



Review

Electrochemical paper-based biosensors for point-of-care diagnostics: Detection methods and applications

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Abstract

Personalized health care (PHC) includes personalized, preventive, predictive and participatory approaches that are significant in new diagnostics. This personal health care requires fast, accurate and minimally invasive diagnostic tools that make it possible to evaluate and monitor the process of disease by diagnosing specific disease biomarkers. Point-of-care testing (POCT) involves a wide range of diagnostic tools that meet this purpose. Electrochemical paper-based devices (ePADs) have been introduced as simple, inexpensive, portable and disposable measurement devices to be used in many POCT applications, especially in handling emergencies and outpatient as well as remote usages. Electrochemical detection is a real quantitative detection method with better sensitivity, selectivity and detection limits than indirect measurement methods. In recent years, there has been a revolution in quantitative detections by POCT, thanks to the benefits of electrochemical sensors and paper substrates. In this paper, recent developments in ePADs, focusing on the properties of paper, reasons for its use in the devices, techniques of device and electrode fabrications, and their application particularly in clinical diagnosis, are reviewed.

Keywords

Clinical analysis; electrochemical detection; electrochemical paper-based device, medical diagnosis; point-of-care (POC)

Introduction

Although mortality has declined worldwide over the past century, it has been accompanied by a dramatic shift from communicable to non-communicable diseases such as cancer and diabetes and cardiovascular, autoimmune, and respiratory diseases. More than 70 % (41 million) of deaths worldwide are due to non-communicable diseases [1]. Also, based on the report of the World Health Organization, about one billion people worldwide do not receive health care services [2]. To minimize these deaths, the leading agencies, policymakers and especially the United Nations have enacted a number of plans to improve public health [3]. Among the measures taken to foster healthcare systems, one may refer to providing diagnostic kits, services thereafter, and affordable

health care [4]. So, primary diagnosis and personalized treatment in clinics play important roles in controlling diseases and enhancing the survival of patients, especially in retarded areas and in developing countries where patients have limited access to laboratory tests [5]. The need for the reduction of costs as well as accurate, rapid and minimally invasive diagnostic techniques has led to the advancement of point-of-care (POCT) testing [6]. In this regard, certain attempts have been made, such as presenting the World Health Organization guidelines known as the ASSURED guidelines, which postulate Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free and Deliverable services to end-users [7–9]. Patient status tracking may also be performed with POC devices during recovery. For different sensors, especially healthcare sensors, electrochemical paper-based analytical devices (ePADs) can be useful. Through these devices, electrochemical detection occurs with a paper substrate. Among the many benefits of ePADs, the paper applied is low-cost, easily accessible, flexible, eco-friendly, biocompatible and lightweight. It also has a soft matrix with many capillaries that facilitate fluids' self-pumping when they come into direct contact. Additionally, the surface of the paper can be modified with nanostructured materials to increase the detection sensitivity.

Electrochemical methods are increasingly utilized in the development and design of ePADs. This is owing to their advantages over indirect measurement techniques such as the colorimetric technique; they are capable of quantitative detection with better selectivity, sensitivity and detection limits. Electrochemical detection is also advantageous in terms of sensor fabrication cost, power consumption and capacity for miniaturization. In combination with a paper substrate and a compact sensor, an electrochemical detector can serve as an accessible healthcare product to be typically used in loco analysis and POC evaluations [10]. Among the benefits of miniaturization, one may refer to the reduction of the required sample volume, electrode surface saturation due to the high surface-to-volume ratio of the paper-fiber matrix detection region, and the portability of the device [11,12].

Various electrochemical techniques such as potentiometry, voltammetry, conductometry, coulometry, polarography and amperometry can be used to detect electrochemical signals on ePADs. The use of paper substrates in electrochemical detections eliminates the disadvantage of the non-portability of potential acetate devices [11]. An example is one of the first and probably the most widely used POC electrochemical sensors for glucose detection [13–15].

This paper provides a review of some recently developed electrochemical, paper-based devices (ePADs) for the diagnosis of diseases. It also briefly accounts for electrode and device fabrication techniques and the use of paper in detecting devices.

Strategies for choosing a paper substrate

A major advantage of using paper is the reduction of costs, particularly in developing countries that have limited resources. Paper is amenable to different techniques due to its special features. The white color of the paper makes it suitable for use in colorimetric, fluorimetric, and chemiluminescent techniques, or any other assays that can be recognized by the naked eye [16,17] or in smartphone-assisted measurements [18–20]. The high availability and variety of paper, as well as its excellent mechanical properties like lightness, specific stiffness, flexibility and ease of use, are other reasons for using paper as a stable substrate for biosensors [21,22]. In addition, paper is biodegradable and quick for disposal (*e.g.*, by incineration), which provides a good opportunity for creating disposable biological biosensors [23–25]. In addition, ease of transportation, wide availability and storage, portability and ease of patterning through the printing technology make it possible to develop an inexpensive and portable biosensing device for various applications [26–29].

Among the advantages of paper, some can be highlighted for electrochemical biosensing applications, on which we are focused in this article. For example, the intrinsic properties of paper, *e.g.*, the fibrous and porous 3D structure of cellulose fibers, can lead to: a) higher absorption for effective storage (*e.g.*, in antibody, aptamer or enzyme modifications) and delivery of samples which can lead to the increased sensitivity, faster response time and accuracy of time-dependent measurements [30–32]; b) elimination of air bubbles, which is beneficial in sensors especially in microfluidics [21]; c) large specific surface area to enhance the number of immobilized biomolecules [30,33,34]; d) elimination of a need for pumps and electrical power to transport fluids owing to its numerous capillaries [4,21,23,35,36]; e) better electrochemical detection by use of conductive inks easily deposited on paper [37], and f) detection with just very low volumes of samples [37].

Another notable benefit of paper to use in ePAD devices is that it helps to develop origami-like systems. For this purpose, various layers of paper are assembled by folding or stacking to achieve a controlled interaction *via* 3D structure [25]. Obviously, the working electrode is printed on one side of the paper device, and the reference and counter electrodes are printed on the other side. The working electrode can, thus, be modified conveniently without influencing the other electrodes [38,39]. Integrating origami into paper-based diagnostic sensors allows more flexibility and freedom in design and offers such advantages as specific and high sensitivity, fewer operation steps, simplicity of the device operations and, therefore, a decrease of the assay time. Other advantages include good registration and repeatability, controlled test time, multistep processes, reduction of the total amount of the reagent (antibody, enzyme, another substrates) required and, thus, the reduction of the cost, ability to interface with available hand-held devices (*e.g.*, commercial glucometers), conduction of lateral flow assays, good flow control within 3D ePADs for multiplex assays [36]. Therefore, signal amplification reactions are controlled, and target propagation problems are reduced by a lateral current in the channels of the flat paper system. Thus, reagent incongruity is prevented [40], and an extremely homogeneous distribution encompasses all the surfaces of the paper reaction areas [40]. Although, three-dimensional electrochemical paper-based analytical devices (3D-ePADs) possess these advantages, 2D-ePADs are still widely used [15,41,42].

Although the paper makes great platform substrates for electrochemical paper-based devices, it has certain inherent limitations. For example, it cannot be used for quantifying analyses and programmable steps due to the continuous flow that occurs through inactive capillary wicking. Of course, researchers have recently proposed an active paper-based microfluidic device to overcome this limitation [43]. Thick and highly porous papers, such as filter papers, are also problematic. Depending on the fabrication technique, the dielectric paste used to pattern hydrophobic walls cannot keep the capillary wicking of solutions from hydrophilic regions [44]. Also, the ePAD canals formed inside the paper are open at the top and the bottom, increasing external risks such as pollution, leakage of fluids to any surface in contact with the canals and their evaporation. Because the rate of solvent evaporation depends on the relative humidity, the capillary flow into the paper channels can be modified [45]. Fortunately, different kinds of paper are available with various properties that can be selected according to the analytical requirements, the field of utilization, and the electrochemical paper-based device fabrication techniques [46]. Filter, chromatographic and office papers are the most widely utilized substrates for ePADs. Among them, Whatman #1 paper is the most extensively used in laboratories, probably due to its availability, excellent wicking ability and absorption of more aqueous solution by a dry filter paper [34,47]. For more information, one can refer to the recent review research reported by Desmet *et al.* with detailed information on the diffusion coefficient values of analytes with ePADs made of different types of paper, especially

Whatman paper, a comparison of the reported values in solution, the amount of water absorbency by the paper, and the electrochemical techniques used to determine these values [35].

Figure 1 shows some examples of origami paper-based devices [25,48-50].

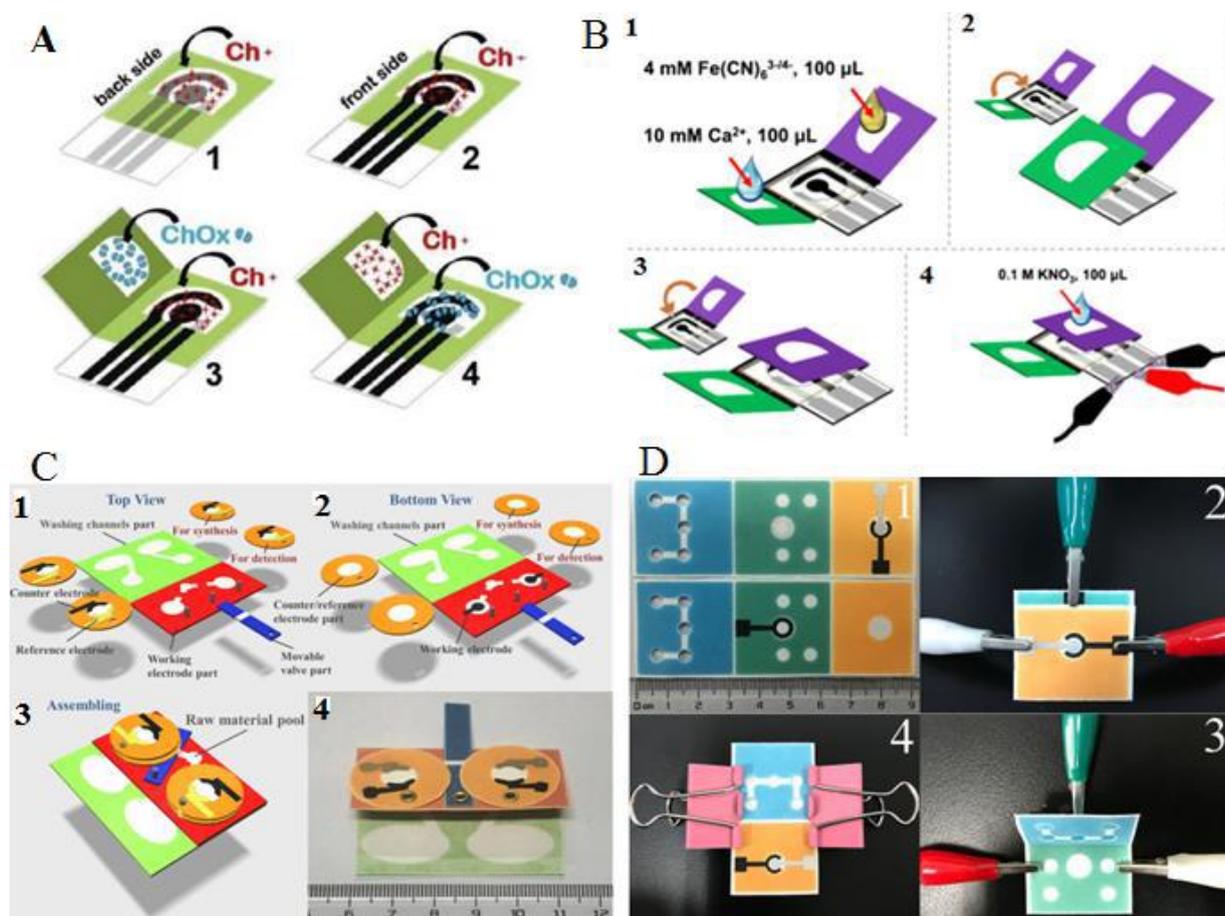


Figure 1. Examples of origami paper-based devices. A) Comparison of one-layer ePADs and origami-ePADs [25] - Reprinted with permission copyright (2019), Elsevier; B) Schematic illustration of the origami-ePADs and preparation of ePADs [48] - Reprinted with permission copyright (2019), Springer-Nature; C) Schematic illustration of design and details of an origami paper-based analytical device (oPAD) [49] - Reprinted with permission copyright (2019), Elsevier; D) Schematic design of device and size, and shape of the lab-on-paper device [50]; Reprinted with permission copyright (2018), Elsevier

Fabrication techniques

Fabrication of electrochemical paper-based devices

Since the production of the first paper-based device attributed to Müller and Clegg [47] and the production of paper-based microfluidics by the Whitesides group, which was the first device for determination of glucose created through a photolithography technique [51], there have been a lot of developments in fabrication techniques and paper-based applications [52]. Generally, the fundamental techniques of PADs fabrication are photolithography [53], wax printing [54,55], inkjet [56], screen printing [57], laser treatment [58], and wet etching [59]. Of course, the fabrication techniques of PADs are similar to those of microfluidic PADs; they are based on the creation of hydrophilic zones for electrochemical detection with hydrophobic borders on paper to confine the flow of fluid inside the desired position. In this regard, hydrophilic and hydrophobic zones can differ based on the volume of the needed solution.

In this section, the most common kinds of PAD and electrode fabrication methods are introduced. According to the literature, wax printing is a very common technique. Wax-based fabrication techniques are high-speed, inexpensive, facile and non-toxic. Generally, the process involves two steps; first, wax is rubbed on the paper through a screen on it, and then the wax melts into the paper through a hot plate to form hydrophobic barriers to the paper [60,61]. Martins *et al.* [10] produced the first wax-printed paper-based electrochemical device for the detection of an oxidative stress biomarker (3-nitrotyrosine, or 3-NT). To build a three-electrode system on paper, they used carbon and silver conductive inks. Sun *et al.* [54] used an industrial solid-wax printer (XEROX Phaser 8560DN, Japan) for the manufacture of a paper-based device. The technique of photolithography, which has been commonly used in the expansion of paper-based devices, is based on hydrophobization, followed by selective paper dehydrophobization. Photolithography has a high resolution and good reproducibility, but it is disadvantageous due to the use of chemical solvents and expensive equipment [62,63]. Kaur *et al.* [53] expanded a microfluidic paper-based cholesterol biosensor. They utilized a patterning filter paper (Whatman#1) to fabricate microfluidic channels by photolithography. A hydrophobic barrier was formed on the filter paper by SU 8 photo-resistant microchannels with the dimensions of 1000 μm (wide) \times 100 μm (thick).

Electrode fabrication techniques

Electrode production is the most important issue of ePADS that determines how successful and efficient the devices are. There are various techniques to fabricate electrodes on a paper bed, *e.g.*, inkjet printing, screen printing, stencil printing, drawing with pencil/pen, sputtering and e-beam deposition [36,64–67] some of which will be explained here. The inkjet printing technique is easy to use, inexpensive and scalable for production. It also has a wide range of scientific and industrial applications, providing a carrier medium for a set of filler materials. Lately, carbon nanotubes (CNT) types of ink, *e.g.*, metal ink, have been applied in inkjet printing. Due to requiring no prefabrication of patterns or masks, the technique makes rapid and low-cost printing possible [68]. Two modes of printing are used in this technique, referred to as drop-on-demand printing and continuous inkjet printing. In continuous inkjet printing, a continuous stream of ink droplets is formed and then deflected by voltage plates. Depending on the applied voltage, the deposition of droplets onto the paper varies *via* the gutter. In drop-on-demand inkjet printing, the injection of ink droplets occurs by pulses from a nozzle. Thermal buckling, thermal resistors, acoustic waves and piezoelectric transducers can generate pulses [69,70]. The principles, applications and advancements of the inkjet printing technology have lately been reviewed by Kholghi Eshkalak *et al.* [71]. In a screen/stencil-printing system intended to obtain a desirable electrode on a paper substrate, the ink is pressed and diffused to the open regions of a screen/stencil. Of course, hydrophobic zones on paper can be created using this technique. A benefit of the screen/stencil printing technique is reliable repeatability, although there may be low resolution, imprecision and high ink waste because of low contact pressure during production [66,68–73]. In one of the reviewed studies [64], the electrodes were created by screen printing, and the test zone was designed through solid wax printing. The biosensor could perform human antigen diagnosis (immunodeficiency virus p24) in serum with a very low detection limit.

Clinical analysis

EPADs have been widely applied for a variety of purposes. Extending extremely specific and accurate point-of-care (POC) devices for diagnosis, environmental monitoring, early clinical essays

and treatment control are significant challenges in developing and developed countries [74,75]. Reduction of the cost and the quantity of the sample, on-site diagnosis and reduction of patient anxiety are the advantages of POC analytical platforms. Accordingly, interdisciplinary research can grow in this context [76,77]. The extremely large surface area of fiber-networks and the power-free fluid flow through a capillary force are the unique features of paper that have made it appropriate for multiple sensing applications, ranging from rather ordinary chemical sensing to specific detection with diverse diagnosis techniques [51,78]. In this regard, some examples of ePAD applications are mentioned here to indicate the ability of the device in various fields.

Cancer

The unique technique of medical detection is the analysis of biomarkers [79,80]. There are many biomarkers to use for diagnosing human and animal diseases [81]. Among them, cancer markers are widely analyzed to evaluate tumor outbreaks. It is of importance to diagnose and control diseases in their early stages [82,83]. Regarding cancers, however, a delay in the diagnosis often leads to the loss of the best opportunity for treatment. Therefore, it is essential to find ways of quick cancer marker sampling and analysis that are less expensive and easier to conduct as well [6,84]. Thus, sensitive, quick and precise identification of cancer cells or carcinogenic biomarkers is a significant part of the studies on ePAD development. Among various cancer tumors, the most malignant one posing the greatest risk to people is lung cancer. The identification of this cancer tumor in its early stages can improve the chance of survival [85]. In this case, the microfluidic paper-based electrochemical DNA biosensor (μ PEDB) presented by Tian *et al.* [84] can be of help. For the sensitive detection of epidermal growth factor receptor (EGFR) mutations in patients with non-small cell lung cancer, microfluidic paper-based analytical devices (μ PADs) are introduced as an alternative to POCT instruments. The attractive features of these modern devices for critical analysis are cost-effectiveness, simplicity of use, compactness, disposability, and low reagent and sample consumption [86,87]. In this study, a paper zone was modified by AuNP layer to create a large surface on a bare PWE and enhance its conductivity to serve as a working electrode, and then polypyrrole (PPy) was polymerized on the AuNP-PWE surface. The electrical resistance of the electrode was found to be $0.8 \mu\Omega$, suggesting a wonderful electrical conductivity. Next, through non-covalent interaction, the single-stranded DNA was adsorbed onto the modified gold electrode surface with the PPy membrane. Using the differential pulse voltammetry method, the interaction between methylene blue (MB) and H_2O_2 was catalyzed by horseradish peroxidase (HRP). This biosensor showed a very low limit of detection (LOD) of 0.167 nM . Because of the importance of diagnosing the disease, another research group designed an advanced wireless POCT system for the detection of neuron-specific enolase. This system consisted of μ PADs, an electrochemical detector, and an Android smartphone. Moreover, for the diagnosis of small-cell lung cancer, neuron-specific enolase (NSE) was of clinical significance. There was a high demand for the point-of-care diagnosis of NSE. For the surface modification of μ PADs, amino-functional graphene, thionine and gold nanoparticles (NH₂-G/Thi/AuNPs) nanocomposites were used. The quality of the POCT system was checked, and the results were stored in the EEPROM memory; they could be demonstrated in real time using mobile Bluetooth [88].

In another study, a gold nanoparticle electrode was used to construct an electrochemical immunosensor for the ultrasensitive diagnosis of the carcinoembryonic antigen (CEA) [89]. The counter and the working electrode were printed using gold nanoparticle ink, while industrial silver ink was used to produce the reference electrode. The hydrophobic channels were also impregnated

on the paper substrates using wax printing. In this study, a mercapto-amine functionalized receptor was developed on a paper-based screen-printed gold (Au) electrode (P-SPGE) for selective CEA sensing and a self-assembled monolayer (SAM) layer was used to covalently bind the active amine groups of the functionalized mercapto-amine receptors to the anti-CEA carboxylic groups.

Differential pulse voltammetry (DPV) was used to quantitatively evaluate the CEA levels, and the detection limit was found to be 0.33 ng mL^{-1} . Wang *et al.* [90] constructed a lab-on-paper system for the extremely sensitive estimation of MCF-7 cells using a polyhedral-AuPd nanoparticle-based dual-mode cytosensor. Regarding the low content of MCF-7 cells in biological samples, there would be a need to enhance detection sensitivity. Therefore, nanoparticles with a wide surface area, superior catalytic activity and excellent conductivity were used for the decomposition of H_2O_2 . In this research, a supersensitive cytosensor was designed based on PH-AuPd NPs and 3D-rGO/PWE modifier. Also, AuNPs were grown on the surface of this modifier to increase the sample area conductivity. An Au@3D-rGO/PWE was made on a suitable lab-on-paper (LPD). Then, MCF-7 cells, and H1 and H2 as two different types of aptamers, were placed on the surface of that electrode (Figure 2). As it emerged, MCF-7 cells could be better detected by the preloading of aptamer H₂ on PH-AuPd NPs.

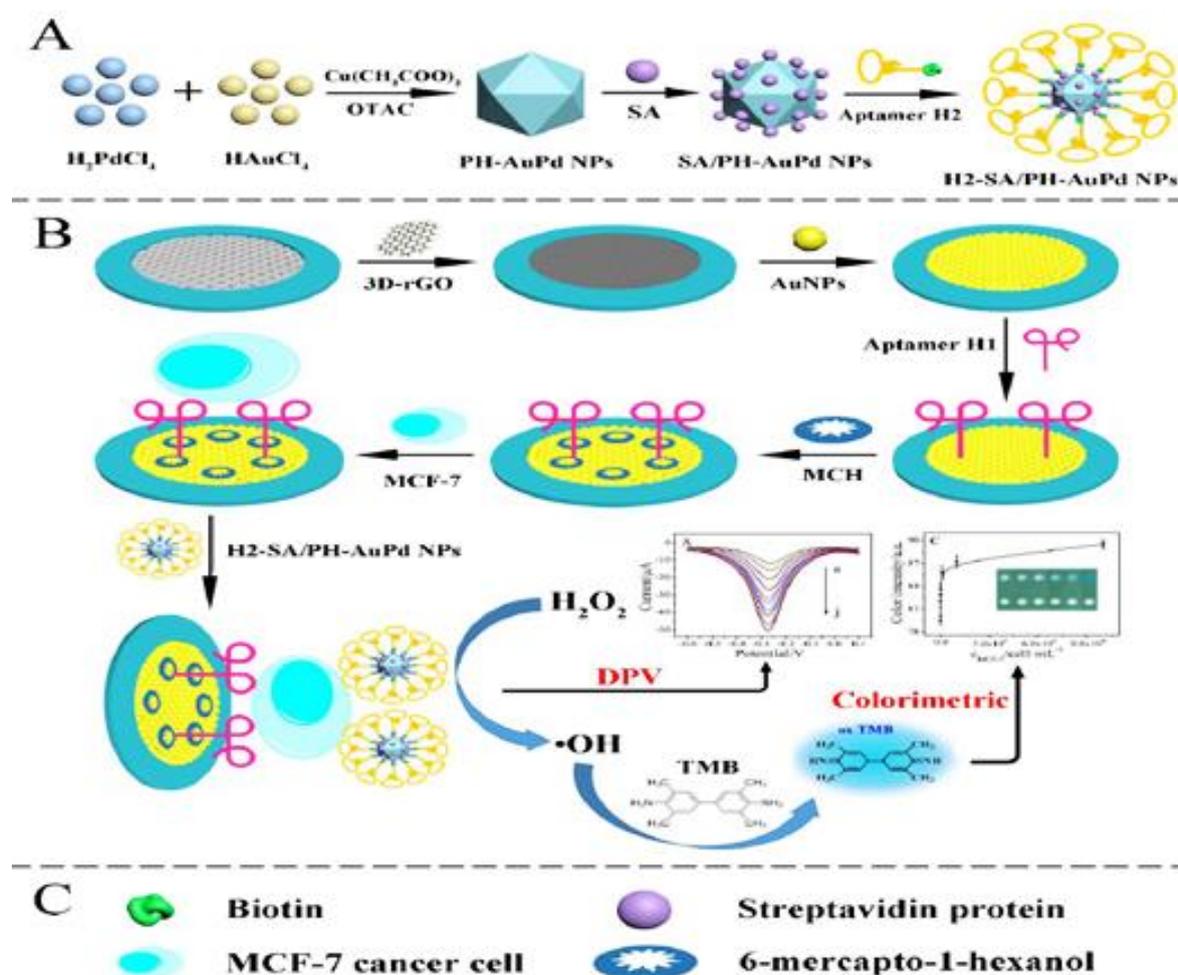


Figure 2. Steps of the electrode fabrication and immobilization of a dual-mode cytosensor: (A) H₂-SA / PH-AuPd NPs preparation process; (B) MCF-7 cells detection and the technique of signal detection [50] - Reprinted with permission copyright (2018), Elsevier

In this research, a novel electrochemical paper-based biosensor was also designed for the highly sensitive detection of microRNA. For this purpose, the target chain substitution was combined with

AuNPs and Cu-MOF (AuNPs@Cu-MOFstab) for their synergetic catalytic effect. All the chain displacement reactions and the electrical signal measurements were done on a compatibly designed origami electrochemical device (OECD). The AuNPs@Cu-MOF nanoparticles catalyzed the glucose oxidation, and a limit of detection of 0.35 fM was observed for miRNA-155. For both healthy individuals and cancer patients, it is possible to detect miRNA-155 in serum with this biosensor [50]. The simple manufacturing of ePADs has allowed the use of relatively sophisticated sensors that are also economically viable to develop. Multiplexed or simultaneous identification of many targets can be carried out by any of these sensors, supplying more accurate information for therapeutic studies [29,91]. A multiplexed system mostly diminishes the amount of the sample volumes needed, the time of the analysis, and the overall expense of the analysis. In this context, Sun *et al.* [54] constructed an advanced rotational paper-based analytical device (RPAD) for the multiplexed identification of prostate-specific antigen (PSA) and the cancer biomarkers of carcinoembryonic antigen (CEA). They made the device by putting three paper disks together using an empty rivet. The *on-off* states of the paper-based vents were conveniently controlled by the rotation of the paper disks. While other vents are disposable, the humidity of these rotational vents can be taken so as to reuse them. They also have a fast response, which makes this system unique in the categories of such devices. The rotational electrochemiluminescence immunodevices proved to have detection limits of up to 0.07 ng mL⁻¹ and 0.03 ng mL⁻¹ for CEA and PSA, respectively.

An ePAD was also designed to determine the cancer antigen 125 (CA125) through the screen-printing method. In this study, nanocomposites of (rGO/Thi/AuNPs) were coated on an ePAD working electrode to immobilize and recognize the signal enhancement of CA125 antibody (anti-CA125). The immunoassay results showed a detection limit of 0.01 U mL⁻¹. In addition, the immunosensor exhibited a good electrochemical performance and a potential for the POCT of other tumor markers [33].

One of the important diagnostic methods in the field of sensors is paper-based electroanalytical strips. In this regard, Cinti *et al.* [92] designed experimental comparisons of sensing breast cancer mutations by signal ON and signal OFF paper-based electroanalytical strips. The signal ON and signal OFF methods were tested in combination with paper-based electrodes. A single strand DNA for H1047R (A3140G) missense mutation in exon 20 was used as the target in breast cancer. The two methods had limits of detection in the nM range and almost similar analytical results and binding constants measured at the nM level. There were, however, some differences in terms of miniaturization and expenses. While both techniques are promising, the signal OFF reflects optimum manufacturing and simplicity of use. In another paper, a mobile trap microfluidic paper-based electrochemical device (Bio-MIP-ePADs) was presented as a novel strategy for the clinical diagnosis of biomarkers [49]. First, a molecularly imprinted polymer (MIP) was electro-synthesized on the standardized region of the instrument, and then it was added to a target analyte as a particular receptor. A non-imprinted polymer (NIP—electro-synthesized polymer without the target analyte) was also constructed in the same manner as an MIP in an identical area but separate from the system. These two identically structured but separate areas included three substrates of Whatman chromatographic paper grade No. 1 patterned by wax printing. In another part of the study, a mobile trap was created to allow the continuous and quick reception and delivery of various liquids needed for the MIP/NIP syntheses and further electrochemical analysis. In this procedure, the carcinoembryonic antigen was used as a model target, and the CEA detection limit was 0.32 ng/mL. Kumar *et al.* [93] presented a nanostructured iron oxide (nFe₂O₃@PEDOT:PSS) nanocomposite and an electrochemical paper-based cancer biosensor using poly(3,4-ethylenedioxythiophene):poly(styrenesulfonate) to detect CEA by the amperometry technique. In addition, another research group [94] reported a

graphene-PEDOT:PSS modified paper-based aptasensor for the diagnosis of this cancer biomarker by electrochemical impedance spectroscopy. Compared to the expensive common electrodes (*e.g.*, ITO, gold and glass carbon), this paper electrode had a better electrochemical efficiency. Table 1 presents a summary of surface modifiers and target biomarkers, as well as the analytical performance of paper-based biosensors for the detection of different cancer biomarkers.

Table 1. A summary of paper-based biosensors for cancer detection (from 2016 to 2020)

Electrode surface modifier	Biomarker	Sample type	Technique	Response range	Sensitivity	Detection limit	Ref.
NH ₂ -G/ Thi / AuNPs	CEA	Serum	DPV ¹	1-500 µg L ⁻¹	-	10 ng L ⁻¹	[95]
PPy/AuNP	EGFR	Saliva	EIS ²	0.5-500.0 nM	-	0.167 nM	[84]
mercapto-amine	CEA	Serum	DPV ¹	1.0–100.0 ng mL ⁻¹	-	0.33 ng mL ⁻¹	[89]
AuNPs@Cu-MOFs	miRNA	Serum	DPV ¹	1.0 fM -10 nM	-	0.35 fM	[90]
Au@3D-rGO	MCF-7	Serum	DPV ¹	50–107 cells mL ⁻¹	-6.8 µA dec ^{-1*}	20 cells mL ⁻¹	[50]
MWCNTs	CEA	Serum	EIS ²	0.1-100 ng mL ⁻¹	-	0.07 ng mL ⁻¹	[54]
	PSA	Serum	EIS ²	0.1-50 ng mL ⁻¹	-	0.03 ng mL ⁻¹	
rGO/THI/AuNPs	PSA	Serum	DPV ¹	0.05–200 ng mL ⁻¹	-2.0 µA dec ^{-1*}	10 pg mL ⁻¹	[96]
rGO/THI/AuNPs	CA125	Serum	DPV ¹	0.1–200 U mL ⁻¹	-0.37 A mL U ⁻¹	0.01 U mL ⁻¹	[33]
GO@Chitosan	CEA	Serum	DPV ¹	1.0–500.0 ng mL ⁻¹	19.3 µA dec ⁻¹	0.32 ng mL ⁻¹	[49]
nFe ₂ O ₃ @PEDOT:PSS	CEA	Serum	CHA ³	4-25 ng mL ⁻¹	10.2 mA	-	[93]
NG/ Thi/AuNPs	CEA	Serum	DPV ¹	0.01-500 ng mL ⁻¹	-2.8 µA dec ^{-1*}	2 pg mL ⁻¹	[97]
PB/PEDOT/ AuNPs	PSA	Serum	DPV ¹	0.05–500 ng mL ⁻¹	-2.8 µA dec ^{-1*}	10 pg mL ⁻¹	
Ag-RGO/CysA-Au NPs	CA15.3	Plasma	CHA ³	15-125 U mL ⁻¹	-	-	[98]
MoS ₂ /AuNPs/AgNW	microRNA 141 and 121	Serum	EIS ²	-	-	0.1 fM	[99]
Graphene-PEDOT:PSS	CEA	Serum	EIS ²	0.76-14 ng mL ⁻¹	-	0.45 ng mL ⁻¹	[94]

¹DPV: differential pulse voltammetry; ²EIS: electrochemical impedance spectroscopy; ³CHA: chronoamperometry; *dec means log of the biomarker concentration

Neurotransmitters

Nervous system disorders (NSDs) have been one of the main public health concerns over the past century [100]. The fast detection of these disorders is very important for more effective treatment and lower costs for patients [101]. The POCT of neurotransmitters is of extreme importance in clinical studies and the fast detection of NSDs. Paper-based electrochemical biosensors, among the various types of POCT platforms, have made enormous progress in the detection of neurotransmitters [102]. Nantaphol *et al.* [103] reported a boron-doped diamond paste electrode (BDDPE) coupled with µPADs to create an electrochemical sensor with high efficiency.

A mixture of boron-doped diamond (BDD) powder and mineral oil was used to prepare a BDDPE. It was easily printed in different geometries of the electrode. Using µPADs, the performance of the BDDPE was investigated through the analysis of heavy metals like Cd and Pb and biological samples like serotonin and norepinephrine. The BDDPE proved to have a lower capacitive current and a wider potential window than conventional carbon paste electrodes (CPEs). These findings indicate the ability of BDDPEs as POC sensors when coupled with µPADs. Also, for biochemical and neurochemical analyses, Trouillon *et al.* [104] designed paper-based polymer electrodes. The electrodes were made by covering of a filter paper with a modified PEDOT: PSS solution. It was found that, unlike planar electrodes, a paper electrode would show better resistance to neurotransmitter fouling as well as protein fouling. A noteworthy finding of the study was that long electrodes are more stable than short ones during the continuous oxidation of serotonin and dopamine. In another research work, Casadio *et al.* [105] applied an electrochemical biosensor based on MIPs for noradrenaline thermal diagnosis in aqueous solutions. In order to generate modified screen-printed electrodes (SPEs) with MIP, MIP polymer particles were added to screen-printed inks, and they were mixed together. The performance of the MIP-SPEs was evaluated through the heat-transfer method

(HTM), an easy and cheap detection method based on thermal resistance. Among the advantages of polymer-based systems, one may refer to their high potential in pharmaceutical applications due to their features such as portability, simplicity, and low cost [106]. An electrode of this type has excellent flexibility to adapt to MIP layers [107], and it can be targeted toward other disease markers. As a POC sensor, the system creates a good opportunity for pharmaceutical tests. Cinti *et al.* [108] designed a paper-based electrochemical biosensor to detect nerve disorders. In this study, the paper provided an effective surface on which to place reagents (*i.e.*, enzymes). The electrode was printed, and the environmental samples were measured for the recognition of paraoxon as a nerve agent simulant. This system made it possible for the simple and low-cost monitoring of a polluted site without a need for any chemicals or sample preparation, allowing for paraoxon detection down to $3 \mu\text{g L}^{-1}$. Therefore, the fabrication of this device can be easily expanded for different types of user-friendly stand-alone biosensing platforms.

Viruses

Certain detection methods such as enzyme-linked immunosorbent assay (ELISA) for IgM antibodies [109], reverse transcriptase-polymerase chain reaction (RT-PCR) for RNA [110] and electrochemical aptasensor using an AuNP-modified screen-printed carbon electrode (SPCE) for the detection RBD protein S SARS-CoV-2 [111] are used to fight the spread of viruses. The implementation of these methods is time-consuming and costly. It also needs experienced staff in the laboratory. Considering the drastic proliferation of emerging viruses, like the coronavirus (COVID-19), which has killed many people around the world recently, there is an urgent need to establish affordable POC technologies for accurate, rapid, and sensitive screening of patients suspected of viral infections. In the meantime, ePADs provide amazing sensitivity and excellent selectivity and are easily miniaturized [112]. Zhao *et al.* [113] constructed a portable μ PAD platform for the electrochemical multiplex detection of the antibodies of the hepatitis C and human immunodeficiency viruses in serum. This platform includes an electrochemical microfluidic paper-based immunosensor array and a multi-channel potentiostat and is capable of performing assays in eight samples at the same time within 20 min. The multiplexing function of this device enables it to generate multiple measurement data from a single run for the human immunodeficiency virus (HIV) and hepatitis C virus (HCV) markers. In addition, its wireless module can transfer the results to a remote telemedicine site. A unique combination of paper-based microfluidics and mobile devices makes the platform low-cost, portable, high-throughput, and user-friendly. The identification of HIV and HCV antibodies in the mouse serum with the detection limits of 300 and 750 pg mL^{-1} could be done by this biosensor. In another development, an economical and handmade paper-based device was designed for the electrochemical detection of the influenza virus [21]. The detection of this virus by means of this paper-based, label-free electrochemical immunosensor actually occurred for the first time. The paper was modified with a spray of hydrophobic silica nanoparticles and used in a stencil-printed electrode. This paper-based biosensor was highly hydrophobic. Single-walled carbon nanotubes were used to modify the stencil-printed carbon electrode, and chitosan improved its sensitivity. Through glutaraldehyde cross-linking, antibodies were also immobilized. The immunosensor displayed a good selectivity and a low limit of detection (113 PFU mL^{-1}) for the H1N1 virus. This simple device was employed as a disposable and inexpensive biosensor to detect pathogenic microorganisms, especially in developing countries.

In another study, an electrochemical biosensor was constructed based on paper-based peptide nucleic acid to determine human papillomaviruses (HPVs) [114]. An anthraquinone-labeled pyrrolidiny peptide nucleic acid (acpcPNA) probe (AQ-PNA) and a graphene-polyaniline (G-PANI)-

modified electrode were used to create this novel electrochemical biosensor. Due to properties such as wide potential range, low cost and fast response time, carbon is appropriate to be used in ePAD DNA biosensors. Nevertheless, the limited relationship sensitivity of micro-scale electrodes, as a section of ePADs, is a significant impediment. To solve this problem, graphene, which has a large specific surface area and unique electrochemical properties, was utilized as a carbon-based nanomaterial to modify working electrodes through the inkjet printing technique. The AQ-PNA probe was also immobilized on the electrode surface through electrostatic attraction. The DNA biosensor was then employed to find a synthetic 14-base oligonucleotide target with a sequence corresponding to HPV type 16 DNA. Square-wave voltammetry was used to measure the electrochemical signal response of the AQ label before and after hybridization, as shown in Figure 3. The detection limit of the biomarker was 2.3 nM under optimal conditions.

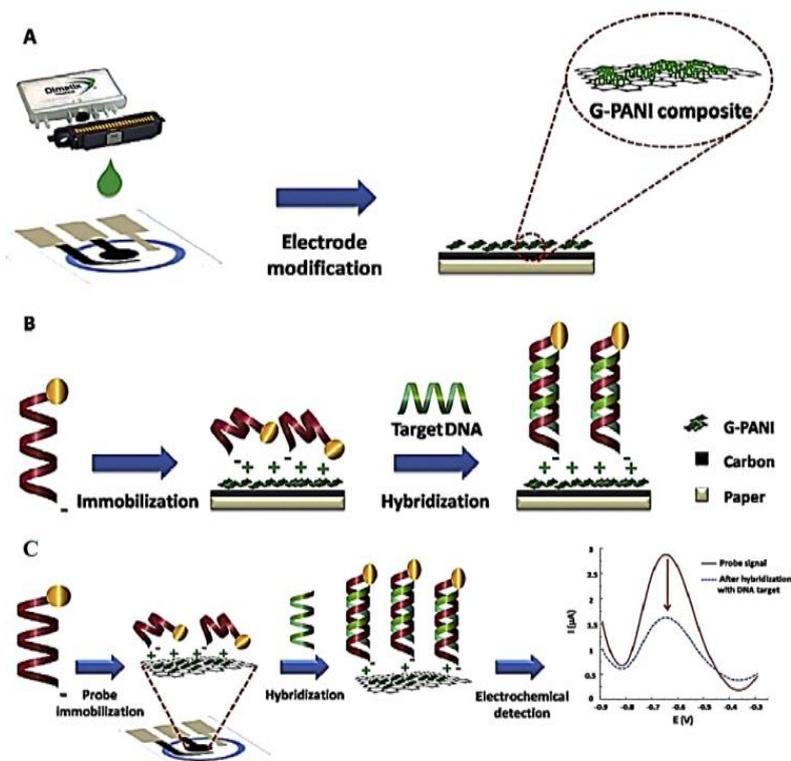


Figure 3. Schematic display of: (A) electrode modification procedure, (B) immobilization and hybridization of the electrochemical paper-based DNA biosensor device, and (C) electrochemical detection of the AQ label utilizing square-wave voltammetry technique before and after hybridization [113] - Reprinted with permission copyright (2017), Elsevier

Viral pathogens pose serious health threats around the world, but the common sensing methods are often insufficient and too slow to deal with those threats. To tackle the problem, an EIS ePAD analytical device was designed by Channon *et al.* [115] for the rapid detection of virus particles in minutes. The researchers applied easy patterning techniques to find the impediments on cellulosic paper to efficiently integrate it to functionalized Au microwire electrodes. In this respect, dithiol modification could produce a strong base layer to cross-link to antibodies *via* carbodiimide coupling. Any considerable change of impedance was considered as a sign of the virus particles captured onto the antibody-modified electrode. The proposed system and the intelligent electrode modification strategy can provide grounds to determine different intact viruses and other biological targets. The technique will ultimately allow the multiplexed POC diagnosis of viral infections in a rapid and sensitive manner.

Proteins

There is an increasing need for the mass production of biosensors that are able to measure protein biomarkers rapidly and efficiently in both clinical and biological research. In this regard, Boonkaew *et al.* [38] designed an origami paper-based electrochemical immunosensor to detect the C-reactive protein (CRP) in a certified serum sample. A filter paper (Whatman No. 1) and the wax printing method were used to manufacture the device. The Adobe Illustrator CS6 software was also employed to make an oPAD pattern. In this study, a screen-printed carbon electrode (SPCE) was modified with graphene to improve its sensitivity, and then gold nanoparticles were electro-deposited onto the G/SPCE, followed by the assembling of a monolayer of L-cysteine. Finally, the anti-CRP was adsorbed and immobilized on the modified electrode (Figure 4). $[\text{Fe}(\text{CN})_6]^{3-/4-}$ as a redox probe and SEM and cyclic voltammetry were used to validate the modification of the electrode. This method was low-cost, disposable and portable. Also, a label-free paper-based electrode was developed for the quantification and determination of the standard bovine serum albumin (BSA) protein [116]. The biosensor included a specific antibody, carbon nanotubes (CNTs) and cellulose filtration paper. Its limit of detection was found to be 2.89 ng/mL, which is similar to that in the typical ELISA technique for BSA measurement. The electrochemical technique employed in that research takes approximately 10 minutes to perform, therefore greatly decreasing the time of analysis compared to the usual ELISA method. In another research, gold nanoparticles were used to modify a screen-printed electrode (SPE). For this purpose, poly (2-methacryloyloxyethyl phosphorylcholine) (PMPC-SH) with thiol-terminated material was self-assembled on the surface of the electrode. This paper-based electrochemical device was used for the diagnosis of the C-reactive protein (CRP). The nonspecific adsorption of the protein was minimized on the PMPC-modified electrode. Albumin, myoglobin and bilirubin proved not to interact with this system. It was used for CRP identification in a licensed human serum. It is, indeed, a promising sensor for the electrochemical detection of CRP using highly sensitive, inexpensive and disposable materials [48].

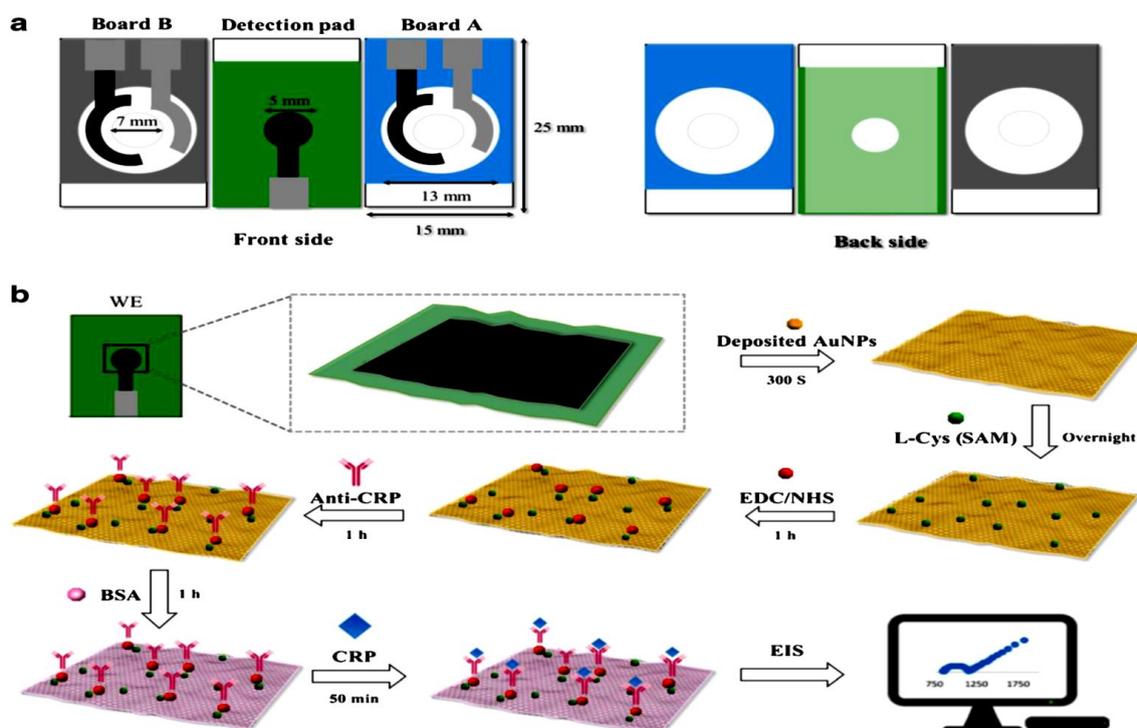


Figure 4. a) Schematic display of origami paper-based analytical device (oPAD) and its components; b) steps of electrode preparation and immobilization for detection of CRP by an immunosensor [37] - Reprinted with permission copyright (2019), Springer-Nature

To see how protein immobilization occurs in screen-printed graphite layers, Wróblewski *et al.* [117] investigated six different protein immobilization procedures, including physical adsorption, electrochemical carboxylic group generation, graphite functionalization with succinic anhydride, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide activation, and graphite functionalization with 3-(triethoxysilyl) propylsuccinic anhydride. The experiments showed that the best results would be obtained when graphite powder was functionalized before the preparation of the screen-printing paste. To verify the efficiency of the chemical functionalization process in the presence of functionalized groups, EDX and XPS analyses were conducted. Generally, coating a big surface with a high yield is easy through the printing technique, and it is possible to use different substrates, hard ones such as ceramics or glass, as well as flexible ones such as polymer films, textiles or paper.

Clinical practices

Butyrylcholinesterase (BChE) is localized in the muscles, brain and other tissues [118]. Its biological role is unknown, but recent studies have shown that BChE hydrolysis affects fat metabolism. It is, thus, called 'hunger hormone' [119]. This material has been used as a short-acting blocker of the acetylcholine receptor in anesthesia [120]. The first 3D paper-based printing device to measure BChE in human serum samples was made by Scordo *et al.* [121]. The screen printing and wax printing techniques were used to manufacture this paper-based sensor. To measure the BChE activity, butyrylthiocholine was applied as an enzymatic substrate. A thiocholine by-product was also recognized by means of an ePAD biosensor modified with the Prussian Blue and Carbon black nanocomposite.

In another study, a 'pop-up' electrochemical paper-based analytical device (pop-up-EPAD) was designed by Wang *et al.* [36] It was used for the analysis of beta-hydroxy-butyrate (BHB), which is a key biomarker of diabetic ketoacidosis. The device was a clever and advanced invention influenced by pop-up greeting cards as a (3D) pop-up system. It proved to have the ability to directly measure BHB in the blood. Amor-Gutiérrez *et al.* [122] constructed a multiplexed paper-based electrochemical device with inexpensive materials such as paper, multifunctional connector headers and carbon ink. To easily merge a sampling step, the paper-based electrochemical platform was combined with a glass fiber tape. It could perform eight simultaneous measurements. Both tasks, *i.e.*, sampling and simultaneous measuring, were designated to bioenzymatic glucose biosensors. They showed awesome reproducibility and dealt with a wide linear range of concentrations. In another study [123] for the determination of Hemoglobin A1c (HbA1c), a sensitive electrochemical nanobiosensor was developed. It was used to measure glucose concentration in people with diabetes. The nanobiosensor was made with a paper graphite sheet as an electrode and modified with a nanocomposite of rGO-gold. The nanocomposite increased the surface area and provided an appropriate substrate through the strong covalent bonding of a thiolated DNA aptamer as a bioreceptor on the electrode surface. So far, many paper-based biosensors have been developed on the basis of the molecular recognition of single-strand DNA (ssDNA), antibodies (Ab) and antigens coupled with enzymatic reaction. Two label-free integrated μ PADs were developed by Wang *et al.* [124] and Ruecha *et al.* [125]. To immobilize antibodies, the devices were modified with multi-walled carbon nanotubes (MWCNTs), thionine (THI), gold nanoparticles (AuNPs) and polyaniline (PANI). The fabrication of the microfluidic channel of the paper-based sensors was done through a wax-printing technique. The modified electrodes were successfully applied to the sensitive, specific and POC diagnosis of two antibodies, 17 β -estradiol (17 β -E2) and interferon-gamma (IFN- γ), in

human serum. It is to be noted that 17 β -E2 plays an important role in regulating reproduction in human beings, while IFN- γ has a critical role in the diagnosis of tuberculosis susceptibility.

In another study [12], the clinical biomarkers of uric acid (UA) and creatinine (CNN) were estimated simultaneously in urine samples with high recovery values. The device for this purpose was an ePAD which consisted of two spot sensors in the same working electrode. For the direct oxidation of UA, the surface of spot 1 was modified with quantum dots of graphene and CNN oxidation. Also, the surface of spot 2 was modified with quantum dots of graphene, creatininase and ruthenium electrochemical mediator. The ePAD was entirely created by cutting a filter paper with a cheap domestic cutter printer. A microfluidic channel was made through the electrode by the production of a screen-printed electrode on a polyester film. These two biological biomarkers were simultaneously characterized sensitively and selectively through square-wave voltammetry.

E. coli O157:H7 is one of the most important foodborne pathogenic bacteria, which can often lead to such diseases as bloody diarrhea, hemolytic uremic syndrome and even death [127]. The existing methods for the detection of *E. coli* O157:H7 mostly lack sufficient sensitivity and mainly have a low detection limit. Burrs *et al.* [128] presented the first graphene paper functionalized with fractal platinum nanocauliflowers to detect *E. coli* O157:H7 and to perform the electrochemical biosensing of small molecules (*e.g.*, glucose). The researchers illustrated the synthesis of platinum nanocauliflower-graphene hybrids on a nanocellulose paper to be used in POC biosensors. The platinum surface was functionalized with either aRNA aptamer through covalent linking or glucose oxidase through chitosan encapsulation. The response times were found to be 12 minutes for *E. coli* and 6 seconds for glucose, which were similar to those of commercial electrode sensors and silicon biochips. Table 2 presents a summary of the analytical characteristics of various paper-based sensors for clinical detection.

Table 2. Analytical characteristics of paper-based biosensors for clinical detection (from 2016 to 2020)

Electrode surface modifier	Analyte	Sample type	Technique	Response range	Sensitivity	Detection limit	Ref.
AuNPs/CNT _s	Bisphenol A	ABS plastic toys	LSV ¹	0.2 – 20.0 mg L ⁻¹	-	0.03 mg L	[129]
RGO-CuNP/GCE	CFA	Urine	DPV ²	9.9 - 91.7 μ M	-	367 nM	[130]
AuNPs	Glucose	Coke	CV	0.5–10.0 mM	240 μ A mM ⁻¹ cm ⁻²	148 μ M	[55]
Graphite pencil	Ascorbic acid	Commercial tablet	SWV ³	0.5-3.0 mM	0.47 μ A M m ⁻¹	70 μ mol L ⁻¹	[131]
PB/CB	Glutathione	Serum	CHA	1-10 mM	0.102 μ A M m ⁻¹	60 μ M	[132]
	Metallocenes	Serum	CV	-	-	1 ppt	
CNT	Glucose	Serum	CV	-	-	1 ppt	[133]
	1,2-benzanthracene	Serum	CV	-	-	10 ppt	
Carbon-ink	NSAID	Tap water	LSV ¹	0.1 - 5.0 μ M	0.85 μ A μ M ⁻¹	70 nM	[134]
Silica	Dexamethasone	Herbal medicine	DPV ²	10-500 mg mL ⁻¹	19.3 μ A dec ⁻¹	3.59 mg mL ⁻¹	[135]
	Prednisolone	Herbal medicine	DPV ²	10-500 mg mL ⁻¹	19.3 μ A dec ⁻¹	11.98 mg mL ⁻¹	
ZnONPs/PEDOT:PSS	Hydrazine	Water	CHA ⁴	10-500 μ M	0.14 μ A μ M ⁻¹ cm ⁻²	5 μ M	[24]
PET	Glucose	Sweat	Amperometric	0.0-1.9 mM	35.7 mA mM ⁻¹ cm ⁻²	5 mM	[136]
Silver nanostructure	Glucose	Serum	CHA ⁴	3-3000 μ M	4610 μ A mM	1.1 μ M	[137]
Pt/Nafion/GOX/Nafion	Glucose	Saliva	Potentiometric	316-3160 μ M	-93.2 \pm 1.8 mV dec ⁻¹ *	120 μ M	[138]

¹LSV: linear sweep voltammetry; ²DPV: differential pulse voltammetry; ³SWV: square wave voltammetry; ⁴CHA: chronoamperometry; *dec means log of the biomarker concentration

Conclusion

The literature shows a recent enrichment of studies on ePADs in terms of quality and number. Among all the available biosensing systems, paper-based devices have proved to be highly encouraging owing to their transportability, easy accessibility, low-cost fabrication, amenability for reagent integration, capillary flow properties, and easy patterning. Besides, the natural polymeric materials used in them are eco-friendly and safe to discard. Therefore, such biocompatible materials are used as solid electrochemical sensor substrates. Despite their advantages, ePADs have some

limitations in terms of stability, lifetime, multiplexing capabilities and reproducibility. With regard to the unique properties of paper, the use of paper-based miniaturized sensors has widely expanded for point-of-care diagnosis in miniaturized settings. The miniaturization of devices is an issue that attracts increasing attention now that electronic measurements or spectrometric functions can be integrated in smartphones. It has been made easy to attach miniaturized printed electrodes to biomolecules, thus improving their analytical efficiency from the perspective of sensitivity and selectivity. Accordingly, the number of analytical experiments conducted outside the laboratory has substantially grown in recent years. This advanced technology widely helps low- and middle-income countries. Despite the tremendous achievements in this field, paper-based electrochemical biosensors require more research with a focus on a) achieving reproducible quantitative results, b) developing new methods for the fabrication and modification of electrode materials and paper substrates, c) developing advanced systems with enhanced functions but simplified platforms, d) using easy and low-cost fabrication methods for mass production and successful commercialization, and e) producing portable and cheap systems in order to extend their applications in remote areas. Moreover, further studies should be conducted on the development of POCTs and the fabrication of ePADs that can be used easily by everyone at any level.

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