REVIEW

Bio-analysis of Saliva Using Paper Devices and Colorimetric Assays

Adhan Narasimhan[1,2] · Harshil Jain[1,2] · Kowshika Muniandy[1,2] · Raja Chinnappan[2,3] · Naresh Kumar Mani[1]

Abstract

In light of the evolving nature of various diseases, time becomes a crucial factor in diagnosis and identifying the underlying causes. A point-of-care device provides a rapid diagnosis of a disease without using complex and advanced instruments, which are costly and difficult to transport. A paper-based device is a relatively frugal solution wherein the paper is used as a substratum in which the reactions are carried out. These methods are non-invasive, and the sample collection is relatively easy. Saliva is one such body fluid in which various biomarkers are present for numerous diseases. Bioanalysis of saliva has attracted more attention recently due to its non-invasiveness and robustness. Exploiting the discovery of clinical biomarkers from salivary analysis has the potential to revolutionize the healthcare sector by providing crucial information to monitor the health status of individuals and disease progression which enables personalized treatment. This review provides the limitation of the traditional methods in clinical applications and highlights the significance of saliva as a non-invasive biological fluid that is a source of multiple biomarkers associated with various diseases. It also provides insights into the different paper-based colorimetric microfluidic devices developed against salivary biomarkers in the past decade. The major challenges in the point-of-care application and the future prospects have been discussed as well. Further, we also emphasize the importance of this approach in dental disease diagnosis which is least explored and holds potential applications.

Keywords Paper-based assay · Salivary analysis · Non-invasive · Point-of-care · Body fluids · Biomarkers · Qualitative detection

1 Introduction

Clinical diagnosis and health monitoring are critical parts of healthcare management, which incites the treatment decision and track of a patient's health progress [1]. The accuracy and efficiency of clinical diagnosis have improved

Adhan Narasimhan, Harshil Jain and Kowshika Muniandy have contributed equally.

✉ Naresh Kumar Mani
naresh.mani@manipal.edu; maninaresh@gmail.com

1 Microfluidics, Sensors and Diagnostics (μSenD) Laboratory, Centre for Microfluidics, Biomarkers, Photoceutics & Sensors (μBioPS), Department of Biotechnology, Manipal Institute of Technology, Manipal Academy of Higher Education, Manipal, Karnataka 576104, India

2 College of Medicine, Alfaisal University, Riyadh 11533, Saudi Arabia

3 Tissue/Organ Bioengineering & BioMEMS Lab, Organ Transplant Centre of Excellence, Transplant Research & Innovation Department, King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia

significantly due to advancements in medical technology. A wide range of diagnostic tools such as blood tests, imaging, wearable devices, remote monitoring, and real-time electronic health records have been developed [2]. Though these tools are more accurate and qualitative, the analysis is time-consuming, requires laboratory setups, expensive sophisticated equipment, and well-trained professional operators. Rapid analysis is a critical factor that could facilitate the timely treatment of patients. Modern biosensor technology tools overcome these limitations [3]. One such potential diagnostic tool that provides prompt and reasonable precise outcomes is paper-based microfluidics [4]. Paper-based devices have numerous benefits, including their affordability, user-friendliness, rapidness, reliability, portability, and most significantly, their biocompatibility [5–16]. In addition, thread-based devices were also leveraged for wide range of sensing applications [17–35]. Generally, invasive and non-invasive are the two types of clinical sample collection methods that are used for bioanalysis [36]. Invasive techniques involve penetration to the skin and body tissues. They have their own set of disadvantages, which include discomfort to the patient, time taken for

wound healing in case of incision, potential puss formation and development of infection in the cut area, time taken for result analysis, cost and economics of the equipment used to analyze the sample and poor ease of use [37–40]. These disadvantages create a void to be filled using a fast, effective, and reliable detection technique where non-invasive sampling and paper-based devices play a significant role [41]. In non-invasive sampling, the samples are collected from the patient without penetrating the dermis or skin layer. The sample collection is usually from saliva, urine, gums and gingival crevicular fluid (GCF), tears, and sweat. Overall, the patient samples containing the biomarkers to be detected undergo appropriate diagnostic procedures, and their levels are compared to those of normal levels. Additionally, to diagnose a health status or condition, healthcare professionals leverage other monitoring and imaging techniques. These include heart rate monitoring, electrocardiograms, blood pressure tracking, magnetic resonance imaging (MRI Scans), computed tomography (CT scans), and other methods [42–47].

In general, various conventional methods are used for detecting biomarkers including Electrophoresis [48], Chromatography [49], polymerase chain reaction (PCR) [50], enzyme linked immunosorbent assay (ELISA) [51], Spectrophotometry [52], Chemiluminescence [53], and Electrochemical methods [54]. These methods are the most accurate and reliable in determining the concentration of the biomarkers. Chromatography uses preferential separation of compounds in determining the desired biomarker [55], whereas electrochemical methods use change in the electrical signals in the presence of the target biomarker and signal change is utilized for the quantitative detection of the biomarker [56]. Chemiluminescence and spectrophotometry involve the emission and absorption of light. In chemiluminescence, the biomarker is usually allowed to react with another luminophore that resulting in light emission [57], which is detected by the light sensor. Spectrophotometry involves the determination of compounds using their specific absorption wavelength [58]. ELISA is an immunoassay where the emission of light marks the detection of the compound due to the binding of fluorescent-tagged antibodies to the biomarker [59, 60]. Despite their accuracy and reliability, conventional methods have several drawbacks, including time-consuming processes, high costs, inconvenience, and challenges in transporting and storing samples. Additionally, the required instruments for analysis are often available only at specialized facilities, making the process even more cumbersome [61].

Saliva is a viscous fluid that is secreted by the salivary glands, which are present under the jaw and near the ear [62]. The fluids produced by salivary glands help with the digestion and breakdown of food. They also protect the mouth's lining from becoming dry and eliminate any harmful bacteria that may enter through the mouth by using peroxidases and enzymes [63, 64]. Saliva consists primarily of water with various enzymes including lipases, lysozyme, amylase, maltase etc. [65, 66]. Saliva has an average pH of 6.7, making it slightly acidic. The change in the pH could also be an indicator for diseases such as gingivitis [67]. Saliva also consists of a relatively large number of white blood cells and other electrolytes and antibodies such as IgA [68]. Saliva and urine are two of the most convenient and effective bodily fluids for non-invasive diagnosis, as they both contain numerous biomarkers that are associated with different diseases and their stages. When the levels of biomarkers in the blood increase, they get secreted in the saliva, and hence saliva also contains biomarkers from the blood such as certain viruses, glucose, and lactate [69, 70]. In addition, saliva is constantly secreted, making saliva the most viable source for collecting samples for disease diagnosis using non-invasive methods [71]. In spite of many advantages, using saliva as a biofluid for sample collection has some drawbacks. One of them is the safety of the medical practitioner who handles the sample, as it may be contaminated with a bacterial or viral disease [69]. Proper disposal of the used sample after diagnosis is essential. Improper disposal methods lead to contamination. In cases where patients experience excessive salivation, the collected sample may not be suitable for diagnosis due to the potential for significant dilution of their saliva. The schematic illustration showing invasive and non-invasive methods for sample collection, different salivary biomarkers and conventional methods for detecting the salivary biomarkers is given in Fig. 1.

In recent years, microfluidic paper-based analysis devices (μPADs) have seen remarkable development. Compared to conventional tests, they are economical and can be used to diagnose various diseases [72]. Paper-based microfluidics system that consists of patterned paper, with small volumes of fluid moving through multi-channel designs within the paper substrate, using capillary action [73–76]. They are compatible with testing biological samples such as serum, urine, whole blood, nasal swabs, and saliva [77, 78]. Paper-based microfluidics use various approaches for detection, namely, colorimetric and electrochemical methods [79–83]. Other methods of detection include electrochemiluminescence and chemiluminescence [84, 85].

The colorimetric method is widely used for detecting myriads of analytes. This approach involves observing a color change when the desired analyte is present [86]. Colorimetry is arguably the simplest form of detection as it does not involve the requirement of any complex instruments to detect the change observed. The 'color change', aspect of colorimetry is defined by the laws of physics and the color observed is a result of an electron transition from a higher state to a lower state. The tuning and construction of the color detection algorithm however is a complex process as
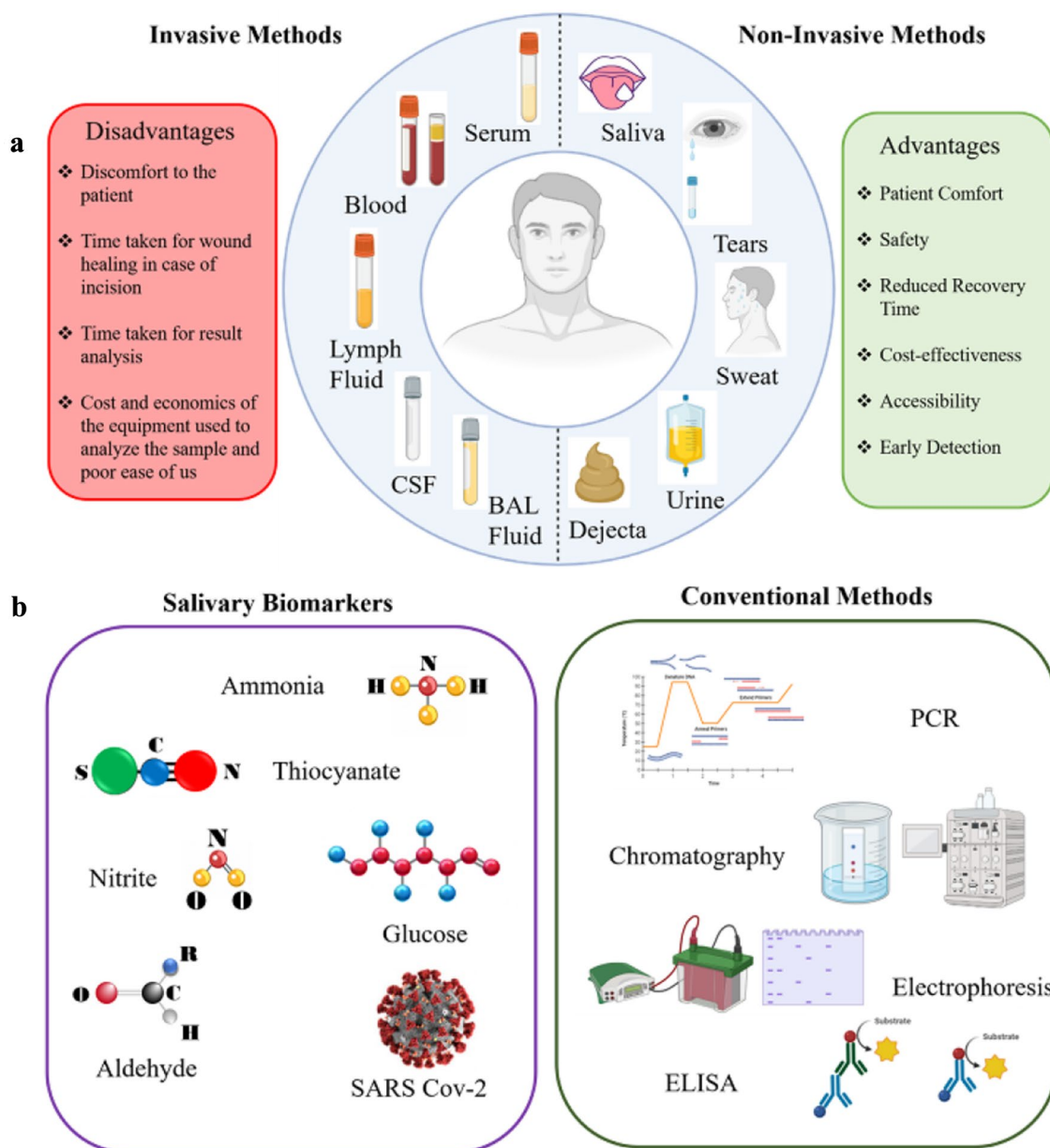
Fig. 1 Schematic illustration of **a** invasive and non-invasive methods for sample collection, **b** various salivary biomarkers and conventional detection methods

the creators need to specify the hex value range in which the application should output a set of defined remarks for those ranges. The software not only needs to be able to distinguish color but also be able to detect the intensity of the output which can convey the quantity of the compound that is detected. In colorimetry, the color change is initially plotted on a graph to determine a standard concentration slope, this concentration slope is obtained by testing a set of linearly varying concentration ranges. The standard curve obtained by this method is also known as the calibration curve, using which the detected output is quantified. The minimal concentration of the analyte that can be detected

using a device is termed as its limit of detection, below which there would be no visible or significant color change observed. In most devices, the limit of detection is usually less than the lower range specified. This is done to eliminate false positive results that may arise due to impurities present in the sample.

There are two distinct approaches available for creating μPADs; they are physical deposition and chemical modification, respectively. Standard manufacturing methods include polydimethylsiloxane (PDMS), wax printing, inkjet printing, and laser cutting. Other manufacturing methods, such as thermal embossing, photolithography,

and hydrophobic silylation, are also present [87, 88]. According to the World Health Organization (WHO), there is an urgent need for diagnostic methods that are not only affordable but also sensitive, specific, user-friendly, robust, rapid, equipment-free, and deliverable to end-users (ASSURED Criteria). Among the various available diagnostic tools, paper-based analytical devices (PADs) have emerged as an up-and-coming option. This is because PADs can meet all the aforementioned criteria, providing an efficient and convenient solution for diagnostic testing. In addition to their ease of use, PADs also offer the advantages of low manufacturing costs and easy disposal, making them an increasingly popular choice in the field of diagnostics [89]. This review article focuses on the application of paper-based devices that deploy colorimetric methods for detecting biomarkers from the salivary fluid. Lateral flow devices for detecting biomarkers in saliva have not been discussed in this paper as it is beyond the scope of the paper, and readers of this article are advised to lean towards other review papers regarding the information on different testing methodologies [90].

2 Detection of Biomarkers Using Paper-Based Systems

Biological marker or Biomarker is a collective term used for diagnosing any disease or condition in the human body. Biomarkers have many types, such as biochemical, pathogenic and single molecule or ions. These biomarkers are used for diagnosing oral diseases and early prognosis of other conditions such as cancer and periodontitis in the human body. Biochemical markers are chemicals or molecules that are produced or released at the time of disease, either at the initiation of the disease or during its progression [91]. These markers are critical indicators as their levels in the body rise or fall from the normal levels during illness. By measuring the levels of specific biomarkers, both the individual and practitioner can determine the appropriate countermeasures to take. Most of the biochemical biomarkers are present in the blood, while some can be found in tissues of tumors, stool, saliva, and urine [92]. Biomarker diagnosis from urine and saliva is the most easy and non-invasive sampling. Herein, the potential biomarkers were identified from the salivary samples using paper-based assay systems. Various biomarkers such as thiocyanate, nitrite, ammonia, cyanides, aldehydes, lactoferrin, and lactates are specific to wide range of diseases. In this section, we highlight different colorimetric detection approaches used for detecting aforementioned biomarkers (Table 1).

2.1 Thiocyanate

Thiocyanate, or SCN^- is a conjugative base of thiocyanic acid. It is an anion, and it functions as a human metabolite [92]. It is often found in human urine and saliva samples and can be used as a biomarker to indicate different disease diagnoses and progression. Thiocyanate is one entity of the dissociation products of glycosinolates and cyanide [93]. Thiocyanate also functions in the host defence mechanism by being a part of the lactoperoxidase microbicidal pathway [94]. Since thiocyanate is an anion, it combines with other cations, such as ammonia, sodium, potassium, etc., to form ionic compounds that may negatively affect the body. For example, prolonged and repeated exposure to ammonium thiocyanate is found to cause nausea, headache, and vomiting. Potassium thiocyanate can cause headaches and, if inhaled, can lead to respiratory tract infection. The presence of thiocyanate in saliva has been associated with different diseases and could serve as a promising biochemical biomarker for detecting them. Paper-based microfluidics provides easy and cheap detection of thiocyanate, and many papers have been published linking thiocyanate to disease progression and their detection using paper-based microfluidics. Pena-Pereira F et al. successfully developed a colorimetric assay for in situ detection of thiocyanate from saliva. The assay uses $Fe(NO_3)_3$ as a reagent, when it reacted with thiocyanate in the presence of nitric acid, $Fe(NO_3)_3$ turns into $Fe(SCN)_3$ [iron(III)-thiocyanate], a red-colored complex. The wax printing method was used to prepare the hydrophobic areas, and the detection limit was 0.06 mmol/L of thiocyanate [95]. Jirayu Sitanurak et al. improved the economy of mass-producing these paper-based devices by preparing a new and cheap method for printing the hydrophobic barrier in Whatman filter paper using T-shirt ink (Fig. 2). The reagent used was $Fe(NO_3)_3$, and the detection limit for thiocyanate was 1 mmol/L [96].

Yu et al. used spraying-based technology for the fabrication of the device. The filter paper was seated on top of a patterned acrylic plate, and later the lacquer was spray painted on the filet paper forming a pattern of the plate. Separate bottom and top layers were fabricated simultaneously, and then they were sandwiched together to form a 3D layer. Ferric nitrate was utilized for the detection, and the limit of detection was 0.074 mmol/L [97]. In another study, Pungjunun et al. developed an improved $\mu_{pump}PAD$, a microcapillary grooved device engraved by LASER capable of dual-mode sensing using both colorimetric and electrochemical detection. These devices have a more comprehensive range of thiocyanate detection, and for colorimetric-based detection, $Fe(NO_3)_3$, was used and the limit of detection was 6 μmol/L [98]. The schematic illustration showing the $\mu_{pump}PAD$ assembly and assay procedure of $\mu_{pump}PAD$ is given in Fig. 2.

**Table 1** Different salivary biomarkers and their detection using paper- based devices

No.	Biomarkers	Diseases	LOD	Chemicals used	Fabrication	References
1	Thiocyanate	Smoking Indicator, Periodontitis	6 µmol/L	Fe(III), CTAB, and HNO_3	Wax Printing	[98]
			1 mmol/L	(0.6 µL of 0.5 mol/L Fe(III) in 1 mol/L HNO_3	Ink Screen Printing	[96]
			60 µmol/L	10.1 g of $Fe(NO_3)_3$ $9H_2O$ in 20 mL Water + 2 mL of nitric acid	Wax Printing	[95]
2	Nitrite	Gingivitis, Periodontal Diseases, Carcinogenic Nitrosamines with a potential to cause mouth and lung cancer, Bowel cancer	0.05 µmol/L	Griess reagent–naphthylethylenediamine dihydrochloride and sulphanilamide suspended in phosphoric acid	Whatman Filter Paper	[103]
			14.8 µmol/L		Wax Printing	[97]
			9.6 µmol/L		Spraying Patterning of 3D Mpad	[106]
			10 µmol/L		Wax Printing	[101]
			1 µmol/L		Inkjet Printing	[102]
			7.8 µmol/L		Corona Treated Paper	[104]
			7 µmol/L		Paper Cutting	[128]
			0.1 mg/dL		Wax Printing	[105]
3	Ammonia	Kidney & Renal Problems	11.3 mg/L	Silver nanoparticles modified by thiomalic acid and maltol bromothymol blue indicator (BTB)	Cut from PTFE and Laminated	[107]
			0.03 mg/dL		Laser Jet Printing	[108]
4	Cyanide	Pseudomonas aeruginosa	0.053 mg/dL	5,10,15-tritolylcorrolate of cobalt (III) triphenylphosphine	Anion exchange and drop casting	[113]
5	Aldehydes	Oral Cancer Risk	6.1 µmol/L	MBTH + $FeCl_3$	Wax Printing	[115]
6	Lactoferrin	Inflammatory Bowel Disease	110 µg/mL	2-(5-Bromo-2- pyridylazo)-5-diethylaminophenol + Fe^{3+} complex	Wax Printing	[117]
7	Lactate	Potential Heart Risk Assessment, Measurement of Training Efficiency	0.814 µmol/L	lactate oxidase + Horse Radish Peroxidase	Wax Printing	[121]
8	Glucose	Diabetes Meletus	29.65 µmol/L	Glucose Oxidase + Horse Radish Peroxidase	Ink Printing on paper towel	[106]
			0.3 mmol/L	Glucose Oxidase + Horse Radish Peroxidase	Wax Printing	[128]
			27 µmol/L	Glucose Oxidase + Horse Radish Peroxidase	Paper Cutting	[127]
			0.37 mg/dL	GOx-HRP coupled with 2,4,6-tribromo-3-hydroxy benzoic acid	Wax Printing	[126]
			47 µmol/L	Gox-HRP with 3,3',5,5'-tetramethylbenzidine	Wax Printing	[129]
9	SAR-CoV-2	COVID-19	0.1 µg/mL	Monoclonal antibody anti-SARS-CoV-2	Wax Printing	[145]
			200 genomic copies/µL	Heat-inactivated SARS-CoV-2	Grade 222 chromatography paper (Cellulose reaction pad)	[148]

2.2 Nitrite

Nitrite is an intermediate in oxidative and reductive processes such as nitrification and denitrification [99]. Nitrite is generally accepted to be the compound responsible for the preservation of foods along with other compounds like table salt. Long-term studies in mice have shown that feeding on a nitrite-deficient diet would lead to metabolic syndrome and cardiovascular death after 22 months [100]. Oral bacteria can convert nitrate into nitrite, which can be absorbed into the digestive system. Nitrites, which are linked to various diseases, can be detected as biomarkers in blood, urine,

Fig. 2 Protocol for simultaneous detection of thiocyanate and nitrite in artificial saliva (reproduced with permission from Ref. [96]. Copyright 2019, Elsevier)
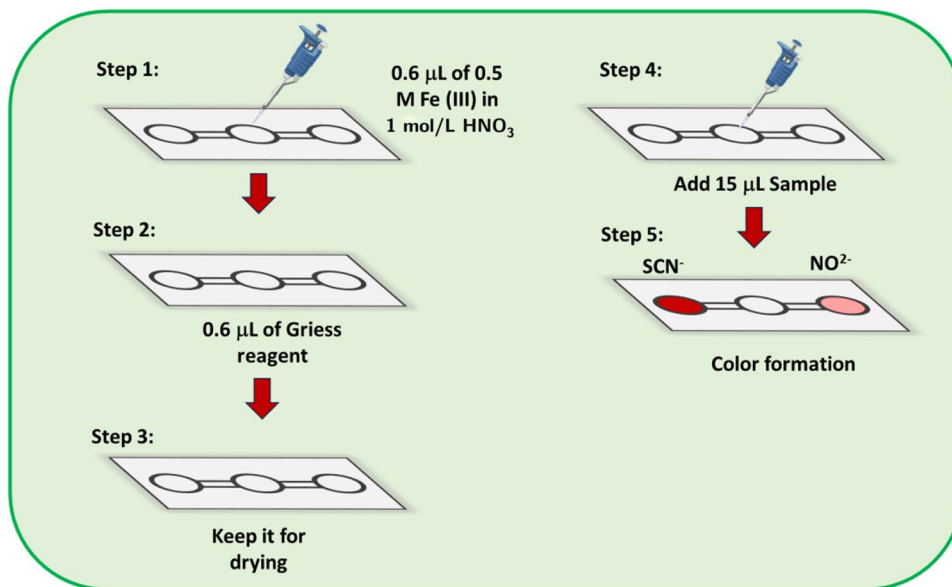
and saliva samples. Prolonged nitrite exposure has almost similar symptoms to thiocyanate, including nausea, vomiting, headache, and abdominal cramps. Nitrite can be easily detected by Griess Reagent, which is a mixture of naphthyl ethylenediamine dihydrochloride suspended in water and sulphanilamide in phosphoric acid, and a lot of paper-based microfluidic devices have been formulated based on that.

Bhakta et al. is the pioneer in colorimetric detection of nitrite using paper based microfluidic devices. They used wax printing to form the hydrophobic layer and used Griess Reagent to detect nitrite, which turns into a dark pink color when interacting with nitrite. The observed limit of detection was 10 µmol/L [101]. Jayawardane et al. developed the microfluidic device with inkjet printing instead of wax printing (Fig. 3a). They also studied the functionality of the device at different temperature conditions and concluded that it could work in various temperatures ranging from room temperature to -20 °C. The optimum time allowed for color formation was found to be 5 min using Griess reagent and the limit of detection was 1 µmol/L [102].

Ferreira et al. improved Jayawardene's work by developing a paper-based device that is capable of detecting 0.05 µmol/L of nitrite. They used a two-layer approach where one layer consist of Griess reagent, and the other was empty, allowing for more contact to facilitate the reaction (Fig. 3b). These layers were then laminated on both the top and the bottom, which is the most delicate step that decides the reproducibility of the device, as misalignment of the layers would vastly decrease the efficiency of the device. The sample was placed on the top layer, and the color change was photographed for quantification using a software [103]. Yu et al. simultaneously detected thiocyanate and nitrite using the same 3D microfluidic device with Griess reagent

as an indicator. The limit of detection for this method was 9.6 µmol/L [97].

Jiang et al. implemented a new fabrication technique for constructing the hydrophilic pattern in the paper. After immersing it in an OTS-hexane solution, they used corona treatment on the filter paper. The device was flower-shaped and has 8 arms to facilitate the simultaneous detection of multiple salivary samples. The results of samples taken from the same person via this device and ion chromatography methods were very much comparable. The limit of detection for this device was 7.8 µmol/L [104]. Noiphung et al. developed a paper-based device using wax printing capable of detecting both pH and nitrite. The device had two arms for nitrite detection and three arms on the opposite end for pH detection. Nitrite detection was done in saliva samples of humans in Australia, and they found a linear correlation between nitrite concentration and concentration of C-reactive protein in saliva. The detection was done using Griess Reagent, and the limit of detection was 0.1 mg/dL [105]. Chiang et al. used a combination of wax printing and 3D printing technology to fabricate the paper-based device, which has multiple advantages like the ability to print any design pattern of the µPAD, higher resolution of the patterns printed and the hydrophilic channels are not vulnerable to organic solvents. Griess reagent was used for the detection of nitrite, and the limit of detection was 14.8 µmol/L [106].

2.3 Ammonia

Ammonia and other nitrogen compounds such as nitrite and nitrate are the critical nutrients from which plants get their nitrogen. Nitrogen cannot be absorbed from the atmosphere and hence it has to be given in different forms,

Fig. 3 a Schematic representations of the developed (1)2D, (2) 3D microfluidic paper-based analytical devices (μPADs) and card containing one used (3) 3D μPAD. The components are labelled as follows: DZ (Detection Zone), TC (Transport Channel), RC (Reduction Channel) and IS (Interleaving Sheet, Cellulose Triacetate (reproduced with permission from Ref. [102]. Copyright 2014, American Chemical Society). **b** The schematic illustration showing the μPAD for (1) nitrite, (2) nitrate detection and (3) device after sample placement. The layers are indicated as follows: L1 (top layer of the laminating pouch), L2 (bottom layer of the laminating pouch), E1 (empty layer), G1 (Griess reagent layer), Z (zinc embedded layer), E2 (empty layer), and G2 (Griess reagent layer) (reproduced with permission from Ref. [103]. Copyright 2020, Elsevier)
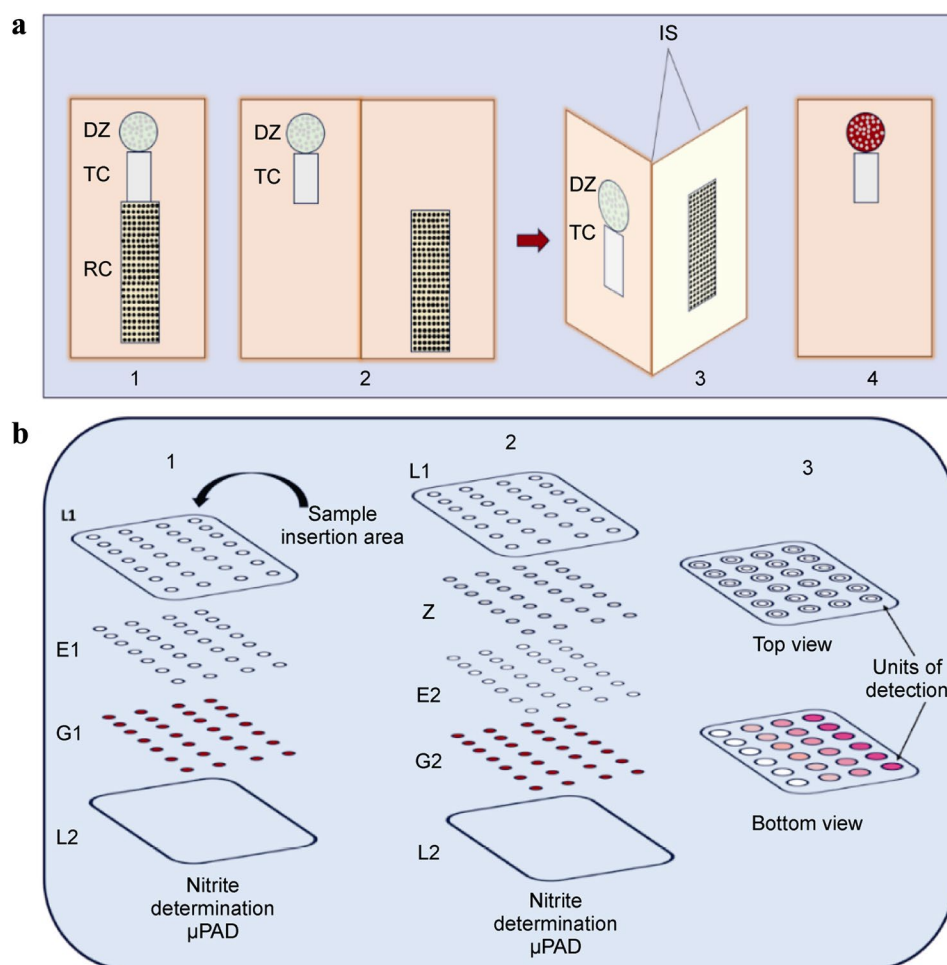
such as ammonia. Although a small quantity of ammonia can be ingested orally, it is typically produced in the gut by bacteria that digest protein compounds to release ammonia. Ammonia has a distinctive foul-smelling odor and a sour taste. The human kidneys are tasked with eliminating ammonia from the body. However, if they are unable to perform this function, an excessive buildup of ammonia can occur, which may eventually lead to the presence of ammonia in the mouth. Elevated ammonia levels in the blood can cause severe complications like brain damage, confusion, tiredness, and even possible coma and death. Ammonia in saliva can be determined by point-of-care devices, which are helpful in the early and cheap diagnosis of any probable kidney diseases. Sheini et al. developed a colorimetric assay for detecting ammonia in biological samples such as saliva. It is an origami paper-based device consisting of silver nanoparticles capped by thiomalic acid. The reaction is a hydrolysis reaction of urea into ammonia and water, and the silver nanoparticles capped with thiomalic acid accumulated ammonia where the color of the mixture turned from yellow to brown. The limit of detection for ammonia was observed to be 0.03 mg/L [107]. Thepchuay and team worked on a paper-based device that had stacks of 3 circular disks for the detection of ammonia where one layer is the sample layer and the second had sodium hydroxide solution, a separation layer that is permeable made of PTFE, and the third layer had bromothymol blue, the detection reagent which changes color to blue when exposed to ammonia. Laser-jet printing was used to print the hydrophobic barriers, and the limit of detection for this device was 0.03 mg/L [108].

2.4 Cyanide

Cyanide is the name given to a compound that contains the functional group $C\equiv N$. The most commonly referred to substance when the term cyanide is generally used is hydrogen cyanide which has a chemical formula of HCN. Hydrogen cyanide is a toxic compound and is fatal to the organism [109, 110]. Cyanide is used in manufacturing industries to manufacture textiles, paper, and plastics and is used in pest extermination. It is a significant health concern for those who are working in these industries must be fully protected before handling cyanide [111]. Cyanide is also a bi-product produced by the organism Pseudomonas aeruginosa which

gets secreted in the saliva and can be used as a biomarker for diseases using an easy, non-invasive test [112]. Lvova et al. developed a paper-based sensor to detect hydrogen cyanide in saliva. He used drop-casting on Whatman filter paper using an anion exchange process to form detection spots, using 5,10,15-tritolylcorrolate of Cobalt (III) triphenylphosphine as detecting agent. When it comes to contact with a cyanide group, this reagent change its color from red to light yellow as the concentration increases (at 6500 K temperature). The limit of detection was found to be 0.053 mg/L [113].

2.5 Aldehydes

Aldehydes are chemical compounds with the functional group CHO, where the carbon is double-bonded to an oxygen. These compounds are highly reactive and prone to cause damage to the body if not eliminated properly. Aldehyde dehydrogenase is usually involved in the metabolism of aldehyde. It oxidizes the aldehyde into carboxylic acid, which is less harmful to the body and is later metabolized by the liver and used up by the muscle cells. Blocking of the enzyme via drug uptake and constant exposure to pollution could result in aldehyde accumulation, and these further can cause damage to the cells and cause carcinogenic [114]. Hence, detecting aldehydes in saliva can be helpful in diagnosing salivary aldehyde dehydrogenase activity.

Ramdzan and team fabricated a paper-based device for the detection of aldehydes from saliva using 3-methyl-2-benzothiazolinone hydrazone and iron (III) chloride which is characterized by a blue color due to the formation of formazan dye when reacts with an aldehyde. The device

had a 3D design with two overlapping layers where one consisted of 3-methyl-2- benzothiazolinone hydrazone and had 15 detection zones of 8 mm diameter each. The second layer had 15 reagent zones with a 4 mm diameter each and consisted of iron (III) chloride. The sample addition is done at the first layer. The limit of detection was 6.1 μmol/L [115]. The schematic illustration showing the development of a microfluidic paper-based analytical device for the determination of salivary aldehydes, Fig. 4.
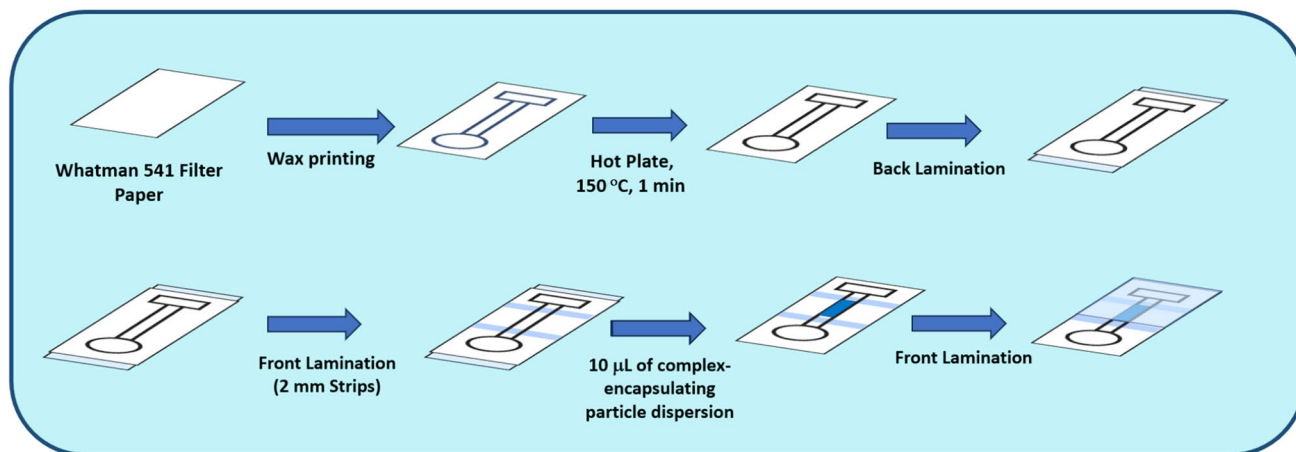
2.6 Lactoferrin

Lactoferrin is a protein usually found in milk and helps absorb iron from the digested food to the bloodstream. Lactoferrin from breast milk given to infants also has antimicrobial activity and helps in fighting infection [116]. Lactoferrin in saliva is responsible for the antimicrobial effect of saliva; hence a higher than usual concentration of this protein in salivary sample indicates a probability of having periodontal diseases and other oral diseases. High lactoferrin concentration in saliva can also indicate inflammatory bowel disease as its level correlates with the disease severity. Kudo and his team developed a point-of-care device for detecting lactoferrin in biological samples such as saliva. They used 2-(5-bromo-2-pyridylazo)-5-diethylaminophenol as a reagent, when it reacted with lactoferrin, results in the displacement of Fe^{3+} ions and hence a color change to dark blue or purple. The complex was encapsulated in poly(styrene-block-vinylpyrrolidone) particles, immobilizing the complex to the paper (Fig. 5). The device was fabricated using wax printing, and the limit of detection for lactoferrin was 110 μg/mL [117].

Fig. 4 Schematic representation of the development process for a microfluidic paper-based analytical device designed for the determination of salivary aldehydes (reproduced with permission from Ref. [115]. Copyright 2016, Elsevier)
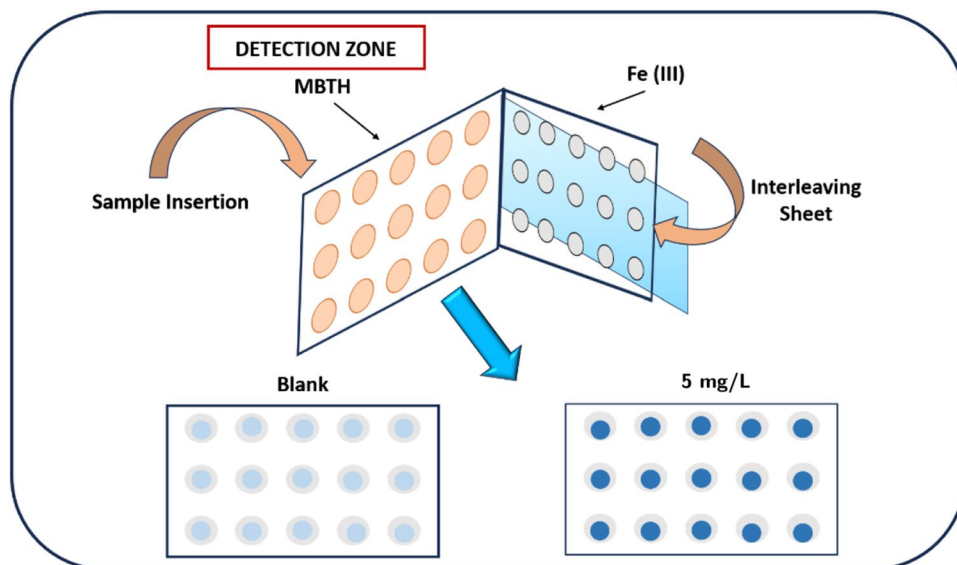
Fig. 5 Fabrication procedure for μPADs for distance-based readout of Lactoferrin (reproduced with permission from Ref. [117]. Copyright 2020, Elsevier)
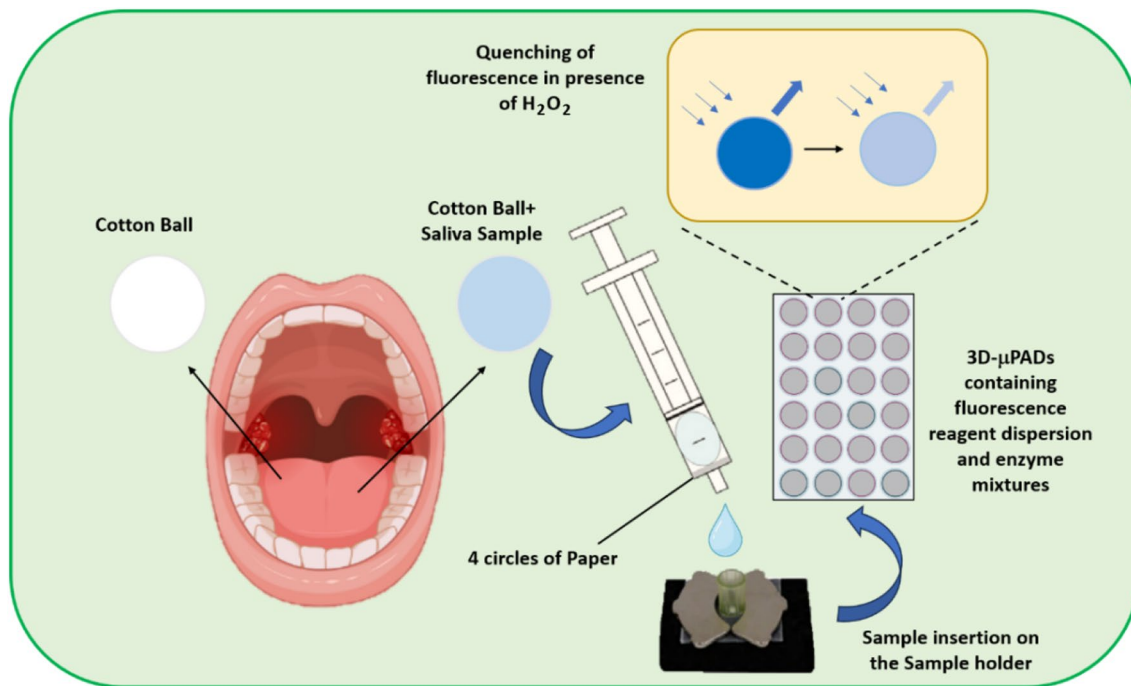
2.7 Lactate

Lactate or Lactic Acid is a biomolecule that is made by muscle cells and red blood cells and is usually present in low concentrations in the blood, but rises when the oxygen concentration is lower as they get formed by muscle cells as a by-product of glucose oxidation in an oxygen-limited condition [118]. A higher lactate presence in blood could imply poor metabolism by the body as the oxygen is not used correctly for glucose breakdown [119].The condition does not mean a problem with a particular organ, as the failure of different organs also results in a rise in lactate levels in the blood. It implies that there could be a problem with either one or many of these organs, such as the heart, liver, and kidney [120]. The rise in blood lactate level will reflect in the mouth and saliva; hence, saliva can be used for assessing blood lactate levels using point-of-care devices to diagnose various disease conditions. Rossini and team developed a device to detect lactate in saliva samples. They fabricated the device using wax printing. Fluorescent nanoparticles were used as detection probes along with lactate dehydrogenase and horse-radish peroxidase. Carbon dots were used to synthesize these nanoparticles with citric acid and tyramine as precursors. These solutions were mixed with PBS to form the reagent impregnated into the filter paper. The fluorescence quenching was visible after UV light was shown on the paper. The limit of detection for lactate was 0.814 μmol/L [121]. The Schematic illustration showing the paper microfluidic device using carbon dots to detect glucose and lactate as shown in Fig. 6.

2.8 Glucose

Glucose is an essential carbohydrate used by the body to generate ATP, which is the energy currency of the cells

[122]. Glucose is broken down into carbon dioxide and released by the cells, utilizing the bond break energy to form ATP molecules. Carbohydrates are the primary source of glucose, and they are broken down by different enzymes, such as maltase, to form glucose. Excess glucose in the body is converted into fat, and some are stored as glycogen [123]. In diabetes, there is an elevation in blood glucose levels due to either the ineffectiveness or complete absence of insulin, which is responsible for regulating the levels of glucose in the body. Prolonged elevation of glucose levels in the body can give rise to a range of health-related complications, including heart disease, kidney failure, impaired wound healing, and even loss of consciousness [124]. Hence the diagnosis of high glucose presence in the blood is essential and can be helpful in the early diagnosis of diabetes. Glucose can be easily detected by using Glucose Oxidase and Horse Radish Peroxidase, which is implemented in point-of-care paper-based microfluidic devices for easy detection [125].

Santana-Jiménez et al. developed a low-cost bi-enzymatic paper-based sensor for the detection of glucose. They used glucose oxidase-horse radish peroxidase and 2,4,6-tribromo-3-hydroxy benzoic acid, giving a usable range of detection from 0 to 180 mg/dL of glucose in saliva. Wax printing was used to fabricate the device, and the limit of detection was 0.37 mg/dL [126]. Tansu Gölcez and team integrated an offline smartphone app along with the paper-based device for easy detection and quantification of glucose in saliva. The application can utilize both RAW and JPEG images and converts them into RGB values using MATLAB, which is later used to plot the calibration curve. They used glucose oxidase, horse radish peroxidase, and chitosan for improved detection. They used ink-printed paper towels to form the hydrophobic barriers. The limit of detection was 29.65 μmol/L [127].
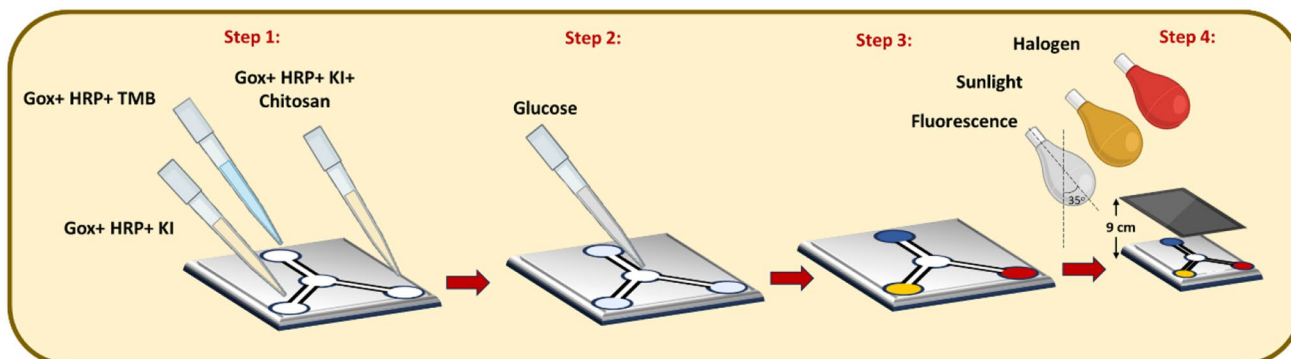
Fig. 6 Schematic illustration showing paper microfluidic device using carbon dots to detect glucose and lactate (reproduced with permission from Ref. [121]. Copyright 2021, Elsevier)

Chiang et al., and de Castro et al., detected nitrite and glucose simultaneously µPAD. They used glucose oxidase and horse radish peroxidase as reagents for detection, and their limits of detection were 29.65 µmol/L and 0.3 mmol/L for nitrate and glucose respectively [106, 128]. Mercan et al. coupled the classic Gox-HRP reagent with 3,3',5,5'-tetramethylbenzidine as a chromogenic agent to help improve detection. The colorimetric output of this paper-based device was then analyzed using a smartphone application that uses a machine learning algorithm trained using multiple data sets of photos from different smartphones. Hence this application can be used on a wide range of mobile devices. A

linear LDA correlation of 98.4% was achieved using TMB, and the limit of detection was 47 µmol/L [129]. The schematic illustration showing glucose detection using µPAD and smartphone as depicted in Fig. 7.

3 Detection of COVID-19 Biomarkers Using Paper-Based Systems

As of September 2022, the COVID-19 pandemic is responsible for over 610 million confirmed cases and over 6,508,521 deaths worldwide (As per the WHO's COVID-19

Fig. 7 Schematic illustration showing glucose detection. The color change in the µPAD detection zones was photographed using a smartphone under fluorescent, sunlight and halogen light sources (reproduced with permission from Ref. [129]. Copyright 2021, Elsevier)

Dashboard). SARS-CoV-2 coronavirus is the causative organism of the pandemic [130]. The subfamily and family of coronaviruses is Coronavirinae and Coronaviridae, respectively [131]. In March 2022, the World Health Organization declared the outbreak a global pandemic [132, 133]. Owing to the pandemic, a tremendous impact has been seen on global health. Additionally, a significant social and economic impact was seen owing to several lockdowns and closures worldwide. The virus is known to transmit rapidly by respiratory droplets and aerosols; early detection is crucial [134]. Fast, accurate, low-cost diagnostic tools have become a prerequisite to tackling the COVID-19 pandemic. The nucleic acids present in the samples are analyzed using various molecular detection methods, which in turn aids in identifying the virus. Commonly, laboratories worldwide have been using the method of real-time reverse transcriptase (RT-PCR) for the detection of the coronavirus [135, 136]. A primary disadvantage of using RT-PCR as a detection method is the possible occurrence of false negative cases [86]. Apart from nucleic acid tests, bioanalytical detection methods such as immunoassays work on quantifying antigen–antibody interaction. However, they have low specificity when compared to tests that recognize the RNA sequences of the virus [137]. Lateral flow immunoassays (LFIAs) and enzyme-linked immunosorbent assays (ELISAs) are the most extensively used methods to detect antibodies against SARS-CoV-2 [138]. Recent studies have shown that the CRISPR-Cas 13-based assay has a higher detection capacity compared to the RT-PCR assay [139]. However, the test's sensitivity might be hindered as the viral load keeps changing and is not constant [86]. The pandemic saw µPADs being established to provide an efficient and low-cost solution to point-of-care (POC) necessities. This was obtained by combining microfluidics with various paper-based approaches [140]. The detection of nucleic acids or protein targets usually makes use of fluorescence or colorimetric detection approaches [141]. There are different detection targets for SARS-CoV-2, namely, antigens, virus genes, and antibodies. The present paper-based diagnostic methods can be classified under the above-mentioned detection targets. Present viral infections are detected by viral antigen and gene tests. Serological tests account for the detection of prior infections [142, 143].

Saliva-based detection, nasopharyngeal swab, and serum are frequently used to detect SARS-CoV-2. However, sensitivity poses to be a challenge in saliva-based detection [144]. To tackle this, Fabiani et al. established a paper-based immunoassay using magnetic beads. The platform uses 96-well wax-printed paper to support the immunological chain. The method uses color images aided by a smartphone with Spotxel free-charge app for visualization. The effectiveness was ensured by collecting the nasopharyngeal swab specimens of the respective patients and testing them using

the RT-PCR method. The assay could also detect the delta variant of SAR-CoV-2 by the NGS method. A colorimetric approach was used, with the limit of detection being 0.1 µg/mL [145]. The Scheme of the smartphone-assisted paper-based device for detecting SARS-coV-2 in saliva samples is depicted in Fig. 8.

In the case of SARS-CoV-2, point-of-care tests are upcoming, however, they are associated with some key disadvantages, such as reduced scalability and the requirement to process the samples, which could prove to be time-consuming [146]. Davidson et al. developed a paper-based device for detecting nucleic acids present in samples with the help of loop-mediated isothermal amplification (LAMP). It produced a colorimetric response. It was then optimized for SAR-CoV-2 detection in humans' saliva, which does not require pre-processing. RT-LAMP is a technique characterized by the amplification of nucleic acids and is conducted at a constant temperature [147]. The device consisted of Grade 222 chromatography paper. It was 100% specific with a limit of detection of 200 genomic copies/µL. It could also detect multiple targets and pathogen detection by just varying the primer sets of LAMP [148].

4 Conclusion and Future Prospects

Saliva is one of the potential body fluids to monitor the diseases and overall health of the body. This non-invasive diagnosis of biomarkers from salivary samples are simple, easy, and low-cost. This review outlines the constraints of conventional methods (Table 2) in clinical applications while emphasizing the importance of saliva as a non-invasive biological fluid that contains numerous biomarkers linked to different diseases This review highlights the state of the art of recent advancements in the diagnosis of salivary biomarkers using different methodologies. Additionally, it offers an overview of the advancements made in the past decade regarding paper-based colorimetric microfluidic devices designed for detecting salivary biomarkers. The focus of this paper was on the salivary biomarkers as well and their detection using paper-based systems. As the area of paper-based electronics advances, we may anticipate more completely integrated and reusable sensor systems. Paper-based devices have a great and mostly unexplored potential, making them an attractive and cost-effective alternative for future applications. Compared to the small molecule and nucleic acid-based biomarkers, identification, and characterization of new proteome composition and human saliva would help find more possible protein biomarkers. Protonic biomarker diagnostic would integrate the biomarker into medication, therapeutic followed by ultimate progression of the diseases. Detection of these biomarkers is more convenient, cost-effective, easy, and rapid. The bioanalysis report
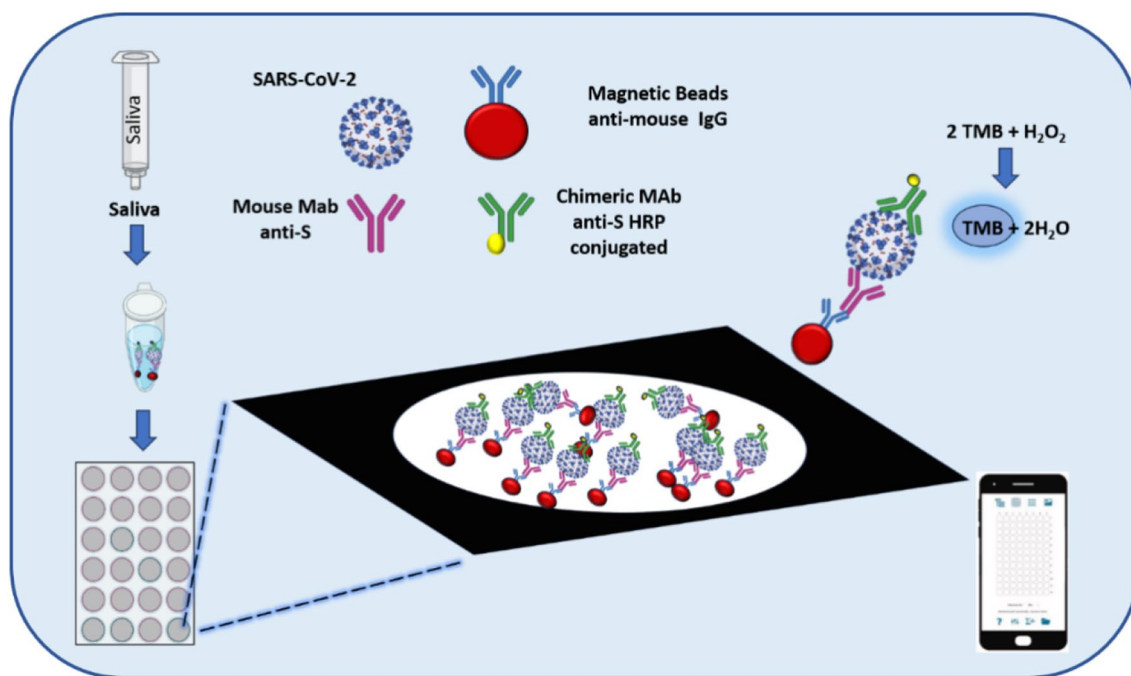
Fig. 8 Scheme of the smartphone-assisted paper-based device for detecting SARS-coV-2 in saliva samples (reproduced with permission from Ref. [145]. Copyright 2022, Elsevier)

would be an alarm to the patients to have a possible treatment before it reaches the advanced stage. Despite many advantages, such as being easily associable for point-of-care testing and assured, they have still many challenges to overcome for the real sample analysis. such as non-specific interaction, low sensitivity, and low detection limit. However, the sensitivity of the sensors can in tuned for better performance by integrating the advanced nanomaterial into the detection systems. On the other hand, loop-mediated isothermal amplification (LAMP) methods can be used to improve the sensitivity of the nucleic acid-based biomarkers enabling point-of-care testing with more sensitivity. Identification of disease-specific new biomarkers such as microRNA (miRNA) would facilitate easy diagnosis of the diseases. For example, a simple pH test paper-based sensor has been developed for the sensitive detection of miR by Feng et al. by rolling circle amplification (RCA) technique with minimal laboratory setting [149].

The emergence of 3D printing technologies has revolutionized the fabrication of these devices, moving beyond the conventional 2D design to incorporate three-dimensional structures. This advancement allows for the inclusion of features such as detecting multiple samples and multiple biomarkers from a single sample. Furthermore, significant progress has been made in various fabrication techniques employed for developing these devices. In the past, wax printing and inkjet printing methods were commonly employed in paper-based device manufacturing due

to their cost-effectiveness and simplicity of production. However, these methods have certain limitations, such as uneven sample penetration through the paper, resulting in incomplete filling of the detection zone. Additionally, they may face challenges in detecting certain biomarkers, such as pathogens and antibodies, which may not be compatible with wax or inkjet printing techniques. To overcome these limitations, novel techniques have been developed that combine the traditional wax and inkjet printing methods with the implementation of capillary membrane action, as well as the utilization of aptamers and nanoparticles. These innovative approaches aim to enhance the detection limit and enable the detection of previously untargeted biomarkers.

While numerous paper-based devices rely on smartphones for result interpretation, it is important to note that the majority of these devices are utilized in developing nations and under-developing regions. Consequently, it is crucial for these devices to be designed in a way that allows them to function independently without the need for external equipment such as smartphones or computers for result interpretation. One approach to achieving this is by incorporating a grading sheet into the device, which displays the range of colors it can generate and their corresponding values. This can be accomplished by including a separate sheet alongside the device that outlines all possible colors and their associated values, providing a quantification of the biomarker concentration in the sample. However, creating an accurate grading sheet requires multiple standard graphs

Table 2 Conventional detection methods and their advantages and disadvantages compared to PADs

No.	Conventional methods used for detection	Biomarkers detectable	Mechanism	Advantages compared to pads	Disadvantages compared to pads	References
1	ELISA	COVID, Periodontitis–*A. actinomycetemcomitans* Gingivitis–*P. gingivalis*	Antibody based assay wherein the tagged antibodies bind to the protein of interest and emit detectable photo signal	Precision detection of various proteins and microorganisms Differentiate between similar compounds Works across all biological samples	High cost for testing. High capital cost for acquiring and setting up the instruments. Poor portability to remote places in need for rapid testing.	[157] [158]
2	PCR	COVID, Periodontitis–*T. forsythia*, *A. actinomycetemcomitans* Gingivitis –*P gingivalis*	Targeted gene amplification, achieved by multiple cycles of amplification of the gene of interest. The gene (if present) can later be visualized by running the sample through gel electrophoresis	Ability to detect diseases even at low concentrations Highly useful in mass testing Helpful in finding gene disorders which can be both inherited as well as acquired	Slow turnover time per result. Involves complex protocols and appropriate knowledge to handle the instruments. Cannot be mass produced in case of emergency.	[159] [160]
3	Chromatography	Ionic biomarkers such as nitrite, cyanide, thiocyanate and ammonia	Capillary action based separation, analytes move through the paper depending on their affinity to the solvents used	Combination of multiple solvents allow for detection of different analytes Separate and isolate a compound from a mixture		[161] [162] [163]
4	Electrophoresis	Cancer causing biomarkers such as thiocyanate and nitrite	Separation of analytes based on their charge and size in a voltage gradient. Separation medium can be varied according to the compounds which are to be studied	Simultaneous analysis of multiple compounds Ability to set different gradients based on temperature, voltage, and size which allows detection of various compounds		[164] [165] [166]
5	Chemiluminescence	Lactate, peroxides and oral cancer biomarkers	Light emission from a chemical reaction that can be detected to confirm the presence of the compound	High sensitivity and helpful in identifying specific reaction centres in a large sample		[167] [168]

and analyses to be conducted beforehand. It is important to acknowledge that human error can significantly impact the interpretation of color, potentially leading to incorrect diagnoses. Consequently, scientists often rely on smartphones or computer-based result analysis to mitigate these errors. Further research and development are needed to address these challenges and enhance the usability of paper-based devices without external equipment. The current use of paper-based devices deployed in identifying biomarkers in saliva can be extended to study and analyze biomarkers in Gingival Crevicular Fluid or GCF. GCF is a fluid secreted by the gums, and it mainly contains antibodies, serum, diseased cells, and microbes [150–152]. It also has various biomarkers such as nitrite, C C-reactive proteins, and uric acid, to name a few. Analyzing this fluid can help identify any prevalent gum diseases, such as periodontitis and gingivitis. These diseases appear to have similar biomarkers to those detected in the saliva, like nitrite and nitric oxide [153, 154]. Hence, they can be used to diagnose dental diseases using GCF as a biofluid instead of saliva. Dental plaques and other oral microbial disorders can also be detected and analyzed using paper-based devices as the advancement in the area of 3D printing and that of integrating aptamers and anti-antibodies, which are tagged with a dye into the paper combined with lysing reagents have enabled them to detect DNA and RNA. Comprehensive investigations into symptom-based approaches for the detection of dental diseases are yet to be conducted. Exploring the predominant symptoms and utilizing biomarker detection can assist in localizing or narrowing down the specific type of acquired disease. For instance, periodontitis manifests in various symptoms, including tooth sensitivity, gum pain or bleeding, altered taste sensation, halitosis (bad breath), and gum swelling, among others. By combining these symptoms with the detection of relevant biomarkers like nitrite and nitric oxide, the diagnosis of the disease can be facilitated. The primary advantages of this approach lie in its simplicity and rapid detection capability. Further research in these areas has the potential to yield significant benefits.

Though the sensitivity of many biosensors is high from the proof of concept report, it is highly challenging to reproduce the same sensitivity from the real sample due to the counter interaction of the biological component in the fluid with the sensing elements, which leads to false conclusions. Therefore, bioanalysis data from a single biomarker is not sufficient for a physician to make a decision. Bioanalysis of multiple biomarkers from the same sample using multiplex sensors facilitates improvement in the accuracy of the report [155, 156]. At this stage, these biosensing methods are immature, and more experimental research has to be carried out to improve their sensitivity, specificity, and multiplex capacity for the performance of sensors for real sample applications.

Declarations

Conflict of interest The authors declare that they have no known financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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