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Printable biosensors towards next-generation point-of-care testing: paper substrate as an example

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Abstract

Printable biosensors have gained numerous exciting advancements towards downstream applications in fundamental biomedical research, healthcare, food safety, environmental monitoring and governance, and to name a few. Particularly, paper-based printable biosensors have gained rising popularity in providing affordable platforms due to their merits, such as cost-effective, accurate, simple, and efficient detection of diseases for clinical diagnosis. In addition to advantages and opportunities in point-of-care detection, printable biosensors are also facing challenges. Herein, this review aims to provide a systematic summary of the development of printable biosensors, with a special focus on paper-based printable biosensors. Different types of substrates for printable biosensors are highlighted with a focus on paper substrates which have superior properties like low-cost, simple, flexible, lightweight, recyclable, etc. In addition, current printing technologies to fabricate paper-based sensors, including wax printing, photolithography, screen printing, inkjet printing, and laser printing summarize, are discussed, together with strategies for biomolecular fabrication on substrates and transducers. Finally, we also discuss the challenges and possible future perspectives, hoping to provide researchers and clinicians with informative insights into paper-based printable biosensors for smart and effective point-of-care detection.

Keywords: Printable biosensors, point-of-care testing, microfluidic paper-based analytical devices, challenges

1. Introduction

A biosensor is an analytical device that uses a biological recognition system to investigate or detect molecules, often including three main parts: 1) a biorecognition element with high specificity and selectivity to the target analyte; 2) a transducer to convert the resulting signal from the interaction between analytes and recognition elements into a measurable and quantifiable signal, and 3) a signal processor to report the analyte signal[1]. Biosensors play an irreplaceable role in biomedical diagnosis, treatment and disease progression monitoring, drug discovery, food control, and environmental monitoring. The current widespread of COVID-19 has largely emphasized the importance of developing biosensors for point-of-care testing (POCT) to provide timely prevention and control of the pandemic. POC biosensors allow the quantitative detection of clinically relevant biomarkers in biological fluids outside conventional laboratories, thus facilitating an early diagnosis and prompt treatment[2]. Compared with labor- and time-consuming traditional diagnostic methods, POCT has been characterized to be ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free and Deliverable to the end user)[3]. Biosensors are traditionally fabricated in a stepwise manner, therefore it is time and cost-consuming when considering designing a new type of biosensors. Although the maturing of 3D printing technology is making up for this shortcoming, the need for large-scale industrial production makes printable biosensors especially essential and attractive in POCT. There are many advantages for using printing technologies to fabricate biosensors, such as simplifying fabrication steps, reducing fabrication costs, decreasing variations caused by stepwise fabrication, simplicity in use, and so on[4]. Printed strips like pregnancy test strips and electrochemical glucose test strips have been successfully integrated into the current clinical applications. Hence, as an emerging and attractive area, printable biosensors hold great potential in POCT.

As the development of wearable technologies and digital healthcare management, printable biosensors have progressed significantly, various printable biosensors have been invented in both academia and industry. Although some existing literatures have reviewed the printing technologies[5], different types of biosensor according to the detection strategies[6] [7] or the bio-element immobilization methods[8], most reviews are limited to a single topic and lack an overall overview of the area. More importantly, most reviews have ignored the challenges and future perspective of the paper-based printed biosensors. Herein, we comprehensively and systematically discussed the most advanced printable biosensor development in the recent years, from substrate materials and substrate fabrication, to printing technologies and their applications. Paper is further selected as the model substrate to discuss printing technologies to generate POC biosensors. Based on printing techniques and printable properties, we further display a more comprehensive view of how the paper-based biosensors are fabricated. Subsequently, surface treatment of paper surfaces and assay immobilization strategies with different signal readouts are highlighted. Finally, challenges and the perspectives associated with printable biosensors are discussed. This review shed a light on the printable biosensors towards cost-effective point-of-care or point-of-need detection in

modern analytical science.

2. Substrates for printable biosensors

2.1 Substrate materials suitable for printable biosensors

The substrate is the main component of the biosensor to accommodate biological molecules recognition, achieving sensing purpose[9]. Today, printing technology is one of the most promising and compatible manufacturing approaches enabling cost-effective and large-scale fabrication. Also, the effective utilization of materials in the development process makes the print-on-demand approach extremely attractive[4]. Generally, some basic requirements of suitable substrates for printable biosensors should be considered, including dimensional stability, surface smoothness, low coefficient of thermal expansion, conductive and barrier properties for moisture and gases, solvent resistance, etc.[2, 10]. If we extend the definition of "printing" that conventionally means depending on inks and printers to a wider definition that includes all additive/computer controlled techniques to fabricate patterns, circuits, and active or passive electronical/biological components of biosensors, then actually, various materials can meet the requirements of substrate to some extent and be applied in different fields in printable biosensing. Common materials include glass[11], silicon[12], ceramics[31], metal[13], polymer[14], bio-derived materials[15] and paper, using different printing techniques.

Among all these materials, polymer materials like polydimethylsiloxane (PDMS) polymethyl methacrylate (PMMA), polyethylene terephthalate (PET), polyethylene naphthalene (PEN) and many other thermoplastic polymers are the most used substrate materials for now[16]. More and more polymer based tattoo-like biosensors have been applied for healthcare diagnosis[17]. Polymer materials have high flexibility, stability and relatively good biocompatibility. Also, the price and initial investment of polymer based POCT devices was low. Polymer based materials could also be air permeable, for example, Kim et al. recently introduced a soft poroelastic biosensor for simultaneous epicardial recording and imaging[18]. This substrate material has unique structural properties, which yielded a robust coupling to living tissues, enabling high-fidelity recording of spatiotemporal electrophysiological activity and real-time ultrasound imaging for visual feedback. However, polymer based substrate still has many imperfections. For example, polymer materials are relatively difficult to be functionalized, especially considering the possible change of properties that functionalization may induce to the materials. As a specific example, in recent years, conductive polymers have been used to produce biosensors[19], however, due to steric effects, the presence of any group would increase the chain-to-chain distance and decreases interchain conductivity, which will decrease the biosensor's performance[20]. Also, many polymer materials cannot be degraded, which could induce waste management crisis[21].

Although materials like glass, silicon, ceramics and metal have poor flexibility, they represent an alternative solution to plastic films when high temperature or complex structure (micro/nanoscale) fabrication is required during microfluidic biosensor

manufacturing[13]. Such materials could also be printable by taking depositing solution-based functional materials at designed locations to form desired patterns or circuits, or laser cutting/ablation as printing process. Actually, silicon-based technologies such as micro electrical-mechanical systems offer many advantages for the fabrication of miniaturized Lab-on-Chip, especially if combined with plastic materials[4]. However, these materials as biosensor substrates are expensive, energy-consuming, time intensive, not compatible with fragile bioreceptor molecules[22] and not bio-degradable[23]. Therefore, such materials are seldomly used for large-scale biosensor fabrication, let alone POCT purposes.

In recent years, bio-derived materials, including silk proteins[15], polysaccharides, gelatin, chitin and chitosan [24] have received much attention as substrates for printable and wearable biosensors for their green and sustainable properties like biocompatible and biodegradable. These materials are easy to be functionalized with bioreceptors and combined with synthetic polymers [15b, 25]. Similarly, textiles substrates including wool, cotton, or synthetics like nylon, polyester, polyaniline etc., also become emerging materials for printed wearable biosensors due to their excellent properties such as intrinsic flexibility, durability, and stability[26]. Photolithographic and inkjet printing are the most common printing methods that have been applied to the pattern/circuit fabrication process. However, these bio-derived materials also have many disadvantages, including high cost, lack of sufficient physical strength and potentially irreversible damage, resulting in their limitations in working as wearable sensors[27].

2.2 Paper-based printable piosensors

In addition to materials introduced above, paper, as an emerging alternative biosensor substrate, has gradually gained popularity for printable biosensor fabrication, due to its low cost, simplicity, flexibility, portability[28] and easy disposition, high-throughput and volume manufacturability[29]. Paper has been used as a substrate material in analytical testing for centuries. However, only until 2007 did Martinez et al. report the first microfluidic paper-based analytical devices (μPADs), demonstrating the potential of paper in POCT, especially in limited resource settings[30]. Although paper itself (without printing) could be directly used for some simple colorimetric biosensing purpose[31], most paper-based biosensors are still based on printing techniques because printing is extremely suitable for large-scale fabrication requirement on paper substrate and allows for more advanced uses and designs. Among various paper-based biosensors, lateral flow assay (LFA) is still the most popular μPADs in POCT due to its versatile applications, although it is normally used for qualitative or semi-quantitative analysis [10]. Compared with LFA, μPADs combine the simplicity of paper strips, and the complexity of the conventional POC devices that are adaptable for multiplex and quantitative analysis, and are keeping on being the spot light of printed biosensors.

Paper is a cellulosic material that has many unique and excellent properties for fabricating biosensors, such as low cost (a simple μPAD typically can be manufactured for under $0.01) and rich surface chemistry (paper is chirality and can be modified with various functional groups to introduce proteins, DNA, or small molecules[32]). Paper is

easy to be stored and transported[33] due to its enhanced mechanical properties like high specific stiffness, lightweight (~10 mg/cm²) and low thickness (0.07-1 mm). Its high flexibility and deformability facilitate the complex 3-dimensinal (3D) structural formation and accommodate different printing techniques. As one of paper's most important features[34], the porous structure brings high air permeability, free diffusion of gas[35], reagents storage[69], filtration of sample and allows for separating analytes by chromatographic method[70]. In addition, the high surface-to-volume ratio of paper increases the number of enzyme molecules or colourimetric probes that can be immobilized[36], leading to improved detection sensitivity for colorimetric methods[37]. Also, because of capillary action and hydrophilicity, paper allows for the passive transportation of liquids, facilitating LFA with high energy efficiency[38]. The unique characteristics of paper, such as its eco-friendly nature with easy decomposition[32], impressive biocompatibility[7] [39] and potential to provide a strong contrast to a coloured substrate[40], give it advantages as a substrate for colorimetric tests.

However, the limitation of the paper-based printable biosensor is also obvious. First, paper devices are fragile and susceptible to tearing[35]. It is non-reusable because the cellulose membranes' porous structure is deformed and loses the ability of capillary wicking by repeated wetting/drying cycles during treatment[41]. Also, paper-based kits are often limited to qualitative or semi-quantitative analysis, lacking the sensitivity, accuracy and multiplex analysis capabilities offered by conventional analytical instruments[1]. More importantly, the principles behind fluid flow, biomolecule immobilization and biorecognition on paper are complex and remain elusive [42]. That being said, numerous ongoing works are dedicated to improving paper-based biochips. For example, to improve the paper-based biosensing sensitivity, signal enhancement techniques, changing the readout type, or the use of instrumentation have all been applied[43]. However, those modifications could contribute to the complixicity and increased cost. A balance between the production cost and modifications of paper-based biosensors with desirable function should be considered during the development stages.

Particularly, due to the paper's excellent flexibility and foldability, 2-dimensional (2D) structured μPAD emerged in 2008 followed by the 3D structured biosensors[44]. Compared with 2D devices that only allows for LFA, 3D paper-based microfluidic devices that combine lateral and vertical flow components offer several advantages including shorter analysis time and greater spatial discretion. The early 3D structured paper-based biosensors (Fig. 1A) did not require complex fabrication procedures, and yet have been effective in colorimetric-based bioassays and ELISA[45]. Today, with the rapid development of integrated detection techniques, there have been many improvements compared with the original ones. Generally, the second generation technology was first developed by Josephine's group in 2011 using the principles of origami[46]. Origami structured 3D μPADs (Fig. 1B) could provide a one-step reaction procedure, as products formed in multiple layers can serve as reactants in subsequent layers[45a, 47]. Also, this design can effectively eliminate problems of reagent diffusion by lateral flow in the channels of planar paper devices and thus avoid the incompatibilities of reagents located in different zones[47-48]. Josephine's group reported

the first 3D origami paper device for potentiometric biosensing, called NoSlip (Fig. 1C) in 2016. By folding and unfolding the paper structures, their device could perform versatile potentiometric functions[49]. In 2018, Elizabeth et al. developed an interesting LEGO-block like library of prefabricated modular components that connect-and-react for the easy engineering of paper analytical devices. Each component, called Asynchronous Modular Paperfluidic Linear Instrument-free (Ampli) (Fig. 1D), was designed to contain paper-based fluidic elements preformed into shapes that passively store, transport, split, and mix reagents[50]. Their design enables user-driven design on demand, without the need for subsequent fabrication equipment. More recently, Cao et al. reported a sensitive wax/screen printed μPAD with electrochemical signal readout (Fig. 1E). The patterns were fabricated on cellulose paper and then the pre-patterned paper was folded for four times to form five stacked layers[51]. Their design is a good example of 3D printed skin based wearable biosensor.

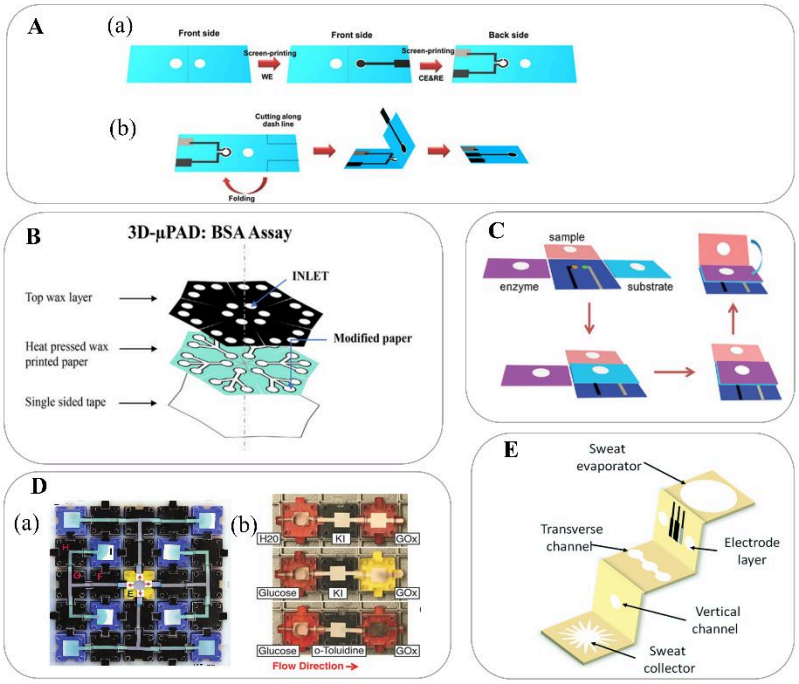

Fig. 1 3D paper-based biosensors: (A) (a) Fabrication procedure of paper-based electrochemical device for human IFN-γ detection, and (b) origami folding sequence[48], reprinted with permission from Elsevier; (B) Depiction of the different layers and materials used in the BSA assay 3D-μPAD fabrication[45a], reprinted with permission from wiley; (C) a 3D origami potentiometric paper-based device[47], reprinted with permission from wiley; (D) (a) LEGO-block system structure biosensor: Ampli, and (b) Glucose reaction circuit with swappable components shows ability to select and fabricate different reaction pathways[50], reprinted with permission from wiley; (E) Layered structure of 3D paper-based microfluidic electrochemical integrated device[51], reprinted with permission Royal Society of Chemistry.

Different types of paper, including chromatography papers[52], filter papers[53], nitrocellulose membranes[54], glossy papers[55], office papers[56], paper towels[57], graphene paper[58] et al., have their unique properties and have been applied for various

fields. The comparison of different types of papers has been discussed in previous reviews[7]. Among them, chromatography paper seems to be the most advantageous, with a flat arrangement for smooth fluid flow and simplicity for mass production, while office paper is rendered to be the most popular option with its wide availability and low-cost[59]. Generally, the pore size and thickness of the paper determine the ultimate usability because they impact the fluid flow and the strength of the device[60]. For most PADs fabrication, reduced thickness of paper substrates is preferable as thinner paper substrates and requires less wax or ink, resulting in a cost reduction. In recent years, multiple detection approaches, such as colorimetric techniques, chemiluminescence, electrochemiluminescence, fluorescence, and electrochemistry, have been employed for paper-based detection platforms, which will be discussed in Section 4.

3. Different printing technologies of paper-based biosensors

Different conductive and non-conductive materials could be used to fabricate printable biosensors via various printing techniques. It should be noticed that the 'printable' here should be extended to more generalized definition which refers to 'all additive/computer controlled techniques to fabricate patterns, circuits, and active or passive electronical/biological components of biosensors'. In this case, the patterning of reaction zones and detection areas and the possible modification process related are one of the most important steps in fabrication of printed paper-based devices. Generally, there are two methods to fabricate the reaction zones and detection zones. The first method is to define hydrophobic zones in the cellulose substrate using hydrophobic materials (like wax). The second method is to cut the paper to define the physical boundaries of the delimit channels and the detection zones[61]. In this section, we will discuss the main methods that are commonly used to fabricate and modify the liquid flow channels on the paper surface, including drop casting, vacuum filtration, inkjet printing, wax printing, screen printing, stencil printing, laser printing and photolithography[7] and their updated applications. Noticeably, Ataide et al. reported an excellent review focusing on the printing technologies for electrochemical paper-based biosensors[61].

3.1 Drop casting

Drop casting is a simple method without the requirement of sophisticated equipment and is frequently used to deposit nanomaterials onto the electrode surface of printed biosensors[62] (Fig. 2A) to modify the electrode. However, the nanomaterials' homogeneity, ratio to additives and mask choice for patterning should be thoroughly considered during sensor fabrication[61]. Papamatthaiou et al. demonstrated an electrolyte gated field-effect transistor (FET) DNA biosensor to selectively detect the complementary DNA sequence. The transistor channel was formed by drop-casting graphene ink on the paper surface and peptide nucleic acid probes were immobilized on the graphene channel to enable the label-free DNA detection[63]. Their study demonstrates the potential for integrating FET sensors into highly sensitive quantification Lab-on-Chip diagnostic platforms.

3.2 Vacuum filtration

The vacuum filtration is a method to modify the 3D structure of the paper-base of the printed biosensors by filling the paper's 3D structure with desired materials. The vacuum filtration method mainly involves using a vacuum to push the printing materials (typically ink) into a porous material. This method is popular in fast and scalable electrode production and is widely applied to fabricate electrodes on paper substrate with carbon materials like graphene, graphite, and carbon nanotubes. The main drawbacks of vacuum filtration are the high time and material consumption to achieve homogenous filling, and the requirement of masks for the patterning process[61]. To solve those issues, Ponlamuangdee et al. reported a simple and cost-effective fabrication of a paper-based Surface-Enhanced Raman Scattering (SERS) substrate by coating poly (diallyldimethylammonium chloride) and gold nano stars on the filter paper using a vacuum filtration system (Fig. 2B). The paper-based SERS substrates were produced within an hour[64], and simplified the vacuum filtration processing techniques in paper-based biosensors.

3.3 Screen-printing

Screen printing is one of the most used technique in paper-based biosensors to produce conductive tracks (channels) on paper surface. One of the earliest screen-printed paper-based microfluidic electrochemical biosensor can be dated back to 2009 that Dungchai et al. used photolithography method to build microfluidic channels and applied screen-printing technique to fabricate electrodes on paper substrate[65]. Screen printing uses templates to create hydrophobic patterns, which is cost-effective, reproducible, and therefore suitable for large-scale production. Typically, the fabrication of screen-printed electrodes (SPEs) includes the following steps: (i) selection of screen-mesh template to generate the desired pattern, (ii) selection of appropriate substrate material, (iii) selection and preparation of the printing media (i.e., inks/paste), (iv) printing on the solid substrate, (v) drying and curing of the printing media[66] (Fig. 2C). The surface of SPEs can be modified with a range of compounds such as antibodies, enzymes, DNA strands, polymers, metals, or electrochemical mediators to fulfill the specific analytical requirements[42b]. In most reports, the three electrodes (working, counter and reference electrodes) are screen-printed onto a paper substrate based on the template. Carbon inks or pastes are generally used for printing the working and counter electrodes, while Ag/AgCl materials are applied as reference electrodes[67]. Compared with other fabrication methods, screen-printing is well established and reliable. Also, it has relatively low-cost and simple pattern process, together with the ability to use various types of inks and the fabrication of multiple devices in one step. One of the main disadvantages is the need to use a template for each designed pattern. Additionally, ink composition optimization requires efforts. Maier et al. reported the first real-time and calibration-free disposable paper-based electrochemical wearable sensor that can monitor exhaled H_2O_2 in artificial breath[67]. Their device can be integrated into a commercial respiratory mask for exhaled breath testing and could also be extended to continuous monitoring of other analytes in exhaled breath by modifying the sensing electrodes, which may further contribute to the early detection of respiratory infectious diseases.

Li et al. [68] introduced a characterized crystal structure of cobalt oxides functionalized MoS_2/reduced graphene oxide onto a screen-printed electrochemical sensor. The electro-catalytic activity of the modified working electrode demonstrated superior catalytic activity towards glucose oxidation with a lower LOD of 30 nM. Their study opens the way to take non-enzymatic glucose electrochemical sensing toward POCT. A more recent example also reported by the team, introduced a highly integrated sensing paper for real-time analysis of sweat[69]. The printed HIS paper was folded into a multi-layer structure to form a 3D sweat diffusion path and was assembled by combining hydrophobic protecting wax, conducting electrodes and the incorporated MXene/methylene blue (Ti_3C_2Tx/MB) active materials. This 3D structure provides efficient pathways for the diffusion of sweat and facilitates the fixation of enzymes and the accessibility of electrolytes. Their study demonstrates a miniaturized, low-cost, and flexible solution for various biochemical platforms, including wearable bioelectronics.

3.4 Wax-printing

Wax-printing is another common method in paper-based printed biosensor to fabricate the channels/circuit of the sensors. It should be emphasized that the earliest wax printed devices are fabricated by Whitesides' group in 2009, and at that time they have already built a comprehensive and effective process of wax-printing biosensor fabrication and the analytical model to account for the spreading of molten wax in paper[70]. Lin et al. also introduced the simple process of wax-printed biosensor fabrication based on Whitesides' group's study. They simplified the procedure and the device could be easily made by DIY (do it yourself)[71]. Wax printing is one of the most used methods for μPAD production. Compared with screen printing, it belongs to digital printing methods, which means it could directly transfer computer images onto paper substrates and do not need template[72] (Fig. 2D). Therefore, it is extremely suitable for simple colorimetric device fabrication. However, wax printing is limited to have relatively low resolution printing and the printed patterns are unstable at high temperatures. So, it is usually combined with screen-printing techniques to create more complicated structures in electrochemical devices. Basically, wax printing method creates microfluidic channels by depositing wax patterns on paper-based devices, followed by heating treatment to form hydrophobic barriers on the paper to control fluid flow. After heating, the wax will penetrate through the paper and create a wax barrier. As heating is the critical step for wax fabrication, various factors including heating temperature and heating time during this step will affect the reproducibility of the device[61, 73]. Wax printing is inexpensive and efficient, producing approximately 100-200 μPADs in a single batch using a commercial printer with a heating source, which makes it suitable for various analytical and diagnostic biomedical applications[74]. It is also environmentally friendly and compatible with various biological materials. We used was-printing on filter paper to prepare the μPADs for hCG detection without using the expensive nitrocellulose membrane, which has similar performace to LFA based pregency test strips[75]. Chen et al. proposed a novel wax-printed LFA for α-fetoprotein enzyme-linked immunosorbent assay, which is an alternative approach for an automated and one-step enzyme-linked immunosorbent assay. They designed four wax-

delayed channels that could delay the flow time for 11s compared to the flow time of the non-delayed channel (Fig. 2E). Their device is able to detect α-fetoprotein with LOD of 1 ng mL^{-1} and assessed with the naked eye within 10 min, which indicates the potential of the wax-printing technique to apply for POC automated ELISA[76].

In response to COVID-19 pandemic, Laura et al. developed a novel paper-based immunoassay to measure SARS-CoV-2 in saliva. They used magnetic beads to support the immunological chain, taking 96-well wax-printed paper plate as a platform and realized the color visualization by using a smartphone and related App. The calibration curve demonstrated a dynamic range up to 10 μg mL^{-1}, with a LOD equals 0.1 μg mL^{-1}[73]. Their device overcomes the drawbacks of the commercially available immunochromatographic antigen kit able to identify only patients with high viral load.

3.5 Inkjet-printing

Inkjet printing is a digital and non-contact printing technique to fabricate the deposited patterns, and can be mainly divided into continuous and drop-on-demand[65] (Fig. 2F). Compared with screen-printing and wax printing methods, it could streamline the fabrication process by reducing cross-contamination and is easier to be customized using computer-aided design (CAD) software. Additionally, inkjet printing requires less ink compared to screen printing[66]. The requirements for fabrication equipment and inkjet materials are the main drawbacks of this technique, as the unsuitable viscosity and surface tension of materials could cause inkjet clogging problems. Suess et al. proposed an inkjet-printed aptamer-based biosensor to detect fluoroquinolone ciprofloxacin. They presented a ciprofloxacin-binding RNA aptamer developed by systematic growth of ligands by exponential enrichment (SELEX). They also demonstrated that RNA aptamers can be inkjet-printed, dried, and resolved while keeping their functionality consistently intact[33]. Their study demonstrated the potential of RNA as the recognition molecules used in printable biosensors. To improve the performance of the inkjet-printing, Bai et al. reported the effects of pressure wave propagation on enzyme activity from the aspects of wave superposition, wave amplitude, resulting mechanical stress, and protein conformation change using pyruvate oxidase as the model enzyme. They found that the mechanical stress could increase pyruvate oxidase activity by 14.10% during the inkjet printing process. They also investigated the enhancement mechanism, concluding that mechanical activation or mild proteolysis could change the conformation of pyruvate oxidase and improve its activity[67]. Their study contributes significantly to modulating the activity of other enzyme-based inks, benefiting the development of biosensors.

3.6 Stencil-printing

The stencil printing is a simple and inexpensive method to create the printed pattern on the paper surface. Instead of using high-definition conventional materials, the stencils electrodes could be homemade by using adhesive tape and transparency film to create electrode design masks[77] (Fig. 2G). So, there're huge advantages in cost-effectiveness compared to other fabrication methods. However, the disadvantage is that it requires a high viscosity ink, which may reduce the electrodes' durability and limits this

technique's development[78]. Li et al. reported the first E-μPAD with ZnO nanowires (NWs) synthesized in situ for electrochemical enzymatic detection of glucose in human serum. The reaction zone is formed on a paper substrate by wax patterning, and electrodes are patterned on top of the reaction areas by stencil printing of conductive inks. Glucose oxidase is immobilized on the ZnO NWs by simple addition and drying. It is the first study to modify the μPADs with ZnO nanomaterials for biosensing providing superior sensitivity (8.24 μA mM^{-1} cm^{-2}) and LOD (59.5 μM) to existing commercial meters[79].

3.7 Laser-printing

Laser printing is commonly used for fabricating channels on low-cost thermoplastics devices and is comparatively less applied in paper-based devices. The laser printing method can mainly be categorized into laser scribing and cutting[80]. Basically, the fabrication principle is using a laser to create etched channels on paper to accelerate wicking speeds in μPADs. It is especially suitable for large-scale device production because of its easy operational process, the limited requirement of masks, reagents and environmental control capability. However, it does require specialized equipment[80] and could only be applied to certain types of paper (Fig. 2G), such as imaging card paper, chromatography paper, and multipurpose printing paper[81]. Sidharth et al. explored different laser settings to determine the optimal configuration and found that simply cutting a slit into the paper created the fastest wicking channels. The slit acted as a macro capillary, allowing fluid to bypass the paper and speed it up[82]. Another promising emerging strategy for laser printing application is to use laser treatment to convert cellulose into graphitic carbon, which is an ideal electrode material for electrochemical paper-based devices. This process is known as the pyrolysis of paper[81]. The disadvantage of this technique is that the pyrolyzed paper is relatively brittle and easily cracked during handling. de Araujo et al. published the first study introduced using CO_2 laser to pyrolyze the surface of the paperboard to produce a conductive porous non-graphitizing carbon material[83]. As another interesting example, Clark et al. described the formation of fluorescent derivatives via laser-thermal engraving. After treating the chromatography paper using a CO_2 laser engraver, a blue fluorescent pattern could be observed when the substrate was irradiated with UV light under selected experimental conditions[84]. Their study reveals the potential of a novel and effective laser printing method for optical biosensor fabrication.

3.8 Photolithography

Photolithography technique is more commonly used in fabricating desired patterns on biopolymer, plastic polymer and glass-based biosensors[85]. When use paper as a substrate, photolithography involves the hydrophobisation of paper using a photoresist to produce complex micro/nanoscale structures/patterns with high-resolution spatial topography (<10 nm)[86]. During photolithography fabrication, photoresist like octadecyl trichlorosilane and ultraviolet resin was poured onto the paper. After baking using a laser printer, the paper is covered with a patterned transparent film, and then the unpolymerized photoresist is removed using acetone, followed by drying the paper

with air plasma to develop hydrophilic areas[7] [85] (Fig. 2H). The drawbacks of photolithography method are that the entire paper substrate must be treated, which is time-consuming, with high chemical consumption. It also requires special facilities such as photoresists, UV light sources, oxygen plasma and a clean room[53]. The photolithography technique is normally used in paper substrates combined with screen-printed technology. Chen et al. designed a μPAD based on photolithography and screen-printing technology to detect human chorionic gonadotropin, introducing the electrochemical immune filtration analysis into μPADs for the first time[87].

3.9 Pen-plotter

In recent years, the pen-plotting techniques has attracted more and more attention due to its ease-of-implementation to build patterns on the paper surface. In 2016, Gallibu et al.[88] and Nuchtavorn et al.'s[89] team have reported their studies about printing and patterning designs on paper using inks with hand-writing or pen plotter (drawing machine) to fabricate paper-based devices. The Gallibu et al. described a easily fabricated μPADs, use commercially available Sharpie ink permanent markers to draw patterns on chromatography paper to detect glucose colorimetrically[88]. The Nuchtavorn et al. reported using a desktop digital craft plotter/cutter and technical drawing pens to draw fluidic brakes. Their effort showed the possibility and the potential of using marker ink to create barriers on paper rapidly and designing in a high degree of freedom[89]. Ghaderinezhad et al. introduced a more elaborate paper feeder device to control the paper delivery process during drawing[90]. They have also discussed the cost and resolution offered by this fabrication method. More recently, Lee et al. introduced their work on a technique to control capillary flow on paper by inducing roadblocks on the flow path with water-insoluble ink drawing on the paper and using the void formation process between a wetted paper and a sheath polymer tape to create timers[91]. Their work is essential to control the step-by step process for the LFA equipment while applying a simple method.

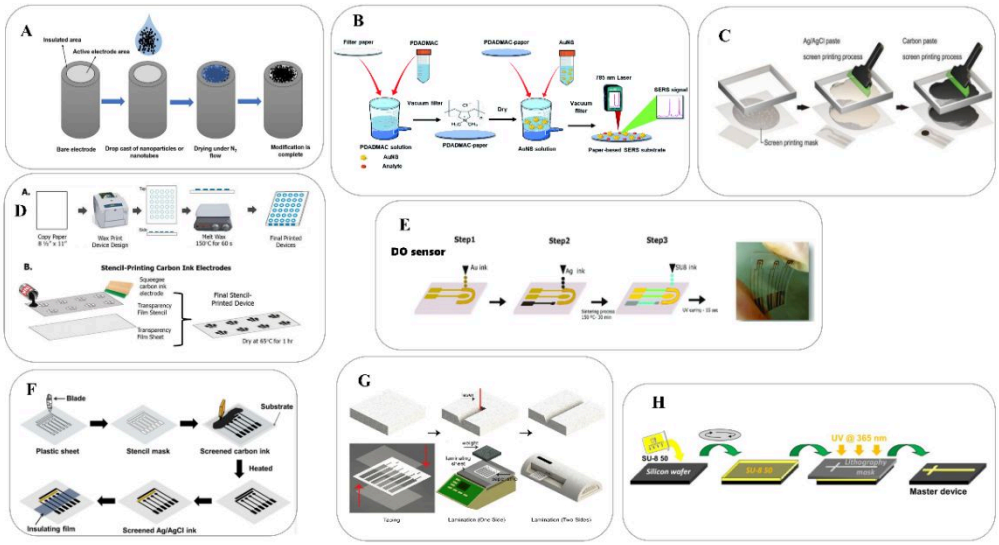

Fig. 2 Different printing technologies of paper-based biosensors. (A) A schematic showing the drop casting of nanoparticles or nanotubes onto an electrode[92], reprinted with permission from Elsevier; (B) Vacuum filtration: schematic showing each step of plasmonic paper preparation process[64], reprinted with permission from Royal Society of Chemistry; (C) Schematic illustration of fabrication of pH sensors based on a screen printing process[66], reprinted with permission from SpringerOpen; (D) Examples of fabrication schemes for production of (a) wax printed paper-based well devices and (b) stencil-printed transparency film-based carbon electrodes[72], reprinted with permission from IntechOpen; (E) Schematic illustration of fabrication of on inkjet-printing process[93], reprinted with permission from MDPI; (F) Stencil-printing: schematic illustration of the multichannel graphite electrodes fabrication[77], reprinted with permission from MDPI; (G) Laser-printing: Schematic summarizing fabrication of laser-etched grooves on paper[80], reprinted with permission from Optica Publishing Group; (H) Fabrication of microfluidic devices using photolithography[85], reprinted with permission from Royal Society of Chemistry.

4. Fabrication of paper substrates with biorecognition elements

Immobilizing biological elements onto a surface should be stable, permit diffusion of substrates and products, and allow excellent electron transfer. This section will mainly discuss the various immobilization methods, including physical adsorption, entrapment, encapsulation, covalent conjunction and affinity-based attachment, to immobilize the bio recognition element like functional groups, proteins or nanomaterials to the paper surface. It should be emphasized that although the focusing point of this Section is not the printing techniques, all the immobilization method could be applied in printable paper-based biosensor fabrication.

Noticeably, it is essential to apply surface treatment method to improve the paper surface performance and help biorecognition molecular immobilization. For example, a variety of surface chemistries, including divinyl sulfone chemistry, diazonium chemistry or polymer chemistry, have been proposed to facilitate the covalent immobilization of biomolecules onto paper[94]. Common surface treatments could change paper surface's properties like hydrophobicity, charge, and ability to form

hydrogen bonding in supramolecular interactions with coating agents[95]. These improvement in paper properties could help paper-based devices perform better in sample storage and collection, sample separation, sample preconcentration and detection[96]. However, Cao et al. raised a unique statement that the functionalization strategies for paper may not be necessary. They mentioned that most published work took it for granted that hydroxyl groups of the paper surface need to be transformed to other functional groups to enable covalent immobilization of biomolecules by common bioconjugate techniques. However, it has been neglected that the paper's properties of low concentrations of carboxyl groups may facilitate the covalent immobilization of biomolecules onto paper surface[94] Their opinion may point out a direction for future study.

4.1 Physical adsorption

Physical adsorption as the most straigtforward method to immobilize bio-recognition elements, is a spontaneous and fast process involving ionic interactions, hydrogen bonding and van der Waals interactions. The disadvantage of physical adsorption is the weak bondingstrength, leading to the possibility of surfactant molecule release. Also, high loss of biomolecules and randomly oriented adsorption limited the use of physical adsorption methods. A simple addition of surfactant is an alternative treatment to chemical modification. For example, the oxygen plasma treatment can clean the filter paper and generate uniform net negative charges on the surface, which can then effectively adsorb chitosan polymers from chitosan acetate solutions via electrostatic interactions. Nanotechnology has been widely applied to modify paper-based assays based on physical adsorption strategy. Compared with conventional methods, altering biosensors with various nanomaterials and nanostructures has huge potential and could improve critical parameters such as sensitivity, dynamic range, the limit of detection, selectivity against interfering species and reduced sample volume for analysis[55, 97]. Nanomaterials like gold nanoparticles (AuNPs)[98] (Fig. 3A), carbon dots and quantum dots are currently the most used nanomaterials for modification. For example, Tugba et al. reported a novel graphene paper-based electrode consisting of graphene modified with a conducting polymer, glucose oxidase (GOx) and AuNPs for glucose detection. A conducting polymer was spread over graphene-coated papers and used as a matrix for glucose detection. GOx was immobilized with AuNPs via a physical adsorption technique to finalize the desired biosensor[99].

4.2 Entrapment

Entrapment is a non-covalent coupling method when many biosensing factors like antibodies and enzymes could be entrapped in buried mediators such as conductive polymer materials. Polymer materials like polyacetylene, polyaniline, pirlindole polyquinone and polypyrene are commonly used as buried mediators due to their excellent biocompatibility. Compared with the physical adsorption method, entrapment has more stable bonding. It is also easy to operate as compared to covalent conjunction method. Particularly, sol-gel glass has been used to immobilize biomolecules within its porous optically transparent matrix with demonstrated functional activity[100]. For

example, silica sol-gel possesses a silicate network and good biocompatibility to entrap the antibody with enhanced attachment. Roda et al. utilized a bilayer film of polyelectrolytes (Poly (allyl amine hydrochloride/poly (sodium 4-styrene sulfonate)) to entrap Lox/HRP/TMB on paper substrate (Fig. 3B). Hydrogen peroxide produced by these oxidases reacts with the chromogenic reagent 3,3′,5,5′-tetramethylbenzidine (TMB) to form a colored product, in the presence of the enzyme horseradish peroxidase (HRP)[101]. This method is the popular enzymatic bioassay exploiting analyte-specific oxidases.

4.3 Encapsulation

Encapsulation is another non-covalent coupling immobilization method, which is different from entrapping. Taking enzymes as an example, the main difference between entrapment and encapsulation methods is that entrapment involves immobilizing enzymes by physical entrapment inside a polymer or a gel matrix (no semipermeable membranes are needed) while encapsulation refers to the process of spherical particle formation wherein a suspension is enclosed in a semipermeable membrane. Also, the binding force of the entrapment is usually weaker than encapsulation and therefore enzyme leakage could have occurred. Carbon materials are also suitable for encapsulation. Shoseyov et al. reported using a paper-based dipstick biosensor that utilizes peptide-encapsulated single-wall carbon nanotubes (SWCNTs) for protease detection (Fig. 3C). A significant drop in the photoluminescence (PL) of the SWCNTs could be detected upon enzymatic digestion of the peptide. As the emitted PL is in the near-infrared region, The designed biosensor has a good signal-to-noise ratio in biological fluids[102]. Their device is capable of detecting the abnormal levels of trypsin activity in urine samples and can be used for early diagnosis.

4.4 Covalent conjunction

Direct chemical modifications through covalent bond formation are the most important surface chemical treatment. Because of the lack of functional orientation and the burying of the active sites, the overall performance of non-covalent immobilization could impair sensitivity and immunological activity, limiting their practice use. However, while covalent attachment allows forming a stable bond between the functional groups of the protein and those of the substrate, the covalent reactions are typically slow and laborious. Additionally, the required experimental conditions can be detrimental to the substrate's protein and electronic properties, causing reproducibility challenging [103].

Covalent conjunction is vital for DNA application. Carbodiimide has been used as in situ coupling reagents as early as the 1960s[104]. Since then, DNA sequences have been coupled to micro- and nanocrystalline cellulose, nonwoven cellulose fabric, and regenerated cellulose[105]. Various methods have been applied, including epichlorohydrin- and bis(epoxide)-mediated coupling, reductive amination, photo-cross-linking, and cellulose-binding DNA aptamers[106]. Capture probes are required to capture the target DNA on the surface for immobilization, and aptamer is one of the most used probes. Often, an aptamer is a single-stranded nucleic acid that can

selectively bind to the target biomolecule based on the tertiary structures (Fig. 3D). Due to its unique property, the aptamer is ideal as a molecular recognition element rather than other probes like antibodies for various biological diagnostic devices[107]. Many nanomaterials with high surface-to-volume ratios have been applied in aptamers modification. For example, the modified sulfhydryl group at the end of aptamers could form Au-S bond with nanomaterial AuNPs[108]. In addition, the signal changes from the combination of aptamers and analytes can be amplified by nanomaterials. The key principle is that dissociation from the aptamer and aggregation of AuNPs causes colorimetric and electrochemical change. One of the popular strategies is to introduce amine groups on one terminus of the aptamer to immobilize the carbonylated magnetic beads (MBs) via EDC/NHS coupling. In contrast to antibody immobilization, this results in highly oriented immobilization of aptamers, suggesting that only the terminal amine group participates in the coupling procedure. Use streptavidin-coated MBs and biotin-labelled aptamer for the attachment is also a possible method[109]. MBs modified with various chemical groups are the most important emerging covalent junction strategies for paper substrate immobilization[110]. To provide different specificities, MBs can be functionalized with various reactive groups, such as amines, carboxyls, epoxyls, and tosyls, which can be used to immobilize high-affinity ligands such as aptamers, proteins, antibodies, etc. For example, Goswami et al.[111] designed aptamer-coated MBs to capture biomarkers on a paper substrate following colorimetric and fluorometric analysis.

For protein immobilization, binding by noncovalent interactions like van der Waals onto pure cellulose is too weak and the ionic strength and pH in the detection environment may strongly affect the connection stability. In contrast, covalent immobilization ensures the biomolecule of interest in the cellulose has the most stable binding and the most uniform distribution[112]. Also, covalent connections can be achieved almost exclusively at the C6 position because most of the free hydroxyl groups in cellulose are involved in interchain hydrogen bonding[113]. Harald et al. explored four different peptide conjugation strategies and evaluated two conjugation enzymes (sortase A and microbial transglutaminase) for directed target protein coupling[113]. Another important consideration for protein immobilization on paper substrate is retarding its specificity and immunological activity to overcome the detection limit. It can highly affect the device's performance if electron transfer is not guaranteed or if the protein undergoes major conformational changes that alter its functionality[114]. Barsaski et al. introduced a novel strategy for the covalent immobilization of protein (GOx as example) onto lithography patterned paper surface. They first use the photo-crosslinked the benzophenone-containing copolymers onto paper, then covalently bound enzymes to the surface by transamidation. Their method displayed a novel immobilization method, onto the cellulose fibers without compromising its activity, therefore greatly improved the reaction performance[115]. Valentina et al. reevaluated four different peptide conjugation strategies for functional protein immobilization on paper, and two conjugation enzymes, sortase A and microbial transglutaminase, are used for directed target protein coupling. Their work showed a relatively comprehensive view for protein immobilization strategies evaluation and could be

devoted to achieve high protein loads using biofunctionalized papers[113] (Fig. 3E).

4.5 Affinity-based attachment

The affinity attachment method is a cost-effective alternative when considering large-scale fabrication or the immobilize target structure is labile. The principle is to create strong affinity bonds between the affinity tags at a specific position (normally far from the active site of the native proteins), the enzyme structure and a solid support functionalized with the complementary affinity ligand[116] (Fig. 3E). Avidin and streptavidin combing with the biotin group has high affinity and could form a stable system. Therefore, biotin-avidin or immobilizing intermediate binding proteins like protein A or G followed by the capture of antibodies are commonly involved in affinity-based immobilization. Kang et al. demonstrated a simple surface modification method based on a PDA/protein G mixture. Protein G can bind to the Fc region of antibody and expose the binding site of the antibody to antigen specifically. Thus, oriented antibodies can have better antigen-binding capacity compared to randomly oriented antibodies[117]. Besides, nanoparticles could also be used for capturing antibodies using affinity attachment method. For example, avidin-modified AuNPs can be used to capture the biotinylated antibody[118], in which process SH-PEG-COOH served as the linker between AuNPs and avidin. Rica et al. overcame the irreversible binding of nanoparticles to cellulose matrices, and presented that AuNPs modified with avidin or antibodies can be transferred from a dry reservoir to wet paper[119]. Other metal nanoparticles and Quantum dots could also be immobilized on the paper surface using affinity attachment method[120]. Ihalainen et al. reported the fabrication of alternate layers of biotin/streptavidin/biotinylated-CRP-antigen/anti-CRP antibody, which are grown on inkjet-printed gold electrodes on disposable paper-substrates. They designed four layers for the antigen/antibody affinity immobilization. Specifically, they immobilized the bio-CRP antigen onto the MBP thiol/streptavidin layer covered electrodes then followed by the immobilization of target analyte, anti-CRP antibody, on the recognition layer[121]. As a pioneer work, they also verified the formation of consecutive layers of supra-molecular protein assembly using an electrical technique.

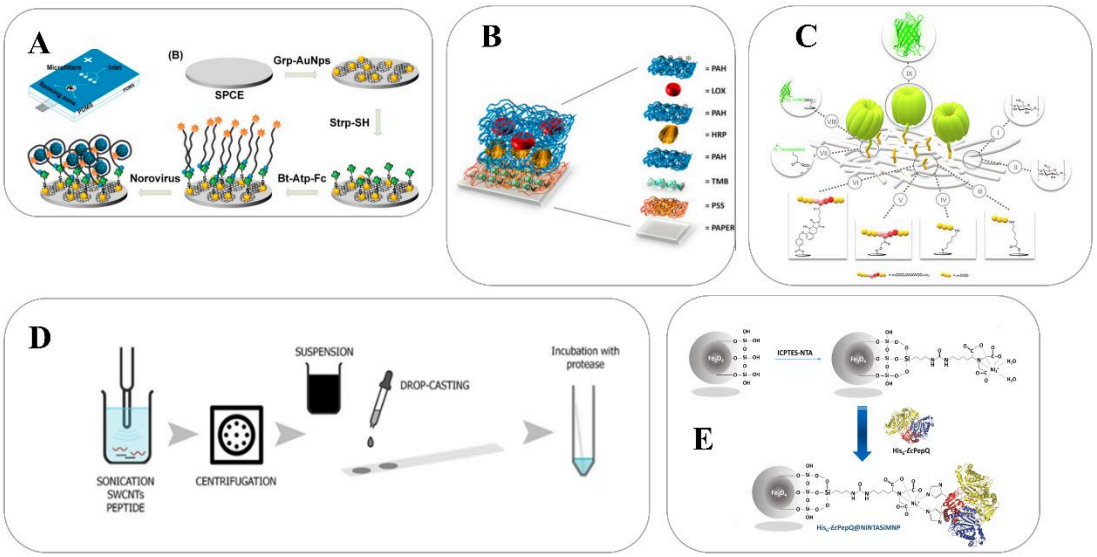

Fig. 3 Immobilization methods for biomolecules. (A) Immobilized AuNPs via pH physical adsorption[98], reprinted with permission from American Chemical Society; (B) Utilized PAH/PSS bilayer film to entrap Lox/HRP/TMB on paper substrate[101], reprinted with permission from Ref. with permission from elsevier; (C) Overview of the evaluated strategies for functional protein immobilization on paper: (I) Cotton linters or (II) TEMPO-oxidized cotton linters as cellulosic supports. (III, VI) Different strategies for the immobilization of an enzyme recognition sequence as an immobilization anchor. (V) Oxime ligation with a synthetic peptide possessing an aminooxy functionality at a lysine side chain. (VI) Esterification with a photoenol moiety and coupling of a synthetic peptide. (VII) Connection generated through ligation of a C-terminal DIPGQGMTG sequence on a protein with the N-terminal GGG sequence on paper. (VIII) Connection generated through sortase-A-catalyzed ligation of a C-terminal LPETGG sequence of a protein with the N-terminal GGG-sequence on paper. (IX) tGFP as model protein (PDB: 1EMA)[113], reprinted with permission from American Chemical Society; (D) Encapsulate peptide on SWCNT for protease detection[122], reprinted with permission from MDPI; (E) Affinity immobilization of a bacterial prolidase onto metal-ion-chelated magnetic nanoparticles[123], reprinted with permission from MDPI.

5. Types of paper-based printable biosensors and their transducers

5.1 Optical paper-based printable biosensors

The basic principle of optical biosensing systems is converting biological interactions to optical signals such as luminescence, fluorescence, reflectance, and absorption. Optical systems deliver signal and information via light (e.g., amplitude, intensity, polarization, frequency, wavelength, and phase of the light), resulting either in visible cues of analyte discovery or the ability to collect and analyze the biorecognition with minimal invasiveness. Therefore, it could achieve direct and real-time monitoring and label-free requirements. Advantages of optical biosensing include reduced interference from testing sample on electric signals, minimal requirements for electrical insulation,

and easier multiplexed detection implementation. Optical biosensors can be mainly divided into direct and indirect types. The former relies on the transducers' optical properties change that induced by the binding of the analyte (e.g., surface plasmon resonance (SPR), reflectometry, interferometry, and photoluminescence)[76] (Fig. 4A). The latter relies on the variation of labeling agents such as fluorescent or photoluminescent quantum dots (QD), gold or silver nanoparticles, and dyes during sensing process[124]. Basic optical signal detection methods used in paper-based biosensors include colorimetry, fluorescence, surface-enhanced Raman spectroscopy (SERS), luminescence and chemiluminescence, which will be highlighted in the following sections.

5.1.1 Colorimetric paper-based printable biosensors

Colorimetric methods detect the presence and concentration of testing analytes by evaluating the formation or change in color via i) direct imaging combining cameras or mobile devices with quantification software such as MATLAB, or ii) traditional spectrophotometers by measuring the absorbance of the sample[7]. Colorimetric signal is widely used in paper-based biosensors because analytes can be easily reflected in color that the naked eye could observe. Also, colorimetric detection has improved the reproducibility compared with electrochemical biomarker detection methods[125]. The key obstacle of colorimetric biosensors is to design the assay that could transfer signals into color change. In the traditional colorimetric enzymatic reaction, the enzyme HRP and its chromogenic substrate, such as TMB and 2'-azinobis (ABTS), are commonly used, but instability affects their performance[126]. Flavia et al. reported a simple but sensitive (LOD equals 50 μM) wax-printed paper-based device for the tear glucose measurement using TMB as the chromogenic reagent[127] (Fig. 4B). Teepoo et al. provided a possible solution by integrating the paper device, enzymes, chromogenic reagents and corresponding substrates to generate a screen-printed colorimetric products, it will display different colors (brown, blue and pink) for different substance (sucrose, fructose and glucose) [128]. Also, traditional enzyme reactions could cause heterogeneity of the detection results due to the insufficient interaction between the paper substrate and the enzyme or chromogenic reagents. As a result, researchers introduced nanomaterials like metal nanoparticles, magnetic nanoparticles, carbon-based nanostructures[129], conjugated polymeric nanovesicles, quantum dots[130] and nanozymes to work as an indicator for colorimetric signal to overcome enzyme heterogeneity, and increase the peroxidase-mimetic activity. For instance, Reza et al. reported a simple laser-cutting μPAD used peroxidase-mimetic Pt-decorated Ni-doped nitrogen-rich graphitic nanotube (Pt/Ni@NGT) to replace peroxidase for the detection of glucose. The nanostructure within the material exhibited excellent biocompatibility with highly efficient immobilization and retention of GOx[131]. Reza et al. also reported another laser-cutting μPAD using DNA-templated Ag/Pt nanoclusters (DNA-Ag/Pt NCs) to mimic peroxidase activity to detect miRNA-21[132]. AuNP is another popular choice as color indicator. AuNPs have extremely high extinction coefficients, and thus concentrations in nM scale are enough for visual observation. Secondly, the color change is easily detected upon aggregation of AuNPs[133]. Particularly, their SPR is

influenced by its surrounding matrix's properties, and thus, analyte-triggered aggregation of AuNPs results in a bathochromic shift of the SPR band. Due to interparticle surface plasmon coupling effect of AuNPs, it changes the color of the solution from red to blue[134]. In a recent study, Lais et al. developed a screen-printed LFA for simultaneous and fast determination of Alzheimer's blood biomarkers (fetuin B and Clusterin). They immobilized selective antibodies to targeted biomarkers on AuNPs to form the AuNP-Ab structure and deposited on paper pads[135]. Compared to the conventional stick format, their design allows the usage of a 3-fold less volume of sample and lowered production costs.

In addition to the enzymatic reaction, colorimetric compounds such as Prussian blue (PB) may change color upon a redox reaction and are commonly used in transmitting colorimetric signals. For example, Bahram et al. designed an electrochromic reaction on a laser-printed paper surface using the external potential to oxidize glucose to generate H_2O_2, which resulted in the oxidation of $K_3Fe(CN)_6$ and the consequent formation of PB. They used the intensity of the blue color in the reporting cell as a colorimetric signal that can be digitally read through a digital camera[136].

5.1.2 Fluorescent paper-based printable biosensors

Fluorescence-based technique involves the detection of luminescence from fluorophores, resulting from interactions with the sample molecule[7]. The fluorophore molecules could be small molecules, proteins or nanoparticles, and are normally used to label proteins, nucleic acids or lipids by excitation of the fluorophore molecule under an appropriate length of light. Fluorescence detection often includes excitation light sources, fluorophore particles, wavelength filters and detectors[137]. Compared with colorimetric biosensors, fluorescent methods provide higher spatial and temporal sensitivity, and a wider detection range for potential quantitative detection. In recent years, several paper-based fluorescent probes have been fabricated on the paper substrate, and various fluorescent materials like fluorescent dyes, quantum dots, carbon dots and metal nanoclusters have been applied in fluorescence sensing[138]. For most fluorescent biosensors, fluorescent dyes are required to change colors or be quenched after adding analytes[139] (Fig. 4C). Fluorescent dyes are widely used as the indicator in fluorescent paper-based biosensors for its easy surface modification and good biocompatibility. Rhodamine, fluorescein, cyanine and coumarin are the most common molecular organic dyes that have been broadly used in fluorescent biosensing[140]. Sikes et al. described a wax-printed colorimetric biosensor with fluorescein as an indicator, incorporating liposomes into polymerization-based signal amplification (PBA). Their novel design allowed for 30-fold signal enhancement compared to conventional PBA[141]. However, the use of fluorescent dyes has reduced in recent years due to fluorescent dyes' low fluorescence quantum yield, weak resistance to photo-bleaching and broad emission bands. To solve these problems, Liang et al. designed a wax-printed flower-like silver (FLS) platform to reduce the paper background fluorescence and improve sensitivity by silver-enhance FRET efficiency. Fluorophore-functionalized DNA1 (DNA1-N-CDs) was covalently combined with FLS, which was hybridized with a quencher-carrying strand (DNA2-CeO_2) to form an FLS-enhanced fluorescence

biosensor[142]. Through this method, the growth of FLS in μPADs not only reduced the background fluorescence but also improved the surface enhanced fluorescence detection of miRNAs.

Fluorescent probes are molecules that absorb light of a specific wavelength and emit light of a different wavelength. Because the fluorescent probes use a single emission wavelength, so the fluorescence intensity either decreases by quenching or increases by removing the quenching effect in the presence of the analyte. The major disadvantage of fluorescent probes is that they are easily affected by environmental factors. QD, carbon dots (CDs) and metal nanoclusters are the most used fluorescent probes nowadays. Compared with the organic dyes, QD's stable fluorescence and high stoke shift properties enable the spectroscopy detection at a low signal intensity, which makes the fluorescence detection easier[134]. For instance, nitrogen-doped graphene quantum dots and folic acid-pPdAu/GO was used as fluorescent quencher and recognition element for cancer cells[130]. Unlike QD, CDs possess high quantum yields, broad excitation and tunable emission spectra, together with AuNPs, GO, and MoS_2 as the efficient fluorescent quenchers[129]. Wang et al. reported a fluorescent paper-based sensor (FPS) constructed on a hybrid PDMS/paper platform where cellulose papers were covalently functionalized with CDs as fluorophores via Schiff base chemistry[143]. Compared with the CDs solution-based sensing system, this FPS achieved an improved sensitive assay of FA than the conventional strategy, which gave a higher LOD of 9.1 μmol L^{-1}[144]. Metal nanoclusters generally are tunable with the strong fluorescence emission. For example, Xu et al. mentioned that conventional fluorescent materials, such as fluorescin, organic dyes and QDs, are usually excited by high-energy ultraviolet (UV) or visible (vis) light. However, UV-vis excitation often causes autofluorescence and light scattering on the paper substrate, which could affect detection sensitivity. Therefore, Lanthanide (Ln^{3+})-doped upconversion nanoparticles (UCNPs) were employed as a fluorescence biolabel alternative, since UCNPs could avoid the autofluorescence and scattering light induced by UV-vis excitation due to its NIR-excitation nature. Xu et al. fabricated a novel fluorescence test paper by printing UCNPs conjugated with carcinoembryonic antigen (CEA) antibody on the paper substrate using the digital printing method (UCNPs as "ink") for CEA detection[145].

5.1.3 Ratiometric paper-based printable fluorescent biosensors

The principle of ratiometric fluorescence biosensor is based on measuring intensities at two or more wavelengths of an excitation or emission spectrum to detect changes of local environment. It facilitates the quantitative determination of target analytes and the fluorescence imaging of toxic substances associated with various human diseases[146]. Compared with optical biosensors introduced above, ratiometric fluorescent biosensors usually have high sensitivity and inherent reliability, reflecting the self-calibration provided by monitoring two (or more) emissions. Various ratiometric sensing probes based on small fluorescent molecules have been developed. For example, internal charge transfer (ICT) based probes have been used for heavy or transition metal ions (Zn^{2+}, Cd^{2+})[147], and Disulfide-carbamate based naphthalimide derivatives have been widely exploited as ratiometric fluorescent probes for biological thiol detection[148].

Wang et al. designed a paper-based ratiometric fluorescence nanosensor for the selective detection of Cu^{2+} by covalently connecting the carboxyl-modified red fluorescent cadmium telluride (CdTe) QDs to the amino-functionalized blue fluorescent CDs. The hybrid CDs-QDs probe was printed on a microporous membrane using the digital printing method (probe solution as ink)[149].

Mobile smart devices have been widely applied to improve colorimetric detection. Jung et al. described an automatic and accurate determination of saliva alcohol concentrations using smartphone-based colorimetric imaging combined with wax-printed paper biosensor[150](Fig. 4D). More recently, Morales-Narváez et al.[151] engineered a fluorescence reader with paper materials and a smartphone camera using screen/stencil printing techniques, leading to an advantageous device for quantitatively interrogating quantum dot nanocrystal concentrations. Their efforts greatly facilitate the colorimetric signal readout process in POCT. Chu et al. also reported a similar ratiometric fluorescent paper strip device for visual quantitative sensing of pesticide. They combined the red/blue-emitting quantum dots with nanoparticles (silica and AuNPs) to realize the signal recognition and readout[152] (Fig. 4E).

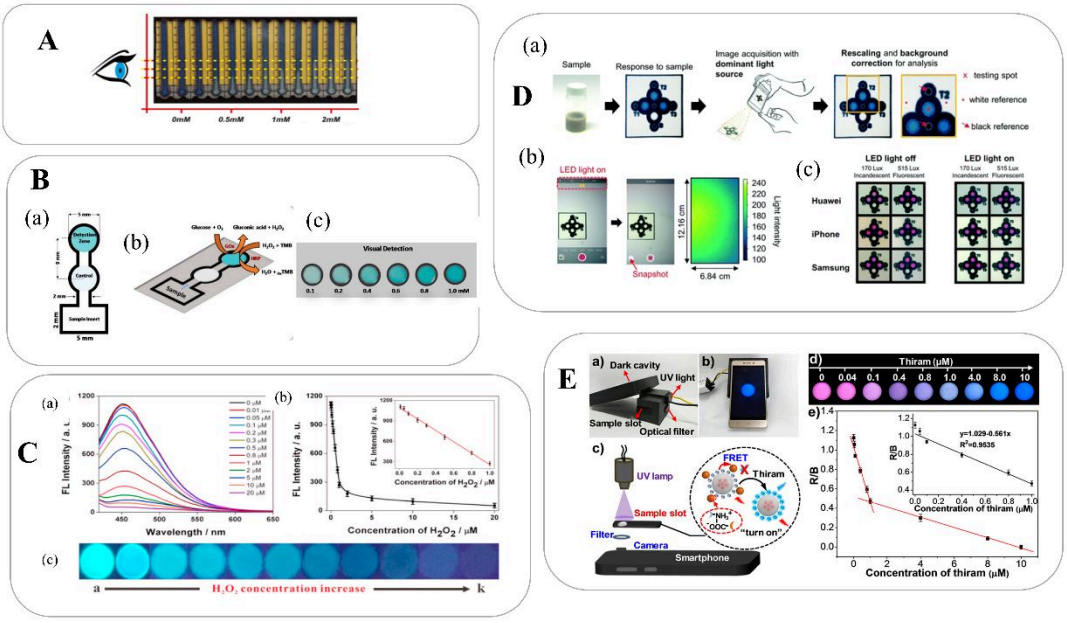

Fig. 4: Colorimetric paper-based biosensors. (A) Distance-based detection: A photograph of the set of (iron (III) chloride/iron (III) modified paper strips after the color change[76], reprinted with permission from Elsevier; (B) Presentation of a paper-based colorimetric biosensor for tear glucose measurements: (a) (b) the layout of μPAD used for glucose colorimetric assays and a simplified view of the enzymatic reaction mechanism. (c) color scale for visual detection of tear glucose levels using TMB [127], reprinted with permission from MDPI; (C) Fluorescence responses of the as-proposed platform in the presence of H_2O_2 with different concentrations in solution state: (a) fluorescent spectra; (b) fluorescence intensity at 460 nm, inset: linear plot of FL intensity versus H_2O_2 concentration. (c) Photographs of the paper-based sensor under 365 nm UV illumination upon the addition of H_2O_2 with different concentrations[139], reprinted with permission from MDPI; (D)

Determination of the concentration of a sample using a smartphone-based colorimetric device: (a) Schematic showing the determination of the concentration of a sample using a smartphone-based colorimetric device. (b) Screenshot showing the spatial light distribution from the integrated LED light source indicating where the device should be placed. (c) Images of devices taken at different lighting conditions with the LED light on and off using different phones[150], reprinted with permission from Royal Society of Chemistry; (E) Smartphone platform using a ratiometric fluorescent paper strip for visual quantitative sensing: (a) (b) 3D structure of the smartphone sensing platform. (c) Platform's inner structure and the principle for detection of thiram. (d) A series of fluorescence images under UV light with different concentrations of thiram. (e) Ratio of R/B values versus concentration of thiram in the range of 0-10 μM[152], reprinted with permission from American Chemical Society.

5.2 Electrochemical paper-based printable biosensors

Electrochemical paper-based biosensors involve measuring the presence and quantity of analytes using potentiometric or amperometric method. Conductometric sensors can also measure the capability of analytes to produce current between electrodes[7]. Generally, electrochemical paper-based biosensors have the advantages of low power consumption, high portability, and is capable of quantitative measurement. Electron transfer takes place on electrochemical electrodes, which could be fabricated using various materials. Current research mainly focused on the electrode materials to improve the electrochemical signal and stability. Based on applied electrochemical methods, Ataide et al. have categorized the electrochemical paper-based biosensor into amperometric sensor, voltametric sensor, potentiometric sensor, impedimetric sensor, coulometric sensor and electrochemiluminescence sensor, with detailed information of their properties and characters[61]. Liu et al. have also comprehensively reviewed the engineering strategies for enhancing the performance of electrochemical paper-based analytical devices in recent years[43a]. Hence, we will only focus on the complementing methods of electrode surface treatment and briefly introduce the chemical engineering of electrochemical paper-based biosensors.

5.2.1 Electrode treatments

The electrochemical detection has become one of the most used PADs detection principles, due to its desirable features, including high sensitivity and rapid response. Three electrodes including working electrode (WE), counter electrode (CE) and reference electrode (RE) are usually needed for EC analysis. After adding analyte solution on the sample zone, the analyte will bind to the detection antibody at the conjugate zone and then bind to the capture antibody at the test zone; Under capillary action driving force, the excess conjugates will be migrated to the absorbent zone[43a]. The first paper-based electrochemical PAD is produced by Dungachi et al. for the simultaneous determination of glucose and lactate in real samples using photolithography and screen-printing technology[153]. The electrode of printed electrochemical biosensors are usually printed using conductive inks like carbon inks. Various paper-based biosensor fabrication methods have been thoroughly discussed in Section 2, and these methods could also be applied to electrode fabrication. Table 1

presents a comprehensive review of printed paper-based biosensors based electrochemical signal readout. Here, we will mainly focus on electrode treatment methods and materials. The ferro-/ferri-cyanide redox couple is stable and shows reversible electron transfer kinetics, therefore is used as a standard redox probe for the electrochemical characterization of electrodes. Fernández-Abedul et al. reported a simple screen/stencil-printed paper-based electroanalytical device using a traditional enzyme cocktail (GOx, HRP and potassium ferrocyanide) to detect glucose. They used enzyme cocktail GOx, HRP and the ferrocyanide as mediator of the electron transfer[154]. Apart from potassium ferrocyanide, nanomaterials like oxide nanoparticles, metal nanoparticles, semiconductor nanoparticles and composite nanoparticles or nano-hybrids are also widely used to improve the performance of paper-based electrodes for various purposes[155]. Most used materials in enhancing electrochemical biosensors include [156] carbon nanotubes (CNTs), graphene nanosheets (GNS) and single-walled carbon nanotubes (SWCNTs)[157] for their high electrical conductivity. Surface plasmon-related electromagnetic like AuNPs or AgNPs is favoured for their high active surface area, enabling many biorecognition molecules. Other metal nanomaterials like the homogeneous triangular Au nanosheets (T-AuNSs) are also used to enhance the electro-active surface area of paper[158] because of their excellent conductivity and large electro-active surface area for immobilization. Various metal oxides like iron oxide (Fe_3O_4) nanoparticles, zinc oxide (ZnO) and copper oxide (CuO) have also been utilized for electrochemical paper-based biosensor. Malhotra et al. incorporated iron oxide nanoparticles (nFe_2O_3) with poly(3,4-ethylenedioxythiophene) polystyrene sulfonate (PEDOT:PSS) to improve electrochemical signal stability and achieved high sensitivity of 10.2 $\mu A\ ng^{-1}mL\ cm^{-2}$ in the detection range of 4-25 $ng\ mL^{-1}$ for CEA[159]. It should be mentioned that they didn't apply printing method, yet simply fabricated a reduced graphene oxide modified conducting paper for their electrochemical detection. Their study is an excellent example showing that even for paper-based electrochemical biosensors' fabrication, printing of circuit is not always necessary.

The stability of the enzyme immobilized on the electrode should also be considered. Metallo phthalocyanines, such as cobalt(II) phthalocyanine, copper(II) phthalocyanine and iron(II) phthalocyanine, have excellent electrocatalytic activity. However, their low conductivity and inadequate electrochemical activity have limited their further application. As a result, Kalcher and co-workers[160] reported a wax/screen-printed non-enzymatic glucose sensor on paper-based analytical devices for the first time with cobalt phthalocyanine, graphene and an ionic liquid.

5.2.2 Electroactive probes

Quantitative analysis of biomolecules could be effectively reached through monitoring the proportionally labeled electroactive probes. Due to their favourable redox properties, ferrocene derivatives are still the most used electroactive probes in electrochemical biosensors[161]. Several available strategies that exploit ferrocene derivatives as electroactive substances have been proposed, such as modifying ferrocene derivatives to the end of molecular beacons, labeling target ssDNA used versatile coupling linkers

with ferrocene derivatives before/after hybridization and using “Click” chemistry to connect acetylene ferrocene to azide-modified DNA. Methylene blue (MB) is another traditional electroactive probe most used in electrochemical paper-based biosensors. For example, Arduini et al. covalently immobilized special peptide with electroactive probe MB, mimicked the substrate of Botulinum neurotoxins (BoNTs) on a screen-printed electrode. In the presence of BoNTs, methylene blue on the peptide will be cut, resulting in the alteration of the electro property of the biosensor. This labelled peptide-based biosensor demonstrates excellent potential for DNA detection. With the help of MB, radiometric strategy can also be utilized in the electrochemical paper-based device[162]. Yu et al. combined the radiometric technique with MB to detect manganese super oxide dismutase gene, based on a T-AuNSs modified screen-printed paper electrode. They modified MB with hairpin for capture probe and used ferrocence for signal probe. In the presence of the target gene, the signal probe labelled with ferrocene undergoes the conformational change from close to open state. This resulted in the generation of residual DNA that can be recognized by the capture probe. In the end, the oxidation peak current of Fc increased while the peak current of MB was unchanged because of the unchanged distance between MB tags and electrode[158].

Noble metal nanoparticles are also commonly used as probes to expand the surface of the electrode, improve conductivity serving as charge carriers and catalysts for the electrochemical analysis, and amplifying the detected signal. For example, AuNPs can be used as an electroactive probe. Li et al. designed a sandwich-structure immunoassay system for rapid early quantitative detection of pancreatic cancer with a new biomarker, pseudopodium-enriched atypical kinase one, SGK269 (PEAK1). The immunosensor was constructed by covalently immobilizing screen-printed paper electrodes with the versatile nanomaterial graphene oxide for the incorporation of antibodies.[163]. Carbon nanomaterials (CNF, GO, multi-walled carbon nanotubes (MWCNTs)) can also amplify the signal by being modified onto the electrode surface to enhance electron transfer and increase the surface area[164]. Cai et al. reported immobilizing anti-E2 antibody on a wax/screen-printed working electrode coated with MWCNTs/thionine(THI)/AuNPs nano composites for the detection of 17β-estradiol (17β-E2). The THI molecules here were non-covalently attached to the MWCNTs through π-π stacking interactions and serve as an electrochemical mediator. The excellent electrical conductivities of MWCNTS and AuNPs have accelerated electron transfer for the signal amplification and improve the sensor’s sensitivity[165].

Other novel electroactive probes like PNA could also be used in electrochemical PADs. PNA has been used for the detection of miRNA for many years. The change from uncharged PNA to the negatively charged duplex of PNA and miRNA can be monitored with positively charged ruthenium (III) hexamine using differential pulse voltammetry[166]. Overall, the limitations of electrochemical sensing schemes are related to the accuracy, stability and reproducibility due to the variations in the electrode surface.

6. Challenges and perspectives of printable biosensors in POCT

The printable biosensor is extremely suitable for POCT purpose, which has gained popularity in recent decades, due to advances in printing technologies that have made devices more reliable, easier to use and more cost-effective. Particularly, the recent COVID-19 pandemic has shown the importance of POCT for rapid diagnosis, providing patients with timely and appropriate treatment. A rapid and precise diagnosis could also help reduce the misusage of antimicrobials, preventing the potential of antimicrobial resistance[167]. Integreated with intelligent signal readouts, we believe that the POCT will become one of the most important application fields of printable paper-based biosensors in the coming future. However, printable paper-based biosensors still have challenges in POCT. The major issues associated with paper-based platforms include sample processing, lack of reproducibility, long-term instability, limited clinical accuracy, all of which need to be addressed for adoption as a practical biosensing device[42b]. It's well known that printable assays for POCT are normally less sensitive than in the laboratory and often subject to potential interferences. In addition, Shaw pointed out that the inexperienced and untrained individuals who perform the test also contribute to the accuracy of POCT[168]. So, for future studies, the following aspects should be considered more wisely to improve the printable paper-based biosensors for POCT uses.

6.1 Printing techniques

Although various printing techniques have been studied and widely applied in the biosensor fabrication process, many challenges still exist. Generally, more efforts should be put in the requirement for fabrication equipment and ink materials composition optimization, as the unsuitable viscosity and surface tension of materials could cause printing failure[169]. Also, for some specific printing techniques like laser cutting, photolithography or screen-printing, only selected types of paper are suitable or customized masks maybe needed. Nowadays, some of progresses have already been made in the ink materials and printing techniques improvement. One of the widely applied technologies is the graphene-based printing ink modification. Graphene-based ink is of particular interest in recent years due to its advantages of facilitation in the low-cost development of electronic and optical devices. Cue et al's study showed that for their inkjet-printed paper-based analytical device, when the concentrations of enzyme-graphene ink with varying from 0.05 to 0.2 mg μL^{-1}, the sensitivity of their biosensor has been improved greatly for detection of glucose, lactate, xanthine and cholesterol[115]. They attributed the improvement in sensitivity to the change in the rates of enzymatic reaction and electron transfer. Also, the conductivities of all graphene electrodes increased significantly by 25-30 times as the ink loading increased from 0.05 to 0.4 mg μL^{-1}. This maybe the result of the increase in electron carriers with increasing ink concentration. Liquid metal modified with small organic molecules demonstrates potential as the bioink for printable biosensors[170].

6.2 Sample pretreatment

Analysis of clinical samples like blood, urine and saliva present challenges for complete

integrated printable paper-based sensing platforms as the sample pretreatment method and procedure could be time-consuming and equipment-dependent. For example, unprocessed blood contains cells, proteins and other components. Therefore, pretreatments like chemical reagents and external filtration are required[42b], leading to the high demand of cost-effective and simple pretreatment techniques. Previous literature has comprehensively reviewed the advances in sample pre-treatment methods for paper-based devices, indicating that removing large matrixes and retrieving target analyte (e.g. DNA, RNA, or protein) from the sample is vital for diagnostic accuracy[171]. Xu et al. demonstrated a platform named POCKET (point-of-care kit for the entire test), which is versatile for analyzing multiple types of DNA in different fields in a sample-to-answer manner (Fig. 5A). The POCKET consists of a chip which integrates the sample preparation with triple signal amplification and a foldable box that uses a smartphone as a heater, a signal detector, and a result readout[172]. Noticeably, the function of the smartphones as heater, permitting the rapid and robust point-of-care DNA amplification even at the cold environment. Their device is an excellent example of taking integration of sample pretreatment into design considerations. As another excellent example, Silva et al. reported the development of a simple colorimetric paper-based analytical device with electrochemical pretreatment for the detection of procaine in seized cocaine samples. To eliminate the chemical interference of benzocaine, they modified the traditional printed electrode pattern by removing the unprinted zone of the pattern circle to create an integrated pretreatment region for oxidizing benzocaine. Their pretreatment design largely improved the detection selectivity while decreased the detection cost[173].

6.3 Reagent stability

Maintaining the stability of assay reagents is fundamental to ensuring the quality, reproducibility, and reliability of diagnostic tests, especially considering the unpredictable user environments of POCT[174]. The common component of biosensors includes antibodies, enzymes, nucleic acids, and organic/inorganic compounds, which are all sensitive to environmental change and require cold storage (4 or -20 °C) for longer lifespan. In contrast, most POC devices have to work in uncontrolled environments, limiting the translation of well-validated diagnostic assays into accessible POCT in the field. To solve this problem, many commercial assays store lyophilized reagents in disposable cartridges for cold-chain-free storage. However, the required centralized manufacturing facilities and costly maintenance/repair could increase the cost and potentially limit the application range. Recently, Han et al. demonstrated a possible solution by introducing a polyvinyl alcohol (PVA) film-based reagent-storage platform for POC biosensors. This method incorporates premeasure, premix, and prepackage reagents, which can deliver assay reagents without cold storage and simplify quality diagnosis[175]. According to their study, the reagent filming holds excellent potential for streamlining and strengthening biochemical assays, providing a highly feasible approach to advancing cold-chain-free diagnostics.

6.4 Reproducibility

Compared with many other substrate-based biosensors, paper as a substrate is much more unstable under varied clinical environments, and therefore are more likely to have high batches variations affected by temperature and humidity. In addition, other reasons, including complex samples, probe instability[53] and uncontrolled physical properties of paper including porosity, permeability, and capillary flow rate, could also induce the instability of the test result. Actually, most existing paper-based sensor devices require recalibration to limit the impact of batch variation. LFAs are dependent on immobilized proteins and antibodies as ligands, and multiplexing assays pose a challenge due to batch variation and antibody cross reactivity[176]. Choi et al. reported using a portable temperature-humidity control device to provide an optimum environmental requirement for sensitivity improvement in LFAs. They found that with optimum experimental conditions (55 °C, >60% RH), the testing LFA was able to improve the sensitivity of almost 10-fold using dengue viral DNA and HIV DNA as model analytes[177].

Growing attention has been put to reproducibility problems of printable paper based POC devices. Shrivastava et al. mentioned that mobile POCT systems for field use would probably need internal biosensing parameter controls to ensure stability and reproducibility, which is a substantial obstacle to the calibration-free requirement [178]. Hong et al. described a method of covalent binding of proteins to cellulose paper, overcoming the poor producibility problems due to the limitation of physical protein adsorption on PADs. This method includes the periodate oxidation of paper, forming a Schiff base, and reductive amination. They identified aldehyde and imine groups formed on paper using FT-IR analysis. This covalent bonding approach enhanced the binding force and binding capacity of proteins[179]. Taken together, this immobilization method is a good example that contributes to the commercialization of PADs with high reproducibility and sensitivity.

6.5 Multiplex detection

Another critical aspect of printable paper-based biosensors is using techniques to detect multiple analytes, because correlations among physiological levels of multiple analytes is essential for many diseases early and accurate diagnosis[178]. The main advantage of multiplexed analysis is the capability to detect multiple biomarkers qualitatively or quantitatively in a single sample. Also, the multiplex assay could save time and sample input, and reduce variations of multiple single plex assays. However, even the most popular format used in POCT multiplex-detection, namely LFA with multi-test lines, is still insufficient due to the confined sensing domain, the limited sensitivity, the challenge of cross-reactivity interference and the high-throughput requirement[180]. With this in mind, Masterson et al. reported a label-free nano plasmonic-based biosensor, multiplex screening test for COVID-19 that can quantitatively detect 10 different biomarkers (6 viral nucleic acid genes, 2 spike protein subunits, and 2 antibodies) with the limit detection of 89 aM. Their detection approach shows the high-throughput capability by quantifying both IgG and IgM antibodies directly from COVID-19-positive patient plasma samples in a single instrument run[181]. Yin et al. reported a sandwich structure snail-shaped microfluidic chip for the multiplex detection

of cardiac markers including cTnI, CK-MB, and Myo with high sensitivity and a short detection time using the chemiluminescence method[182] (Fig. 5B). For cardiac diseases like acute myocardial infarction diagnostic, multiplex detection is particularly important for the early and rapid diagnosis, disease grade assessment, and evaluation of therapeutic effects. By integreated with smartphone analysis, a screen printed electrode was used for simultaneous detection of insulin and glucose in saliva for diabetes early diagnosis[183]. With the aid of assay development, future printable paper-based device is possible to realize the robust high throughout POCT purposes by integrating with the advances in the techniques like micro/nanofabrication, 3D printing and printed circuit board [43b].

6.6 Sensitivity

High sensitivity determines the success of a biosensor. For example, ultra-sensitivity (typically < 1 pM) would enable the sensor to detect cytokine at an ultra-low level from background noise, which permits reliable detection using only a small input volume of biofluids without analyte enrichment. However, the aim of POCT is to provide simple and fast detection, which requires no or limited sample treatment, minimal sample volume, limited signal amplification, and all these requirements would ultimately affecting the specificity and sensitivity of biosensing[180]. As a result, POCT has relatively low detection sensitivity compared to other diagnostic methods. To build a more practical device, the balance between sensitivity and simplicity is important and many efforts have been devoted to address the challenges. Today, multidisciplinary study dramatically improved biosensing performance by providing analyte specificity through biorecognition, probe elements for biomolecules or selective membrane for ions, to provide a quantifiable signal. These progressions have been tested in the COVID-19 diagnosis. In addition, Shrivastava et al.'s review also provides a update of the sensitivity progress in recent years[178].

Applying signal amplification strategies on printed biosensors is an important strategy to improve sensitivity. Signal-amplification strategies in PADs could be mainly divided into nanomaterial-based, nucleic acid-based, and PADs based signal amplifications[43b, 184]. A detailed and comprehensive review of the signal amplification strategies for paper-based devices is summarized by Liu and her colleagues[43b]. Here we will just briefly summarize the nanomaterial-based signal amplification and nucleic acid-based signal amplification strategies that could be applied to printable paper-based biosensors. Various NPs, such AuNPs, MNPs, and QDs, have been adopted in nanomaterial-based signal amplification methods to improve signal intensity[185]. The SERS method is a common and powerful sensing technique in nanomaterial-based signal amplification that generates and amplifies the inelastic light scattering of molecules when being adsorbed on metals like gold or silver[186].

Nucleic acid-associated signal amplification (NAAT) has also been applied for highly sensitive printable paper-based POC diagnostics of pathogens or virus[187]. The conventional polymerase chain reaction (PCR) is the most extensively used technique to rapidly process DNA samples, but it requires precise temperature control for

reactions, which is difficult to realize for POCT purposes. Alternatively, various isothermal signal amplification methods have been developed, including rolling circle amplification (RCA), loop-mediated isothermal amplification (LAMP), strand-displacement amplification (SDA) and recombinase polymerase amplification (RPA)[188]. Especially, the CRISPR/Cas based biosensing systems have been demonstrated to have diagnostics capability with high specificity and super sensitivity, and has already been applied to recent COVID-19 pandemic diagnosis and aptamer-based PADs fabrication[189].

Shirin et al. described a 3D microfluidic paper-based electrochemical NAAT (Fig. 5C). They used off-the-shelf gold plasma-coated threads to integrate electroanalytical readouts. All steps (including sample incubation, rinsing and detection) are integrated to allow time-sequenced reactions by constructing movable stacks structures of filter paper. Their device could complete the NAAT in 95 min, with a LOD of 0.06 pM target synthetic DNA, and was able to detect 1 ng μL^{-1} O. cf. ovata genomic DNA[190]. Their team also presented a woven biosensor based on a thiol-self-assembled monolayer for NAATs that integrates microfluidics structures with an electroanalytical readout[191] (Fig. 5D). Their efforts facilitate the printed wearable digital analytics using NAATs and other advanced electroanalytical paper- or textile-based devices.

6.7 Signal readouts

Based on the worldwide usage of smartphones, connected and digital diagnostics platforms and smartphone-based image recognition technologies have developed rapidly. External devices are possible to improve the sensitivity and selectivity of paper-based assays and provide real-time monitoring and diagnosis of patients. Particularly, the incorporation of mobile attachments for printable paper-based devices significantly improved the qualitative and quantitative digital signal readouts, providing an alternative for early disease monitoring on-site and in remote locations without specialized instrumentation. So far, LFA smartphone-based POCT has been developed for immunoassays, colorimetric and electrochemical sensing, and SPR-based biosensors[42b]. With the popularity of mobile smart devices, barcodes as new digital intervention have also been widely applied in healthcare settings in recent years due to their ability to allow the precise simultaneous detection of multiple targets from patient samples. Dyed beads[192], QDs[193], or shape-encoded microparticles are common techniques employed in fabricating such encoding assays. Up to now, such barcode detection has already been applied to water quality testing[194], medication supervision, toxins in agriculture[195], etc. The aggregation-induced emission probe was also successfully applied in PADs for POCT[196]. If integrated with GPS systems, barcode could tag data with geolocation and time to enable mapping of results, which would help centralized tracking of diseases and outbreaks[197]. The decoding of the barcodes is equally important as barcode development itself, as most decoding methods depend on costly and bulky devices. Many portable barcode decoding devices such as mobile optical devices, smartphones, and barcode scanners have been applied in recent years. Future studies should focus on developing more robust smartphone apps that allow on-site analysis while providing swift transfer and data storage to keep track of records[198],

and engineering the optical pathway associated with smartphone detection[199].

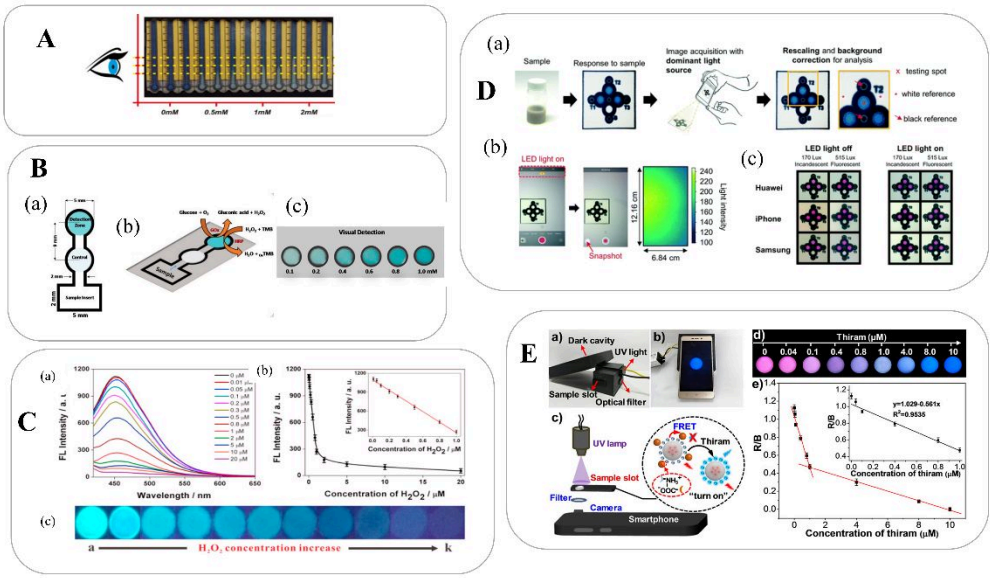

Fig. 5 (A) POCKET: ultraportable multiplex-detection for DNA with high sensitivity and stability[171], reprinted with permission from American Association for the Advancement of Science; (B) Snail-shaped microfluidic chip (SMC) for cardiac markers multiplex-detection[182], reprinted with permission from Frontiers; (C) Paper based electrochemical NAAT with integrated thread electrodes[200], reprinted with permission from American Chemical Society; (D) Electroanalytical woven textile-based microfluidics, thiol-self-assembled monolayer for NAATs: (a) schematics of immobilization of methylene blue conjugated stem-loop (S-L) DNA probes on the clean gold microwire surface. (b) schematics of the mechanism of RPA and providing ssDNA target using lambda exonuclease enzyme for the detection step. (c) and (d) schematics of the woven E-DNA sensor into Coolmax-cotton weave. (e) gel electrophoresis image of RPA amplified S. epidermidis genomic DNA. (f) photo of the real E-DNA sensor integrated into the woven microfluidic devices [191], reprinted with permission from Wiley.

7. Conclusions

The fast development of nanotechnology, microelectronic techniques and microfluidic systems has enabled the integration of various detection methods into portable POC devices. These technologies also made specific POC detection with low sample volumes, low cost, increased speed and sensitivity, and ability of multiplex detection possible[138]. This review summarized the latest progress and development of paper based printable POCT devices. The correlation between paper properties and paper selection is presented, together with the introduction of different fabrication methods, including drop casting, vacuum filtration, inkjet-printing, wax-printing, screen-printing, stencil-printing, laser-printing and photolithography. According to transducers, we generally divided paper-based microfluidic biosensors into optical biosensors using colorimetric, fluorescent and ratiometric fluorescent sensing strategies and electrochemical biosensors. Developing better fabrication and immobilization methods

for signal stability and proper reagents for signal regulation is required for the prospect of paper-based biosensors. To improve paper-based biosensors behavior, different paper surface treatment methods including physical adsorption and covalent conjunction are most used. Various reagents and materials used to improve device performance have been discussed. Challenges for paper based POCT strategy always exist, but many noble cross-filed solutions and progresses have also been provided and tested. In view of future POCT development, the challenges that have discussed in the above sections including reproducibility, sensitivity, multiplex-detection and digital signal readout would still be the essential problems remained to be solved, however, we can still see the promising future of printable biosensors in POCT, such as smart helath monitoring at home.

Table 1 Printed paper-based biosensors with electrochemical signal readout.

CV: cyclic voltammogram; EIS: Electrochemical impedance spectroscopy; DPV: differential pulse voltammetry; SWV: square wave voltammetry; BPE/LED: bipolar electrode based light emitting diode; PEDOT: poly(3,4-ethylenedioxythiophene); rGO: reduced graphene oxide; GO: graphene oxide: AuNPs: gold nanoparticles; GQD: graphene quantum dots; MB: methylene blue; Fc: ferrocene; Thi: Thionine; TEPA/PB: tetraethylene pentaamine; CoPc/IL/G: Cobalt (II) phthalocyanine/ionic liquid/graphene; pPNA：pyrrolidinyl peptide nucleic acid; CEA: carcinoembryonic antigen; NSE: neuron-specific enolase; PEAK1: pseudopodium-enriched atypical kinase one, SGK269; ECL: electrochemiluminescence; CA125: cancer antigen 125.

Analytes	**Paper types**	**Printing techniques**	**Electrodes materials/modifiers**	**Mediators of the electron transfer/redox transition**	**Electrochemical methods**	**Linear range**	**Limit(s) of detection**	**Reference**
Manganese superoxide dismutase (MnSOD) gene	Whatman No.2 chromatography paper	Screen-printing/ Wax printing	Triangular Au nanosheets	Signal probe labeled with Fc	EIS, CV	10 nM-1200 nM	3.91 nM	[158]
Glucose	Whatman grade 1 chromatography paper	Screen-printing/ Wax printing	WE: carbon ink as substrate, ZnO-nanowire decorated	PEDOT	CV	/	4 mM	[201]
	Chromatography paper	Laminating	Copper foil tape, carbon conductive tape	/	BPE/LED	10 mM-10 mM	1.79 mM	[202]
	Filter paper (Whatman No. 1)	Screen-printing/ Wax printing	WE: carbon ink; CE: carbon ink; RE: carbon ink	CoPc/IL/G	CV	0.01-1.3 mM	0.67 μM	[203]
	Whatman No. 1 filter paper	Screen printing	WE: carbon ink; CE: carbon ink; RE: silver/silver chloride	rGO-TEPA/PB	CV	0.1 mM-25 mM	25 μM	[204]

			ink				
	Whatman Chr 1	Screen printing	WE: carbon ink; CE: metal wires; RE: metal wires	Ferrocyanide	CV	0.1-15 mM	3Sb/m criterium [205]
Influenza Virus	Whatman chromatography paper 4#	Stencil printing	WE: carbo ink CE: carbon ink, RE: silver/silver chloride ink	/	CV, DPV, EIS	10-104 PFU mL^{-1}	113 PFU mL^{-1} [157]
Ferritin	Filter paper grade No.1	Screen-printing/ Wax printing	WE: Graphene; CE: Graphene; RE: Ag/AgCl	Potassium ferricyanide	DPV	0.004-4pM	0.4 fM [206]
Alprazolam	Whatman No. 1 chromatography paper	Laser printing	WE: carbon ink; CE: carbon ink; RE: silver/silver chloride ink	MB	CV, EIS	0.001-5nM	0.001 nM [207]
Mycobacterium Tuberculosis (MTB) oligonucleotide		Screen-printing/ Wax printing	WE: carbon graphene ink; CE: carbon graphene ink; RE: silver/silver chloride ink	pPNA	EIS	2-200 nM	1.24 nM [208]
17 β -estradiol (17 β -E2)	Whatman chromatography paper No. 1 (pure cellulose paper)	Screen-printing	WE: carbon ink; CE: carbon ink; RE: silver/silver chloride ink	Thi	CV, DPV	0.04-400 pM	0.04 pM [165]

Human papillomavirus	filter paper (Whatman No.1)	Screen-printing/ Wax printing	WE: caron ink, CE: / carbon ink, RE: silver/silver chloride ink		SWV	10-200 nM	2.3 nM [166]
miRNA-21	Photographic paper	Writing pen-on paper technology	Ag@Au core – shell/GQD nano-ink	/	CV, SWV	5 pM-5 μM	5.0 pM [209]
Prostate specific antigen (PSA)	Whatman chromatography paper No. 1	Screen-printing	WE: carbon ink; CE: carbon ink; RE: silver/silver chloride ink	Thi	CV, DPV	0.05-200 ng mL^{-1}	10 pg mL^{-1} [210]
miR-141 and miR-21	filter paper	Screen-printing	WE: paper (modified with MoS2/AuNPs/AgNW); CE: platinum wire; RE: silver/silver chloride ink	MB and Fc	SWV	1 fM-1 nM	0.1 fM [211]
Carcinoembryonic antigen (CEA) and neuronspecific enolase (NSE)	Whatman NO. 1 chromatography paper	Screen-printing	WE: carbon ink, CE: carbon ink; RE: silver/silver chloride ink	Thi for CEA; PEDOT for NSE	DPV	0.5-300 fM for CEA; 0.7 fM-7 pM for NSE	0.01 fM for CEA; 0.1 fMfor NSE [212]
Follicle-stimulating Hormone	Whatman chromatography paper	Screen-printing	WE: carbon ink; CE: carbon ink; RE: silver/silver chloride ink	r-GO/Thi/AuNPs	CV, DPV	1-100 mIU mL^{-1}	1 mIU mL^{-1} [213]

Pseudopodium-enriched atypical kinase one, SGK269 (PEAK1)	Chromatography paper (Whatman 1 chr)	Stencil printing	WE: carbon ink; CE: carbon ink; RE: silver/silver chloride ink	GO/AuNPs	DPV	0.04 fM-40 pM	0.04 fM [163]
miRNA-155	Filter paper	Screen-printing/ Wax printing	Carbon ink modified with AuNPs layer	Au-BPE	ECL	1 pM-10 μM	0.67 pM [214]
Cancer antigen 125	Whatman No.1 chromatography paper	Screen-printing/ Wax printing	WE: carbon ink; CE: carbon ink; RE: silver/silver chloride ink	rGO/Thi/AuNPs	DPV	0.1 U mL^{-1}-100U mL^{-1}	0.01U mL^{-1} [215]

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Printable biosensors towards next-generation point-of-care testing: paper substrate as an example

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