2018 © The Japan Society for Analytical Chemistry

Reviews

Trends in Paper-based Electrochemical Biosensors: From Design to Application

Vivian Bee Chin LEE,*† Noor Faizah MOHD-NAIM, Eiichi TAMIYA,*** and Minhaz Uddin AHMED***

**Biosensors and Biotechnology Laboratory, Integrated Science Building, Faculty of Science, Universiti Brunei Darussalam, Jalan Tungku Link, Gadong BE 1410, Brunei Darussalam*
***PAPRSB Institute of Health Sciences, Universiti Brunei Darussalam, Jalan Tungku Link, Gadong BE 1410, Brunei Darussalam*
****Department of Applied Physics, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan*

Electrochemical bio-sensing using paper-based detection systems is the main focus of this review. The different existing designs of 2-dimensional and 3-dimensional sensors, and fabrication techniques are discussed. This review highlights the effect of adopting different sensor designs, distinct fabrication techniques, as well as different modification methods, in order to produce reliable and reproducible reading. The use of various nanomaterials have been demonstrated in order to modify the surface of electrodes during fabrication to further enhance the signal for subsequent analysis. The reviewed sensors were classified into categories based on their applications, such as diagnostics, environmental and food testing. One of the major advantages of using paper-based electrochemical sensors is the potential for miniaturization, which only requires relatively small amount of samples, and the low cost for the purpose of mass production. Additionally, most of the devices reviewed were made to be portable, making them well-suited for on-site detection. Finally, paper-based detection is an ideal platform to fabricate cost-effective, user-friendly and sensitive electrochemical biosensors, with large capacity for customization depending on functional needs.

Keywords Paper-based device, electrochemical detection, biosensor, nanomaterials, 3D origami, 2D sensor, 3D sensor

(Received August 26, 2017; Accepted November 20, 2017; Published January 10, 2018)

1 Introduction 8
2 Paper Design and Fabrication 9
2·1 Paper substrate
2·2 Designs: 2D and 3D devices
2·3 Construction of barrier: hydrophobic zone
2·4 Electrodes characterization: fabrication and surface modification
3 Applications 14
3·1 Clinical diagnostics
3·2 Environmental
3·3 Food
4 Conclusions and Outlook 17
5 Acknowledgements 17
6 References 17

Vivian Bee Chin LEE pursued her Bachelor's degree in 2016 at Department of Chemistry from Universiti Brunei Darussalam. She worked at Biosensors and Biotechnology Laboratory, Department of Chemistry, Universiti Brunei Darussalam as research assistant (2016 –) under the supervision of Dr. Minhaz Uddin Ahmed where she continues her research on various fabrication and development of paper-based electrochemical device incorporating several nanomaterials with protein.

Noor Faizah MOHD-NAIM obtained her Bachelor's degree in Biotechnology, Master's degree in Biomedical Research and Ph.D. in Clinical Medicine Research at Imperial College, London. Her Ph.D. thesis centered around cell biology, molecular biology and protein biochemistry. She is currently working at PAPRSB Institute of Health Sciences, Universiti Brunei Darussalam as a lecturer under the Biomedical Science course. Her current research interests include cancer biology, autophagy and biosensor.

† To whom correspondence should be addressed.
E-mail: minhaz.ahmed@ubd.edu.bn

1 Introduction

The advent of portable on-site testing devices, alternatively known as point-of-care (POC) devices, has initiated the growth of a fast-growing field of research that has various applications, clinically and industrially.[1] This is driven by the numerous advantages possessed by POC devices such as low fabrication cost, rapid data output, requirement of smaller sample volume, easy sample handling, portability, disposability and user-friendliness. There are various platforms for point-of-care devices, for example, the polymers polyethylene terephthalate has been used in measuring blood ammonia,[2] and poly(dimethysiloxane) has been employed for colorimetric and electrochemical detection of enterovirus 71.[3] Glass, ceramic, and plastic are other platforms for POC devices. Increasingly, paper has become an attractive platform for POC devices due to its various benefits.

A POC device that uses paper as a platform is otherwise known as a paper-based analytical device (PAD), whereby the cellulose fibers that make up a large proportion of paper act as a solid substrate.[4] Due to its many advantages, such as high sensitivity, portability, low production cost, ease of operation and potential for customization and modification, PADs have experienced an increase in popularity in its use to detect analytes, in clinical, biochemical and environmental settings.[5] There has been a constant demand for quicker and simpler analytical methods using PADs, with enhanced signal response and low detection limits.[6] Various analytical methods have been employed when utilizing PADs, including immunoassays such as colorimetric, localized surface plasmon resonance, fluorescent, electrochemical, chemiluminescent and photo-electrochemical. With the advancement of technology, the detection methods used in PAD devices have moved from the more traditional colorimetric-based detection, which is capable of generating clear, qualitative and semi-quantitative results with detection performed with the naked eye or fully quantitative with the aid of a simple spectrophotometer,[7-9] to the more sensitive electrochemical techniques, which utilize carbon paste or metal micro-wires electrodes as sensors to produce highly quantitative results.[10]

Electrochemical techniques are being increasingly used in the fabrication and design of PADs due to the potential for miniaturization, low sensor fabrication cost, compatibility with advanced microfabrication technology and high sensitivity that is made possible by better conductivity.[11] The electrochemical reading on paper substrate is highly comparable to results produced by the more conventional benchtop techniques, such as UV-Vis spectroscopy, liquid chromatography and inductively coupled plasma–mass spectrometry, in terms of sensitivity and specificity. Potentiometry, coulometry, polarography and amperometry can be used to determine electrochemical signals on PADs. However, in order to push more electrochemical PADs into commercial markets, development of multiplexed tests on PADs are highly desirable.[12] This is because sample treatment steps such as collection, storage, separation, extraction, concentration and analysis can be carried out in a single multiplexed sensor, which will further facilitate electrochemical detection.[13] Ultra-sensitive and high-throughput electrochemical detection of cancer biomarkers, such as carcinoembryonic antigen (CEA), alpha-fetoprotein (AFP), cancer antigen 125 (CA 125) and carbohydrate antigen 153 (CA 153) have been made possible by fabricating sensors in a multiplexed manner.[14]

Depending on the target analytes, the sensor fabrication techniques employed could vary from the construction of a barrier wall and electrode to further electrode modification using nanotechnology. "Wall" fabrication of the biosensors, or more specifically the discrimination of hydrophobic and hydrophilic areas on the biosensor, is essential to prevent the back-flow of solutions and to allow reactions to be carried out in a confined zone. Fabrication could be performed by free-hand cutting or wet etching. Once the paper substrate of choice is identified, paper can be cut to the desired size by cutting or punching method to define the working area or confined zone, which can be designed accordingly based on the range of the PAD functions needed. Depending on the volume of sample solution required for the reaction, the size of the working area can be adjusted accordingly. Generally, the smaller the volume, the smaller the working area needed. Alternatively, equipment-based techniques, such as wax printing, inkjet printing, laser printing, plasma treatment, flexographic printing, photolithography or screen-printing, could be utilized. In terms of the assembly of electrodes, a wide variety of materials can be used such as carbon, silver, gold, graphite, nickel, and epoxy. The electrodes can be produced *via* methods such as screen-printing, inkjet printing, or pencil drawing onto hydrophilic areas.[15] Moreover, to further enhance the sensitivity and specificity of the PAD sensor, nanotechnology modifications on the surface of working electrodes have been shown to improve the signal. Some examples of nanomaterials used are nanocomposites bonded with gold nanoparticles, silver nanoparticles, palladium nanoparticles, platinum nanoparticles, carbon nanotubes, graphene oxide, fullerene or iron oxide superparamagnetic nanoparticles.[16] Several research work involving nanomaterials in PAD fabrication exhibited an improvement in sensitivity and specificity, resulting in the detection of target analytes on a pictogram scale.[17]

In this review, selected published work related to PADs combined with electrochemical detection from the year 2012 to 2017 were reviewed. Our main focus is the various methods utilized for the fabrication of PAD sensors with an emphasis on Whatman chromatographic paper-based sensors that incorporate

Eiichi Tamiya is an innovator and an educator in the field of Nanotechnology and Biosensor, was awarded his Ph.D. from Tokyo Institute of Technology in 1985 and his BSc from Osaka University in 1980. Currently he holds the position as a Professor at the Faculty of Engineering of Osaka University, Japan. His research interests include biochips and biosensors food and environmental monitoring, nano-technology based bioscience and bio-engineering, environmental biotechnology and the design and creation of molecular recognition materials.

Minhaz Uddin Ahmed is a Senior Assistant Professor of Analytical Chemistry and Biotechnology at the Universiti Brunei Darussalam. He obtained his Ph.D. in Chemical Materials Science from Japan Advanced Institute of Science and Technology (JAIST) in 2008 under the supervision of Prof. Eiichi Tamiya. His research focuses on developing reliable and simple methodologies that could allow bioanalytical detection to be carried out for various applications, particularly regarding aspects of food safety, healthcare, and environmental protection, which deserve public awareness.

Table 1 Various types of paper employed as supporting substrate for electrochemical PAD in recent literature

No.	Paper substrate	Target	Types of electrochemical analysis	Reference
1	Whatman RC60 regenerated membrane filter	Bromide, iodide, chloride	Cyclic voltammetry	78
2	Whatman No.1 filter paper	CEA and CA125	Differential pulse voltammetry	35
3	Whatman chromatography paper 3 mm	Staphylococcus aureus	Differential pulse voltammetry	79
4	Nitrocellulose membrane	Acetaminophen	Cyclic voltammetry and differential pulse voltammetry	27
5	180 gsm office paper	Total and glycated haemoglobin	Electrochemical impedance spectroscopy	38
6	Art paper	Bisphenol A	Linear sweep voltammetry	30
7	Labor (67 g/m^2) filter paper	Phosphate	Cyclic voltammetry and chronoamperometric	70
8	PVDF filter membrane	White blood cell	Cyclic voltammetry and differential pulse voltammetry	80
9	Filter papers (qualitative, 102, 15 mm)	Glucose	Cyclic voltammetry and chronoamperometric	81
10	Cellulose acetate filter paper	Small molecules	Cyclic voltammetry and amperometric	82
11	Mixed cellulose ester (MCE)	Nitrite	Cyclic voltammetry	75

Table 2 Fabrication techniques used for construction of PADs using Whatman grade 1 chromatographic filter paper in recent literature

No.	Fabrication technique	Detection method	Target	Sample type	Application	Reference
1	Plotting	Colorimetric	Protein or glucose	Urine	Diagnostics	83
2	Flexographic printing	Colorimetric	Glucose	Synthetic samples	Diagnostics	84
3	Pen in wax	Colorimetric	Salmonella typhimurium DNA	Synthetic samples	Diagnostics	81
		Electrochemical	Glucose	Human serum	Diagnostics	
4	Photolithography	Electrochemical	Human chorionic gonadotropin	Human serum	Diagnostics	47
5	Laser printing	Electrochemical	Dopamine	Human urine and blood serum	Diagnostics	62
6	Laser printing	Electrochemical	Glucose	Human blood	Diagnostics	39
7	Wax printing	Electrochemical	K-562 cell	Clinical samples	Diagnostics	54
8	Wax printing	Electrochemical	CEA and CA125	Human serum	Diagnostics	35
9	Wax printing	Electrochemical	CEA	Clinical serum	Diagnostics	57
10	Wax printing	Electrochemical	HPV	DNA samples	Diagnostics	59
11	Wax printing	Electrochemical	Ketamine	Alcoholic and non-alcoholic beers drinks	Food	32
12	Wax printing	Electrochemical	Lead and cadmium	Mud and seawater	Environmental	29
13	Screen printing	Electrochemical	Ferricyanide	Synthetic samples	—	85

electrochemical-based analysis such as cyclic voltammetry, square wave voltammetry, chemiluminescence and electrochemiluminescence. We will also highlight various PAD sensor designs ranging from the 2-dimensional (2D) to 3-dimensional (3D), including the different types of electrode materials that could be utilized as well as other materials that could be employed for surface modification of the electrodes. Moreover, the various applications of PAD sensors will be discussed before finally concluding with a discussion of the challenges faced in the process of fabricating PADs.

2 Paper Design and Fabrication

2·1 Paper substrate

Paper has become an increasingly common substrate used in microfluidic devices due to its porosity, liquid wicking rate and fiber surface affinity to different analytes.[4] Different materials of paper substrates possess different properties such as grades, sizes, flow rate, thickness and pore size. Therefore, depending on the target analytes, the requirement of the paper substrate will vary. The choice of paper material is important in order to fully utilize the characteristics of the paper substrate of choice to enhance the sensitivity of analysis. A wide range of supporting paper substrates such as chromatography, filter, blotting and office paper of different grades, each of which possesses different characteristics, can be utilized depending on the specific application needed.[18] In Table 1, we have summarized various paper substrates that have been utilized for different targets along with the electrochemical analysis type used as PADs in the more recent literature. Generally, for the fabrication of PADs involving techniques such as wax printing, wax screen-printing, laser printing, inkjet printing, and photolithography, paper substrates of lower thickness is preferable. Thinner paper substrates require a smaller amount of wax or ink to penetrate through the nitrocellulose membrane to create a hydrophobic zone within the PAD, which reduces the cost of production. The popular paper substrate of choice has been chromatography filter paper. Several examples of paper substrates used in electrochemical detection are office paper, art paper, and cellulose acetate filter paper. Besides these, PVDF filter membrane, nitrocellulose membrane and mixed cellulose ester (MCE) membrane can additionally be used. For the full list of paper substrates that have been used for various electrochemical analysis, please refer to Table 1.

One of the most commonly used paper substrates in the construction of paper-based analytical devices is the Whatman grade 1 chromatographic filter paper. The paper's popularity is due to the high alpha-cellulose content, smooth surface, structural uniformity and absence of additives, which guarantee its quality, reproducibility and uniformity.[15] Another significant advantage of using Whatman grade 1 chromatographic filter

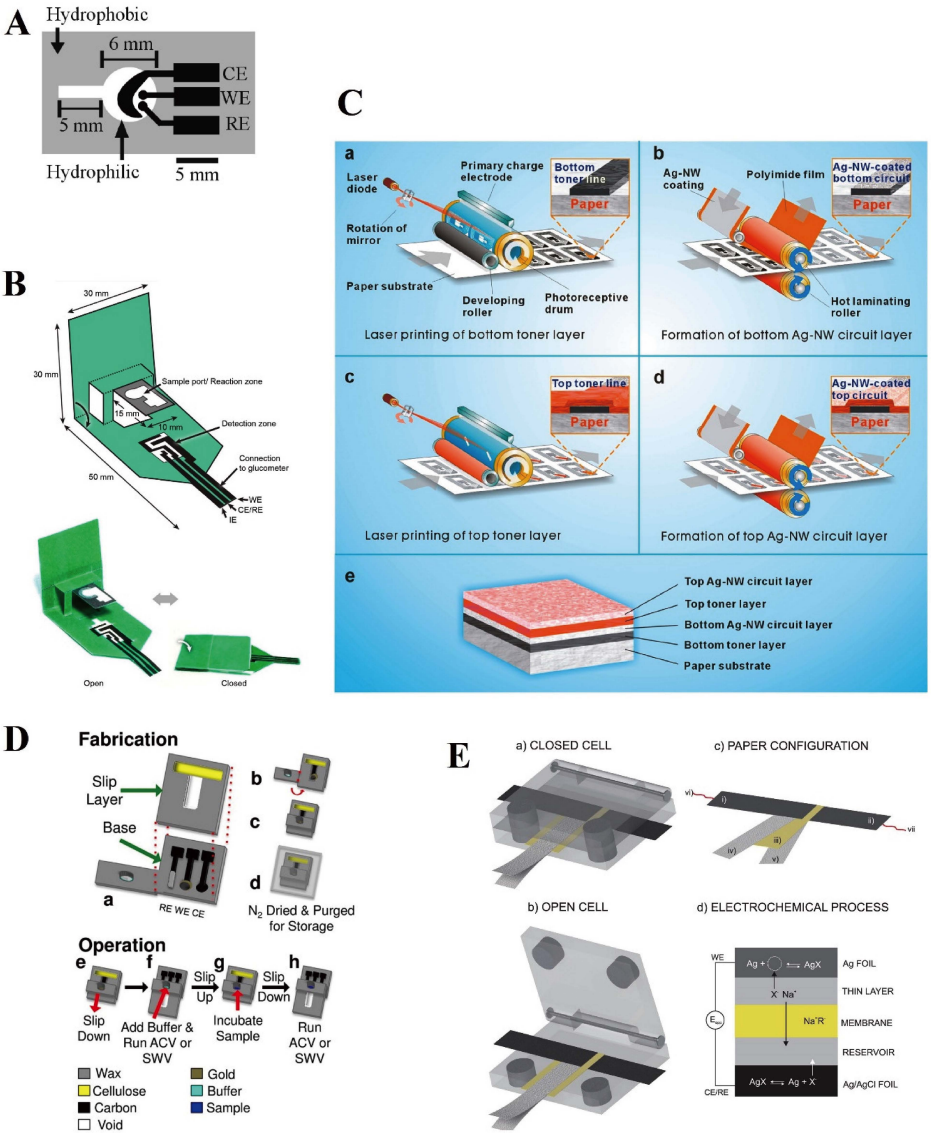

Fig. 1 Schematic representation of existing PAD designs. (A) A 2D electrochemical paper-based microfluidic device for the detection of glucose, lactate, and uric acid *via* photolithiography.[21] (B) A "pop-up" electrochemical paper-based analytical device for analyzing β-hydroxybutyrate.[40] (C) Paper-based multilayer circuits for development of paper-based devices.[46] (D) Quantitative detection of oligonucleotides and proteins *via* paper electrochemical device.[53] (E) Paper-based detection of halide utilizing thin-layer coulometric sensor.[78] Figures are reproduced with permission from American Chemical Society.[21,40,46,53,78]

paper is its suitability for immobilization of enzymes, proteins and DNA due to its high degree of non-specific binding towards biomolecules. In Table 2, we have summarized various studies that utilized different fabrication techniques for the production of paper-based devices and detection of distinct targets, incorporating different electrochemical detection methods using Whatman grade 1 chromatographic filter paper. It appears that fabrication through wax printing is most commonly employed in electrochemical detection, with the target tested varying from clinical samples to food samples to environmental samples. Laser printing is used to analyze target analytes such as dopamine and glucose in clinical samples *via* electrochemical detection. Screen printing and photolithography, on the other hand, is employed for the detection of targets such as ferricyanide and human chorionic gonadotropin. Furthermore, plotting techniques and flexographic printing were also employed for the fabrication of paper-based colorimetric devices. One of the latest fabrication techniques used is the incorporation of wax in a ball pen that includes a heating unit for the dual detection of colorimetric and electrochemical analysis.

2·2 Designs: 2D and 3D devices

During the early days of its invention, electrochemical sensors adopted the 2D concept originally introduced by Whiteside and co-workers,[19] which allowed the simultaneous detection of multiple analytes using a single sample reservoir (Fig. 1A). Over time, 3D sensor designs were developed to allow for more complex operations (Figs. 1B – 1E).[20] To note, for either 2D or 3D sensors, the counter electrode (CE) is always designed to be slightly bigger than the working electrode (WE) and reference electrode (RE) to allow for unlimited current transfer within the current circuit. In addition, the WE is usually placed close to the RE to minimize the effect of encompassed resistance between the WE and RE (Fig. 1A).[21] The main difference

between 2D and 3D sensors is how electrodes are displayed. In a 2D sensor, the three electrodes system is fabricated onto a single piece of paper, commonly on a circular or a channel-like hydrophobic test zone.[22,23] In a 3D sensor, the paper is folded to form an origami configuration, whereby the WE is constructed on one segment of the paper while the CE and RE are prepared on another.[24,25] One advantage of adopting the 3D design over the 2D sensor is that a 3D sensor shows highly homogenous coloration of the paper reaction zones, whereby fluid can move freely in both horizontal and vertical directions to occupy the whole surface area.[26]

A 2D electrochemical PAD was reported for use in the determination of acetaminophen in the presence of ascorbic acid in pharmaceutical samples.[27] A nafion-modified nitrocellulose membrane was incorporated to obtain a negatively-charged membrane, a modification that was performed after the membrane was wax-printed and baked. In addition, a single-walled carbon nanotube (SWCNT) was integrated into the design to enhance the performance of the PAD. The PAD was a combination of four components in the following order: a SWCNT film, a spacer, a wax-patterned nitrocellulose membrane and a cover film. It was square-shaped (2 cm by 2 cm) and fixed in position by laminating to secure the membrane position on top of the spacer, in order to minimize contamination and sample evaporation. It has a conformation of 700 μM in width and a spacing between electrodes of 300 μM with a reliable electrode production confirmed. The device appeared to be able to detect multiple analytes simultaneously with high selectivity.

Another 2D paper-based microfluidic device was developed for the detection of heavy metals and glucose.[28] A piece of chromatographic paper or polyester-cellulose film was used to fabricate a three-electrode system by screen-printing either carbon ink or Ag/AgCl, followed by patterning of the channels by photolithography or wax printing. These specific materials were chosen because the surface of the electrodes did not deform after being wetted in fluids. The patterned paper was taped onto the substrate, whereby the electrodes were supported by double-sided adhesive tape in order to achieve conformal contact. The distance between the paper channel and the WE in the contact area was 4 mm by 4 mm. Glucose was detected *via* chronoamperometric method, while heavy metals were detected by square-wave anodic stripping voltammetry.

A 2D lab-on-paper for heavy metal detection was designed *via* layer-by-layer fabrication of the detection chip.[29] Channels (3 mm wide and 25 mm long, widened in the end part) were wax printed onto filter paper and baked before further preparation of the electrochemical detector. Three electrodes were fabricated by screen printing (500 μm wide by 500 μm long by 4 μm thick). The WE and CE were screen printed using graphite ink, followed by the printing of the RE using a layer of silver/silver chloride ink. A second layer of paper was wax printed, but not melted, to enclose the channel at the bottom of the paper in order to avoid liquid leakages. Adhesive glue was used to attach both layers of paper together and an absorbent pad (5×10 mm) was placed at the end of the channel to increase both the amount:volume of the sample and sensitivity. As a result of this specific design, the platform was not only able to detect heavy metals, but it was also able to act as a sample pretreatment material to analyze the usually turbid real samples.

Determination of glucose and total carbohydrate concentration by a paper-based amperometric sensor for the analysis of foodstuff was previously described.[31] The 2D biosensor design operated by using two WEs, one RE (aqueous Ag/AgCl) and one AE (graphite). The first WE was made up of a 2-mm diameter Ti disk coated with a tiny drop of copper conductive ink suspension. The ink coating on the Ti disk electrode prevented the underlying Ti metal from corroding under experimental conditions and to allow excellent conductivity at the interface with the environment. Ink containing mixtures of graphite powder and Cu nanoparticles was deposited onto the second WE, a non-conventional Ti disk. A polystyrene solution was incorporated in the preparation of ink as a polymeric binder and as a barrier to capillary rise. The best formulation for the ink for the detection of glucose was found to be: 50 mg/mL Cu, 75 mg/mL graphite and 22 mg/mL polystyrene.

Furthermore, Narang and colleagues developed an ultrasensitive 2D device for the electrochemical sensing of ketamine using Whatman No. 1 filter paper.[32] The layout of the device, including the electrodes, were fabricated by stencil template constructed by precision cutting tools in order to provide better control over the dimensions of the electrodes and to reduce problems such as inconsistency with morphology and surface area per electrode. Commercially available conductive carbon ink was used to fabricate the WE and CE, with the hydrophobic barriers constructed out of wax using a wax pen for the definition of hydrophilic channels on paper. The AE was fashioned out of a Pt wire. The circular WE was drop-deposited with nanocrystal of zeolites and graphene-oxide nanoflakes (Zeo-GO). Detection of ketamine, at different concentrations as well as in real samples, was carried out using cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS).

As the field of biosensors developed, 3D sensor designs are being increasingly featured in various research. In a 3D sensor, the auxiliary and sample pads are prepared on different segments of a single piece of paper. The WE is designed on the auxiliary pad, while the CE and RE are fabricated on the auxiliary pad. The auxiliary and sample pads are then folded and stacked together in order to produce a 3D device.[15] Simultaneous detection of carcinoembryonic antigen (CEA) and alpha-fetoprotein (AFP) was reported using a 3D electrochemical PAD.[33] Hydrophobic barriers on the PAD were wax printed followed by baking, with the outer and inner circles measuring 15 and 8 mm in diameter, respectively. The outer circle acts as the hydrophobic barrier, while the Ag/AgCl RE, carbon CE and carbon WE (5 mm in diameter) were screen printed onto the inner circle. A couple of modifications on the working electrode were introduced for the purpose of signal amplification and improved electron conduction, namely nanoporous particle-modified WE (NPPt-PWE) and cysteine-capped flower-like gold nanoparticles (Au-NPs). Cys-capped flower-like Au-NPs could carry metal ions to form metal tracers to directly label antibodies. Detection of CEA and AFP was recorded through amperometric response *via* differential pulse voltammetry. Another 3D electrochemical PAD for the detection of the cancer antigen 125 (CA125) was created by Li and colleagues.[34] The wax-printed biosensor design was comprised of one auxiliary pad and one sample tab of the same size (30.0 mm by 30.0 mm). The carbon WE, 4.0 mm in diameter, was screen printed onto circle A, whereas carbon CE and Ag/AgCl RE were screen-printed half ring-like onto circle B. A dual-signal amplification strategy was employed *via* the use of a dense Au and flower-like Ag bimetallic (Ag@Au-PWE) conducting layer on the cellulose fiber surface in the working zone as a platform for antibody attachment and glucose oxidase-functionalized palladium-gold nanoparticles (Pd@Au NPs) as a catalyst to amplify the signal. The 3D PAD was fixed with a simple home-made device-holder and later connected to the electrochemical workstation to record ECL responses using cyclic voltammetry (CV).

Following this study, an improved 3D PAD version was

devised for the detection of two low-abundance cancer biomarkers, CEA and CA125.[35] The design of the sensor was comprised of two zones, the auxiliary and working zones, both of which were fabricated by wax printing. The auxiliary and working zones were of the same size of 12.0 mm by 20.0 mm by 0.18 mm. The carbon CE and Ag/AgCl RE were screen printed onto the auxiliary zone. The carbon WE was screen printed onto the working zone and modified with Au nanorods through a seed-mediated growth approach to enhance the conductivity and enlarge the surface area of reaction, acting as the sensor platform. The AuNRs-PWE was coupled to Au/BSA-metal ion tracers for further conjugation with antibodies. These dual signal amplification strategy allowed for simultaneous electrochemical detection of CEA and CA125. The device was then folded with the help of a simple home-made device-holder and connected to the electrochemical workstation. DPV scan was then employed to record the amperometric responses.

Ge and colleagues reported a 3D origami paper device for the electrochemical detection of K-562 cells.[36] The structure of the device was comprised of two layers of A4 size pure cellulose paper and were patterned in bulk by wax printing. On both sheets, a wax patterned circular working zone of dimensions 8.0 and 6.0 mm in diameter were fabricated to construct the paper auxiliary zone and paper sample zone, respectively. Both circles were centered at the same position on both papers. Ag/AgCl RE and carbon CE were screen printed onto the auxiliary zone, while the sample zone contained the screen printed carbon WE. The WE was then further modified by growing Au-NPs on its surface in order to improve surface area and conductivity. The printed paper was then folded so that the three-electrode system was in contact and connected *via* the filled solution. DPV peak current was regarded as the signal produced by the biosensor.

A highly sensitive 3D PAD for the determination of Pb^{2+} was demonstrated by Wang and colleagues.[37] Hydrophilic working zones were defined on paper by wax printing, followed by baking. Two circular working zones were prepared, the sample zone (6.0 mm in diameter) and auxiliary zone (8.0 mm in diameter). The Ag/AgCl RE and carbon CE were screen printed using screen mesh onto the auxiliary zone, while the carbon WE was screen printed onto the sample zone. The WE was later modified with Au nanoparticles, which acted as a sensor platform, and DNA functionalized iron-porphyrinic metal-organic framework, which acted as signal probes for the successful detection of Pb^{2+}. The paper sheet was then cut into smaller individual origami PADs. The gap between the sample tab and auxiliary pad was defined as the fold line of about 1 mm in width. Subsequently, the three-electrode system would be connected on the paper electrochemical cell upon the addition of working solution and measurements were read *via* the DPV method.

Boonyasit and colleagues reported a multiplexed 3D PAD utilized for the measurement of multiple diabetes markers.[38] The wax printed paper was initially treated by heating to form blue-coloured hydrophobic and insulating barriers. Upon cooling, the three-electrode areas were printed along with conductive pads onto the hydrophilic zones. Dual carbon WE and CE were screen printed onto the defined hydrophilic areas and cured by baking. Ag/AgCl RE were screen printed onto the same paper sheet followed by baking and cooling. A customized magnetic paper sheet was used to adhere the dual screen printed electrode onto the wax patterned paper layer prior to assembly. A permanent magnet was used to align and assemble the dual screen printed electrode layer to a bottom magnetic paper layer and a top magnetic-paper lid, which contained two circular wells underneath the design. Wax patterned paper created an area that surrounded the three electrodes, which acted as an insulator, creating a reservoir after the alignment with the top magnetic-paper layer with two circular holes. The surface was further modified with haptoglobin (Hp) and 3-aminophenylboronic acid (APBA)-modified eggshell membranes (ESMs) to enhance the sensitivity of the device.

Another 3D biosensor containing fully pencil-drawn electrodes was described by Li and colleagues for the detection of glucose.[39] The chromatography paper was cut into A4 size (210 mm by 297 mm) and the sensor contained a detection zone and an enzyme zone (7 mm in diameter). Before the electrodes were drawn on, patterns of WE, RE and CE were designed on the detection zone to ensure consistency when drawing on paper using 6B graphite pencils. The patterns were printed and heat-treated to form the hydrophilic areas. The paper was cut into pieces after cooling. This was followed by pencil-drawing the electrodes (WE: 3 mm by 2 mm, RE & CE: 2 mm by 2 mm) on the detection zone. A certain range of sheet resistance was obtained by repeatedly depositing the graphitic layers.

A "pop-up" biosensor was created for the detection of β-hydroxybutyrate (Fig. 1B).[40] The microfluidic channels were defined by wax printing on chromatography paper, followed by stencil-printing of the three electrodes system and circuits using graphite ink. A solution of enzyme and cofactor were added into the reaction zone and dried in the dark. A razor was used to cut the paper along guided lines and folded. Individual test strips of 50 mm by 30 mm by 30 mm were formed and a series of "through cuts" and "half-cuts" were performed over guiding lines. The straightforward and reproducible fabrication techniques ensured that the devices produced had consistent overlap between the reaction zone with the dimension of 15 mm by 10 mm and detection zone. A mediator and glucose oxidase (GOx) were then immobilized onto the respective zones for the detection of glucose.

2·3 Construction of barrier: hydrophobic zone

In order to construct a microfluidic channel, the porous network of the cellulose matrix of paper was patterned by hydrophilic barriers in order to define the hydrophilic zones and the path of the hydrophilic fluid solutions.[15] Additionally, hydrophobic patterning prevents the back-flow or overflowing of solution from the paper sensor.[41] The specific area of hydrophobic and hydrophilic zones will vary depending on the volume of the solution required. Additionally, certain detection methods may require a different ratio of areas of CE, WE and RE. Several techniques could be employed for the fabrication of hydrophobic barriers on paper matrix such as wax, inkjet, flexographic, laser, wax-screen and screen-printing, as well as photolithography, plasma treatment, laser treatment, and wet etching.[42]

For the purpose of electrochemical detection on PADs, the most commonly used fabrication method during the start of PAD research is inkjet printing, which moved to wax printing, followed by cutting, laser printing, photolithography and screen printing. Each fabrication method has its own pros and cons. Wax printing is highly preferred compared to other fabrication techniques due to its superior generation of hydrophobic and hydrophilic zones on cellulose membranes. It is also a relatively straightforward technique with absolute flexibility in forming pattern designs.[43] Inkjet printing techniques are able to produce similarly precise and flexible design when compared to wax printing, and has been described to be cost-effective in generating microfluidic channels on paper substrates. However, inkjet printing involves additional fabrication steps as it requires sequential layering of different materials.[44] Wax printing

involves printing wax onto cellulose membranes using a commercial printer, which are capable of printing several pages within a minnte. The cellulose membrane is then heated, causing the wax on the surface to melt, spreading vertically and laterally and creating hydrophobic barriers across the thickness of the paper.[45] In screen-printing, a framed screen was constructed with a pattern or mask through which wax or ink is pressed through the screen onto paper. If wax is used, the wax is subsequently melted so that it penetrates through the paper to form hydrophobic barriers. However, this heating process could add to production cost, and, in such cases, readily-available polymers, such as varnish paint solution or roof sealant could be used as alternatives to be screen printed onto paper substrates for the formation of hydrophilic channels and reservoirs.[43]

Yet another popular method is laser printing, which is an electrostatic digital printing process that involves a laser beam and solid powdered ink, otherwise called toner. The laser beam scans back and forth across a photoreceptive drum in the printer and builds up static electricity that attracts the toner. The toner is later permanently fused onto a sheet of paper. This solvent-free method is convenient for printing of electronic circuits on normal office paper (Fig. 1C).[46] Alternatively, photolithography could be employed, a technique that relies on UV lamp exposure through a photomask of paper saturated by a light-sensitive material, or photoresist. Solvent is used to remove uncured photoresist to leave hydrophilic channels surrounded by hydrophobic photoresist.[47] However, this method is expensive with the involvement of costly photomasks and instruments. Rather than printing, instruments such as a laser cutter could be utilized to create hydrophobic barriers of desired sizes.[48] The cutting technique could also be performed by free-hand as paper substrate is cut into desired patterns without requiring the formation of hydrophilic channels within hydrophobic walls.[49]

2·4 Electrodes characterization: fabrication and surface modification

In an electrochemical PAD, the 3D network of cellulose fibers of paper acts as the base for deposition of redox and conductive materials of the electrodes.[15] The selection of specific electrode material determines its conductivity and sensitivity, as well as the cost of the device and the possibility of adopting specific immobilization procedures. The materials used for the fabrication of RE, WE and CE may vary from each other depending on the type of analysis required, the target component and the samples to be analyzed.[50] Electrodes could be constructed using conducting pastes of materials such as gold, platinum, carbon, silver, graphene and heavy metal. Carbon paste is most commonly used in the fabrication of WE and CE in the screen printing technique. This is due to the lower interference of carbon compared to other materials. Meanwhile, silver paste is mainly utilized in the fabrication of RE due to its stability and consistent potential for electrochemical detection, resulting in a superior signal production.[10]

It is preferable for electrodes to be fabricated using mass-production techniques, such as screen printing and inkjet printing, as they permit mass production with the use of highly reliable repetitive printing tool.[15] Screen printing uses a customized screen to pattern specific paste of conducting material onto paper devices, whereas inkjet printing involves the deposition of pico-liter-sized droplets onto paper substrate controlled by a computer, which requires expensive equipment.[51] Alternatively, a couple of other simple, inexpensive and equipment-free electrode fabrication methods are pencil-drawing using graphite pencil lead or free-hand painting using carbon ink on the hydrophilic zones of paper devices.[10] These methods, however, are not suitable for the purpose of mass-production.

Fabricated electrodes could be further modified with micro- or nano-sized objects, which could improve the sensitivity, stability and repeatability of analyte detection with PADs. This is achieved by attaching recognition agents to mobile particle surfaces, leading to a higher number of recognition elements when compared to planar surfaces. Furthermore, the binding capacity to particles can be strengthen and the speed of target binding can be increased when compared to stationary surfaces. Moreover, enzymatic amplification of a signal is no longer necessary upon micro- or nano-modification.[20] Target analytes that are in a very small amount in the sample may require modification with a more complex nanomaterial ranging from 1 to 100 nano-meters in size.[52]

One of the simpler modifications involves nanoparticles (NPs), which further expands the surface of the electrode.[10] When metal NPs are used, they act as charge carriers and catalysts for electrochemical reaction, enhancing conductivity. Increasing both the electrode surface area and conductivity result in desirable signal amplification. The nanoparticles themselves could be enzyme-immobilized for further signal augmentation, but may have limited stability due to the shorter reaction time, unless they are further modified for increased stabilization.[20] Successful modification of an electrode surface can be verified, confirmed or characterized by some common characterization techniques, such as scanning electron microscopy (SEM) for surface composition, transmission electron microscopy (TEM) for internal composition and electrochemical impedance spectroscopy (EIS) to determine the response of an electrochemical system to an applied potential.

Nobel metal nanoparticles (NMNPs), such as gold nanoparticles (AuNPs), represent one of the commonly employed modifications for electrochemical and electrochemiluminescence detections on PADs. Apart from amplifying surface area and conductivity, they also enhance biocompatibility.[5] AuNPs could additionally be used for aptamer capture *via* gold-thiol chemistry for the detection of DNA and protein (Fig. 1D).[53] Moreover, AuNPs can be used in combination with materials such as graphene, which has been exploited for the detection of K-562 cells, one of the most aggressive human chronic myelogenous leukemia cell lines.[54] In another report that aimed to detect multiple cancer cells, AuNPs were conjugated to palladium and concanavalin-A bioconjugates in order to improve detection.[55] In order to detect acetaminophen in the presence of ascorbic acid, conjugation of AuNPs to polyglutamic acid and single wall carbon nanotubes was implemented by Lee and colleagues.[27] Wang and colleagues reported the immobilisation of AuNPs with silver in order to increase the resistance for detection of carcinoma antigen 125 (CA 125).[56] Alternatively, determination of CA 125, as well as carcinoembryonic antigen (CEA), could be achieved through the modification of WE with Au nanorods and metal ion-coated Au/bovine serum albumin nanospheres. Finally, to detect CEA, modification of WE with amino functional graphene (NH_2-G)/ thionine (Thi)/gold nanoparticles (AuNPs) was shown to improve detection sensitivity and eliminate the requirement of labeling of antigens or antibodies.[57] Aside from Au, other non-Au nanocomposites such as platinum nanoparticles (PtNPs) have been reported. In a study that investigated the detection of CA 125 and CEA, electron conduction was improved by modifying the electrode surface with a nanoporous platinum particle that was used as matrix, with metal ions loaded on L-cystein capped flower-like gold nanoparticles as signal amplification label.[33]

Aside from NMNPs, non-NMNPs such as graphene and carbon nanotubes have been described in other PADs in which the WEs were modified. Zeolites nanocrystals and graphene oxide nanoflakes were incorporated onto the electrode surface of a paper-based sensor for ketamine, which exhibited an amplified signal due to the enhanced surface area of the nanostructures and high electron transfer kinetics.[32] A research work by Cinti and colleagues introduced carbon black (CB) and prussian blue nanoparticles (PBNPs) modification on the electrode of their paper device, which was used to detect the presence of hydrogen peroxidase generated in the presence of ethanol in beers.[58] Li and colleagues modified a gold nanoparticle layer with multi-walled carbon nanotubes (MWCNTs) for use in the detection of bisphenol A (BPA), whereby MWCNTs exhibited significant enhancement effects to BPA oxidation.[30] In another work that aimed to detect human papillomavirus in order to examine the primary stages of cervical cancer, an anthraquinone-labeled pyrrolidinyl peptide nucleic acid (acpcPNA) probe (AQ-PNA) combined with graphene-polyaniline (G-PANI) was used to modify the electrode surface in the electrochemical biosensor, and together they aided in enhancing sensitivity, conductivity and electron transfer rates.[59]

3 Applications

3·1 Clinical diagnostics

The demand for effective point-of-care tests and devices (POCT) is ever expanding worldwide, not only driven by the latest discoveries in biomedicine and therapeutics, but also due to global trends such as the increase in the aging population in developed countries and the change in world demographic transition.[15] The development of paper-based sensors for the detection of metabolites, such as glucose, is one of the most popular success story of PADs used as POCT. This is, in part, due to the direct association of glucose to diseases such as glucose metabolism disorders and islet cell carcinoma.[39] In addition, other metabolites, such as uric acid and ascorbic acid, are also common target analytes for electrochemical PADs. Moreover, protein, nucleic acids and cells are a few components that are analyzed in order to diagnose human health.[60] Examples of how electrochemical PADs have been utilized for clinical diagnostics are elaborated in the following section.

A simple PAD sensor that did not incorporate any nanomaterials modification was fabricated for the determination of acetylcholinesterase (AChE).[61] Graphene ink was used to screen print the WE and CE onto a paper substrate, whereas the RE was screen printed using Ag/AgCl ink. A graphene screen-printed electrode was chosen over a carbon screen-printed electrode due to the higher currents displayed by graphene. Barriers to create the detection zone were created by wax printing. Signal detection was based on the formation of thiocholine (TCh) upon acetylthiocholine chloride (ATCh) hydrolysis by the enzyme AChE. Electrochemical analysis was performed by cyclic voltammetry and amperometry. The sensor exhibited a limit of detection of 0.1 U/mL, with a linear range of 0.1 and 15 U/mL. The sensor was shown to be applicable for the determination of AChE in blood samples.

As mentioned previously, electrochemical detection of an aggressive human chronic myelogenous leukemia cell line, K-562, was made possible by a biosensor constructed by Ge and colleagues.[54] In this biosensor, 3D Au nanoparticles/graphene composite was added onto the working electrode, which resulted in a higher specific surface area that allowed for more concanavalin A (Con A) attachment as well as enhancement in electronic transmission. Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were used to examine the performance of the sensor whereby the linear range and limit of detection for K-562 cells were found to be 1.0×10^3 to 5.0×10^6 cells/mL and 200 cells/mL, respectively. The prepared biosensor provided a novel point-of-care testing method in areas of cellular detection and diagnosis of disease.

Detection of the diabetes markers, total and glycated haemoglobin (HbA1c), was facilitated by a simple 3D electrochemical PAD composed of dual screen-printed electrode on wax-patterned paper coupled to multilayer magnetic paper.[38] The electrodes were haptoglobin (Hp)-modified and 3-aminophenylboronic acid (APBA)-modified eggshell membranes (ESMs) to aid in lowering the charge transfer resistance (Rct). The device exhibited a decrease in data acquisition time, and overall assay time by a factor of 15 compared to the conventional EIS measurements. The limit of detections of total and glycated HbA1c were 0.08 g dL^{-1} and 0.21%, respectively, which offers outstanding sensitivity within a clinically relevant range. The successfully fabricated sensor offered impressive benefits for onsite clinical monitoring of the glycaemic status in individuals with diabetes, which was suitable for near-patient monitoring in remote areas.

Human chorionic gonadotropin (hCG), a biomarker presented in blood and urine of pregnant women, was made detectable *via* a paper-based electrochemical immunosensor.[47] The hydrophilic test zones on the electrodes were activated by periodate oxidation for the consequent conjugation of antibodies. Primary signal antibody functionalized gold nanoparticles (GNP/Abs) and alkaline phosphatase conjugated secondary antibody (ALP-IgG) were employed for the detection of hCG. The fabricated sensor bypassed multiple incubation and washing steps by taking advantage of the permeability of the paper-based SPEs. The GNP/Abs and ALP-IgG modifications amplified the signal to obtain high sensitivity and selectivity. Differential pulse voltammetry (DPV) was performed to detect the electrochemical signal on the device, which exhibited a detection limit of 0.36 mIU mL^{-1} and the linear range of hCG concentration of 1.0 to 100.0 mIU/mL. The biosensor showed great potential for the detection of other biomarkers in medical diagnosis.

A recent attempt to revamp the already popular glucometer was undertaken by Li and colleagues, who fabricated an electrochemical PAD that employed pencil-drawn electrodes for glucose bio-sensing in human blood samples.[39] Pencil lead was made of fine graphite powders bound together by clay. One of the advantages of using this electrode fabrication technique on chromatographic paper was the ease and swiftness of the alteration of design and geometry as desired. Moreover, the direct-writing method of fabricating electrodes on paper results in low-cost PADs with promising outlook for future applications in resource-limited regions. Ferrocenecarboxylic acid (FCA) was added to the detection zone and glucose oxidase (GOx) was added to the enzyme zone. The two zones were then brought into contact by folding, creating the FCA/GOx/PAD. The analysis was performed using cyclic voltammetry, in which the linear range of detection for glucose concentration was between 1 to 12 mM and the detection limit was 0.05 mM. The fabricated device was cheap, flexible, portable, disposable, and environmentally friendly, offering great advantages for practical use under resource-limited environments.

Another electrochemical PAD, which exploited the pencil-drawn electrode design, was constructed for the detection of dopamine.[62] Pencil drawing on filter paper served as conducting tracks for electrochemical analysis. Detection of dopamine was

studied by using DPV to assess the sensitivity of the developed PA. The linear range of dopamine concentration was observed between 0.1 to 700 μM with a limit of detection of 0.04 μM. The interference from ascorbic acid (AA) and uric acid (UA) were studied and based on the results, both did not appear to interfere in the detection of dopamine. In short, the results showed the significant promise of the fabricated sensor as an alternative approach for real sample analysis of dopamine.

An electrochemical PAD for the purpose of white blood cell counting was fabricated and analyzed by DPV generated by a portable electronic potentiostat system. This system was coupled to a smartphone *via* Bluetooth communication to view the voltammetry test signal.[80] The electrochemical PAD was able to rapidly quantify the concentrations of WBC covering the common physiological and pathological range of 200 to 20000 μL^{-1} with high repeatability and a low coefficient of variation of 10%.

A noninvasive detection of epidermal growth factor receptor (EGFR) mutations using paper as a biosensing platform was previously described.[63] The sample zone was modified with AuNP-PWE and pyrrole. The electrode surface was modified with oligonucleotides and the electrochemical signal was obtained by analyzing DNA hybridization reaction *via* horseradish peroxidase (HRP) as a label. Signal detection was made possible by mixing template DNA (tDNA) with detection probe in the sample zone followed by electrochemical impedance spectroscopy. The biosensor showed good linear relationship of target DNA ranging from 0.5 to 500.0 nM, with a low limit of detection of 0.167 nM. In this study, saliva samples were utilized, which reduced the risk of blood transmitted diseases and was painless for patients while at the same time provided real-time information on EGFR mutation status.

Graphene, polyvinylpylidone and polyaniline (G/PVP/PANI)-modified paper-based biosensor for the detection of cholesterol in human serum was fabricated *via* electrospraying.[22] The sensitivity of the biosensor was enhanced by the presence of a small amount of PVP in the nanocomposites, which extensively amplified the dispersibility of graphene and improve the electrochemical conductivity of electrodes. Cholesterol detection was determined by cyclic voltammetry and chronoamperometry, which exhibited a linear range of 50 μM to 10 mM with the detection limit of 1 μM. In this study, the interference was eliminated by the use of sodium dodecyl sulfate (SDS).[64] The prepared PAD demonstrated good stability after a storage period of two weeks, retaining 89.1% of the initial response. The developed sensor served as an alternative tool for cholesterol screening in medical diagnosis due to its ease of use, low cost, ease of disposal and portability.

Determination of neuron-specific enolase (NSE), a biomarker for small cell lung cancer, was made possible *via* a wireless POCT system.[65] Amino functional graphene, thionine and gold nanoparticles (NH_2-G/Thi/AuNPs) were used to accelerate electron transfer and amplify the signal. The NSE concentration was determined by differential pulse voltammetry (DPV) *via* a wireless POCT system and the results were automatically generated and stored in an Android smartphone. Results were saved in the file format "txt" which could be viewed on the smartphone through Bluetooth in real time. This biosensor displayed a good linear relationship ranging from NSE concentration of 1 to 500 ng/mL, with the limit of detection of 10 pg/mL, which was lower than that obtained through the electrochemical impedance spectroscopy (EIS) method. In addition, the wireless POCT system was in agreement with reference data obtained from the ELISA method with the relative errors ranging from –6.4 to 3.79%.

A "pop-up" electrochemical PAD was constructed and fabricated to detect β-hydroxybutyrate (BHB), a diabetic ketoacidosis biomarker detection (Fig. 1B).[40] This single-use PAD contained a sample port, a reaction zone for storing the enzyme and a detection zone spatially isolated from the first two zones. The device changed from "off" to "on" configuration when the sample port, reaction zone and detection zone were in contact through modest mechanical pressure by folding the device between thumb and forefinger or by placing some weight on top of the device. It is additionally possible for the device to be incorporated into a glucometer, which initiated the amperometric measurement of BHB once the sample reached the electrodes. This set-up achieved a limit of detection of 0.3 mM and linear dynamic range of 0 to 6 mM. Five key functions were supported by the 3D pop-up bionsensor: controlled timing, good repeatability, greater accessibility due to incorporation of glucometer, reduced cost and electrochemical measurement triggered by folding.

A sensitive electrochemical PAD was developed for the simultaneous detection of metals such as Pb^{2+}, Cd^{2+} and Zn^{2+} in human serum for clinical application.[66] A graphene-polyaniline (G/PANI) nanocomposite electrode was fabricated in the presence of polyvinylpyrrolidone (PVP) by reverse-phase polymerization. The performance of the modification was characterized by scanning electron microscopy (SEM) and cyclic voltammetry (CV). Graphene was incorporated with PANI to enhance the electrochemical properties of the nanocomposite. Measurements from the fabricated sensor were determined by square-wave anodic stripping voltammetry with a detection limit of 1.0 μg/L for Zn^{2+}, and 0.1 μg/L for both Cd^{2+} and Pb^{2+} with linear working range of 1 to 300 μg/L. This demonstrated the potential of developing low-cost alternatives for the heavy metal determination in human serum.

3·2 Environmental

Although the original purpose of developing PADs was for use as biomedically-related POCT, their use has expanded into environmental research in an effort to combat the impact of pollution on the environment.[10,12] Onsite analysis was made more accessible through the fabrication of electrochemical PADs in comparison to the more traditional method of electrochemical detection that involves complicated and bulky instruments, which are not portable for the purpose of onsite analysis. Environmental analysis usually scans for heavy metals and toxic contaminants, which are often linked to excessive pollution from surplus of insecticides, pesticides and factory waste. Additionally, monitoring of the quality of drinking water is essential to inspect for the presence of any contaminants and ensure that the water is safe for consumption.[67]

Quantification of concentrations of various analytes such as heavy-metal ions or glucose was demonstrated using a microfluidic electrochemical PAD.[28] Glucose detection was carried out by spotting enzyme on the surface of the paper microchannel before the addition of analytes for chronoamperometric analysis. Analysis of glucose in artificial urine revealed a linear range of 0 to 22.2 mM, with the limit of detection of 0.22 mM and sensitivity of 0.43 $\mu A\ mM^{-1}\ mm^{-2}$. For heavy metal ions analysis, bismuth (500 μg/L) was deposited *in situ* along with Pb^{2+} ranging from 0 to 100 ppb. The limit of detection was approximately 1.0 ppb as determined by anodic stripping voltammetry. The biosensor for heavy metal ions analysis was reusable by replacing the blotting paper pad. Dissolved analytes were removed due to continuous wicking before the next cycle deposition of metals. Hence, the fabricated device should be useful for public health, environmental

monitoring and other applications.

The existence and toxicity level of heavy metals such as Pb^{2+}, Cd^{2+}, Cl^{-}, Zn^{2+}, and Bi^{3+} in the environment have been monitored using electrochemical and colorimetric PADs.[68] A disposable electrochemical PAD was fashioned out of stacked gold nanoparticles modified with carbon nanotubes for the determination of bisphenol A (BPA).[30] BPA has been described as a hormone disruptor and could lead to cancer and developmental disorders. It was suspected that BPA could enter the environment through waste and into the human bodies through end-user products, such as food-storage or packaging materials, toys, bottles or cans.[69] The linear sweep voltammetry (LSV) was used to obtain a readout for BPA levels and demonstrated a wide linear range of detection between 0.2 to 20 mg/L with the limit of detection of 0.03 mg/L. A high resistivity against interferences was displayed using the constructed PAD, which was able to detect BPA that leached out from samples such as acrylonitrile butadiene styrene (ABS) plastic toys and polycarbonate (PC) drinking bottles.

A study by Medina-Sanchez and colleagues reported the fabrication of an eco-friendly electrochemical lab-on-paper to be used for the detection of heavy metal such as Pb^{2+} and Cd^{2+}.[29] The platform of the device behaved as a sample pretreatment material because of the filtering properties of paper. The signal from the PAD was analyzed using a computer-controlled potentiostat/galvanostat by square wave voltammetry. The quantification range demonstrated for lead and cadmium in aqueous samples was from 10 to 100 ppb, with a limit of detection of 7 to 11 ppb, respectively. They additionally tested the PAD using complex samples such as mud and seawater without prior sample pretreatment, such as filtration, and found that the detection efficiency was satisfactory as the paper itself possessed some filtering capability.

Another published work investigated the level of phosphate in river water *via* a screen-printed electrochemical PAD, which generated electroactive phosphomolybdic complexes that were measured.[70] The hydrophilic area was modified with molybdate ions, potassium chloride and sulfuric acid to allow for the reaction with phosphate, followed by the electroanalytical enzyme-free determination of phosphate level. The performance of the device was studied using cyclic voltammetry, whereby the PAD exhibited a wide linear range of up to 300 μM and limit of detection of 4 μM. The storage stability was studied by storing modified sensors for 30 days at room temperature without any exposure to light and no sign of decrease in signal was detected with the initial response maintained completely. These developed sensors have met the requirements set by WHO guidelines, such as affordability, sensitivity, portability, ease of use for the affordable, sensitive, specific, user-friendly, rapid, equipment, and delivered (ASSURED) devices, which render them suitable for rapid and simple analysis.[71]

3·3 Food

Besides using biosensors as biomedically- and environmentally-related POCT, PAD have also been developed for the determination of food safety. Food safety includes handling, storing and preparation of food for prevention of infection as well as ensuring that food maintain enough nutrients for a healthy diet.[72] Contamination of food may occur at any point during food production or distribution, with food producers carrying the biggest responsibility in ensuring and monitoring food safety. Focus has been placed on simple, low-cost and miniaturized devices for the chemical analysis of products, such as food and beverages.[73,74]

An electrochemical sensing platform for analyzing nitrite based on a simple and efficient vacuum filtration system was constructed.[75] With the aid of mixed cellulose ester (MCE), the WE was modified with reduced graphene oxide and gold nanoparticles to further improve nitrite detection. This proved to be a promising platform when compared to various reference methods due to the relatively wide linear range and low detection limit demonstrated by the biosensor. Electrochemical oxidation and determination of nitrite was examined by cyclic voltammetry, whereby the detection limit was calculated to be 0.1 μM. Real samples such as milk were precipitated by $ZnSO_4$ prior to analysis, which was later centrifuged and filtered in order to remove interference from existing proteins and fats. The accuracy of the proposed system was found to be acceptable with 96.0% recovery, and the reliability was confirmed with UV-visible measurements from two methods that were in good agreement with the analysis of the sample.

Ketamine, a drug commonly used in medical science to maintain a state of anaesthesia, could produce a broad range of sedative effects and is often used illegally to spike alcoholic drinks at a higher than permitted dosage.[76] Ketamine was reported to be successfully detected electrochemically using a PAD modified with zeolites nanoflakes and graphene-oxide nanocrystals (Zeo-GO) in beverages such as whiskey and juices.[32] Zeo-GO was used to produce an amplified sensing signal when compared to unmodified electrode and could be attributed to the large surface area and increased electron transfer kinetics for ketamine. The electrochemical performance of the device was tested using cyclic voltammetry. The linear range of detection of ketamine concentrations ranges from 0.001 to 5 nM/mL, with detection limit of 0.001 nM/mL. In brief, the developed PAD had great advantages over the traditional three-electrode system as preparation was easier, electrodes were inexpensive to fabricate, and it was portable and disposable.

Determination of glucose and total carbohydrates in foodstuff was described by Terzi and colleagues, as previously mentioned. An amperometric PAD that incorporated electrically conductive ink based on Cu nanoparticles, graphite and polystyrene increased the sensitivity of the sensor.[31] A combination of Cu, graphite and polysterene ink were employed for electrode fabrication. Based on the amperometric results, the most suitable formulation for Cu, graphite, and polystyrene were 50, 75 and 22 mg/mL, respectively. The estimated total concentration of carbohydrate was found to be in good agreement with the value on the label of the soft drinks being investigated and estimates obtained through conventional chromatographic method. Also, the resulting sensor could be used either by deposition of a drop of solution or by dipping the cell into the sample.

Ethanol is one of the ingredients in characterizing the strength of beer, and the detection of ethanol in beers was made possible by employing a PAD that was nano-modified by carbon black (CB) and prussian blue and electrochemically screen-printed.[58] The enzymatic reaction between alcohol oxidase (AOx) and ethanol produced hydrogen peroxidase, which was detected by the nanocomposite that acted as an electrocatalyst within the biosensor. The sensitivity of the PAD was determined by cyclic voltammetry and chronoamperometry whereby the scan rate for cyclic voltammetry experiments varied from 20 to 1000 mV/s for each electrode type. The PAD demonstrated a fast and sensitive response after only 40 s. The developed sensor allowed quantification of ethanol up to 10 mM with sensitivity of 9.13 μA/mM cm^2 and the limit of detection of 0.52 mM. Interference study was carried out in the presence of methanol, acetic acid, glucose and ascorbic acid, which may be found in commercial beers as a result of the fermentation process and

antioxidant capacity. It could be observed that methanol appeared to produce an interfering response but was not as effective as ethanol. Moreover, this PAD demonstrated that office paper could be utilized as a substrate in biosensor development, making it appropriate for on-site analysis with potential integration with existing devices.

A paper-based enzymatic sensor that combined carbon ink and metal wire electrodes was developed for the determination of glucose in real food samples.[77] Gold-plated pins were employed to connect the paper-based WE as well as to act as the RE and CE. In addition, it worked as a clip to support the paper in between. A mixture of GOx and HRP enzymes was deposited onto the carbon WE as recognition element. Potassium ferrocyanide was utilized as a mediator for electron transfer and glucose could be detected as ferrocyanide was reduced. Enzyme specificity was demonstrated when the biosensor was investigated for potential interference by fructose and ascorbic acid but no interference in signal was detected. The biosensor achieved a linear range of 0.3 to 15 mM of glucose, making it suitable for the quantification of glucose in real food samples. Moreover, the biosensor was found to be stable for up to one week.

4 Conclusions and Outlook

The sensitivity and selectivity of PADs for certain target analytes could be improved in many different ways. The development of materials for electrode modification has augmented both electrical conductivity and surface area of PADs. In electrochemically-based PADs, antibody carriers could serve as catalyst, while functional nanomaterials acted as signal tags by utilizing DNA or enzyme-based signal amplification approaches. A target with high binding affinity could further enhance the incorporated immune recognition element. Electrochemical detection is likely to be increasingly employed in the future compared to other quantitative detection methods, due to properties such as simple instrumentation, efficient analysis, high sensitivity, and high selectivity. Despite the initial development of PADs for diagnostics application, the research and development of electrochemical PADs has been extended and applied to environmental and food monitoring. New strategies are continually being explored to further enhance sensitivity and lower the cost of PAD production with aims to eventually eliminate the use of expensive portable bench top potentiostats for reading electrochemical signals. As an alternative, smart phones could be connected to a circuit board that serves as the source of data transmission for paper-based electrochemical analysis. Future studies should move towards instrument-free techniques and non-equipment based readouts. Finally, the improvement of stability, accuracy and shelf-life of PADs could encourage future commercialization to allow for early diagnosis of diseases, detection of food contamination and environmental hazards.

5 Acknowledgements

V. B. C. L. and M. U. A. would like to acknowledge the financial support for the project given by the Brunei Research Council of Negara Brunei Darussalam through Grant BRC-10.

6 References

1. M. Safavieh, M. K. Kanakasabapathy, F. Tarlan, M. U. Ahmed, M. Zourob, W. Asghar, and H. Shafiee, *ACS Biomater. Sci. Eng.*, **2016**, *2*, 278.
2. N. T. Brannelly and A. J. Killard, *Talanta*, **2017**, *167*, 296.
3. Y.-Hou, J.-J. Wang, Y.-Z. Jiang, C. Lv, L. Xia, S.-L. Hong, M. Lin, Y. Lin, Z.-L. Zhang, and D.-W. Pang, *Biosens. Bioelectron.*, **2017**, *99*, 186.
4. Nilghaz, L. Guan, W. Tan, and W. Shen, *ACS Sensors*, **2016**, *1*, 1382.
5. M. Hasanzadeh and N. Shadjou, *Mater. Sci. Eng., C*, **2016**, *61*, 979.
6. S. A. Lim, H. Yoshikawa, E. Tamiya, H. M. Yasin, and M. U. Ahmed, *RSC Adv.*, **2014**, *4*, 58460.
7. S. Roy, I. A. Rahman, and M. U. Ahmed, *Anal. Methods*, **2016**, *8*, 2391.
8. G. G. Morbioli, T. Mazzu-Nascimento, A. M. Stockton, and E. Carrilho, *Anal. Chim. Acta*, **2017**, *970*, 1.
9. Dhiman, P. Kalra, V. Bansal, J. G. Bruno, and T. K. Sharma, *Sens. Actuators, B*, **2017**, *246*, 535.
10. J. Adkins, K. Boehle, and C. Henry, *Electrophoresis*, **2015**, *36*, 1811.
11. S. A. Lim and M. U. Ahmed, *RSC Adv.*, **2016**, *6*, 24995.
12. N. A. Meredith, C. Quinn, D. M. Cate, T. H. Reily III, J. Volckens, and C. S. Henry, *Analyst*, **2016**, *141*, 1874.
13. R. H. Tang, H. Yang, J. R. Choi, Y. Gong, S. S. Feng, B. Pingguan-Murphy, Q. S. Huang, J. L. Shi, Q. B. Mei, and F. Xu, *Crit. Rev. Biotechnol.*, **2016**, *37*, 411.
14. Y. Wu, P. Xue, K. M. Hui, and Y. Kang, *Biosens. Bioelectron.*, **2014**, *52*, 180.
15. C. M. Silveira, T. Monteiro, and M. G. Almeida, *Biosensors*, **2016**, *6*, 1.
16. S. A. Lim and M. U. Ahmed, *Biosens. Bioelectron.*, **2015**, *70*, 48.
17. S. A. Lim and M. U. Ahmed, *Food Chem.*, **2016**, *206*, 197.
18. F. Arduini, S. Cinti, V. Scognamiglio, D. Moscone, and G. Palleschi, *Anal. Chim. Acta*, **2017**, *959*, 15.
19. W. Martinez, S. T. Philips, M. J. Butte, and G. M. Whitesides, *Angew. Chem.*, **2007**, *46*, 1318.
20. J. C. Cunningham, P. R. DeGregory, and R. M. Crooks, *Ann. Rev. Anal. Chem.*, **2016**, *9*, 183.
21. W. Dungchai, O. Chailapakul, and C. S. Henry, *Anal. Chem.*, **2009**, *81*, 5821.
22. N. Ruecha, R. Rangkupan, N. Rodthongkum, and O. Chailapakul, *Biosens. Bioelectron.*, **2014**, *52*, 13.
23. G. Wu and M. H. Zaman, *Talanta*, **2015**, *134*, 194.
24. L. Li, J. Xu, X. Zheng, C. Ma, X. Song, S. Ge, J. Yu, and M. Yan, *Biosens. Bioelectron.*, **2014**, *61*, 76.
25. C. Fischer, A. Fraiwan, and S. Choi, *Biosens. Bioelectron.*, **2016**, *79*, 193.
26. S. Liu, W. Su, and X. Ding, *Sensors*, **2016**, *16*, 2086.
27. S. H. Lee, J. H. Lee, V.-K. Tran, E. Ko, C. H. Park, W. S. Chung, and G. H. Seong, *Sens. Actuators, B*, **2016**, *232*, 514.
28. Z. Nie, C. A. Nijhuis, J. Gong, X. Chen, A. Kumachev, A. W. Martinez, M. Narovlyansky, and G. M. Whitesides, *Lab Chip*, **2010**, *10*, 477.
29. M. Medina-Sanchez, M. Cadevall, J. Ros, and A. Merkoci, *Anal. Bioanal. Chem.*, **2015**, *407*, 8445.
30. H. Li, W. Wang, Q. Lv, G. Xi, H. Bai, and Q. Zhang, *Electrochem. Commun.*, **2016**, *68*, 104.
31. F. Terzi, B. Zanfrognini, S. Ruggeri, N. Dossi, G. M. Casagrande, and E. Piccin, *Sens. Actuators, B*, **2017**, *245*, 352.

32. J. Narang, N. Malhotra, C. Singhal, A. Mathur, D. Chakraborty, A. Ingle, and C. S. Pundir, *Biosens. Bioelectron.*, **2017**, *88*, 249.
33. L. Li, Q. Kong, Y. Zhang, C. Dong, S. Ge, and J. Yu, *J. Mater. Chem., B*, **2015**, *3*, 2764.
34. W. Li, H. Yang, C. Ma, L. Li, S. Ge, X. Song, J. Yu, and M. Yan, *Electrochim. Acta*, **2014**, *141*, 391.
35. C. Ma, W. Li, Q. Kong, H. Yang, Z. Bian, X. Song, J. Yu, and M. Yan, *Biosens. Bioelectron.*, **2015**, *63*, 7.
36. S. Ge, W. Liu, L. Ge, M. Yan, J. Yan, J. Huang, and J. Yu, *Biosens. Bioelectron.*, **2013**, *49*, 111.
37. X. Wang, C. Yang, S. Zhu, S. Ge, and J. Yu, *Biosens. Bioelectron.*, **2017**, *87*, 108.
38. Y. Boonyasit, O. Chailapakul, and W. Laiwattanapaisal, *Anal. Chim. Acta*, **2016**, *936*, 1.
39. W. Li, D. Qian, Q. Wang, Y. Li, N. Bao, H. Gu, and C. Yu, *Sens. Actuators, B*, **2016**, *231*, 230.
40. C. Wang, J. W. Hennek, A. Ainla, A. A. Kumar, W. Lan, J. Im, B. S. Smith, M. Zhao, and G. M. Whitesides, *Anal. Chem.*, **2016**, *88*, 6326.
41. M. Tenjimbayashi, M. Higashi, T. Yamazaki, I. Takenaka, T. Matsubayashi, T. Moriya, M. Komine, R. Yoshikawa, K. Manabe, and S. Shiratori, *Appl. Mater. Interfaces*, **2017**, *9*, 10371.
42. Y. Xia, J. Si, and Z. Li, *Biosens. Bioelectron.*, **2016**, *77*, 774.
43. J.-Y. Sun, C.-M. Cheng, and T.-C. Liao, *Anal. Sci.*, **2015**, *31*, 145.
44. W. Su, B. S. Cook, Y. Fang, and M. M. Tentzeris, *Nature*, **2016**, *6*, 35111.
45. E. Carrilho, A. W. Martinez, and G. M. Whitesides, *Anal. Chem.*, **2009**, *81*, 7091.
46. G.-W. Huang, Q.-P. Feng, H.-M. Xiao, N. Li, and S.-Y. Fu, *ACS Nano*, **2016**, *10*, 8895.
47. L. Cao, C. Fang, R. Zeng, X. Zhao, Y. Jiang, and Z. Chen, *Biosens. Bioelectron.*, **2017**, *92*, 87.
48. M. A. Mahmud, E. J. M. Blondeel, M. Kaddourab, and B. D. MacDonald, *Analyst*, **2016**, *141*, 6449.
49. D. D. Liana, B. Raguse, J. J. Gooding, and E. Chow, *Sensors*, **2012**, *12*, 11505.
50. M. Abdorahim, M. Rabiee, S. N. Alhosseini, M. Tahriri, S. Yazdanpanah, S. H. Alavi, and L. Tayebi, *Trends Anal. Chem.*, **2016**, *82*, 337.
51. Y. Wu, Y. Sun, F. Xiao, Z. Wu, and R. Yu, *Talanta*, **2017**, *162*, 174.
52. L. Juntao, L. Jinping, W. Yang, X. Huiren, W. Li, F. Yan, and C. Xinxia, *Eur. J. Biomed. Res.*, **2015**, *1*, 9.
53. J. C. Cunningham, N. J. Brenes, and R. M. Crooks, *Anal. Chem.*, **2014**, *86*, 6166.
54. S. Ge, L. Zhang, Y. Zhang, H. Liu, J. Huang, M. Yan, and J. Yu, *Talanta*, **2015**, *145*, 12.
55. L. Wu, C. Ma, L. Ge, Q. Kong, M. Yan, S. Ge, and J. Yu, *Biosens. Bioelectron.*, **2015**, *63*, 450.
56. S. Wang, L. Ge, M. Yan, J. Yu, X. Song, S. Ge, and J. Huang, *Sens. Actuators, B*, **2013**, *176*, 1.
57. Y. Wang, H. Xu, J. Luo, L. Wang, Y. Fan, S. Yan, Y. Yang, and X. Cai, *Biosens. Bioelectron.*, **2016**, *83*, 319.
58. S. Cinti, M. Basso, D. Moscone, and F. Arduini, *Anal. Chim. Acta*, **2017**, *960*, 123.
59. P. Teengam, W. Siangproh, A. Tuantranont, C. S. Henry, T. Vilaivan, and O. Chailapakul, *Anal. Chim. Acta*, **2017**, *952*, 32.
60. M. Dou, S. T. Sanjay, M. Benhabib, F. Xu, and X. Li, *Talanta*, **2015**, *145*, 43.
61. Y. Panraksa, W. Siangproh, T. Khampieng, O. Chailapakul, and A. Apilux, *Talanta*, **2017**, *178*, 1017.
62. W. Li, D. Qian, Y. Li, N. Bao, H. Gu, and C. Yu, *J. Electroanal. Chem.*, **2016**, *769*, 72.
63. T. Tian, H. Liu, L. Li, S. Ge, X. Song, and M. Yan, *Sens. Actuators, B*, **2017**, *251*, 440.
64. P. Rattanarata, W. Dungchai, W. Siangproh, O. Chailapakulad, and C. S. Henry, *Anal. Chim. Acta*, **2012**, *744*, 1.
65. Y. Fan, J. Liu, Y. Wang, J. Luo, H. Xu, S. Xu, and X. Cai, *Biosens. Bioelectron.*, **2017**, *95*, 60.
66. N. Ruecha, N. Rodthongkum, D. M. Cate, J. Volckens, O. Chailapakul, and C. S. Henry, *Anal. Chim. Acta*, **2015**, *874*, 40.
67. H. Zhang, S. Qi, Y. Dong, X. Chen, Y. Xu, Y. Ma, and X. Chen, *Food Chem.*, **2014**, *151*, 429.
68. J. Mettakoonpitak, K. Boehle, S. Nantaphol, P. Teengam, J. A. Adkins, M. Srisa-Art, and C. S. Henry, *Electroanalysis*, **2016**, *28*, 1.
69. Y. Q. Huang, C. K. C. Wong, J. S. Zheng, H. Bouwman, R. Barra, B. Wahlstrom, L. Neretin, and M. H. Wong, *Environ. Int.*, **2012**, *42*, 91.
70. S. Cinti, D. Talarico, G. Palleschi, D. Moscone, and F. Ardiuni, *Anal. Chim. Acta*, **2016**, *919*, 78.
71. M. Urdea, L. A. Penny, S. S. Olmsted, M. Y. Giovanni, P. Kaspar, A. Shepherd, P. Wilson, C. A. Dahl, S. Buchsbaum, G. Moeller, and D. C. H. Burgess, *Nature*, **2006**, *444*, 73.
72. WHO, WHO Food Safety, **2015**, http://www.who.int/mediacentre/factsheets/fs399/en/.
73. *Escarpa*, Royal Society of Chemistry, **2014**, *14*, 3213.
74. W. Martinez, S. T. Philips, and G. M. Whitsides, *Anal. Chem.*, **2010**, *82*, 3.
75. P. Wang, M. Wang, F. Zhou, G. Yang, L. Qu, and X. Miao, *Electrochem. Commun.*, **2017**, *81*, 74.
76. J. Y. K. Cheng and V. K. K. Mok, *Forensic Sci. Int.*, **2004**, *142*, 9.
77. O. Amor-Gutierrez, E. C. Rama, A. Costa-Garcia, and M. T. Fernandez-Abedul, *Biosens. Bioelectron.*, **2017**, *93*, 40.
78. M. Cuartero, G. N. A. Crespo, and E. Bakker, *Anal. Chem.*, **2015**, *87*, 1981.
79. J. Bhardwaj, S. Devarakonda, S. Kumar, and J. Jang, *Sens. Actuators, B*, **2017**, *253*, 115.
80. X. Wang, G. Lin, G. Cui, X. Zhou, and G. L. Liu, *Biosens. Bioelectron.*, **2017**, *90*, 549.
81. Z. Li, F. Li, Z. Liu, M. You, Y. Wen, Z. Qu, X. L. Li, and F. Xu, *Biosens. Bioelectron.*, **2017**, *98*, 478.
82. S. Cinti, C. Minotti, D. Moscone, G. Palleschi, and F. F. Arduini, *Biosens. Bioelectron.*, **2017**, *93*, 46.
83. D. A. Bruzewicz, M. Reches, and G. M. Whitesides, *Anal. Chem.*, **2008**, *80*, 3387.
84. J. Olkkonen, K. Lehtinen, and T. Erho, *Anal. Chem.*, **2010**, *82*, 10246.
85. P. J. Lamas-Ardisana, P. Casuso, I. Fernandez-Gauna, G. Martinez-Paredes, E. Jubete, L. Anorga, G. Cabanero, and H. J. Grande, *Electrochem. Commun.*, **2017**, *75*, 25.