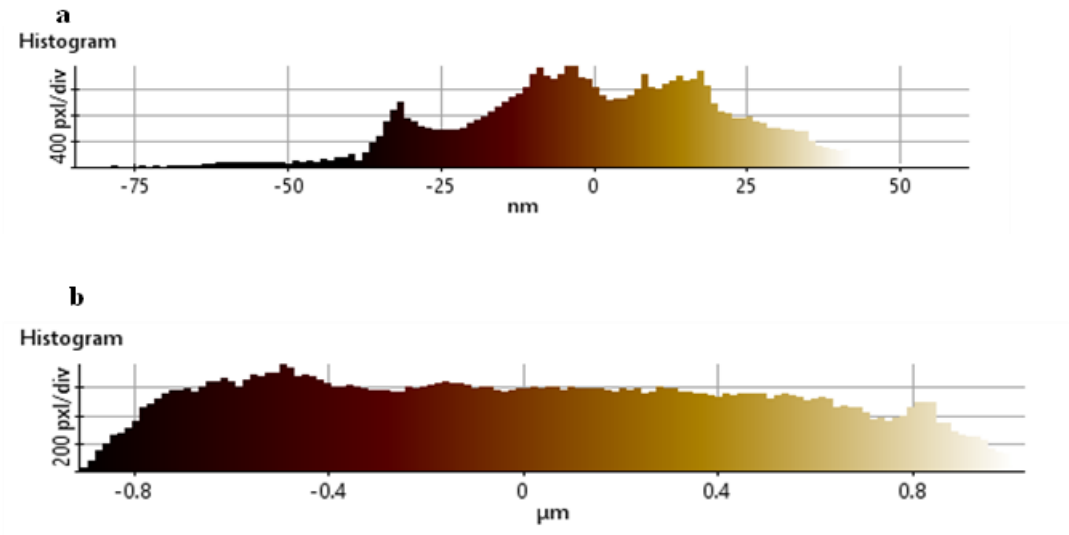

Figure 4.8 (a) Surface height histogram of LF1 (b) Whatman grade 1.

To overcome these major challenges in the present scope of the work, we envisage the combination of LF1 (for plasma separation) and Whatman grade 1 (for uniform reagent distribution) filter paper to fabricate our novel μPAD. A schematic showing Jaffé reaction between creatinine and picric acid in NaOH has been provided in ***Figure 4.9***.

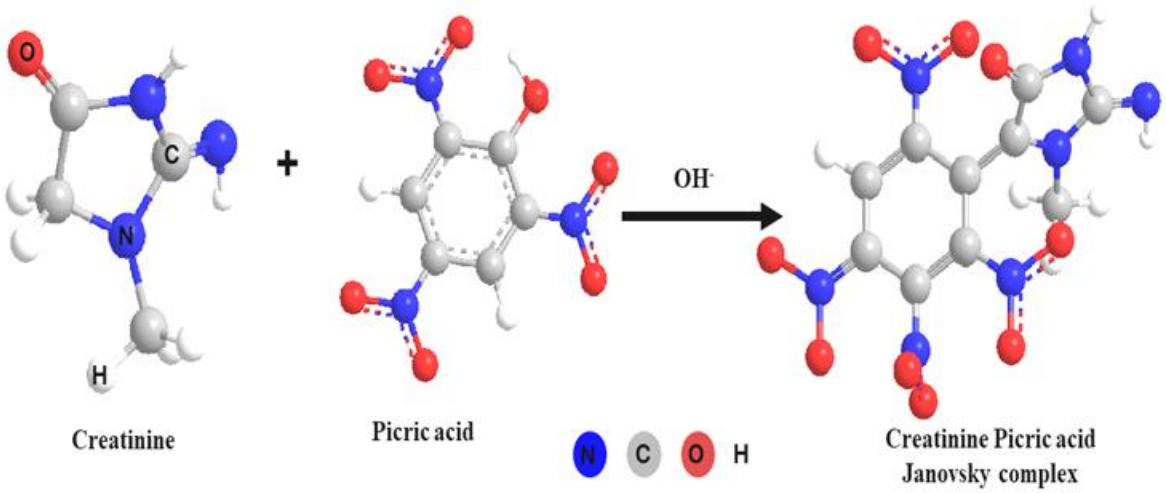

Figure 4.9 Schematic showing Jaffé reaction between creatinine and picric acid in NaOH.

4.4.4 Performance assessment in relation to the conventional approach

Six different concentrations of creatinine such as 0.1, 0.5, 2.5, 5, 10, and 15 mg/dL were prepared to produce the calibration curve. Subsequently, the obtained greyscale intensity is plotted with the known creatinine concentration as shown in ***Figure 4.10****(a)*. An excellent linear correlation is obtained between grey scale intensity and creatinine concentration for five repetitions of each measurement (grey scale intensity = 2.421 × creatinine concentration + 196.2, $R^2 = 0.978$). From linearly fitted calibration curve the obtained value of limit of detection

(LOD) and lower limit of quantification (LLOQ) were found to be 0.219 mg/dL and 0.732 mg/dL respectively (signal to noise ratio three).

For further validation of the accuracy of the developed POC device, 35 blood samples were collected from Sir Sunderlal hospital of Banaras Hindu University (BHU) along with the gold standard test results. Ethical clearance for the same was obtained from Ethical Committee of Institute of Medical Sciences, BHU (Ref. No. ECR/526/Inst./UP/2014/RR-20 dt. 19.5.2020).

First the creatinine concentrations of the obtained samples were measured using our device and after that a comparison of these measurements were carried out against the gold standard Automated Biochemical Analyzer. ***Figure 4.10*** *(b)* depicts an excellent correlation between measurements in our device and measurements in gold standard device. An ideal straight-line with slope as unity is also plotted and the deviations have been found in the order of magnitude of 0.33% leading to the high degree of accuracy and confirming 95% degree of confidence within the error ranges of ± 2.5% as depicted by blue lines in the ***Figure 4.10*** *(b).* It is important to note that only one sample with the creatinine concentration of 1.3 mg/dL (healthy patient), predicted unhealthy from our measurement (1.64 mg/dL); while the rest 33 samples classified correctly in the healthy and patients with ailments categories. Our validations study shows that out 35 clinical samples, 5 samples shows > 5% deviations [1 mis-classified, 1 fall below the LOD of our device, 3 within healthy physiological range, none above the physiological range] while the rest of the samples delineates excellent agreement with the gold standard measurements (<5%).

The non-parametric Chi-square (χ^2) test which has been conducted for 35 blood samples where null hypothesis has been considered as the well-agreement of the measurements from our device with the measurements from the gold standard device. The value of χ^2 from our measured and gold standard data has been found as 0.316. Now with degree of freedom (DOF)

as 34 and level of significance as 5%, χ^2 has been evaluated from the standard table and the same has been found as 48.60. Hence, we observed that χ^2 value in relation to our measurements is well below than its value in the table, hence our null hypothesis becomes true with the said level of significance, further confirming an excellent agreement of our results with gold standard results.

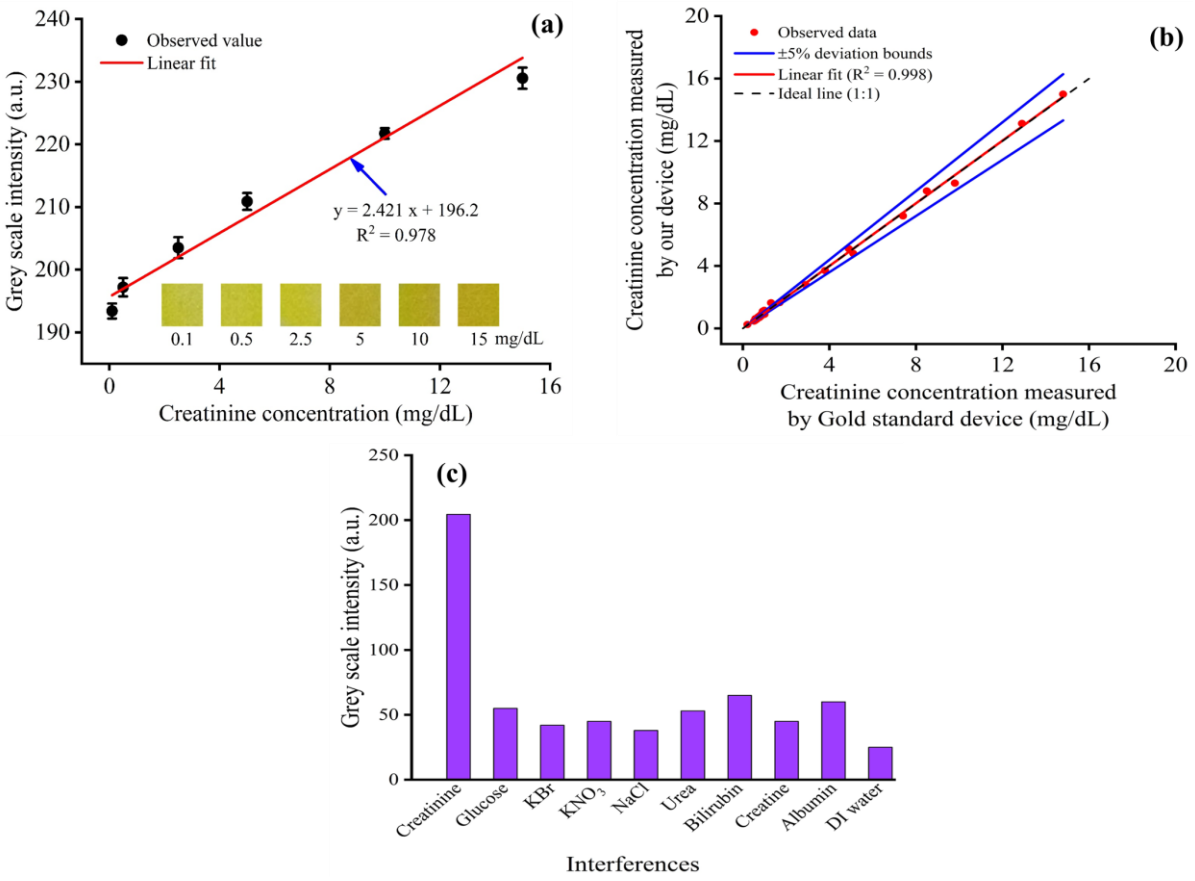

Figure 4.10 (a) Calibration curve for different concentration of creatinine by keeping grey scale intensity on y axis vs creatinine concentration on x axis (b) validation of our fabricated device with gold standard measurement (c) interference analysis of various bio analyte presents in plasma sample.

4.4.5 Performance analysis of the fabricated device

The degree of precision of our measurements were further analyzed through the repeatability and reproducibility of our fabricated device. In order to evaluate the repeatability of the device, multiple readings (n= 5) of different blood samples having creatinine concentration 0.41, 0.52, 0.78, 1, 3.4, 7.8, and 13.5 mg/dL were measured. The selected concentrations cover clinical ranges of renal diseases. The relative standard deviation (RSD) value for different measurements were found to be 4.23%, 2.15%, 1.12%, 3.48%, 2.97%, 1.99%, and 2.29%. The obtained value of RSD is below 5 % which indicates that our proposed device has the potential to replace the gold standard device.

The day-to-day performance of the device is evaluated by repeatedly measuring the creatinine concentration for the blood sample with a creatinine concentration of 4.5 mg/dL in the devices that were fabricated on different days. On the other hand, the device-to-device performance is evaluated by repeatedly measuring the creatinine concentration for the blood sample with the same creatinine concentration in the devices that were fabricated on different days. The relative standard deviation (RSD) from device to device as well as RSD from day to day are 0.59% and 0.78%, respectively. From this repeatability and reproducibility test it can be summarized that our device renders the finest precision like the gold standard device.

4.4.6 Impact of co-analytes

In the present scope of work, alkaline picrate was utilized for the detection of creatinine in blood-plasma samples having some other common interfering agents such as bilirubin, albumin, urea, glucose, NaCl, KNO_3, and KBr which were further considered to carry out interference analysis on the present μPAD platform. 0.7 mM concentration of creatinine as well as 1.4 mM concentration of other analytes were studied for interference analysis. ***Figure 4.10***

(c) depicts bar diagram between different interfering agents and corresponding grey scale intensity. From this figure it can easily be ascertained that our clinical assay is least influenced by any other analyte except creatinine. From this interference study we envisage that our proposed blood test kit can successfully replicate Jaffe' reaction principle in our POC device leading to the perfect miniaturization without any biasness in the accuracy due to the presence of other bio-analytes.

4.4.7 Comparison with existing creatinine measurement microfluidic device

A comparative study has been conducted between our method and existing microfluidic paper device for creatinine measurement as depicted in **Table 4-1**. From **Table 4-1**, we can conclude that our device is single one among all other portable devices which uses whole blood instead of plasma and facilitates the reaction at room temperature additionally integration of RGB sensor in our device makes it portable in the truest sense.

Table 4-1 Comparison between proposed device and available microfluidic paper device for creatinine measurement

	[144]	[145]	[146]	[147]	[142]	**Present method**
Sample type	Urine	Whole blood	Urine	Urine	Urine	**Whole blood**
Reaction	DNBA	Jaffé	Jaffé	Jaffé	enzymatic	**Jaffé**
Assay duration	6 min	6 min	12.5 min	5 min	7 min	**5 min**
Detector	Smartphone camera	CMOS camera	Scanner	Tablet camera	Scanner	**RGB sensor**
LOD	0.82-10.0 mmol. L^{-1}	0.02-0.68	0.46-5.31	0.15-8.85	0.22-2.21	**0.219 mg/dl**
OT	RT	37 ºC	elevated	RT	RT	**RT**
Device cost	low	high	low	high	low	**lower**

4.5 Summary

Most of the clinical paper based clinical assays developed for the measurement of creatinine concentration use separated plasma samples, however, in sharp contrast to reported assays our novel assay uses whole blood directly. The paper device has been fabricated through wax dipping technique, combining LF1 membrane and Whatman Grade 1 filter paper. The later porous substrate stores Picric acid and NaOH in 5:1 ratio for Jaffé reaction to occur. The whole blood is pipetted in sample inlet zone made of LF1 membrane which filters plasma from blood. The filter plasma travels to the reaction PAD (Whatman paper) due to capillary imbibition and reacts with alkaline picrate. The color of alkaline picrate changes prominently from yellow-to-yellow orange depending on the concentration of plasma creatinine.

Usage of 3D box for image quantification, nullifies the effects of ambient light while capturing images, thus, actual image intensities could be analyzed. After that an RGB sensor is placed at the top surface of the box to measure the grey scale intensity of the reaction zone leading to the accurate estimation of creatinine concentration through a linearized relation with excellent correlation ($R^2 = 0.978$). We have validated our biosensor with gold standard device using 35 real blood samples and obtained an excellent agreement ($R^2 = 0.95$). Hence, our device integrated with RGB sensor holds the potential as an alternative to the gold standard tests as an equipment free and accurate alternative suited to cater the needs for POC settings.