Paper-Based Electrochemical Devices in Biomedical Field: Recent Advances and Perspectives

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1. INTRODUCTION

Paper is everywhere around us: we daily come across it in a variety of forms as newspapers, books, magazines, wrapping paper and packing boxes, letters and decorative papers, drawing and printing papers, handkerchiefs and paper towels, toilet paper and so on, even our money is made of paper. This variety of uses reflects the versatility of this material, essentially made of cellulose derived from woods or cotton rags, transformed into sheets through different procedures. Because of this ubiquity, it is not surprising

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that paper could have been conceived as a support for chemical analyses, from past centuries to the very recent development of point of care devices matching the ASSURED criteria [1]. Who among chemists does not know the litmus test for the measurement of pH? It is highly likely that it derived from the collection of essays on experimental history of colours by the famous chemist Robert Boyle in 1664 [2]. Nevertheless, it is since the mid-twentieth century that paper stick for measuring glucose in urine appeared, and from the early eighties, the lateral flow immunochromatographic strips become commercially available, marking a turning point in analyses carried out far from the laboratory. The possibility of carrying out quick and reliable analyses outside the clinical laboratories is definitely a very important goal, both from social and personal point of view, especially because of the reduction of public expenses in the field of prevention and treatment of diseases, but also because avoiding people going and queuing to laboratories, as well as waiting times to get the results of the analyses. The reduction of analysis costs is however the most important characteristic, especially for people living in resource-limited country, where the access to expensive medical apparatuses is often lacking, and the development of novel, cost-effective and easy-to-use diagnostic tools is crucial and could greatly increase the chances for survival of many people. The use of paper as support for the construction of these devices goes just in this direction, because of its low cost, wide availability and eclectic versatility. At the beginning of the 21st century, the Whiteside group started to exploit a new possibility of utilizing paper, by creating on the surface of filter paper microfluidic channels [3], forming hydrophobic patterns and taking advantage of the porosity of cellulose for driving solutions in predesigned directions. They named these tools as "microfluidic paper-based analytical devices" (μPADs) [4], and, from this moment on, scientists saw the possibility to realize microfluidic devices without the need of sophisticated technologies such as soft lithography for example, or the use of micropumps, microvalves, etc. This research area therefore rapidly grew up, so that several reviews dealing with this subject are actually present in literature [5–9]. The irrefutable success of this approach descends from the possibility to carry out increasingly sophisticated analyses while maintaining characteristics of speedy, easiness of use and inexpensiveness. The simplest devices are based on colorimetric readout, because in this case no transducers are necessary, but the results can be visualized by naked eye. Nevertheless, not only chemical compounds have been entrapped on the paper surface, although these latter are capable of

measuring pH, the presence of proteins, blood or bilirubin in urine for example, but also enzymes, prevalently belonging to the oxidase and peroxidase families, or proteins and antibodies, as in the case of lateral flow immunochromatographic assays, the most famous of which is surely the pregnancy test. ELISA tests have also been carried out on paper devices [10] and problems of covalent immobilization of bioreagents and their long-term stability have been faced [11,12]. Florescence and chemiluminescence have also been applied as detection technique for paper devices, and, to increase sensitivity, metal nanoparticles have been employed to obtain a signal enhancement. These optical readouts allowed the use of digital cameras and smartphones as detectors, because they possess both a light source (LED flash) and a digital camera for detection, being thus able to substitute more expensive spectrophotometers. In such a way, it would be possible to carry out analyses in resource-limited locations, and to transfer data to centralized facilities [13]. Another very successful detection technique coupled to paper-based devices is the electrochemical one. This technique already possesses characteristics of high sensitivity, selectivity, simplicity, miniaturization and low cost of apparatuses. The combination of the two principles brought to the realization of the so-called microfluidics paper electrochemical devices (μPEDs) [14,15]. Printing an electrochemical sensor onto a paper substrate can be done with relative simplicity, using screen printing or inkjet techniques for example, and many of the assays that nowadays are carried out in clinical laboratories could be transposed on paper-based devices.

This chapter gathers examples of potentiometric [16] and of numerous voltammetric paper devices, which can be simple as a chemical sensor or complex such as immunosensors and DNA sensors. Nanomaterials have been also exploited for increasing sensitivity and selectivity, obtaining very promising results. The focus will be on clinical applications of these paper-based devices, with the intent of realize practical, cheap, fast, but reliable and robust POCTs (point-of-care tests) that could be really useful to more and more people in more and more contexts.

2. WHICH PAPER?

Depending on the analytical needs and the field of application, different kinds of paper materials can be adopted in manufacturing the electrochemical platforms. There is not a "universal" paper; the selection of the paper's grade depends on the specific assay to be developed. The

main characteristics to be evaluated when fabricating a paper-based electrochemical sensor are summarized below:

1. Chemistry: grade of cellulose fibres, presence of lignin, degree of esterification, nitrogen contents. It might affect the deposition and/or flow of species;
2. Surface area: it affects the loading of reagents, and sensitivity and reproducibility of the assay;
3. Flow rate: it represents the speed of a migrating analyte along the paper-based device. It is critical in reaching a good sensitivity depending on the distance of testing area;
4. Pore size: it is related to the size of particles that can be retained;
5. Porosity: it is defined as the void volume of the paper, and it has not to be confused with pore size;
6. Thickness: thin papers will allow a faster spread of the reagents towards the testing area than thicker papers do; and
7. Cost: economic sustainability needs to be evaluated.

Filter and chromatographic papers are the most used substrates to realize electrochemical platforms for biomedical purposes. They allow producing reagent-free platforms, which represent a great advantage towards the realization of stand-alone and user-friendly devices, particularly useful for those applications in remote areas. Among these, Whatman filter paper No. 1, with a 11-μm pore size, is certainly the most used substrate [14,17–21]. Thanks to its porosity, particle retention and flow rate, it is possible to manufacture electrochemical platforms capable to store the reagents, filter the sample, make a reaction happen and flowing the detectable product towards the electrochemical testing area. Many examples have been reported and, to the date, they represent the largest choice also for the development of colorimetric devices. Some exceptions to Whatman No. 1 have been proposed to realize electrochemical platforms exploiting the three-dimensional (3D) network of cellulose fibres, i.e., Whatman No. 5 (2.5-μm pore size) [22], Whatman No. 81 (cation exchanger paper) [23], Japanese paper [24], standard filter paper (e.g., Cordenons) [25]. However, all these approaches do not differ from each other. Although the features as the pore size, the flow rate and the homogeneity might (slightly) affect the sensitivity and the repeatability of a device, they do not differ in the concept. The same principle has been also adopted by Malon and coworkers, which have developed a method for the determination of lactate saliva by using an electrode printed on cotton fabric [26]. The principal drawback is due to the fact that the cellulosic network can hinder the diffusion of the redox

molecules at the electrode. In these cases, the electrochemical cell is represented by the porous structure of the paper, and the detection limit of the analyte is negatively affected. Although these drawbacks can reduce the application of filter paper in specific application where ultralow detection limits are required, other types of paper can be adopted to create suitable platforms in sensor technologies, exploiting different concepts. Office paper can be used as a sustainable substitute of the classic polyester-based electrodes, i.e., commercial glucometer strips [27]. Although, as reported by the Whitesides' group [28], the cost of an screen printed electrode (SPE) can be tremendously lowered, i.e., from 0.5/1 to 0.014 $/strip by replacing polyester with Whatman No. 1, the use of office paper is capable to provide a further 30% saving over the entire SPE production cost [29]. Due to the increased necessity in lowering the analysis cost and minimizing the production of hazardous waste, other sustainable materials, both economically and environmentally, have been used to replace plastic-based substrates in sensor manufacture. Hydrophobic-coated paper has been used, coupled with polydimethylsiloxane, to detect glucose [30]. A flexible glossy paper substrate (cellulose blended with an inorganic filler) has been also adopted to detect volatile ethanol [31]. These substrates can represent an electrochemical cell similar to the classic plastic-based SPEs. They allow to analyze a drop of sample above the electrode surface, establishing a strict contact between analyte and electrode surface. Fig. 1 displays the two most adopted experimental set-ups when a paper-based electrode is employed.

Considering filter papers, the electrochemical cells are represented by their porosity, and the cellulosic fibres can reduce the diffusion of the analyte. It has been observed by electrochemically comparing the performance of electrode printed on office paper, Whatman No. 1, and polyester,

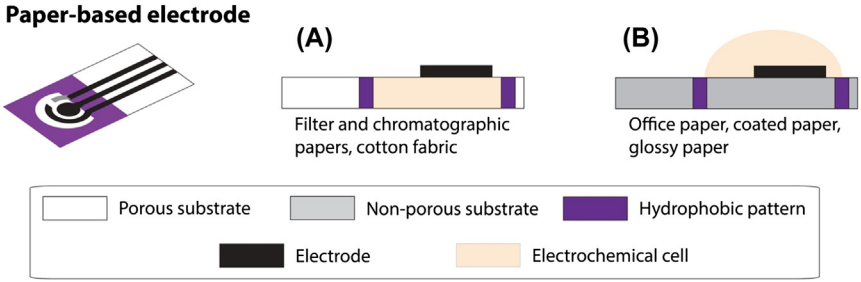

Figure 1 Components and electrochemical cells related to the diverse typologies of existing paper-based electrodes in biomedical analysis. Electrodes obtained onto (A) porous and (B) nonporous substrates.

in presence of ruthenium examine as redox probe. Although the Whatman paper does not show any analyte entrapment, the peak-to-peak separation appears worse than those obtained by using office paper and polyester, while all the interrogated platforms reveal a similar electron rate constant [29]. On the other hand, the use of substrates such us office paper limits the development of reagent-free devices. The only advantage of these substrates is related to their cost-effectiveness and "greener" disposal procedure, when compared with the plastic-based platforms.

3. PAPER PATTERNING

As one of the principal reasons for utilizing paper in biomedical assay is certainly to lower the costs, the high hydrophilic nature of paper might limit its employment, particularly towards the development of electrochemical devices. The easy diffusion of aqueous solution through the cellulosic network could represent an issue, for instance by wetting the electrode contacts at the interface with the potentiostat. To avoid the rise in electrical noise, paper needs to be hydrophobic patterned to define specific testing areas and channels, where reagents can be loaded, analytes can flow, be purified, react, and electrodes can be realized to properly detect the specie of interest. The approach for patterning the paper will depend by the analytical needs, the available facilities and personnel qualification. The most adopted hydrophobic patterning method is wax printing [20,30,32,33]. Firstly, the chosen pattern is designed by using a drawing software package (Adobe Illustrator, CorelDraw, SolidWorks, PowerPoint, etc.). Then the pattern is printed on the paper-based substrate exploiting an office wax printer (generally, Xerox ColorQube). Lastly, the waxed paper is cured in the oven (c. 100°C) allowing the wax to melt and to diffuse through the cellulose fibres network, creating the hydrophobic barriers. Wax printing allows to easily and readily pattern papers, without requiring specialized personnel. However, it is not the best choice for creating complicated patterns, and the wax spreading, being dependent by the paper used, should be considered while developing the analytical device. Nowadays, this method has overtaken the use of photolithography that has been first reported by the Whitesides' group for patterning paper. They reported a rapid procedure for prototyping paper-based microfluidic devices, called FLASH [4], which was firstly applied to colorimetric detection, and then to electrochemical ones [34]. With this approach, paper is initially

impregnated with a photoresist, and by using a mask, through the application of UV light, the photoresist will polymerize and the unexposed one will be washed away, producing sharp channels in the range of 200 μm. However, the requirement of organic solvents, the expensive instrumentations, and personnel expertise, represent the major drawbacks of this approach. These two strategies are the most used towards the development of paper-based electrochemical devices for biomedical assays or to detect relevant biomolecules. Screen-printing technique has been also utilized to create hydrophilic compartment, even if not so many examples have been reported [35,36]. Its simplicity represents the main advantage, but the resolution is not sufficient to realize microsized areas/channels. However, according to the literature, there are a variety of techniques that have been reported for paper patterning, although they have not been applied to electrochemical detection yet. To provide a more complete overview regarding the patterning route that can be followed to develop paper-based electrochemical devices, the following techniques are briefly described: inkjet printing [37–39], cutting [40], plasma treatment [41] and roll-to-roll (R2R) printing [42]. Cutting is a very cheap route to obtain hydrophilic area, but the lack of a "real" hydrophobic/hydrophilic separation requires extra substrates to allow the liquid to flow. Inkjet, plasma and R2R patterning approaches represent a good choice in terms of paper treatment. Differently from photolithography, these methods require low amount of patterning agents, and washing steps to remove solvents are not required. However, the instrumentation involved is not cheap and might be not suitable in resource-limited environments. All the mainly utilized approaches are displayed in Fig. 2, and the advantages and disadvantages are highlighted.

4. DIFFERENT CONFIGURATIONS OF PAPER-BASED (BIO)SENSORS

In the paper-based electrochemical sensor and biosensor field, the first example of paper-based sensor with electrochemical transduction was reported in 2009 by Henry group and published on *Analytical Chemistry* journal [14]. The device was conceived for the detection of glucose, lactate and uric acid in biological fluids using glucose oxidase, lactate oxidase and uricase as biocomponents. In 2010, Whiteside group, the pioneer in colorimetric paper-based sensor, published on *Lab on a Chip* a paper entitled "Electrochemical sensing in paper-based microfluidic devices" [15]. In this manuscript, the authors developed a microfluidic

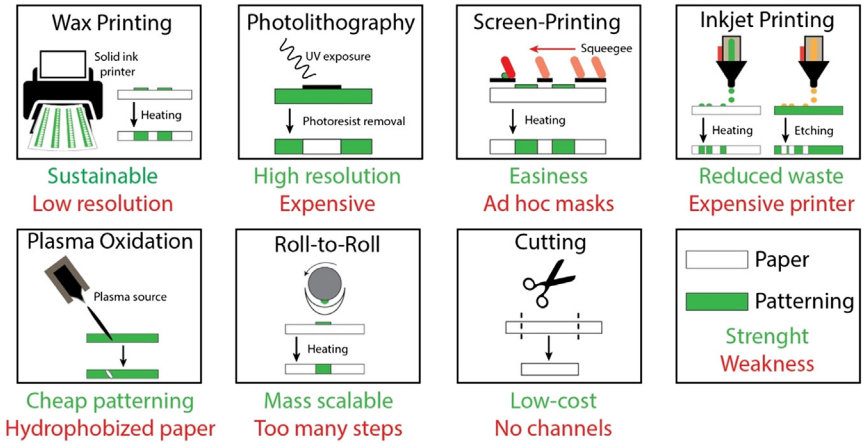

Figure 2 Different routes to pattern paper-based analytical devices.

paper-based electrochemical device capable of quantifying the concentrations of glucose in biological samples including urine and serum. The authors reported that their work was carried out independently of a similar and complementary study by Henry group, highlighting that research on paper-based analytical devices (PADs) was becoming a paramount topic. These first paper-based biosensors were based on 2D configuration capable of both single and multiple detections using a single sample reservoir (Fig. 3). A crucial example of single detection was described by Nie et al. [15], which

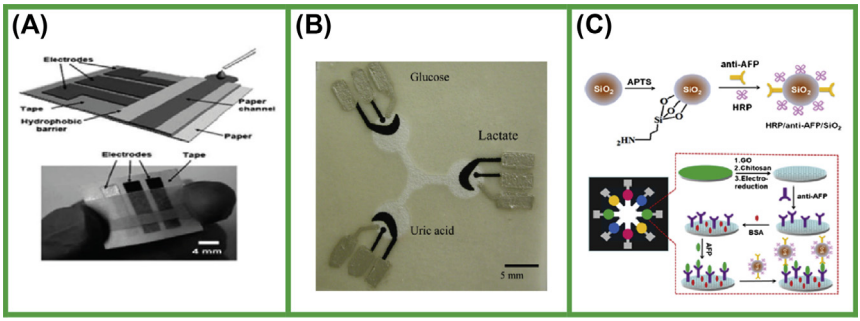

Figure 3 Examples of 2D paper-based analytical device. (A) Scheme of a 2D paper-based electrochemical sensing device for analysis of a single analyte (glucose) [15]. (B) Picture of multiple analysis of glucose, lactate and uric acid loading the sample on hydrophilic area at the centre of the device [14]. (C) Scheme of 2D paper-based electrochemical immunosensors for analysis of four kinds of cancer biomarkers namely alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), cancer antigen 125 (CA125) and carbohydrate antigen 153 (CA 153) [40].

fabricated an electrochemical device consisting of three electrodes printed on a piece of paper substrate and a paper channel, in conformal contact with the electrodes and held in place by double-sided adhesive tape surrounding the electrodes (Fig. 3A). Examples of multiple analyte detection were realized by Henry group [14] as reported in Fig. 3B.

The authors fabricated microfluidic channels on filter paper by photolithography and electrodes by screen-printing technology. Specific enzymes were thus immobilized on the electrodes for the detection of bioclinical markers in human serum samples. This configuration allows measuring different analytes by loading a single sample on the hydrophilic area at the centre of the device, wicking sample into the three separated detection areas. A similar system was developed by Wu et al. [40] for the simultaneous detection of four kinds of cancer biomarkers. In this case, antibodies were exploited for providing the electrochemical detection by a sandwich format chosen with horseradish peroxidase (HRP) and antibody coimmobilized silica nanoparticles and graphene for signal amplification (Fig. 3C).

Beside electrochemical paper sensors based on 2D configuration, several efforts were attained to entrust high innovative and smart 3D formats. For instance, programmable diagnostic devices made from paper and tape [41] as well as origami [42] were designed to offer an easy manipulation of fluidic flow. The first examples were reported for colorimetric detection as reported in Fig. 4, which highlights how to exploit both the vertically and laterally distribution of the fluids, and across each other without mixing through the different coloured solutions [43].

On the contrary, one of the best strategies when using electrochemical transduction is based on 3D and slip geometry, which allows controlled fluid handling and timed reagent delivery. Such devices are called 0-slip PAD and comprise two layers with reagents loaded in different positions that can be or not in contact by layer slipping. The 0-slip PAD approach was exploited for the first time by Crooks group in 2014 [44] for the detection of biomolecules labelled with silver nanoparticles (AgNPs). In addition, this configuration also allowed achieving very low detection limit thanks to two different preconcentration steps. The first preconcentration step is due to the focussing of the magnetic AgNPs labels at a working electrode, followed by the spontaneous oxidation of these labels in the presence of $KMnO_4$. This oxidizing agent is delivered into a channel at a specific time and location by simply slipping a paper layer, with the consequent oxidation of Ag to Ag^+. The second type of preconcentration happens at the working electrode surface, reducing back the Ag^+ to Ag, followed by Ag layer oxidation by

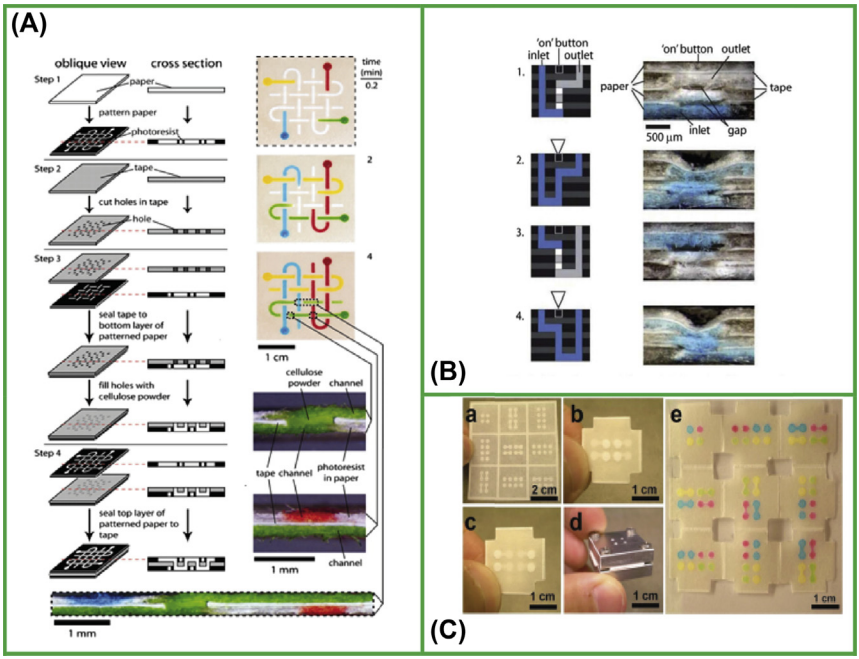

Figure 4 Examples of colorimetric 3D paper-based analytical devices (PADs) that show the distribution of the fluids. (A) Fabrication, pictures of the devices adding red, yellow, green and blue aqueous solutions of dyes to the sample reservoirs, demonstrating that the streams of fluids crossed in different planes without mixing [43]. (B) Scheme and photographs of four buttons: 1 and 3 not compressed, while 2 and 4 compressed using ballpoint pen [41]. (C) Photographs of 3D PAD fabricated photolithography (a–d). Demonstration that the coloured solutions passed through channels and reservoirs without mixing (e) [42].

anodic stripping voltammetry (Fig. 5A). The entire assay was carried in only 4.6 min with low detection of label (767 fM). However, 0-slip PAD suffered from three problems: (1) $KMnO_4$ has poor stability when dried on paper; (2) $KMnO_4$ is a very strong oxidizing agent and can react with other components in the system; and (3) the slip layer is not suitable for automation. To overcome these drawbacks, the same group recently reported a novel 3D paper fluidic device called no-slip PAD, configured for electrochemical detection of biomolecules likewise labelled with AgNPs. In this configuration, the detection of AgNPs is based on galvanic exchange using Ag/Au rather than a chemical oxidant (Fig. 5B). The no-slip resolves the $KMnO_4$ instability as well as the need for layer slipping [45]. Another interesting approach was published on *Journal of American Chemical Society* in 2014

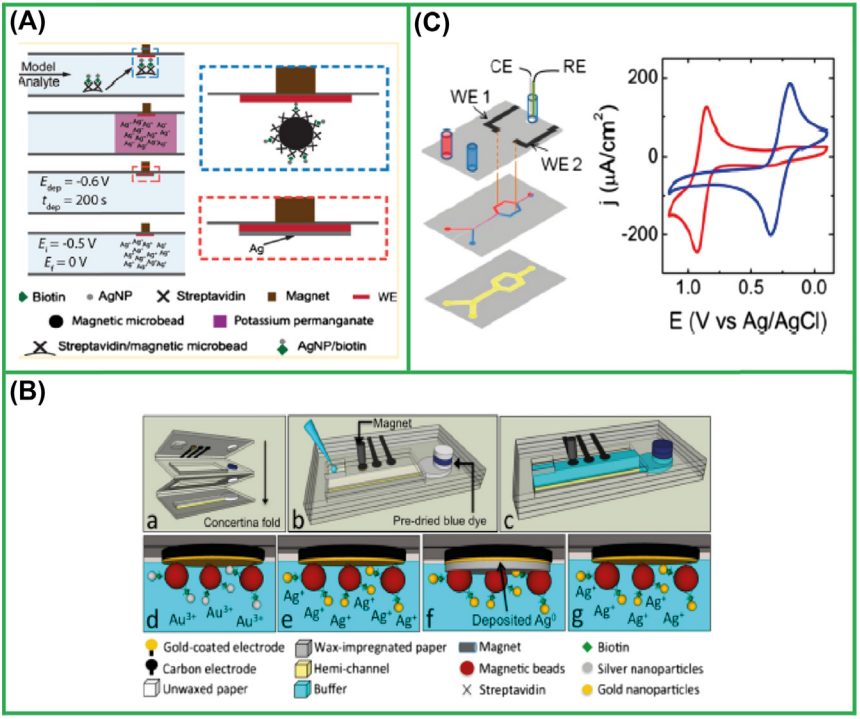

Figure 5 Examples of electrochemical 3D paper-based analytical devices (PADs). (A) Scheme of the 0-slip PAD. The sample flows horizontally across the hollow channel, and the model analyte is concentrated at the working electrode by the magnetic field. Successively, changing the position of the layers, $KMnO_4$ is close to working electrode and MnO_4^- diffuses towards AgNPs, oxidizing them to Ag^+. The Ag^+ is finally detected by anodic stripping voltammetry [44]. (B) Scheme of the no-slip PAD. The sample is loaded at the inlet driven by hemichannel to open channel towards the sink. The predried blue dye is used to indicate when the flow must be stopped and galvanic exchange detection must be initiated (a–c). The mechanism of galvanic exchange is reported in (d–g) [45]. (C) Scheme to quantify the laminar flow in hollow-channel PAD. The red and blue reservoirs were filled with solution of Tris(1,10-phenanthroline) iron(II) sulphate and 1,1-ferrocenedimethanol, respectively. The solutions are flowed for 2 min, and after the flow is stopped and cyclic voltammetry recorded [46].

based on the fabrication of hollow channel in cellulose network [46]. The proposed strategy was able to overcome the drawbacks of the presence of cellulose including low rates of convective mass transfer, significant nonspecific adsorption and a size restriction on the mobility of objects within the cellulose matrix. The authors highlighted that removal of the cellulose fibres from the channels allowed rapid mass transfer yielding reproducible signals. In addition, voltammetric or/and amperometric studies both in underflow

and no-flow have demonstrated that the behaviour is similar to the one obtained using traditional microelectrochemical devices. For instance, a "Y" shaped inlet merging two different solutions into a single channel combined with electrochemical detection was used to quantify the laminar flow.

5. APPLICATIONS OF PAPER-BASED (BIO)SENSORS IN BIOMEDICAL FIELDS

In this section, an overview of the (bio)sensors based on paper with electrochemical transduction described in literature is reported, focussing on the most representative sensors and biosensors applied in biomedical fields.

5.1 Sensors

Research on electrochemical sensors based on paper is gaining momentum in the last years thanks to their high storage and operational stability as well as to their cost-effectiveness and mass production. Most of the sensors have been realized for colorimetric detection of important biomarkers of clinical interest, including thiocyanate in saliva [47], nitrite in saliva [48], glucose, lactate, and uric acid in serum and urine [49]. However, a number of studies have been conducted also towards the design of electrochemical paper-based sensors since electrochemistry can overcome some drawbacks of colorimetric device that, for example, are not capable of analysing coloured samples or providing accurate quantitative analysis.

Important clinical biomarkers have been analysed through this kind of sensors. Whiteside group developed several PADs for the detection of electrolyte ions (i.e., Cl^-, K^+, Na^+ and Ca^{2+}) by ion-selective electrodes exploiting wax-patterned sample and reference zones that each contains a stencil-printed Ag/AgCl electrode [50]. The device was conceived to have measurement zones where sample and reference solutions were added, allowing ionic conductivity between the two solutions and preventing large-scale convective mixing that would shift the potential of the reference electrode or change the concentration of the analyte. For instance, chloride ions detection can be carried out by potentiometry without any modifications of the indicator sensor since Ag/AgCl electrode is proportional to the logarithm of the activity of chloride ions following the Nernst equation. The potentiometric measurements of other electrolytes as K^+, Na^+ and Ca^{2+} required the combination of the sensor with ion selective membranes, which are fabricated using appropriate ionophores. The authors were able to

detect all the electrolyte ions in the range useful for diagnostic uses. Hu et al. [51] described a paper-based nanoporous gold electrode arrays for electrochemical detection of oxygen exploiting the ionic liquid electrolyte 1-butyl-3-methylimidazolium hexafluorophosphate ($BMIMPF_6$). The porous nature of the paper-based sensor allowed the addition of electrolytes from the back of the paper, producing three-phase electrolyte/electrode/gas interfaces at the front of the sensor. This sensor was capable of amperometrically measuring oxygen in 10 s in a linear range from 0.054 to 0.177 v/v %, with a detection limit of 0.0075%. Sensors exploiting the outstanding features of paper have also been applied for different biomedical applications such as the follow-up of drug treatment as well as the quality control of drugs.

A key example was reported by Yu et al. [52] which home-made an analytical device using indium tin oxide (ITO) as working electrode and paper as electrolytic cell to perform electrochemical study of leukaemia K562 cells, to evaluate the cytotoxicity of anticancer drugs. In this case, an ITO glass was combined with punched adhesive tape and filter paper to measure guanine oxidation in cells dropped on a disposable electrode treated or untreated with arsenic trioxide and cyclophosphamide.

Shiroma et al. [53] described the construction of a simple, low-cost and sensitive microfluidic paper-based device combing paper-based chromatography and paper-based electrochemical detection for the detection of paracetamol and 4-aminophenol in pharmaceutical samples. The device consisted of a 2.0-mm separation channel fabricated with wax-printing technique and an electrochemical cell by sputtering technique, obtaining detection limits of 25 and 10 μmol/L for paracetamol and 4-aminophenol, respectively.

5.2 Biosensors

5.2.1 Enzymatic paper-based analytical devices

Enzymes are broadly exploited in paper-based analytical devices thanks to their catalytic abilities to produce both the colorimetric and the electrochemical signals while generating coloured or electroactive products when the target analyte is present. Although colorimetric detection best fits with simpler analytical schemes, electrochemical sensing offers more versatile and quantitative strategies for other applications, such as in turbid and/or coloured matrices. Indeed, the combination of electrochemical detection with enzymes as biocomponents shows a number of advantages in terms of excellent reproducibility, sensitivity and accuracy, as well as flexibility

and foldability in integrated instrumentations, as well as possibility of mass production and affordability.

The applications of enzyme-based electrochemical PADs have been widely described for the detection of clinical biomarkers in biological samples. Dungchai et al. described the first enzymatic PADs using oxidase enzymes to perform simultaneous monitoring of glucose, lactate and uric acid in serum samples [14]. The authors printed by photolithography three electrodes on the hydrophilic and hydrophobic area of paper with the working area of the electrodes on the hydrophilic portion of the device. Thus, glucose oxidase, lactate oxidase and uricase enzymes were immobilized on each printed electrode previously modified with Prussian blue, being a selective catalyst for hydrogen peroxide production. Indeed, H_2O_2 is produced by the enzyme reactions through the transformation of the target substrates, therefore monitored by chronoamperometry at its optimal detection potential, where interferences from endogenous compounds, including uric and ascorbic acid, are minimal (0 V vs. the on-chip Ag/AgCl reference electrode). The three analytes were monitored within a wide concentration range up to 100 mM with relative standard deviations less than 14% ($n = 3$), establishing an acceptable reproducibility for this analytical configuration. Limits of detection of 0.21 and 0.36 mM were obtained for glucose and lactate, making the proposed device capable for clinical diagnosis of all biological samples, in agreement with the normal level of glucose and lactate as well as with the values obtained by traditional tests, demonstrating the feasibility of combining enzymes and paper in the design of microfluidic devices for medical diagnosis.

Noiphung et al. [54] also described the use of glucose oxidase in the design of an electrochemical paper-based analytical device for determining glucose in whole blood samples. They fabricated by wax dipping method a dumb-bell-shaped device containing two blood separation zones with the aim to completely separate whole blood samples with 24%–60% haematocrit without dilution and a middle detection zone where a Prussian blue-modified electrode was screen-printed on alumina and glucose oxidase immobilized (Fig. 6A). Thus, glucose detection was obtained in separated plasma by monitoring the hydrogen peroxide generated from the enzyme reaction, using chronoamperometry at the optimal detection potential for H_2O_2 (−0.1 V vs. Ag/AgCl reference electrode). A linear range was achieved in the range up to 33.1 mM with a %RSD of all glucose concentrations less than 11% ($n = 3$). Human whole blood samples were analysed obtaining results at the 95% confidence interval (pair sample *t*-test;

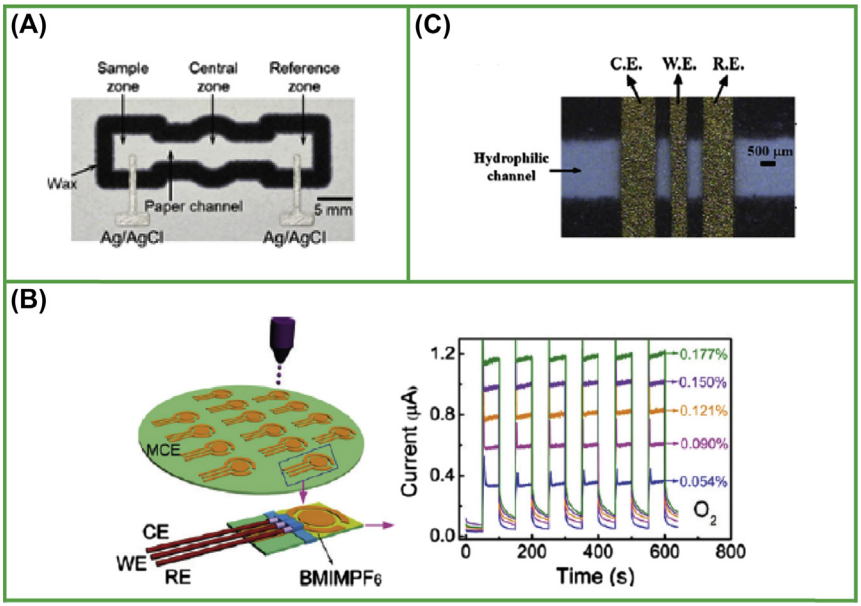

Figure 6 Paper-based analytical devices (PADs). (A) Photograph of a potentiometric PAD for chloride detection [50]. The chromatography paper was patterned by using wax printing technique to delineate (i) sample zone, (ii) central contact zone, (iii) reference zone, (iv) microfluidic channels. Printed Ag/AgCl electrodes are present in the sample and reference zones. (B) PAD for amperometric oxygen detection fabricated by using inkjet printing of gold nanoparticles patterns to fabricated the electrodes and a microsyringe to modify the working electrode with the ionic liquid $BMIMPF_6$ [51]. (C) PAD for paracetamol and 4-aminophenol detection combined with paper-based chromatography [53].

P value = .376) with those generated by traditional spectrophotometric methods based on glucose oxidase and peroxidase reaction.

A number of electrochemical enzyme-based PADs have been realized also for the analysis of urine samples. Li et al. [55] fabricated paper electrochemical devices as test strips combined with a commercial glucometer to detect glucose in artificial urine based on a handheld pressure-assisted ball pen, where conductive inks are capable to flow through the roller ball without the need for any ink pretreatment. Since paper is a porous substrate and can rapidly transfer the sample to the electrode surface, the authors proposed an alternative method for electrode modification, storing reagents ($K_3Fe(CN)_6$ and glucose oxidase) on the filter paper covering the electrode surfaces. The proposed device was able to detect glucose in the range of 2.0–20.0 mM in the sample solutions, with a $R^2 = 0.9982$. Lankelma

[56] realized a novel noninvasive, quantitative assay for the determination of glucose in urine proposing a new design of PAD system for flow-injection analysis. The system comprised a nitrocellulose channel in contact with both the buffer reservoir and a long strip of filter paper dipped in a lower reservoir acting as a sink. The nitrocellulose lied horizontally on a working electrode, which consisted of a thin platinum layer deposited on a Petri dish, while counter and reference electrodes were positioned upstream in the buffer reservoir. This difference in height between reservoir and the sink drove the flow of buffer generated by wicking at a 2 μL/min rate. The flow rate can be tailored varying the thickness, length, width of the paper, as well as varying the height difference between the buffer reservoir and the sink. Nie et al. [15] proposed the use of glucose oxidase immobilized on the top of paper microchannels fabricated by chromatographic and photolithographic patterning or wax printing with two kinds of paper, namely Whatman 1 Chr and polyester—cellulose blend paper (VWR Spec-Wipe). These papers were chosen because of not deforming on the surface of electrodes when wetted by fluids. Chronoamperometric analysis were performed using a +500 mV step potential (vs. a carbon pseudoreference electrode) to detect glucose in urine samples within a concentration range up to 22.2 mM (corresponding to 400 mg/dL), with a detection limit of 0.22 mM (corresponding to 4 mg/mL). The great advantage of such paper-based device is that the paper microchannels are able to confine fluids inhibiting their movements and thus facilitating the chronoamperometric analysis by minimizing the turbulences of the stationary boundary layer near electrodes due to vibration, thermal/density convection or other disturbing sources.

Immobilization methods can considerably influence the shelf life of enzymes, affecting the suitability of commercial paper-based analytical device in terms of robustness, especially during storage and transport when sensor could be exposed to threatening physical conditions (e.g., high temperature). Nery and Kubota [57] described in their review a wide assortment of methods for immobilizing glucose oxidase on paper, including adsorption, gel entrapment, layer-by-layer entrapment, microencapsulation and covalent linkage to better highlight the advantages of different strategy in enhancing the performance of the sensing system. Indeed, storage stability at room temperature can be increased by entrapping enzymes in polymer matrixes able to stabilize the protein structure. A higher enzymatic activity can be obtained by mixing enzymes with bilayer ionic polymers to cover the paper. Finally, immobilization via covalent linkage

can prevent enzymes leakage during washing steps. Beside immobilization methods, fabrication concepts can also affect the performance of the PADs in terms of precision and accuracy. As an example, Malekghasemi et al. [58] proposed multiple instruments and fabrication steps using two different chemicals (hexamethyldisilazane and tetraethyl orthosilicate) and three different methods (heating, plasma treatment and microwave irradiation) to develop electrochemical enzyme-based PADs. In particular, the authors demonstrated that the combination of microwave irradiation with inkjet-printing technique was crucial to fabricate robust and easy-to-use microfluidic devices with fast and cost-effective procedures. The proposed innovative configuration was validated utilizing the rapid urease test as a model system to quantify its limit of detection and to test its response time. According to experimental results, this device was able to sense 0.5 units of urease within 3 min.

Nanotechnology, multidetection and microfluidics have also high potential in enhancing the maturity of enzyme-based PADs for reaching the marketability in biomedical field, in terms of robustness, versatility, sensitivity and sample volume reduction. Paper-based PADs seem to be able of furnishing all these crucial requirements, demonstrating a great potential for driving biosensor technology towards a ground-breaking revolution.

5.2.2 Immuno–paper-based analytical devices

The requirement for faster analyses in immunoassays has moved the research activities to replace enzyme labels with metallic nanoparticles to avoid enzymatic reaction time as well as common substrate with paper, allowing multiple analyses and reagent loading.

Cao et al. [59] introduced an electrochemical immunofiltration analysis in μPADs for human chorionic gonadotropin (HCG) detection. The hydrophilic zones of aldehyde-functionalized screen-printed electrodes were functionalized with capture antibodies and the detection was performed by using a primary antibody functionalized with gold nanoparticles and an alkaline phosphatase conjugated secondary antibody. In detail, the samples moved from the sample application PAD through the conjugate PAD, where gold nanoparticles bioconjugates were immobilized. The HCG antigen reacted and formed the immunocomplex, migrating towards the detection zone. Subsequently, the enzyme conjugated with secondary antibody was introduced into the immunofiltration strip to label the

immunocomplex. Finally, the formed sandwich complex was quantified by differential pulse voltammetry (DPV) adding the enzymatic substrate.

This immunosensor was characterized for the detection of HCG in the linear range 1.0 mIU/mL−100.0 IU mL with a detection limit of 0.36 mIU mL and optimized in real human serum with satisfactory results. Without any doubt, last trends on nanotechnology provided a significant enhancement of PADs performances for their applications in biomedical field. In particular, new smart paper-based devices have been described in literature by taking advantage of the recognized effects arising from the reduced sizes of nanomaterials, including nanoparticles [60], carbon nanotubes [61] and quantum dots [62]. For example, Sun et al. [60] reported a paper-based immunoassay consisting of working electrodes modified with flower-like Au nanoparticles and the capture antibody (Fig. 7A). This immunosensor was incubated with a sample containing a tumour biomarker, followed the antibody labelled with bimetallic nanoparticles. Amperometric measurements were performed to evaluate the immunocomplex formation adding H_2O_2, since the bimetallic nanoparticle tracer catalyzed the H_2O_2 reduction. Under optimized experimental conditions, the proposed immunosensor exhibited excellent analytical performance for enzyme-free detection of carcinoembryonic antigen, with a detection limit of 0.3 pg/mL, showing great promise for clinical application. Multiplexed immunoassay has also recently attracted significant awareness due to its advantages in fast, cost-effective and throughput analysis, in early screening of a wide range of diseases and in the monitoring the response

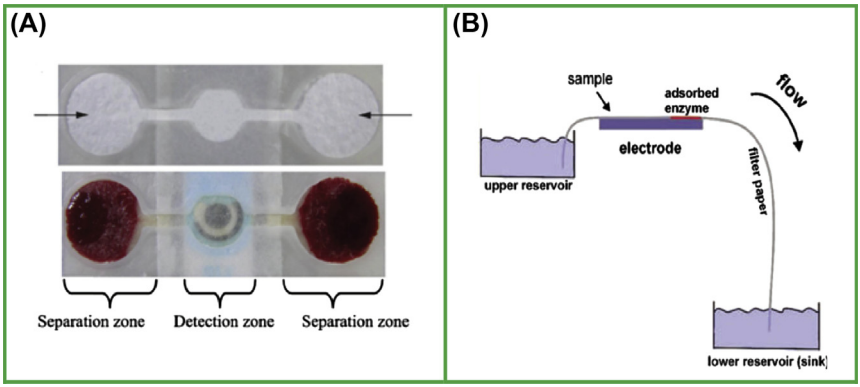

Figure 7 Enzymatic paper-based analytical devices (PADs). (A) Picture of dumb-bell-shaped enzymatic PAD for whole blood separation and glucose detection [54]. (B) Scheme of glucose detection in urine samples using gravity-driven flow [56].

to specific therapies. Ge et al. [63] presented a 24 electrodes array constructed on square paper using a photolithographic method and assembled on the crossing points of 4 rows × 6 columns electrodes, with a paper layer containing the sensing sites and another paper layer hosting printed counter and reference electrodes. This device was able to analyse four tumour markers with limits of detection of 1.5×10^{-4} ng/mL, 3.7×10^{-5} U/mL, 2.6×10^{-5} U/mL and 2.0×10^{-5} ng/mL.

A smart immune-based PAD with multiplexing and telemedicine capabilities was also realized by Zhao et al. [64] for diagnosing HIV/HCV coinfection. The authors presented a portable paper-based platform for multiplexed electrochemical detection of antibody markers of HIV and HCV in serum samples, consisting of an electrochemical microfluidic paper-based immunosensor array and a handheld multichannel potentiostat, which was able to improve the throughput detection. The potentiostat is constituted of a microcontroller unit (MCU; ATxmega32A4, Atmel) with a 12-bit analog-to-digital converter (ADC) and a 12-bit digital-to-analog converter (DAC), a signal multiplexing/demultiplexing unit (74HC4051D, NXP Semiconductors), a signal processing circuit (for converting an electrochemical current into a voltage), a liquid crystal display (LCD; EADOGM163EA, Electronic Assembly), a universal serial bus (USB) to universal asynchronous receiver/transmitter (UART) interface circuit (FT232RL, FTDI), a Bluetooth wireless communication unit and a 9-V battery, with a total cost of $60 (Fig. 7B). This unique integration allowed projecting a portable, low-cost, user-friendly and high-throughput platform supporting the approach of telemedicine.

5.2.3 Nucleic acid paper-based analytical devices

Nucleic acid sensors have received noteworthy consideration thanks to their potential applications not only in clinical diagnostics and genetics but also in forensic field, food/drug sectors and environmental control. Although many traditional biosensors have been developed exploiting nucleic acids, they suffer from some drawbacks related, for example, to the addition of reagents, which in case of paper-based devices can be directly loaded on reservoirs fabricated on the paper. Thus, paper-based biosensors have been widely studied as valid alternative to POC device as they offer simplicity, low power requirements and environmental sustainability. In addition, the combination of electrochemical PADs with DNA hybridization detection approach can provide fast, cost-effective, and highly selective analysis. In this context, the ideal analytical device needs to meet several requirements when

analysing biological fluids, including sample treatment as well as extraction/amplification of the DNA for the analysis [65] (Fig. 7A). To accomplish these crucial requests, several paper-based devices have been designed exploiting optical transduction. For instance, Choi et al. [66] realized for the first time an integrated paper-based DNA sensor incorporating nucleic acid extraction, amplification and visual detection of *Streptococcus pneumonia* in clinical blood samples (Fig. 7B). The author developed a handheld battery-powered heating device for nucleic acid amplification in POC settings, coupled with the optical detection, allowing for very fast analysis (1 h instead of 5 h for the entire sample-to-answer process). Beside PADs based on optical detection, which actually represent the most advanced technology platform currently available, electrochemical DNA-based PADs are beginning to flourish.

A crucial example was published on *Analytical Chemistry* in 2015 by Crooks group [67], which realized a 0-slip PAD to detect a 30-base nucleotide sequence characteristic of DNA from the hepatitis B virus with a detection limit of 85 pM using the metalloimmunoassay above-reported (Fig. 8A). This low detection limit achieved is due to two preconcentration steps performed using silver nanoparticle as labels and magnetic microbeads. In this format, the DNA detection combines simple origami (paper folding) assembly, the open structure of a hollow-channel paper and a convenient slip layer for timing incubation steps. Another approach for designing nucleic acid PADs is based on 3D origami device as the one described by Lu et al. [68].

The authors developed an origami sensor constituted of probe, target DNA and reporter probe conceived as follows:

1. a printed electrode was modified with graphene and gold nanoparticles to immobile the capture probe, exploiting the interaction between the SH group of capture probe (5′-TGG AAA ATC TCT AGC AGT CGT-$(CH_2)_6$-SH-3′) and the gold nanoparticles;
2. an incubation with mercaptohexanol to have a well-aligned DNA monolayer, after the immobilization of capture probe, was carried out;
3. target DNA at different concentrations for a desired time at 37°C was coated on the electrode for the hybridization reaction;
4. the detection was based on a sandwich complex using bioconjugates as amplification label. In detail, thionine (TH) bound to double-stranded DNA (dsDNA) was used as signal tag (TH/D1), together with complementary ssDNA (5′-NH_2-$(CH_2)_6$-ATG TCC CTC AGA CCC TTT-3′) immobilized on nanoporous gold forming an S3-TH/D1-NPG bioconjugates (Fig. 8B).

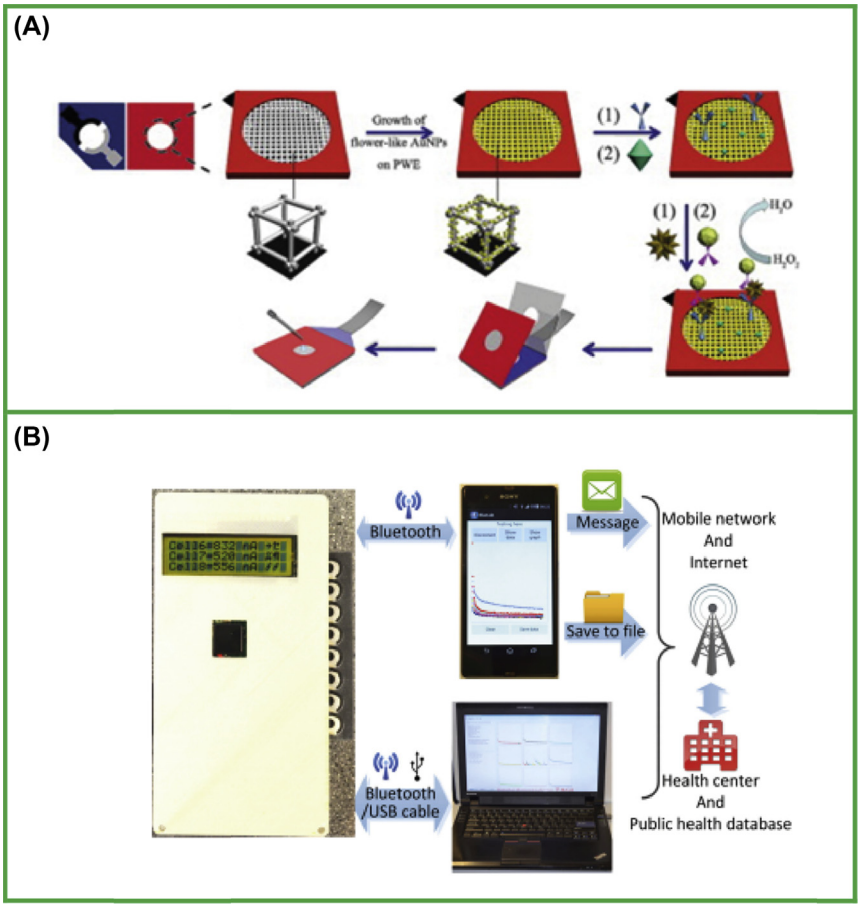

Figure 8 Immuno—paper-based analytical devices (PADs). (A) Origami immuno-PAD for carcinoembryonic antigen detection using working electrodes modified with flower-like Au nanoparticles and the capture antibody labelled with Au-Ag [60]. (B) Multiplexed PAD for the detection of antibody markers of HIV and HCV in serum samples combined handheld multichannel potentiostat able to send data by wireless to PC or smart phone and finally to remote site [64].

Adding the electrolyte, the prepared DNA paper-based sensor was able to perform the electrochemical analysis measuring the signals as proportional to the amount of the reporter DNA, correspondent to the concentration of target DNA. Under optimized experimental conditions, peak currents were linearly proportional to the logarithm of target DNA concentration in the range from 8×10^{-16} to 5×10^{-10} mmol/L, with a limit of quantification of 8×10^{-16} mmol/L. The presence of nanoporous gold with high specific

surface area further increases the amount of TH/D1 and complementary DNA, offering a significant amplification for DNA detection. Beside DNA hybridization, paper-based devices can be also configured with aptamers and peptide nucleic acids as bioreceptors for the detection of analyte of clinical interest. Su et al. [69] developed a paper-based aptamer sensor for human acute promyelocytic leukaemia cells (Fig. 9C).

In detail, Au-paper electrode fabricated through the growth of a gold nanoparticle layer on the surfaces of cellulose fibres in the hydrophilic paper zone of paper electrode was employed as the working electrode. For a selective detection of K562 cancerous cell detection, cell-targeting aptamers KH1C12 were chosen as bioreceptors to functionalize this Au-paper electrode.

To measure the presence of K562 cancerous cell, HRP bioprobe was used for its specific recognition of corresponding glycan on the captured K562 cell surface, and the electrochemical signal due to the HRP catalyzed oxidation of *o*-phenylenediamine by H_2O_2 was measured. The DPV response was directly related to the amount of the immobilized HRP, which depended on the captured cell numbers, giving a detection limit of 400 cells/mL. This origami aptamer-based PAD was successfully applied for in situ anticancer drug screening in a high throughput manner. The same group developed a microfluidic paper origami cyto-device based on electrochemiluminescence (ECL), in which aptamers modified 3D macroporous Au-paper electrodes were employed as working electrodes and efficient platforms for specific cancer cells (MCF-7, HL-60, K562 and CCRF-CEM) capture [70]. Owing to the effective disproportionation of hydrogen peroxide and specific recognition of mannose on cell surface, concanavalin-A conjugated porous AuPd alloy nanoparticles were introduced into this PAD as catalytically promoted nanolabels for peroxydisulfate ECL system. Under optimized experimental conditions, MCF-7 cells were analysed in the range of 450−1.0×10^7 cells/mL with a detection limit of 250 cells/mL. Teengam et al. [71] exploited the peptide nucleic acid acpcPNA probe with the sequence Ac-(Glu)3-CATACACCTCCAGC-Lys(AQ)NH2 (written in the N/C direction, Ac = acetyl; AQ = anthraquinone; Glu = glutamic acid; Lys = lysine), to develop a novel paper-based electrochemical DNA biosensor to detect human papillomavirus (HPV) (Fig. 9D). The sensor was fabricated using inkjet printing technique and modified with graphene−polyaniline and acpcPNA probe labelled with anthraquinone. This biosensor was able to

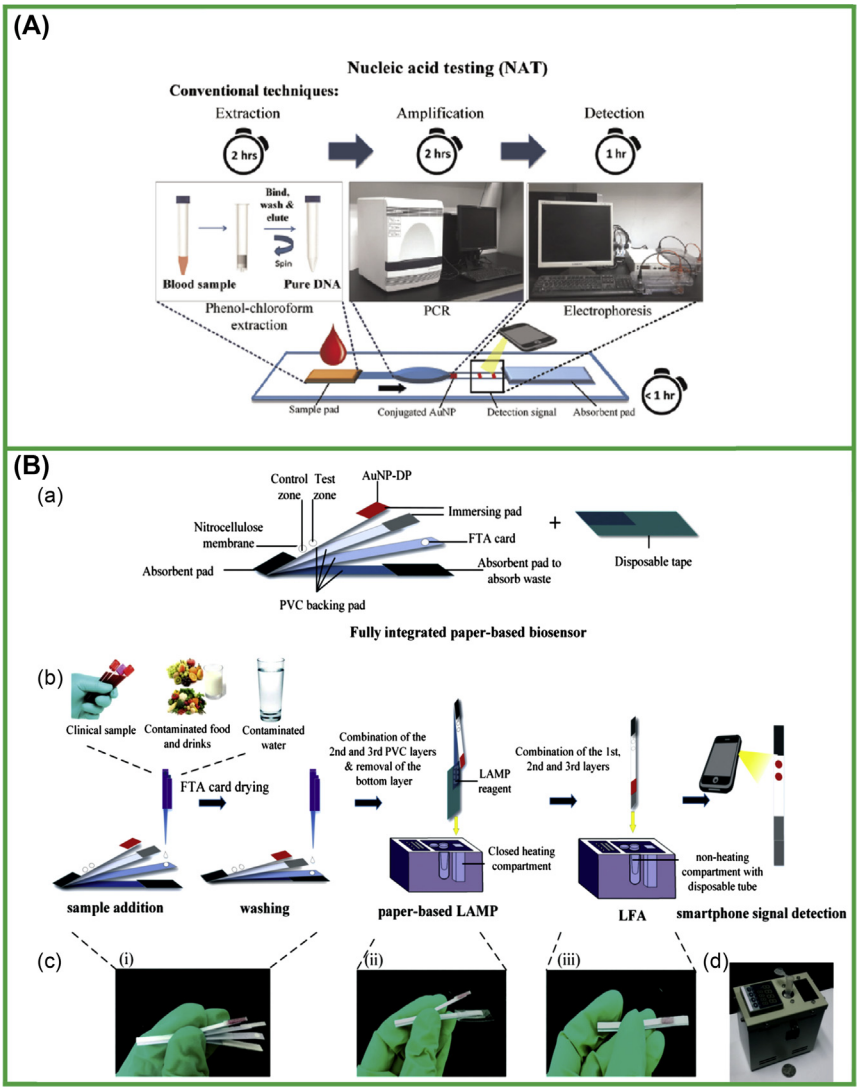

Figure 9 Examples of advances colorimetric nucleic acid paper-based analytical devices (PADs). (A) Ideal-paper-based sample-to-answer molecular diagnostic device [65]. (B) Colorimetric advanced DNA-PADs for *Streptococcus pneumonia* in clinical blood samples [66].

detect a synthetic 14-base oligonucleotide target sequence corresponding to HPV type 16 DNA, measuring the electrochemical signal of the AQ label by square wave voltammetry before and after hybridization, with a detection limit of 2.3 nM (Fig. 10).

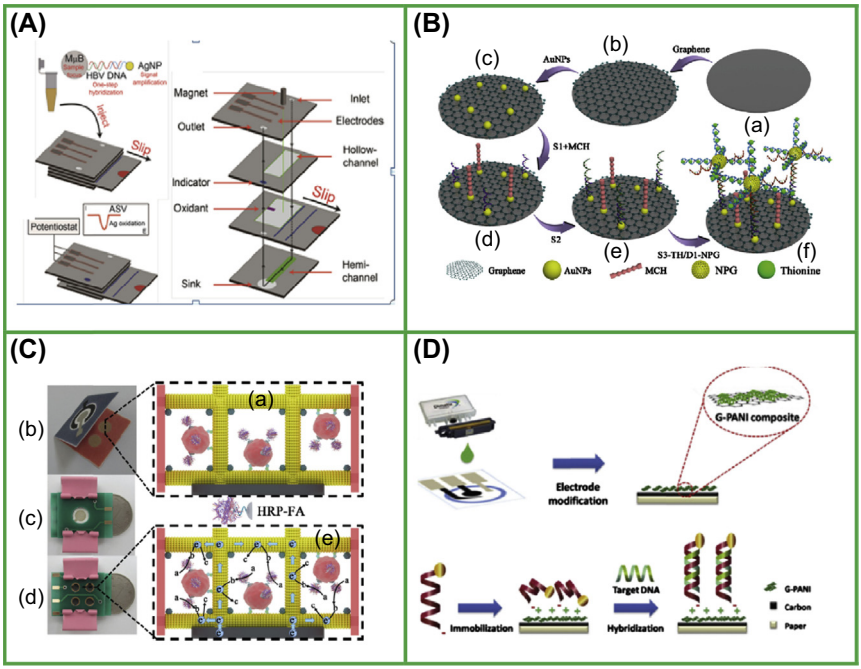

Figure 10 Examples of electrochemical nucleic acid paper-based analytical devices (PADs). (A) 0-slip PAD for the detection of a 30-base nucleotide sequence characteristic of DNA from the hepatitis B virus [67]. (B) Scheme of the mechanism of an origami PAD for the detection of target DNA (5′-ACT GCT AGA GAT TTT CCACACTGAC-TAAAAGGGTCTGAGGGA-3′) [68]. (C) Schemes and pictures of the PADs for the detection of human acute promyelocytic leukaemia cells cancer cell using aptamers as biocomponent and electrochemical detection [69]. (D) Scheme of peptide nucleic acid biosensor for 14-base oligonucleotide target with a sequence corresponding to human papillomavirus type 16 DNA [70].

6. PERSPECTIVES AND CONCLUSIONS

Several research efforts have been accomplished in the last years towards the design of innovative devices capable of fast, accurate, easy and sensitive analysis of clinical samples with the main aim to furnish proficient tools for disease diagnosis and follow-up of therapy efficiency. This goal can be achieved through the design of custom-made electrochemical PADs for biomedical applications, which show outstanding features in terms of reliability, cost-effectiveness, easiness of measurement even in complex matrices (e.g., blood) and environmentally friendless.

Indeed, paper has a great potential as substrate for sensing devices being capable of containing bioreceptors in large concentration, loading reagents in specific reservoirs avoiding pumps or external equipment, as well as providing sample treatment requiring microvolumes of fluid. In addition, functionalization of paper substrate or bioreceptors with nanomaterials can improve the analytical performances of the whole system, as largely observed in literature. Moreover, multiple assays can be developed simultaneously, and integration with telemedicine for healthcare services is a matter of fact. Finally, paper-based devices are portable, ease to use, cost-effective, environmentally friendly, and they can be integrated in user-friendly diagnostic platforms, i.e., handheld glucometer.

Although these remarkable advantages, following the literature reported in this chapter on existing paper-based (bio)sensors, it is clear that extensive development has been made but several others should still be reached.

Further research needs to be considered to overcome some issues associated with:

1. fabrication techniques and incorporation of functional materials on paper surface;
2. evaporation and retention of the sample causing inefficiencies in sample delivery;
3. multiple steps and premixing of samples, which may hinder home-based healthcare;
4. whole-genome/transcriptome amplification, which may suffer from bias and nonspecific products; and
5. validation in biological matrices.

Another requirement to be faced is the development of handheld readout devices, printed on paper to fabricate an entirely paper-based sensing tool easy to dispose of, i.e., incineration. If 2D and 3D PADs have had a high impact on POC field, a device entirely printed on paper can completely change the POC global market having a high impact on both healthcare and environment. Future perspectives should focus on the strict collaboration of different disciplines to fabricate entire ecodesigned and sustainable sensors, giving useful tools in biomedical field without any, or with a minimum, impact on environment.

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FURTHER READING

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