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CHAPTER TWO

Paper-based sensors for the application of biological compound detection

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1. Introduction

Paper-based analytical devices (PADs) received its great attentions as an emerging analytical tool since its outbreak in 2007 first reported by Martinez, M. and group [1]. In that works, various analytes were detected
simultaneously on PADs demonstrating that PADs is very versatile for multiple analyte detection. Moreover, paper is lightweight, flexible, and disposable which is convenient for a fabrication of point-of-care devices or small devices for on-filed analysis. Liquid can be controlled using hydrophobic barrier and transported by capillary force without the requirement of external pump. PADs is also compatible with biochemical applications and consume small amount of samples which is exceptionally benefit for a biological analysis in which the sample size is limited (i.e. sweat, saliva, urine, blood, etc.) [2]. Recently, plenty analytical methods on PADs are demonstrated for biological detection due to its prominent advantages.

Spectrometry (fluorescence, surface plasmon resonance, mass spectrophotometry), electrochemistry, and colorimetry are examples of analytical techniques implemented on PADs [3]. Electrochemistry (voltammetry) and colorimetry are mostly involving, together with cheap and ubiquitous paper-materials resulting in not only cost-effective but also potential analytical devices. Electrochemistry is suitable for PADs due to the ease of fabrication of miniaturized electrodes onto paper, the uncomplicated and portable instruments [4]. Moreover, the working (indicator) electrode can be easily modified with nanoparticles [5], biological probes (i.e. DNA, aptamer) [6], recognizing polymers [7]. The modification increases the sensitivity (for nanoparticles) and the selectivity (for biological probes and polymers) of biological compounds. Therefore, electrochemistry is famous for detection of important biomarkers. Colorimetry is another analytical technique that frequently uses on PADs. The technique studies the presence and quantity of analytes from the colour change and colour intensity, respectively. Due to its simplicity of result interpretation and cost of operation, colorimetry is developed as the detection method [8]. Various methods were used for analyte detection, for example; aggregation [9], antiaggregation [10], and etching [11] are the analytical methods of nanoparticles. Other venerable methods such as complex formation [12] and Griess test [13] were reported in the early development of PADs. The surface of nanoparticles can be modified with specific probe using precious metal and sulphur formation [14] which increases the selectivity of the materials towards target analytes. Thus, nanoparticles were often employed as analytical probes for colorimetric detection of biological compounds. Current applications on PADs concentrates on the development of electrochemistry and colorimetry for biological compounds detection.

The development of detection techniques for biomarkers helps diagnostic and prevention of illness and diseases. Previous reasons proof that PADs is
an excellent choice for these applications. The common methods for biological compounds detection include enzymatic methods, immunoassays, and DNA sensors which are covered in this chapter.

2. **Enzymatic method**

Enzymatic method uses an enzyme as a biological catalyst for numerous reactions that occur in living organisms. Enzymatic technology based on enzymatic reaction possesses several advantages, such as high specificity, mild conditions and easy control. Recently, there are many reports on the application of enzymatic methods in medicine, food analysis, chemical industry, and other relevant fields. In addition, enzymatic method can be combined with paper-based technology for simple, qualitative and low-cost point-of-care applications offering enough capability to be utilized in highly sensitive, fully quantified and multiplexed assays. The first paper-based enzymatic sensor was introduced in 2008 for the detection of proteins and glucose in artificial urine samples, which was fabricated via photolithography [15]. They used glucose oxidase as an enzyme for the oxidation of glucose to gluconic acid and formation of hydrogen peroxide. Hydrogen peroxide was reduced by horseradish peroxidase (HRP) oxidizing iodide to iodine, which resulted in the formation of a brown colour. Simultaneously, proteins were detected using tetra-bromophenol blue (TBP), which electrostatically interacted with proteins to change their colour from yellow to blue. In this part of the chapter, our selected published works related to paper-based devices combined with enzymatic methods from the year 2009 to 2019 were reviewed. Our main focus is the various detection methods utilized for the determination of biological compounds with an emphasis on electrochemical and colorimetric method.

2.1 **Electrochemical detection**

Electrochemical sensor consists of three electrodes: a working electrode, a reference electrode and a counter electrode. These electrodes can easily be screen printed on paper filter using carbon ink and silver/silver chloride (Ag/AgCl) ink. In 2009, our group reported for the first time an enzymatic electrochemical method based on paper based analytical device (PADs) for the simultaneous determination of uric acid, glucose and lactate in biological fluids. By drawing hydrophilic trilobed areas on filter paper through photolithography, PADs assure the homogenous diffusion of the sample volume to the screen-printed electrodes in the lobes. Then, by screen-printing the
working electrodes (WEs) with a carbon ink doped with Prussian Blue and modifying them with different oxidase enzymes (Fig. 1) [16], they achieved sensitive and selective amperometric responses. Corresponding enzymes and electron-transfer mediators were stored in test zones to react with the analytes and produce electrical signals. The limit of detection (LOD) for this device was found to be 0.35 mM for glucose, 1.76 mM for lactate and 0.52 mM for uric acid.

In addition, our group proposed new method for the fabrication of PADs by wax screening methods for the determination of biological compounds. Dungchai et al. proposed wax screen-printing method as a low-cost, simple, and rapid for the fabrication of PADs (Fig. 2) [17]. The effectiveness of wax screen-printing was demonstrated when used for the simultaneous determination of glucose and total iron in control human serum samples using an electrochemical method with glucose oxidase and a colorimetric method with 1,10-phenanthroline.

Moreover, the use of nanomaterials has been widely applied in combination with PADs owing to their unique chemical-, physical- and size-properties. Nanomaterials, including carbon nanomaterials, metal nanoparticles (NPs), quantum dots (QDs) and other functionalized NPs, have been employed for designing PADs. Combination of nanomaterials with biosensors exhibited a great potential for monitoring biomolecules and sensitive detection of target analytes. Nanomaterials can be used as
carriers to load signal markers or directly as signal reporters for sensitively detecting biomarkers and they can accelerate electron transfer reactions when they are used as functional materials on electrode surfaces. Our group successfully synthesized the nanocomposites of polyaniline/nanohematite (α-Fe$_2$O$_3$)/Prussian Blue (PB) [18]. This nanocomposite (PB/CPANI)
has been used for the modification of paper-based sensors which were used for cholesterol detection. The electrocatalytically reduced H$_2$O$_2$ generated during the enzymatic reaction of cholesterol oxidase (ChOx) and cholesterol showed a linear concentration range with good sensitivity and a low detection limit (Fig. 3). Rungsawang et al. [19] developed an electrochemical PAD for the detection of glucose in human blood serum, soft drinks, apple juice and sweet tea. They modified screen-printed carbon electrodes by mixing cellulose acetate with carbon ink and using 4-aminophenylboronic acid as a redox mediator in order to improve the sensitivity towards glucose detection.

In addition, cholesterol paper-based biosensors have been developed as a new, cheap and fast way of diagnosis. Paper-based biosensors offer several advantages compared to traditional substrates such as low cost, high abundance, biocompatibility and disposability. Furthermore, paper-based analysis only requires a small quantity of samples and reagents, which make it suitable for biosensor applications. Graphene (G), polyvinylpyrrolidone (PVP), and polyaniline (PANI) nanocomposite has been successfully prepared by Ruecha et al. and used for the modification of paper-based biosensors via electrospraying (Fig. 4) [20]. ChOx was attached to the G/PVP/PANI-modified electrode for the amperometric determination of cholesterol. Under optimum conditions, a linear range of 50μM to 10mM was achieved.
and the detection limit was found to be 1 μM. It was also concluded that this paper-based biosensor retained 89% of its initial response after a storage period of 2 weeks, indicating a good stability.

Nantaphol et al. used a boron-doped diamond (BDD) working electrode modified with silver nanoparticles (AgNPs) for the development of a μPAD that could sense cholesterol [5]. An electrodeposition method was used to deposit AgNP onto the BDD electrode surface followed by drop casting of ChOx. Compared to the bare BDD electrode, in the presence of the AgNPs, the electrode showed excellent electrocatalytic activity and very low sensitivity for the H₂O₂ reduction. The fabricated device demonstrated a good linearity for the cholesterol concentrations from 0.01 to 7 mM and low detection limit which was estimated as 0.006 mM (Fig. 5).

2.2 Colorimetric detection

Enzymatic colorimetric technique is the most commonly used one as it provides accurate results at a low cost. It involves visually observing the colour change during a reaction and using it for either qualitative or quantitative analysis with the help of the naked eye. In 2010, our group proposed the use of multiple indicators in order to increase the accuracy of paper-based colorimetric assay. The principle of sensor is based on the oxidation of indicators by hydrogen peroxide produced by oxidase enzymes specific for each analyte. Each detection zone comprised several indicators generally classified in two types: one that generated colour on oxidation and the other that would lose colour when oxidized. The device presented consisted of nine...
Fig. 5 The cholesterol sensor based on the coupling of the AgNP/BDD electrode with PAD. Reprinted with permission from Ref. S. Nantaphol, O. Chailapakul, W. Siangproh, A novel paper-based device coupled with a silver nanoparticle-modified boron-doped diamond electrode for cholesterol detection, Anal. Chim. Acta 891 (2015) 136–143.
detection zones positioned around the sample introduction area and was fabricated with photoresist as shown in Fig. 6A and B [21]. With use of mixtures of 4-aminoantipyrine, 3,5-dichloro-2-hydroxybenzenesulfonic acid, o-dianisidine dihydrochloride, potassium iodide, acid black, and acid yellow it was possible to quantify glucose (0.5–20 mM), lactate (1–25 mM), and uric acid (0.1–7 mM). It was found that a single indicator system had an accuracy of around 70%, whereas the multi-indicator system had accuracy above 90%. In 2011, Songjaroen et al. [22] proposed a novel method for the fabrication of paper-based microfluidic devices by wax dipping. The wax dipping

Fig. 6 (A, B) Multiple indicator system for glucose, lactate, and uric acid. (C–E) PADs fabricated by the wax dipping method used for colorimetric applications. Adapted from the respective Refs. W. Dungchai, O. Chailapakul, C.S. Henry, Use of multiple colorimetric indicators for paper-based microfluidic devices, Anal. Chim. Acta 674 (2) (2010) 227–233; T. Songjaroen, et al., Novel, simple and low-cost alternative method for fabrication of paper-based microfluidics by wax dipping, Talanta 85 (5) (2011) 2587–2593.
procedure requires only a hot plate for patterning hydrophobic and hydrophilic areas on paper filter. For application, this PADs was employed for simultaneously colorimetric tests of glucose and protein using an enzymatic assay and the bromocresol green (BCG) method. The colour intensity was monitored from colorless to a blue-green or yellow for protein and glucose reactions, respectively. Images of the colorimetric reaction and design of protein and glucose are shown in Fig. 6C–E.

3. Immunoassay

As we known, PAD has tremendously employed and received considerable interest to be applied as a platform to successfully fabricate a simple, portable and disposable device for various applications. Currently, the utilization of PAD is rapidly growing especially in clinical diagnosis since the main component of paper is made of cellulose-based material, consequently, it is inherent hydrophobicity, and therefore there is no external pump is required to force the solution penetrate through the fibre paper. Likewise, it is biodegradable and compatible, and easy to be disposed of and designed. Furthermore, the surface chemistry of PAD can also be altered depending on a specific application. All these above-mentioned unique properties make PAD especially suitable for the development of medical diagnosis tools in several areas. Although numerous approaches have been successfully established for an immunoassay, electrochemical and colorimetric detections are the widely used techniques among other methods combining with PAD.

3.1 Electrochemical detection

Electrochemical biosensor has been well recognized as a promising method for the selective detection of analyte interest. However, to selectively form immunocomplexes with a target molecule, the analysis requires biological recognition elements such as antibody, aptamer, DNA or synthesized probes. Several detection approaches based on changes in signal such as resistance or electrochemical current have been developed. The general principle of immunoassay involves the immobilization of recognition elements onto the electrode surface. Subsequent formation between the probe and the target molecule causes a change or shift in the response signal. Electrochemical detection on PAD responds to the demand for
user-friendly, portable, sensitive, miniaturized and low-cost sensors to support or substitute traditional assays. This will strongly impact medical diagnostics for various applications.

According to the fabrication of an immunosensing device, electrochemical transducer was first constructed onto PAD with a specific design and pattern. The configuration on PAD was custom-designed to facilitate the preparation or detection steps, in which the solution can be stored and transferred sequentially, resulting in less time consuming and reagent usage. For the fabrication of a recognition surface, the biological probe (antibody) can successfully anchor onto the transducer surface through an activation surface employing EDC/NHS chemistry, nanomaterials and polymers.

Polyaniline (PANI) and graphene (G) are most commonly employed nanocomposite to enhance the detection sensitivity of the developed sensor, also to directly immobilize biomolecule onto the electrode surface [23,24]. The modified sensor provides high surface area for antibody immobilization. Interestingly, the PANI-G modified electrodes exhibit higher sensitivity compared to PANI modified electrodes. The developed system offers several advantages including low cost, low sample volume requirement, disposability, and rapid analysis relative to traditional methods, allowing the platform to be used as an alternative tool for medical diagnosis (e.g. human interferon gamma and CRP) (Fig. 7A and B).

In addition, to closer the real-world application of using PAD for immunoassay, Yakoh et al. describes the device design and fabrication of a stopped-flow sequential fluid delivery platform on a microfluidic paper-based device. This developed device was successfully applied for label-free detection of α-fetoprotein (AFP). This 3D capillary-driven device eliminates the undesirable procedure of multiple-step reagent manipulation in a complex assay [25].

Furthermore, other specific recognition elements such as phosphocholine, haptoglobin (Hp) and 3-aminophenylboronic acid, were also applied to successfully detect CRP and multiple diabetes markers (total haemoglobin and HbA1c), respectively. These recognition elements might be directly or indirectly modified onto the transducer surface to be used as the highly responsive probe to analyte interests. In addition, the dual electrode was further designed onto the customized paper-based device and it was found that the proposed systems could be employed for simultaneously measuring the CRP levels and total haemoglobin and HbA1c using only a single device [26,27] (Fig. 8A and B).
3.2 Colorimetric detection

To the best of our knowledge, most of colorimetric detection on PAD are based on a sandwich-type enzyme-linked immunosorbent assay (ELISA) that require only a single-step application of the sample solution. Colour development on the detection zone can be observed by naked-eye which is varied by the concentration of analyte present in the system. The pattern of PAD can be specially designed and fabricated using a wax-printing technique for automating one-step, delayed or the sequential multistep procedures, where all the reagents are applied at different locations in order to control the fluid travel to the detection region [28–30] (Fig. 9A and B). The capability of the method developed was successfully used to determine

Fig. 7 (A) Fabrication procedure of paper-based electrochemical device for human IFN-γ detection combining with origami folding sequence and (B) The design and measurement of the origami paper-based analytical device (oPAD). Adapted from the respective references N. Ruecha, et al., Label-free paper-based electrochemical impedance immuno-sensor for human interferon gamma detection, Sens. Act. B Chem. 279 (2019) 298–304; S. Boonkaew, et al., An origami paper-based electrochemical immunoassay for the C-reactive protein using a screen-printed carbon electrode modified with graphene and gold nanoparticles, Microchim Acta 186 (3) (2019) 153.
Fig. 8 The fabrication process for the paper-based electrodes (A) and the configuration of the label-free electrochemical impedance system set-up (B). Adapted from the respective Refs Y. Boonyasit, O. Chailapakul, W. Laiwattanapaisal, A folding affinity paper-based electrochemical impedance device for cardiovascular risk assessment, Biosens Bioelectron 130 (2019) 389-396; Y. Boonyasit, O. Chailapakul, W. Laiwattanapaisal, A multiplexed three-dimensional paper-based electrochemical impedance device for simultaneous label-free affinity sensing of total and glycated haemoglobin: the potential of using a specific single-frequency value for analysis, Anal Chim Acta 936 (2016) 1–11.
the levels of biological compounds such as human chorionic gonadotropin (hCG), a model mouse IgG and alpha-fetoprotein (AFP). These PADs demonstrate a user-friendly, easy and quick method for the fabrication of the device, which could be used as a one-step, portable, disposable, low-cost, simple, instrument-free and point-of-care device for the automated immunoassay.

Another work of an immunoassay using colorimetric detection was introduced in 2018 by Songjaroen et al. [31]. This work patterned a cellulose paper to be used as blood plasma separation from whole blood. The device can separate plasma from whole blood and quantify plasma proteins in a single step. The µPAD was fabricated using the wax dipping method (Fig. 10).
The device comprised of a blood separation membrane combined with patterned Whatman No. 1 paper. For blood separation, the blood cells (both red and white) were trapped on blood separation membrane allowing pure plasma to flow to the detection zone by capillary force. The developed device can effectively separate blood cells from plasma within 2 min when blood volumes of between 15 and 22 mL were added to the device. The efficiency of blood separation on the μPAD was studied by plasma protein detection using the bromocresol green (BCG) colorimetric assay. This proposed blood separation on μPAD has the potential for reducing turnaround time, sample volume, sample preparation and detection processes for clinical diagnosis and point-of-care testing.

4. DNA sensor

Nucleic acids, such as oligonucleotides or DNA, are one of the diagnostic indicators which have been extensively used to provide the disease
states. In order to improve the DNA based sensor to be an alternative sensor designed for point-of-care (POC) application, it has been quickly integrated into paper-based analytical devices (PADs) providing a low cost, simple and rapid diagnostic platform especially for developing countries and resource-limited areas. DNA based sensor generally relied on short oligonucleotides designed as a probe for specific hybridization with complementary DNA/RNA. Li et al. [32] used DNA functionalized with magnetic microbeads (MμBs) as a probe for detecting Hepatitis B virus (HBV) (Fig. 11). A slip origami paper-based device (oSlip) based on AgNPs modified electrode was used to carry out Ag⁺ resulting from the rapid oxidation of the AgNPs labels and yielded the detection limit reached to pM level.

The suitable probe is a key parameter for improving selectivity, sensitivity, accuracy, and reproducibility. Although most DNA biosensors employ oligonucleotide as a probe, other alternative probes have been widely used with great success. Peptide nucleic acid (PNA) [33,34], a charge-neutral synthetic DNA mimic which consists of a peptide-like backbone of repeating N-(2-aminoethyl)-glycine units replacing the sugar-phosphate, has been adopted as a biomolecular probe in term of DNA sensing. Owing to its excellent characteristics including resistance to nuclease and protease enzyme and sequence-specific with strong binding to DNA/RNA, PNA has been increasingly applied in bio-sensing with variety methods [35–37]. Nowadays, Vilaivan’s group proposed a new conformationally pyrrolidinyl PNA system (known as acpcPNA) which possesses an α,β-peptide backbone deriving from d-proline/2-aminocyclopentanecarboxylic acid [38]. Compared to Nielsen’s PNA, acpcPNA exhibits the characteristic properties.

Fig. 11 A slip origami paper-based device (oSlip) based on AgNPs modified electrode for HBV detection. Reprinted with permission from Ref. X. Li, K. Scida, R.M. Crooks, Detection of Hepatitis B virus DNA with a paper electrochemical sensor, Anal. Chem. 87 (17) (2015) 9009–9015. Copyright (2015) American Chemical Society.
of antiparallel binding to the target and low tendency to self-hybridize making a stronger affinity and higher sequence specificity binding to DNA. Various PADs based on acpcPNA applications have been reported to date incorporating electrochemical and colorimetric detection.

4.1 Electrochemical detection

Electrochemical DNA sensors have attracted increasing attention as they provide a simple, low-cost and rapid way to achieve high sensitivity. Teengam et al. [39] developed a paper-based electrochemical DNA biosensor using the acpcPNA probe labelled with anthroquinone (AQ) for detecting high-risk human papillomavirus (HPV) type 16 (Fig. 12). Apart from the covalent process, acpcPNA was immobilized onto graphene-polyaniline (G-PANI) modified electrode through electrostatic interaction for eliminating the complicated steps. The electrochemical signal of labelling AQ can be differentiated before and after the addition of complementary DNA and the decreased signal is linearly proportional to the concentration of DNA target.

Although most electrochemical DNA sensors employed electroactive labels or indicators to generate a sensitive signal, a label-free assay would be preferable for reducing time of complicate labelling processes. Teengam et al. [40] reported a label-free electrochemical PAD (ePAD) using acpcPNA as a probe designed for *Mycobacterium tuberculosis* (MTB) detection (Fig. 13). Unlike previous work, the acpcPNA probe was covalently

![Fig. 12 Square-wave voltammograms of immobilized AQ-PNA probe on G-PANI/SPCE before and after hybridization with an equimolar concentration of target DNA. Reprinted with permission from Ref. P. Teengam, et al., Electrochemical paper-based peptide nucleic acid biosensor for detecting human papillomavirus, Anal. Chim. Acta 952 (2017) 32–40.](image)
immobilized onto partially oxidized cellulose paper. The concentration of DNA target was quantified by monitoring the change in the charge transfer resistance ($R_{ct}$) obtained from electrochemical impedance spectroscopy (EIS) before and after the formation of acpcPNA-DNA duplexes. The label-free system is advantageous over the use of electroactive indicators or labels since it eliminates the complicated and time-consuming steps for labelling.

4.2 Colorimetric detection

Colorimetric assays combined with PADs are also appropriate alternative detection mode for DNA sensing. Teengam et al. [41] proposed a colorimetric assay for simultaneous screening of middle-east respiratory syndrome coronavirus (MERS-CoV), MTB and HPV using multiplex colorimetric PADs (Fig. 14). The acpcPNA baring positive charged of lysine at C-terminus was designed as a probe to induce the aggregation of citrate anion-stabilized silver nanoparticles (AgNPs). In the presence of the DNA target, the AgNPs can be dispersed due to the charge repulsion of anionic PNA-DNA duplexes resulting in a detectable colour change. While the semi-quantitative results can be obtained by visually observing the colour change, quantitative analysis
Fig. 14 (A) Design, (B) operation of multiplex paper-based colorimetric device, and (C) the process of acpcPNA-induced AgNP aggregation in the presence of DNA_{com} and DNA_{nc}. Reprinted with permission from Ref. P. Teengam, et al., Multiplex paper-based colorimetric DNA sensor using pyrrolidinyl peptide nucleic acid-induced AgNPs aggregation for detecting MERS-CoV, MTB, and HPV oligonucleotides, Anal. Chem. 89 (10) (2017) 5428–5435. Copyright (2017) American Chemical Society.
can be accomplished by using scanners together with image processing software to carry out colour intensity which is correlated with the DNA target concentration.

5. Other applications

A broad number of chemical substances detection related to biomarkers or physical condition indicators are also demonstrated using a paper-based platform. Various sensing schemes ranging from simple to advanced structures have been designed for a specific purpose. Indeed, most recent paper devices are attempted to integrate complexed functionality such as the ability to perform multiple steps or automated processing into PADs for better performance. As a result, these highly developed PADs could open up new possibilities as alternative tools to conventional analytical methods.

5.1 Electrochemical detection

Many electrochemical PADs (ePADs) have been developed for biological compounds detection. As stated in a previous section, several biosensors are incorporated with the use of biological recognition elements. An enzyme, for instance, is so far used in conventional biosensors. Even though this enzymatic method has been well-established and reliable, the reliance of the fragile enzyme component often resulted in poor storage stability which in turn leading to enzyme inactivation. Therefore, several attempts have been put to use biomimetic materials that can catalyse the direct electrochemical reaction of the analytes of interest. For example, as reported by Boobphahom et al. [42] enzymatic detection of creatinine was recently developed using the paper-based device. The nanocomposite of copper oxide-ionic liquid/reduced graphene oxide (CuO/IL/ERGO) was directly printed onto a screen-printed carbon electrode on PADs using a digital dispensing machine. The soluble copper-creatinine complex is formed during applying the oxidative potential and thus can be detected. Sensitivity and reproducibility towards creatinine detection are greatly improved and enable for detecting creatinine in real acute kidney injury diagnosis.

Compared to other electrode modifications on PADs (drop-casting, electro-spinning, electrospaying and spin coating), the utilization of a digital dispense is demonstrated to be an effective modification method on PADs due to the precise controlled dispensing of the nanocomposites. Likewise, a nonenzymatic method for glucose detection is also of interested.
Glucose, one of the most vital clinical indicators for the diagnosis of diabetes has been reported in several ePADs formats. Glucose oxidase (GOx) is a well-known enzyme for the enzymatic reaction with glucose which produces an electroactive H₂O₂ byproduct. To avoid a short shelf life of enzyme, several reports have been published GOx-like activity materials to alleviate the common problem from enzymatic methods. In 2018, Chaiyo et al. [43] have reported a disposable paper-based sensor using a cobalt phthalocyanine–ionic liquid–graphene (CoPc/G/IL) composite for enzyme-free glucose detection. The results clearly exhibited a superb electrocatalytic activity from the composite towards glucose oxidation to gluconolactone. Not only excellent sensitivity and selectivity but also the advantage in terms of cost and sample-saving obtained from their developed PADs could favour future progress in point-of-care (POC) testing device.

Recently, the fabrication of multiple layers of paper to form 3D μPAD has gained much interest for multiplexed analysis. Using 3D μPAD, more device functionality can be simply applied. Most of the μPAD designs to date have emphasized the use of folding (origami) or stacking paper layers techniques to facilitate the preconcentration, sample manipulation or timed reaction depending on each device design.

Typically, almost all of the chemical assay involves multiple steps and reagents. Therefore, several reports have proposed a paper-based microfluidic device that enables to perform multiple steps/reagents within one single device. For example, Pinyorospathum et al. [44] have reported a label-free paper-based sensor for C-reactive protein (CRP) detection. Although CRP sensors have been widely reported using standard immunoassays, these methods demand the use of expensive antibodies. To overcome this challenge, a selective biomimetic polymer coupled with a folding origami paper was therefore reported. In this report, thiol-terminated poly (2-methacryloyloxyethyl phosphorylcholine (PMPC-SH) containing a mercapto group was used as a recognition probe for trace sensing of CRP. PMPC can be self-assembled onto the electrodeposited gold modified screen-printed electrode through the strong interaction between sulphur and gold affinity. It was also reported that the binding of CRP is dependent on the local concentration of Ca²⁺ and pH of the environment. If the CPR is contacted with Ca²⁺ and further bound with PMPC, the electrochemical signal (using [Fe(CN)₆]³⁻/⁴⁻ as a redox probe) decreases as the CRP-PMPC complex can reduce the accessibility for the redox probe. Using this multiple-step detection, the paper-based device was constructed to simplify the overall procedure into one single device. There are three main zones for
PADs; sample incubation zone (green), electrode zone (black), and analysis zone (purple). The sample incubation zone is where the sample was dropped to incubate with Ca\(^{2+}\) while the analysis zone is where the \([\text{Fe(CN)}_6]^{3-/4-}\) redox probe was dropped for electrochemical analysis step. The device operation consists of 3 steps (Fig. 15). Firstly, the green component was flipped to the middle, and the sample was introduced and left for incubation. Secondly, after the reaction was completed, the green flap was then removed. Then, the middle electrode zone was washed twice to remove the excess. Next, the purple component was folded to the electrode zone and the solution of KNO\(_3\) was introduced to elute the solution of \([\text{Fe(CN)}_6]^{3-/4-}\) to the electrode surface. At this step, the current was monitored by the DPV technique. The LOD was calculated and reported at 1.55 ng\(\text{mL}^{-1}\) with a wide linear dynamic range of 5–5000 ng\(\text{mL}^{-1}\).

Standardly, carbon electrodes have been enormously employed in ePAD format due to their simple fabrication and robustness. However, this kind of electrodes is likely to suffer from the surface fouling effect which in turn results in poor electrode lifetime and sensitivity. On the one hand, boron doped-diamond electrode (BDD); a carbon-based p-type semiconductor electrode is widely known for its high resistance for electrode fouling. Despite its outstanding performance, the currently available forms of BDD electrode are not fully compatible with ePAD platform in terms of device geometry and fabrication process. Until 2017, Nantaphol et al. proposed a BDD powder paste electrode for \(\mu\)PAD [45]. As a demonstration, norepinephrine (NE) and serotonin (5-HT); two neurotransmitters were chosen as model analytes. Previously, 5-HT has been reported to be irreversibly adsorbed on the carbon paste electrode (CPE), making the sensitivity of electrode decreased. For the fabrication of BDD paste electrode, BDD powder was mixed with mineral oil and then filled into a transparency sheet with three channels (the channel was engraved with a laser engraving machine). Then, the double-sided adhesive tape was used to attach the with PADs containing screen-printed reference electrode (RE) and counter electrode (CE) to the as-prepared BDD paste working electrode (WE) as depicted in Fig. 16. As expected, the less severe fouling effect was observed using BDD paste ePAD compared to CPE. Furthermore, electrode pretreatment was also performed to improve resistance to electrode fouling. DPV measurement was further employed for the simultaneous determination of NE and 5-HT. The experimental LODs for NE and 5-HT were found to be 2.5 and 0.5 \(\mu\)M, respectively. This BDD paste electrode was also extended to heavy metals detection (Cd(II) and Pb(II)). Coupled with \(\mu\)PAD,
Fig. 15 Preparation of PADs for CRP detection using differential pulse voltammetry technique. Reprinted with permission from Ref. C. Pinyorospathum, et al., Disposable paper-based electrochemical sensor using thiol-terminated poly(2-methacryloyloxyethyl phosphorylcholine) for the label-free detection of C-reactive protein, Microchim Acta 186 (7) (2019) 472.
this BDD paste ePAD can enhance the mass transport owing to the convection through PAD, thereby enhancing the sensitivity for the metal accumulation. In 2019, Nantaphol et al. also broaden the use of BDD paste electrode for simultaneous detection of NE and 5-HT at different solution conditions within single μPAD [46]. They refer this ePAD system to the Janus, the two-faced Greek god, because of the ability to perform dual electrochemistry at the same time. Since the electroactivity of NE and 5-HT is pH-dependent, performing assay using multiple pH at each compound (NE and 5-HT) optimal conditions would provide the highest detection sensitivity. Overall device fabrication and operation are presented in Fig. 16. Using the optimized conditions, the LODs for NE (pH 6) and 5-HT

![Fig. 16 Schematic for BDDPE fabrication and devices assembly for simultaneous electrochemical detection of serotonin and norepinephrine. Adapted with permission from S. Nantaphol, et al., Boron doped diamond paste electrodes for microfluidic paper-based analytical devices, Anal. Chem. 89 (7) (2017) 4100–4107. Copyright (2017) American Chemical Society. Reprinted with permission from Ref. S. Nantaphol, et al., Janus electrochemistry: simultaneous electrochemical detection at multiple working conditions in a paper-based analytical device, Anal. Chim. Acta 1056 (2019) 88–95.](image-url)
(pH 8) were calculated to be 0.71 and 0.38 μM, respectively. Clearly, with the ability to pattern paper with multiple fluidic channels and store dried reagents in specific zones for condition adjustment, multiple sets of the experiment at differing conditions can be obtained simultaneously. The Janus-e-PAD can reduce the complexity and time of analysis while maintaining the highest degree of sensitivity for each species. This prototype can also be extended to another multiplexed detection (such as enzymatic methods) where analytes require different conditions such as pH, solvent, buffer type, or ionic strength.

In some instances, the electrochemical measurement for the analytes of interest involves multiple steps (reagent introduction step, electrodeposition step, washing step, sample introduction step, and measurement step). It is inevitable for users to deliver reagents/samples as a sequence. As the simplest demonstration, Yager and co-workers have reported a two-dimensional paper-network (2DPN) with multiple inlet legs having different lengths. To start the experiment, this 2DPN was inserted in a shared buffer. Dried colorimetric agents spotted in the inlet legs will sequentially transport to the detection zone in a time sequence. Although this platform could enable sequential fluid delivery, this platform cannot be extended to other reactions since the timing event is programmed by the shape/length of inlet legs. For a better fluid controlling, Yakoh et al. have demonstrated the 3D sequential microfluidic paper-based analytical device (sePAD) [25]. Their developed platforms include two configurations (Fig. 17); flow-through configuration

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Fig. 17 Schematic illustration of the sePAD components and device assembly for electrochemical detection of ascorbic acid and serotonin. Reprinted with permission from Ref. A. Yakoh, et al., 3D capillary-driven paper-based sequential microfluidic device for electrochemical sensing applications, ACS Sens. 4 (5) (2019) 1211–1221. Copyright (2019) American Chemical Society.
for a flow-based electrochemical measurement (such as chronoamperometry) and stopped-flow configuration for a nonconvective electrochemical measurement (such as voltammetry techniques). Basically, the sePAD device contains two paper components; origami paper device (oPAD), and array of movable reagent pad (rPAD). Using this sePAD, it allows reagents to be stored in movable rPAD and transferred sequentially to oPAD for electrochemical measurement. The device operation involves three simple steps: (i) sample spotting and device assembly, (ii) loading buffer to the inlet reservoir, and (iii) pulling the rPAD to introduce reagents or switch off fluid flow. As proof of concept, two biological relevant species (ascorbic acid (AA) and serotonin (5-HT)) were demonstrated. For AA detection, a chronoamperometry was performed using flow-through sePAD configuration. A series of different AA concentrations were sequentially spotted in an rPAD and left to dry. Once assembled, a set of AA working solution was eluted along the channel to the detection zone with only a single manipulation of running buffer. Comparing this sePAD to a static ePAD, this platform only requires a very small sample volume (1 μL) and a self-calibration plot plus sample analysis can be done with just one drop of loading buffer. Another example of the sePAD is the determination of 5-HT using stopped-flow configuration. Typically, the sensitivity for 5-HT detection using a carbon-based electrode is not sufficient to detect. Therefore, several works are prone to deposit the electrode modifying agent such as gold nanoparticles (AuNPs) to enhance the electron transfer capability. However, an electrochemical modification of this metal cannot be directly accomplished on a normal static ePAD as a result of the excess gold residue inside the porous network of paper. To overcome this situation, μPAD was introduced to the system since unbound components can be easily cleaned off following each step. Furthermore, it has been reported that the use of μPAD can improve the sensitivity for the metal accumulation as a result from the increased convective mass transport. Nevertheless, it is important to remark that the contributions of convection during the analysis step are particularly excluded from the electrochemical experiment (such as voltammetry). Thus, the mass transfer is only limited to the contribution of diffusion. Accordingly, a stopped-flow sePAD were elaborately engineered to eliminate the perturbation from the convective mass transport. Using this device arrangement, the movable rPAD can be pulled to a position where a wax barrier (between each hole of rPAD) was vertically aligned to the inlet reservoir in order to stop the fluid. Unlike other electrochemical μPADs where the user is required to cut off the device once the solution passes through the detection zone, or rest for the whole channel
saturation before measurement, the user can manually control the fluid flow without device destruction. In a word, a stopped-flow format employed in the μPAD is allowed to perform both modification step in convectional condition and electrochemical measurement in a quiet solution.

5.2 Colorimetric detection

Colorimetric detection of chemical substances is another promising method for PADs. Due to its simple operation and straightforward interpretation, many colorimetric sensing PADs have been developed until now. As the simplest demonstration, a colorimetric PADs for rapid determination of albumin (AL) to creatinine (CR) ratio in urine samples was presented (Fig. 18A) [47]. AL to CR ratio (ACR) has been used to screen diabetes, which can further refer to kidney disease. In this developed platform, bromocresol green (BG) was used to evaluate the total level of AL and CR, resulting in a colour change from greenish-yellow to bluish-green while Jaffé picric acid (PA) was used to selectively identify the CR level, leading to a colour change from yellow to orange on PADs. Distinguished colour changed can be clearly observed by naked eyes or image processing can be utilized for further semi-quantitative analysis. Using this simple and rapid PADs, it allows the user/patient to estimate the ACR in a urine sample which would be helpful for early diagnosis in POC testing.

The advantages of the colorimetric PADs can also be extended to the microfluidic system as a two-dimensional (2D) or three-dimensional (3D) device. For example, Dungchai et al. [48] have published two methods for the determination of oxidative activity induced by airborne particulate matter (PM) using silver nanoparticles (AgNPs). These two methods are based on the measuring of colour intensity (3D conventional μPAD) and colour length developed along the flow line (2D distance-based device). In this system, glutathione (GSH) was used to study the chemical oxidation induced by PM. The presence of reactive species associated with PM will oxidize the reduced form of GSH to its disulphide whereby the remaining reduced GSH will react with AgNPs and produced a reddish-brown product. The conventional μPAD consisted of three layers: a top layer for sample loading, a middle layer as a flow valve, and a bottom layer containing AgNPs for detection. The colour change was monitored using ImageJ software. For the distanced-based device, a microfluidic flow circuit, which resembles a thermometer, with a series of horizontal baffles was designed. Along the detection channel, AgNPs were spotted. The baffles were patterned to
reduce the flow velocity and increase the reaction time between GSH and AgNPs. The colour will develop along the flow path until all of GSH is consumed. The contification can be further related to the colour length. Overall device operations are presented in Fig. 18. Using 1,4 naphthoquinone

Fig. 18 Schematic illustration (A) colorimetric PADs for detection of albumin to creatinine ratio and (B) μPAD for determination of oxidative activity using Silver nanoparticles. Reproduced from Ref. S. Chaiyo, et al., A novel paper-based colorimetry device for the determination of the albumin to creatinine ratio, Analyst 143 (22) (2018) 5453–5460; W. Dungchail, et al., Determination of aerosol oxidative activity using silver nanoparticle aggregation on paper-based analytical devices, Analyst 138 (22) (2013) 6766–6773 with permission from the Royal Society of Chemistry.
(1,4-NQ) as a model oxidant species for GSH, the LODs at fixed GSH concentration (0.5 nmol) for the conventional μPAD and the distance-based device were found at 3.7 and 20 ng, respectively. The conventional μPAD exhibited a lower detection limit than distance-based device. However, the quantification for the distance-based device can be easily read by the colour length without the need for a complicated scanner or camera for data interpretation. Both methods demonstrate the potential applicability in real applications.

6. Conclusion

This chapter describes different methods for biomarkers determination based on electrochemical and colorimetric techniques. The selectivity of the technique derived from the choice of the biological probe for a certain analyte. The detection methods can be divided mainly into the enzymatic method, immunoassay, and DNA sensor. For the enzymatic method, the biological enzyme is used for converting a specific target into another product which is responsible for the change of the analytical signal. The specificity of immunoassay depends on the reaction between the biological recognition element (i.e. antibody, aptamer, synthesized probe) and biological compounds. The signal changes such as resistance and current can be related to both the presence of the biological targets and their quantity. Similarly, the selectivity of DNA sensor obtains from the specific hybridization between DNA targets and their corresponding complementary DNA/RNA which uses as a bio/synthetic molecular probe. Short oligonucleotide DNA/RNA usually serves as the probe, but a biomimetic probe namely PNA has recently received its attention as well. Moreover, there are other methods such as using the polymer for the recognition site of the protein and using metallic nanoparticles for nonenzymatic assay of glucose which suitable for the determination of biological molecules. Paper is a flexible object that can be folded into different patterns and easily controlled the flow of liquid by creating the hydrophobic/hydrophobia channel. Thus, paper is an excellent material for device fabrication. Furthermore, simple techniques (electrochemistry and colorimetry) can be miniaturized onto paper which is very convenient for the applications of biomarker detection. PADs is also cost-effective, portable, immediately disposable and excellent for point-of-care diagnostic devices that will predominantly corporate in the future of analytical detection.
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