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Integrated Printed Microfluidic Biosensors

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Abstract

Integrated printed microfluidic biosensors are one of the most recent point-of-care sensor developments. Fast turnaround time for production and ease of customization, enabled by the integration of recognition elements and transducers, are key for on-site biosensing for both healthcare and industry and for speeding up translation to real-life applications. This review gives an overview of recent progress in printed microfluidics, from the two-dimensional to the four-dimensional level, accompanied by novel sensing element integration. The latest trends in integrated printed microfluidics for healthcare, especially point-of-care diagnostics, and food safety applications are also explored.

Keywords

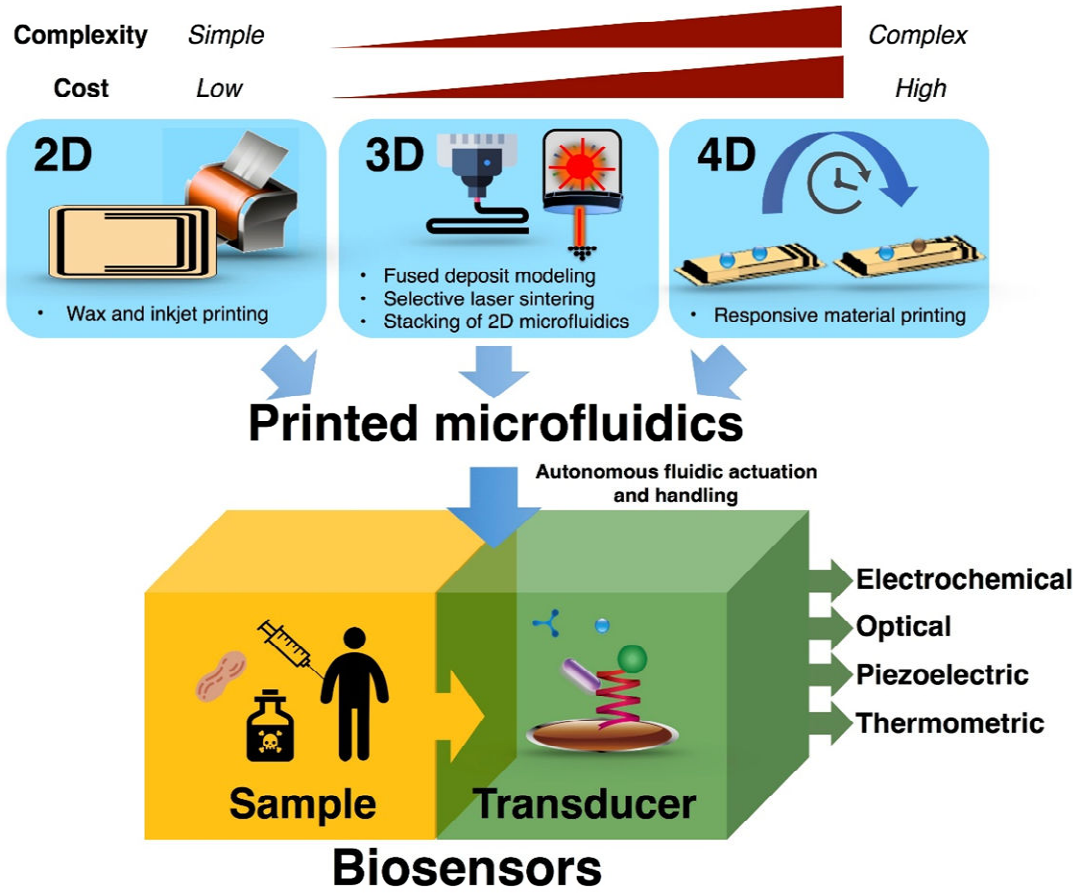
Biosensors; Printed microfluidics; Processable materials; Point-of-care diagnosis; Sample-to-answer; Just-in-time production

Integrated Printed Microfluidic Biosensors

Microfluidic (see Glossary) technology is important in **biosensors** for sample transport, reagent mixing, providing a reaction chamber for loading or immobilization of bio-recognition molecules, initiating bio-reactions and subsequent delivery of the biosensing reagent mixtures to the transducer interface. There are two major approaches for the fabrication of microfluidics: bottom-up and top-down. In the bottom-up approach, the microfluidic device is built from simple elements, e.g. plastic monomers, and the manufacturing is usually performed by printing or drop-on-delivery mechanisms. In contrast, in the top-down approach, the raw material is engraved, e.g. by laser-cutting or milling on acrylic plastic, to produce microfluidic components, channels, chambers and valves, and generally more waste is produced. Printing on the microscopic level, or nanoprinting, has become popular recently for high-resolution rapid prototyping.

Integrating microfluidics in biosensor development has a long history in the diagnostics industry. One conventional example is lateral-flow immunoassay, a biochemical test that measures the presence of protein biomarker using a deposited antibody as a recognition element in paper strip format, and it is commercially available worldwide [1-2]. Figure 1 illustrates the common techniques for fabricating integrated printed microfluidic biosensors. Microfluidics overcome the drawbacks of difficult and time-consuming fabrication, speeding up translation to real-life applications. More importantly, **just-in-time production** of microfluidics, integrated with **recognition elements** and **transducers**, provides a fast turnaround time for production, and facilitates ease of customization for multiple applications, hence speeding up its translation for various applications, especially for portable biosensing which usually requires rapid on-site sample handling steps [3-5]. Here we critically review the fabrication and integration of printed microfluidic biosensors and highlight recent major applications of microfluidic-based **point-of-care (POC)** tests as diagnostics for healthcare and food safety.

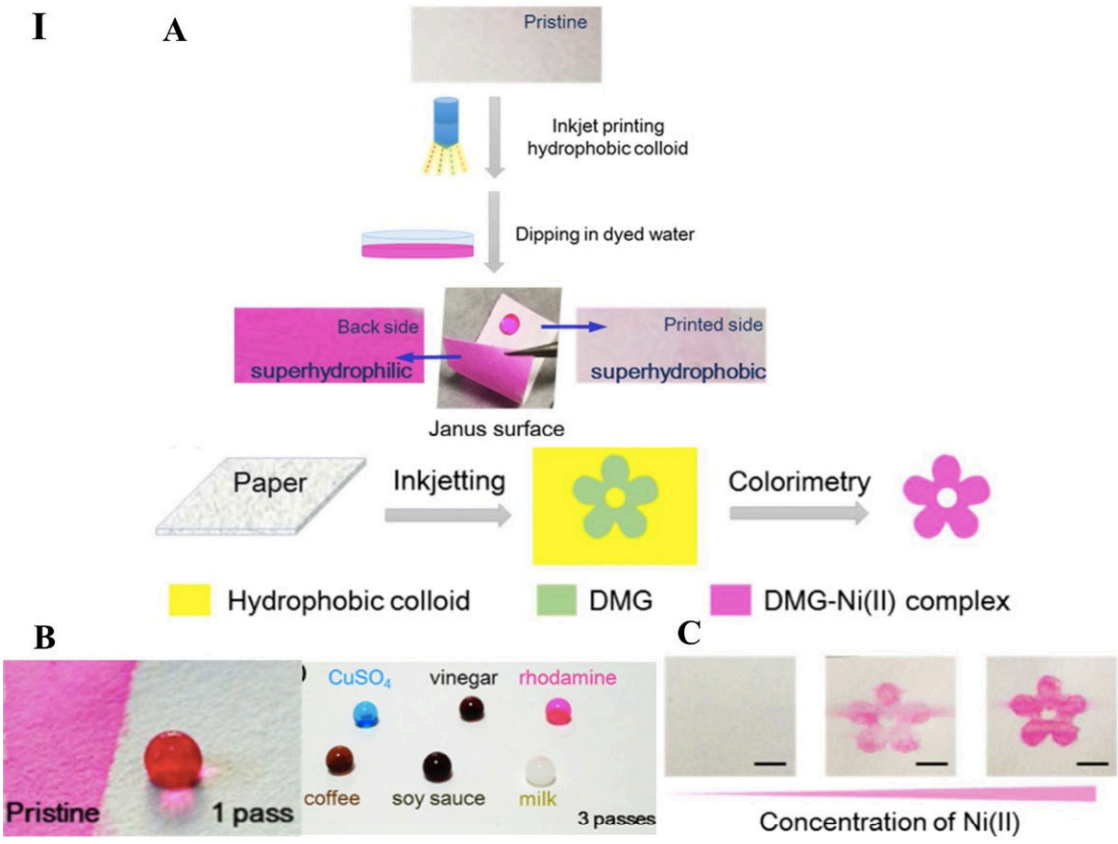
Figure 1. A Summary of Printed Microfluidics and its Biomedical Applications.



Fabrication and Integration of Printed Microfluidics

The fabrication of integrated printed microfluidics starts with the design of microfluidic patterns/features followed by fabrication via an appropriate printing technology, depending on the nature of the material used and ranging from paper, membrane, soft and hard polymers and various responsive materials. The biorecognition element is then immobilized onto the printed microfluidic platform and coupled to a transducer to form a biosensor. Printed microfluidics play a key role in biosensor construction because they significantly influence the final size and performance of the biosensor. Different types of printing methods and integration approaches result in different dimension levels and properties (e.g. laminar flow, diffusion, fluidic resistance and capillary flow) of the resulting microfluidics. Printing procedures and working principles of the 2D, 3D and 4D microfluidic are summarized in Figure 2, and Table 1 gives a brief survey of different microfluidic printing methods.

Figure 2. Fabrication of 2D, 3D and 4D-printed microfluidics. **I.** 2D paper-based printed microfluidics **A.** Inkjet printing on filter papers for hydrophobic-hydrophilic properties for biosensing Ni(II) ions. **B.** Demonstration of hydrophobic-hydrophilic property on the pristine area and consistency of the hydrophobic surface on different reagents after multiple (three) passes. **C.** Performance verification of functionalized printed microfluidics for Ni(II) ion colorimetric assays (scale bar: 5 mm). **II.** 3D-printed microfluidic with 3D chip-to-chip interconnecting layers **A.** Schematic illustration and image (macro- and microscope image) of 3D-printed CCIM interconnects under two independent (red and blue) sets of flow channels crossing up and down between the chips. **B.** Microscopic image on close-up (upper) of the 45-valve array assembled with the corresponding interface chip in clamping fixture, where each row of valves has its control ports connected in series to a pair of CCIMs, and each column has its fluid ports connected in series to a pair of CCIMs for fluid and control channels connected to individual CCIMs (lower). **C.** Schematic diagrams of the 3D printed pneumatically actuated membrane valve in open (upper) and closed (lower) state. Each valve is 300 µm in diameter. **III.** 4D-printed microfluidic with responsive materials. **A.** Printed of liquid crystalline elastomer (LCE) elements with uniaxial orientation. (Left) polymer Ink components; (Right) conceptual representation of the imposed polymer main-chain alignment along the printing direction. **B.** Schematic diagram and **C.** Image shows the thermomechanical response of uniaxially aligned printed-LCE microstructures and results of the change in shape over time upon high temperature trigger. **D.** Photo-responsive polymer gel micro-valves on PDMS microfluidics from printed stamp. **E.** Time-lapse images of microfluidics showing the sequential opening of photo-responsive micro-valves from right to left with time of localized blue light irradiation, which allows the blue dye solution to pass through from main microchannel. Reprinted with permission from references [10, 25, 40, 41].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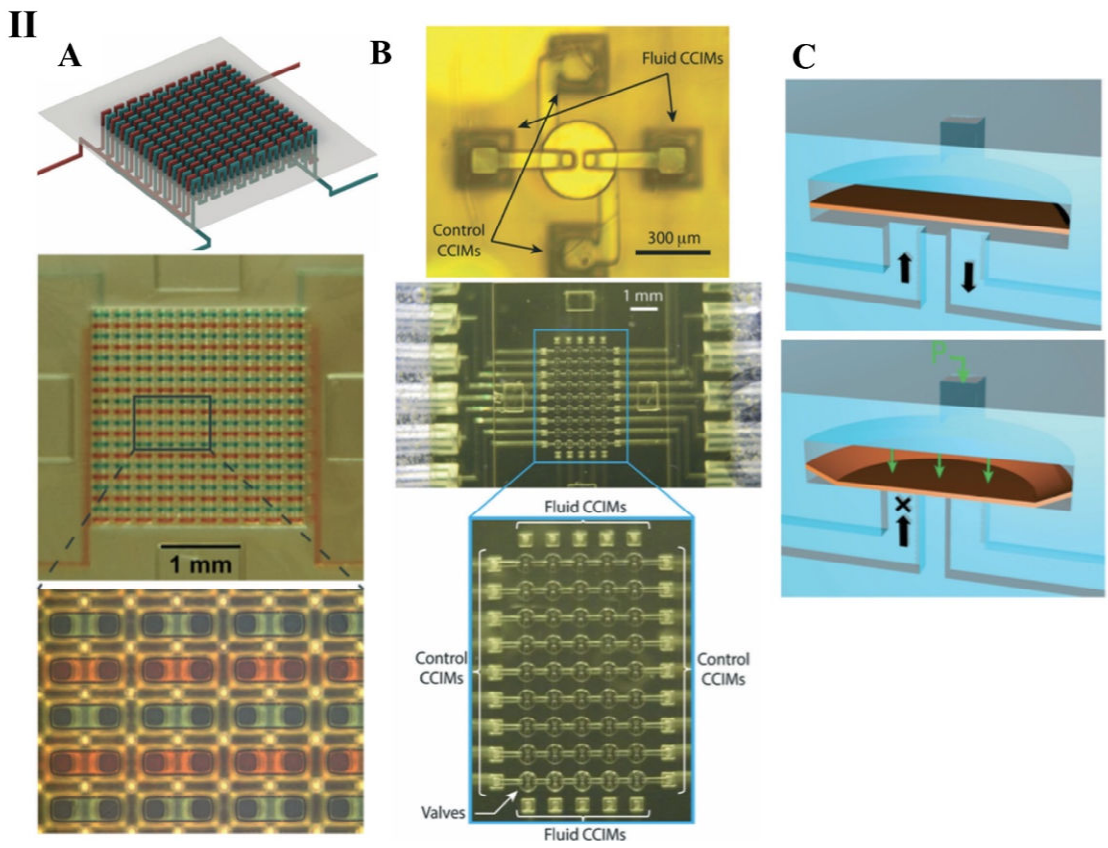
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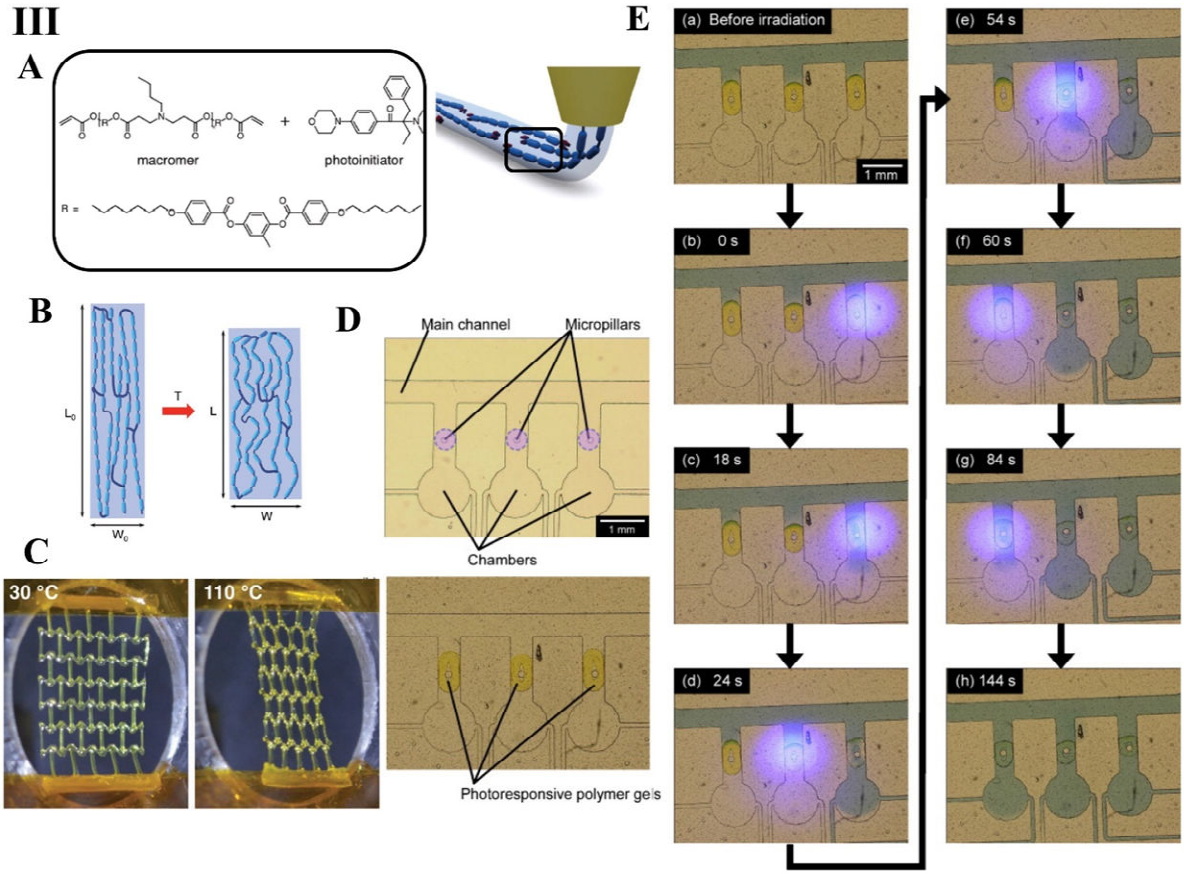


2D-printed microfluidics

Microfluidic printing on a planar surface is commonly used. Most 2D microfluidics share the same principle of fluidic actuation as the lateral-flow assay, which has been well characterized and commercialized. Lateral-flow tests can be considered as the first printed micro-liter fluidic actuation device. Conventional lateral-flow assays, i.e. in paper strip format, are based on nitrocellulose-driven fluidic actuation. Nitrocellulose (NC) is sprayed or printed on the plastic substrate base, where the pore size, thickness and printed density can approximately regulate the flow rate and amount of fluidic retention [1]. However, there is still room for improvement for current paper strip assays, e.g. to deliver versatile control and multiplexing [2].

The introduction of printed microfluidics on paper drastically improved the above situation. Wax printing, or hydrophobic printing, by melting the printed wax onto the target surface and forming hydrophobic barriers, is the most common method in the production of 2D microfluidics [6,7]. Printing unique, designated microfluidic structures for different assays allows all the sensing steps to be performed on paper, which is referred to as **lab-on-a-paper** [8,9]. However, the bottleneck of further development

with wax is the low printing resolution and chemical incompatibility. Non-polar polymers offer some solutions, since they can withstand aggressive cell lysis surfactant agents and organic solvents to form hydrophobic barriers with a high printing resolution to support applications involving, for instance, cell lysis and nucleic acid extraction [10-14].

The utility of printing relies on the quality of the print head. Thermal or piezoelectric print heads consist of a series of nozzles, where the inks made from a wide variety of materials are ejected onto the target surface. A recent advance involving the rapid printing of microfluidic channels confined by fluid walls and overlaid with immiscible fluid, rather than using a solid barrier, enables direct printing of the fluidic and biosensing reagents on an unpatterned surface and further reduces the complexity of integrating the printed microfluidic, recognition element and transducer elements [15]. The ultra-short time from concept to prototype and the low-cost and light-weight of the printers (e.g. office printer) and printing materials improve the transition from laboratory prototyping to large-scale manufacture for on-site biosensing applications.

Micro-contact printing and flexographic printing, i.e. roll-to-roll printing or mask printing, are still being used nowadays because scalable printing, with a "stamp" mask as the template, in micro-contact printing and even continuously printing on a rolling "stamp" are eminently achievable. Structured poly(dimethylsiloxane) (PDMS) stamps are commonly used in micro-contact printing to print both the microfluidic barrier and the pattern of recognition elements on chromatographic paper [16]. Yet, the printing quality relies on the accurate and precise contact focus between the stamp and the target. Different strategies such as pyramidal shaped stamps or magnetic stamps have been developed to overcome the current disadvantage of the print variation, which is higher than for direct inkjet printing [17,18]. Unfortunately, these methods still require a planar- or a roll-shaped stamp, currently fabricated in a complicated manner, thus hindering the speed of prototyping and customization. A combination of inkjet printing and roll-coating is expected to be the solution to further increasing the scale for mass production [19].

3D-printed microfluidics

Microfluidic printing on a 3D level provides an extra dimension for fluidic actuation, which increases the scale of the fluidic network and complexity to make stepwise and multiple biosensing reactions possible. Prior to the availability of highly popular 3D printing, fused deposit modeling (FDM) and pseudo-3D microfluidics (i.e. lamination or stacking of inkjet-printed 2D microfluidics to produce 3D-like channels) was commonly used [12,20-22]. However, the low spatial resolution, irreproducibility and lack of robust stacking techniques limited its practical use in biosensor development.

Additive manufacturing techniques, e.g. FDM is based on the combination of high-speed inkjet-printing and 3D robotic movement with high spatial resolution for layer-by-layer printing on top of a deposited solidified substrate from temperature-dependent liquefied materials, e.g. acrylonitrile−butadiene−styrene (ABS) or **polydimethylsiloxane (PDMS)** to construct a 3D shape within minutes [23-30]. Interconnection methods, e.g. simple integrated microgaskets (SIMs) and controlled-compression integrated microgaskets (CCIMs), have been developed to provide pneumatic connections between microfluidic chips [25]. Moreover, the Lego-like 3D assembly of microfluidics facilitates the creation of reconfigurable multicomponent, complex 3D microfluidic circuits by simply connecting standardized modular interlocking microfluidic element blocks together [30], which reduces the time for trial and optimization of microfluidic actuation during prototyping [31]. When combining the production of the microfluidic with hybrid of pseudo-3D based lamination and FDM, the prototyping remains rapid without the shortcomings of individual methods [32].

An alternative to FDM is direct laser writing, also known as multiphoton lithography, or nanoprinting, which combines the spatial precision of atomic force microscopy (AFM) and localized printing capability by microfluidics, with a nanometer-spatial fidelity and shows a high potential to fabricate a custom designed microfluidic or even nanofluidic biosensor [33]. On the other hand, stereolithography (SL) and selective laser sintering (SLS), relying on the action of a focused laser beam on a photo-sensitive resin liquid and powdered solid substrate, respectively, provide a higher resolution than FDM, for microfluidic fabrication and both of them can be completed in minutes [34-37]. The focused laser beam with a tunable wavelength, power and illumination time enhances the printing resolution down to sub-micron scale. Unfortunately, the photo-sensitive resin commonly used can only withstand a moderate temperature up to 60 ^{o}C without

deformation. The development of thermo-resistive resins such as PDMS resin (3DP-PDMS), with mechanical properties similar to conventional thermally cured PDMS, supported its practical transformation from FDM to SL [38,39]. Although these methods provide better printing resolution, they are impractical for constructing on-site POC biosensors due to the need for a bulky, sophisticated printer which is not readily available.

4D-printed microfluidics

4D-printed microfluidics provide dynamic microfluidics by introducing an additional "time" dimension into 3D-printed microfluidics; it is also called stimuli-responsive microfluidics because the time domain is governed by an external trigger, such as pressure, photo or thermal signals acting on the microfluidics materials and compositions. Reversible shape-morphing behavior upon an external trigger signal affecting soft matter elements in microfluidics increases the flexibility and complexity of microfluidic actuators [40,41]. For example, a thermally-responsive liquid crystalline elastomeric structure altered its geometry when there was a progressive temperature rise from 30 °C to 45 °C, and further changed at 90 °C, enabling the control of fluidic guiding and mixing by the temperature trigger [40]. In addition, photo-responsive polymer is widely used in microfluidic valving systems to provide a fine and accurate control, e.g. valve on/off or flow rate, in fluidic actuation by regulating the time and power of exposure to the laser [41]. The emerging needs and demands for stimuli-responsive printing materials with different properties, such as rigidity, in addition to the currently available soft materials, will shape the future development of 4D microfluidics.

Sensor assembly and integration with biological components

In order for printed microfluidics to perform as a biosensor, integration with biological components, i.e. recognition elements, biochemical reagents, labeling reagents and transducers are required. This integration ranges from state-of-the-art assembly with a commercially available module to novel printing techniques. Integration and immobilization of biological recognition elements, e.g. DNA and protein, onto microfluidic structures rely on adsorption, adhesion, covalent binding and dry pellet attachment. Printing of recognition elements such as aptamers (nucleic acids with a unique secondary structure that specifically binds to a target analyte) produces less interferences under challenging conditions, e.g. high-temperature printing, which often inactivate antibodies [42]. Biosensing components can also be packed in a dry pellet supplement format (using lyophilization and sugar stabilizers) which can be rehydrated before functional use, thus reducing the complexity of device reagent storage [43,44]. **CRISPR**/**Cas9** modules for cell-free reactions with synthetic gene networks is another promising method that can be integrated into printed microfluidics for biosensing [45,46]. The cell-free reactions further reduce the potential biohazard and the instability of living cells or genetically modified organisms. Above all, the rule of thumb of printed biological components is to provide high stability for long-term storage and robust biosensing, e.g. activation of biosensing components upon sample addition, to simplify POC diagnosis in resource limited locations.

Quantitative readout of signal output, including colorimetric, fluorescence or electrochemical signals, in biosensing requires integration with electronic or optical components. The printed circuit board (PCB), a reliable technique to integrate electronic components in the electronics industry, was exploited to create a PCB biosensor in the last decade. PCB embedment into printed microfluidics has been extensively used in digital microfluidics as it provides charge for droplet actuation [47,48]. Printed electrodes provide an alternative to photolithography, e.g. copper electrode patterns, with additional advantages such as the capacity to print electrodes on paper, for measuring quantitative electrical signals, such as voltage and current [6,49]. Electrodes used as recognition elements and transducers can be printed as hetero-structures to deliver improved signals [49]. Flexible and robust design of heater geometry, enabled by ink-jet-printed micro-heaters, provides localized heating for various thermal masses and a steady physiological temperature for initiating

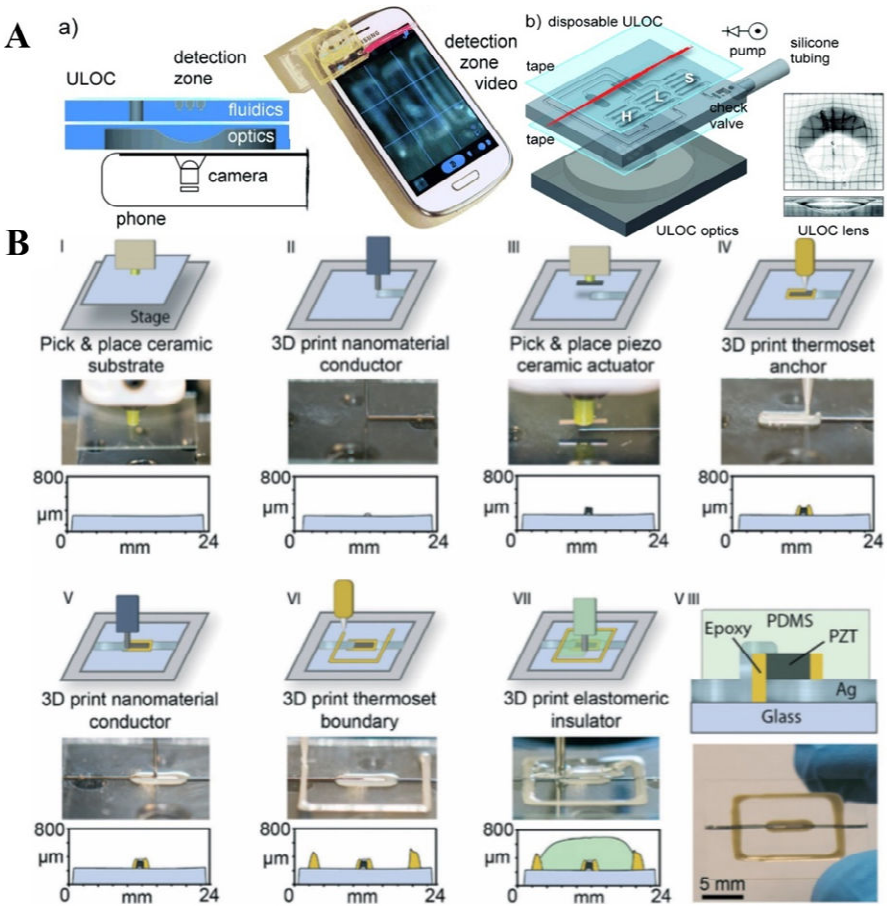
biochemical reactions during biosensing [50]. Furthermore, printed nanoparticles and conductive/insulating inks on flexible substrates, such as paper, plastic sheets, and textiles, offer additional advantages including free bending and stretching, which have high potential in wearable biosensors when integrated with printed flexible 2D microfluidics [51].

Optical components are generally integrated by using commercially available modules, e.g. lasers, optical lens and photodiodes. Integration with mobile phones has been widely reported to detect the visible spectrum using the CCD camera, making it functional as a mobile biosensor [52-55]. The advanced material perovskite has been demonstrated to be amenable to inkjet printing on flexible polyethylene terephthalate (PET) sheets to construct a distributed feedback laser [56]. The development of printed optics with excellent optical properties, e.g. magnification power and transparency, as well as lenses and optical waveguides, will support the promising performance of the integration of printed optical transducers into printed microfluidics biosensors [57].

Other transducers, such as magnetic resonance (MR) spectroscopy and piezoelectric sensors, have been integrated into 3D-printed microfluidics [58,59]. Integrating a silicon photomultiplier, rather than a photodiode into a 3D-printed microfluidic for luminescence assay, greatly improved the **limit of detection (LoD)** [4].

The integration of recognition elements and transducers into printed microfluidics for biosensor construction is summarized in Figure 3. The external cassette or biosensor shell is another concern in building all the components into a usable biosensor rather than just an experimental setup. Conventionally, the lateral-flow assay paper strip was assembled into a plastic cassette made by injection molding, which has a high initial cost of metal mold fabrication. Therefore, 3D printing, such as FDM and SL, is the current trend to produce small- and medium-scaled customized plastic cassettes for new printed microfluidics.

Figure 3. Integration of printed microfluidics with recognition elements and transducers for biosensor construction. **A.** a) Schematics of 3D-printed microfluidics integrated with printed optical lens, silicon tubing for pumping and camera module of smartphone. b) Scheme of the assembly showing the microfluidic that hosts three concentration channels (S: sample; L: low concentration and H: high concentration of calibration solution), a check-valve seat, and a connector to silicone tubing. (Bottom right) Image of the 3D-printed lens showing lens magnification on a millimeter paper and a side view showing the thickness. **B.** Additive manufacturing concept of 3D printing and robotic embedding facilitates the integration of orthogonal in-plane and out-of-plane piezoelectric transducers into microfluidics. Seven fabrication steps (I–VII), illustrated with the schematic and photo of assembly, as well as the height profile, show the 3D printing and embedding processes for fabricating the transducer. (VIII) Cross-sectional schematic (top) and photo (bottom) of the completed integration. Reprinted with permission from references [53, 59].



Successful transformation of printed microfluidics from laboratory test to practical applications depends on some key issues, including the upcoming development and novel integration with printed microfluidics, as well as the challenges in applying integrated microfluidics for biosensing applications. Therefore, some key healthcare and food safety applications will be showcased in the following section.

Integrated Printed Microfluidic Biosensors for Healthcare Applications

Healthcare is one of the most important applications in the biosensor industry. Development of printed POC biosensors for diagnosis, as well as intervention, has been widely researched. Cost-effective just-in-time printed microfluidic POC biosensors, combine sample collection, sample processing and interaction with recognition elements for **sample-to-answer** biosensing. They can provide a rapid and convenient solution to tackle urgent needs in healthcare, such as on-demand fabrication of biosensors for immediate healthcare management in developing countries, including screening of pathogen antigens for outbreak control of emerging diseases and protective immunoglobulin G (IgG) antibodies for global serological surveillance to estimate population-level immunity and efficacy of an immunization program [60,61]. The current application of printed microfluidic biosensors in healthcare is mainly divided into molecular diagnostics, *in vitro* and *in vivo* applications. Table 2 shows examples of these applications with various printed microfluidic biosensors.

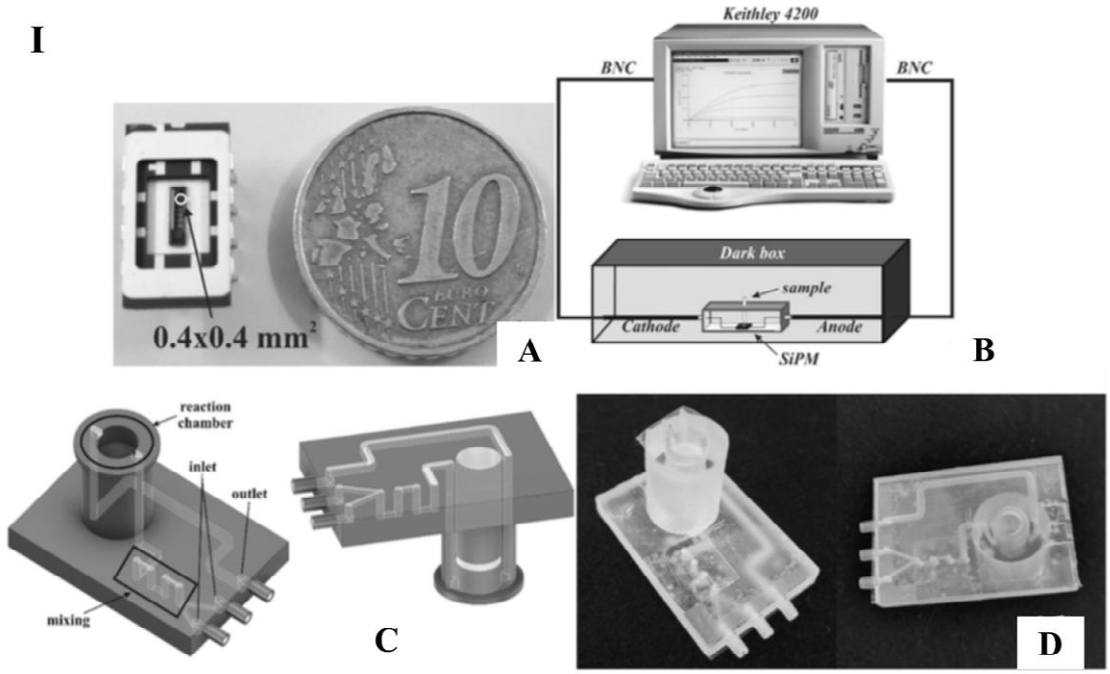
Molecular diagnostics

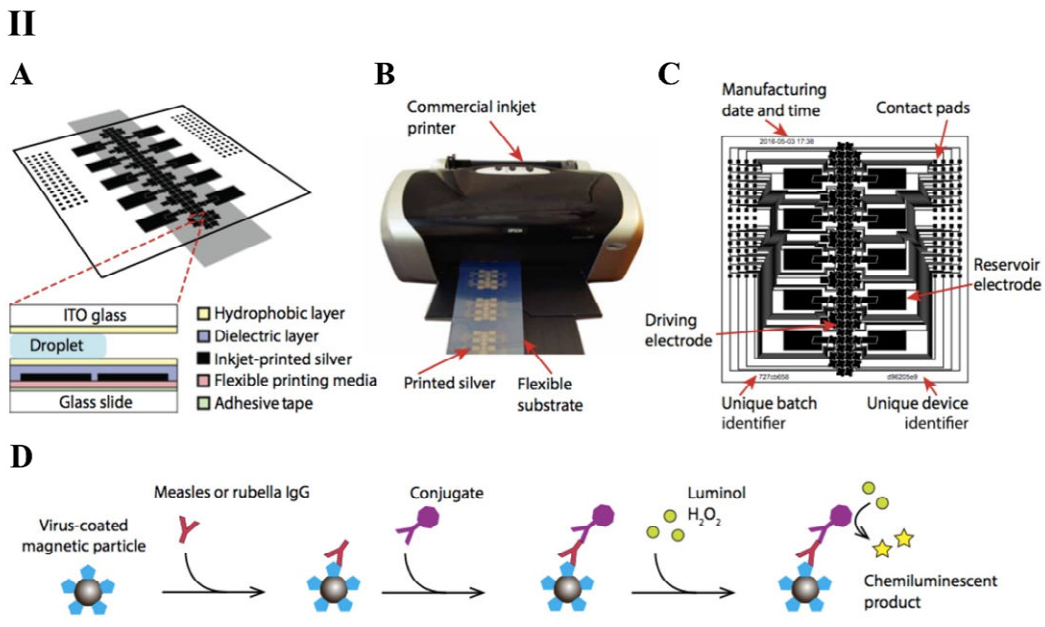
For molecular diagnostics, biosensors detect biologically related small molecules, protein and nucleic acid biomarkers. For small molecule biosensing, the interaction between a small molecule ligand and recognition element is the main process. 2D paper-based hydrophobic printing to produce microPAD's is adequate for optical or electrochemical sensing of small molecules, such as potassium ions, glucose and ATP, or macromolecules, such as the metabolic marker LDH [43,62,63]. Immunological and oncologic protein biomarkers are two important targets in current POC medical diagnosis. Pseudo-3D paper-based microfluidics can be achieved by "origami" folding to increase the throughput and is used for immunoassays to detect tumor biomarkers for cancer diagnosis [64]. The integration of sample-to-answer detection, handling and pre-treatment of clinical samples, such as blood and saliva, requires a complicated

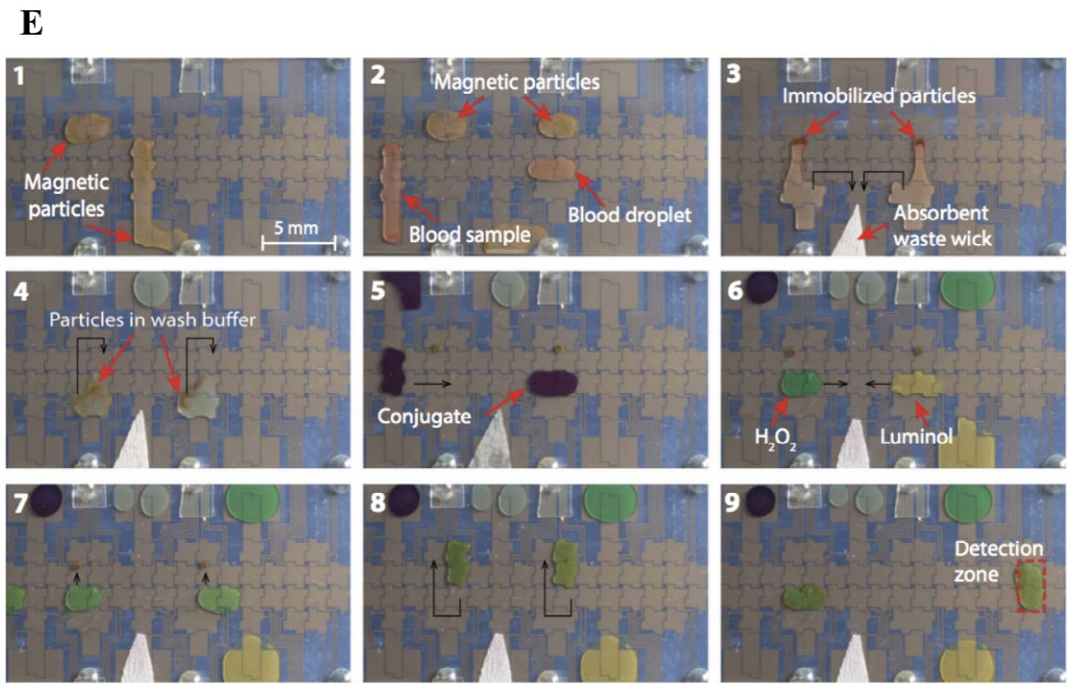
microfluidic structure, while 3D-printed microfluidics is most suitable for the fabrication of complicated microfluidics. Immunoassay, such as the current clinical gold standard **enzyme-linked immunosorbent assay (ELISA)**, requires multiple procedures from sample addition to color product generation, where a combination of 2D and 3D-printed microfluidics is more popular, as illustrated in Figure 4 [3]. Using a recognition element-tagged polymer monolith or paramagnetic beads that capture the target biomarker, as a preconcentration and purification step, prior to biomarker detection in printed arrays, can enhance detection sensitivity [3,65]. Another approach to signal enhancement is using biochemical reactions, such as nano-liposomal amplification and amplification-by-polymerization, which increases the electrochemical signals [66,67].

Figure 4. Printed microfluidic POC biosensors in healthcare diagnosis. **I.** 3D-printed microfluidic integrated silicon photomultipliers (SiPMs) for highly sensitive real-time ATP bioluminescence detection. **A.** Image of the integration of printed microfluidics with SiPM; **B.** Schematic of the setup as a functional biosensor; **C-D.** Schematic and image of the 3D printed microfluidic chip to be assembled in the biosensor. **II.** Inkjet-printed digital microfluidic cartridges integrated with sensing instrument employed in ELISA to detect immunoglobulin G (IgG), such as measles and rubella, for global serological surveillance of vaccination. **A.** Schematic of the DMF from top (isometric-view) and bottom (cross-section) plates assembled to a cartridge with inkjet-printed electrodes (black) pre-coated with dielectric (purple) and hydrophobic (yellow) layers, flexible printing media (peach) substrate fixed to a ITO glass slide (green). **B.** Image of silver flexible electrodes printed with a commercial inkjet printer. **C.** Schematic (top-view) of the DMF device. **D.** Schematic diagram of the ELISA, where paramagnetic particles coated with antigens of measles or rubella virus capture anti-measles or anti-rubella IgG (red) from sample, followed by detection with anti-human IgG-HRP conjugate (purple) and colorless chemiluminescent substrate (luminol and H_2O_2 (yellow-green)), which is converted into product (yellow) by HRP. **E.** Image of fluidic actuation of two assays performed in parallel in a DMF cartridge, where black arrows indicate the direction of droplet movement (From 1-9). Droplets of dispensed particle suspension merged with blood samples are immobilized, while supernatant waste is removed with an absorbent wick. The droplet after washing (blue) and antibody-

conjugate addition (purple) are subjected to chemiluminescent substrate mixing (green). Reprinted with permission from references [3,4].







For nucleic acid biomarkers, nucleic acid amplification tests (NAATs) followed by recognition of amplicon achieves highly sensitive sensing of as low as one single copy of target nucleic acid. A laminated microfluidic formed from polyester-toner has been introduced earlier to perform polymerase chain reaction (PCR), a gold standard NAAT [68]. Further integration of microfluidics with a compact 3D-printed external cassette and a smartphone camera enabled fluorescence imaging for digital PCR detection [69]. Isothermal amplification, such as loop-mediated amplification (LAMP) and rolling circle amplification (RCA) to remove the need for highly accurate thermocycler integration, is preferred in 2D-printed paper-based microfluidics, since paper and hydrophobic barriers can tolerate high temperature. Quantification can be achieved via a visible color change, such as a pH indicator, to detect the reactions that sense any nucleic acid markers, including DNA, RNA and microRNA, within minutes in disease diagnosis, especially for early screening of viral infection and cancer [44, 54, 70].

In vitro and *in vivo applications*

3D FDM microfluidics, which can be rapidly prototyped, are mainly used due to their capacity for complicated microfluidic features for cellular sensing, actuation and manipulation. Wax-printed cellulose filter paper-based microfluidics and microwell arrays, which provide additional cyto-compatibility and convenient biosensing of oxygen concentration due to the intrinsic high permeability, have been employed as a 2D and 3D cell culture systems [71-72]. Cell-counting and culture of living mammalian cells for HIV and inflammation diagnosis have been performed using 3D FDM microfluidics to provide fluidic flow to the cell attachment and counting chamber, respectively [73,74]. In addition, cell manipulation, such as migration and separation, that could increase specificity in downstream biosensing has been performed using stereolithographic printed alginate-hydrogel microfluidic barriers and printed dielectrophoresis, respectively [39, 75]. Printed droplet microfluidics have been shown to dispense picoliter droplets and cells with deterministic control, such as for droplet-based single-cell transcriptome profiling to support the realization of highly quantitative profiling of gene expression across all cell populations simultaneously [76,77]. 3D multicellular spheroid cultures incorporated into microfluidics, that simulate *in vivo* cell-cell interactions, have been demonstrated for the determination of metabolic activity, and can be translated into cell-based biosensors [78,79]. Organs-on-chips achieved by combining printed microfluidics and bioprinting of 3D-cells to mimic the heterogeneous properties, complex vascular structures and physiological responses of real organs, support automated, continual monitoring of extracellular micro-environments, such as pH, O_2 and protein biomarkers for biological study of metabolism and toxicity, and drug screening in the development of personalized medicine [80, 81]. This bridges the gap between *in vitro* cell culture and the animal models or human trial.

Minimally invasive implants are an up-coming trend for applying biosensors *in vivo*. Attachment of a 3D-printed microfluidic, customized based on the 3D organ surface topographical image, on the surface of the kidney was recently demonstrated for minimally invasive ‘microfluidic biopsy’ profiling on the targeted localized region of the organ [24]. A compact SL-printed 3D-microfluidic integrated with FDA-approved microdialysis probes has been used for wireless, continuous monitoring of the metabolite levels, such as blood glucose and lactate of human subcutaneous tissues

[82]. This increases the sensing complexity and throughput compared to the current needle-based biosensors. An implanted microfluidic neural probe with printed flexible polymer has the potential to tackle neurological disorders, including *in vivo* measurement of complex neural circuits and deep brain simulation [83, 84]. It is worth noting that printed microfluidic biosensors should be more biologically compatible for translational use for implanted microfluidics. Unfortunately, most of the 3D-printing materials available for FDM, such as PLA and SLA photopolymers, are highly toxic and environmentally harmful [85-97]. Therefore, biodegradable or food-grade non-toxic printing material, such as alginate and gelatin, or other materials with a non-toxic biocompatible coating, will be possible routes to ensure safety for *in vivo* application [88,89].

Integrated Printed Microfluidic Biosensors for Food Safety Applications

Toxins, produced by bacteria or fungi, as well as harmful environmental chemicals, are increasingly responsible for food poisoning or intoxication. Foodborne pathogens are the most important in food safety, as annually millions of illness and more than 400,000 of deaths worldwide are caused by bacterial contamination, viral infection and toxins from contaminated food and water [90]. In routine screening for outbreak investigation and control, food samples are sent to laboratories during delivery from farm to market [91]. Due to the time-consuming sample transport, contaminated food may have already been consumed before the test. Therefore, printed microfluidic biosensors could provide a solution to current situation by enabling rapid on-site screening.

Detection of toxic and harmful chemicals

A microfluidic channel plate embedded with 3D-printed optical accessory to connect to smartphones for Aflatoxin B1 robust sensing of moldy corn samples meets the testing standards set by authorities in North America, and is suitable for on-site use [55]. Phenol, an industrial pollutant potentially hazardous to aquatic life and human health and contaminating tap water, has been detected using tyrosinase-based electrochemical biosensors fabricated from, for example, multi-walled carbon-nanotubes and gold nanoparticles (GNPs/MWCNT) nanocomposite-screened printed electrode, which provides a large surface area for biosensing [92]. In addition, antibiotic residues in food or falsified antibiotics, which can lead to increased multidrug

resistance in pathogens, could be detected with a simple paper-based 2D wax-printed microfluidics [5,93].

Detection of pathogens

Size-based separation in 3D-printed helical microchannels has been applied to isolate antibody-functionalized magnetic nanoparticle cluster complexes for quantification of *Escherichia coli (E. coli)* in milk [94]. Furthermore, a 2D paper-based biosensor printed with RNA-cleaving fluorogenic DNAzymes (RFDs), a DNA-based enzyme that cleaves fluorogenic substrate upon binding to the target *E. coli* biomarker, delivered increased detection sensitivity [95]. This DNAzyme strategy was also reported for detecting other bacteria such as *Clostridium difficile* [96].

Most of the above applications make use of 2D microfluidics, particularly paper-based 2D microfluidics, because it tolerates slight variation in controlling volume and speed of fluidic actuation. In contrast, addition of bacterial pre-concentration in a magnetic pre-concentrator that increases sensitivity and cell lysis and detection of the bacterial biochemical marker ATP, involves multiple components and steps [97]. Therefore, 3D printed microfluidic is arguably more suitable for such assays.

Concluding Remarks and Future Perspectives

Printed microfluidics have been an attractive choice for the fabrication of the sample/reagent handing interface for various biosensors, thanks to the low cost and relatively short time needed for customization. At present, printed technology has created advanced fluidic actuation using complicated structures in 3D-printed microfluidics, with additional intrinsic sensing using external triggers for dynamic control of microfluidic structure such as 4D-printed microfluidics. The choice of printed methods and dimensions of microfluidics (2D, 3D and 4D) for the development of printed microfluidic biosensors is dependent on the application. In our opinion, a low-cost, just-in-time produced 2D-printed microfluidic is a suitable choice for use as a POC sensor in remote regions for diagnosis, while 3D-printed microfluidics support rapid prototyping in industrial research for fluidic actuation requiring a high precision.

Computer-aided design (CAD) further accelerates the rapid prototyping of printed microfluidics with complex networks. Yet, current microfluidic design is too technical

for researchers other than microfluidics engineers to interpret. In this context, the open-source repository of printed microfluidic design files and specifications, and recently developed feature-based software with graphical user interface (GUI), will simplify the design and fabrication process [98,99]. This will encourage experts of other disciplines, such as biologists, to develop innovative printed microfluidic biosensors.

The choice of suitable printing materials is a current limitation. In our opinion, printed metal [100], conducting polymers [101] and optics [102] with electrochemical and optical properties similar to current molding methods, will become more prominent in the upcoming decade. Transducers, such as electronics [103] and optical waveguides, will be available to be printed directly rather than being used as an additional embedment within a PCB as at present (see Outstanding Questions).

Advancing biotechnologies with respect to the recognition element, such as locked nucleic acids (LNA) and peptide nucleic acids (PNA) that resist nucleases and proteases naturally omnipresent in clinical samples, and antibody fragments or minibodies that bind to targets with higher affinity, are expected to improve biosensing sensitivity and specificity. Although the above recognition elements are currently expensive due to the demand-supply gap and the limits of current synthesis technology, their advantages and the ability of direct printing of these on printed microfluidics will increase the demand and synthesis technology available.

The accuracy of the above POC diagnostics will influence the level of integration of the data into healthcare big data repositories to enable further analysis and use. Biosensor networks on global serological surveillance are to be encouraged exploiting the fast turnaround time and ease of customization of printed microfluidic biosensors used as POC diagnostics.

Considering the upcoming advances in terms of materials, printing techniques and integration methods, we believe that utilizing printed microfluidics as POC biosensors, especially in urban and remote areas with limited access to centralized laboratories, or sample-to-answer readout for downstream treatment will meet urgent unmet needs, and will be much more common in the coming years.

Glossary

Biosensor: a self-contained integrated analytical device that combines a biological recognition element with a transducer used for detection of an analyte in a quantitative or semi-quantitative manner.

Enzyme-linked immunosorbent assay (ELISA): a current gold standard method relying on enzyme-linked antibody for detecting protein markers.

CRISPR/Cas9: a prokaryotic immune system using Cas9 enzyme to recognize and specifically cleave the DNA strand complementary to CRISPR sequence, a family of DNA sequences found in the genomes of prokaryotic organisms.

Just-in-time production: a methodology to streamline production when needed without pre-storage. It aims to reduce times within the production cycle, including the time, space and labor for delivery of stock from inventory.

Lab-on-a-paper: a miniaturized device that combines various laboratory functions on a paper substrate.

Limit of detection (LoD): the lowest concentration of the target that is distinguished from a blank with a stated confidence level.

Microfluidics: the actuation of fluid or droplet with a volume below microliter, typically from picoliter to microliter, in a microenvironment such as microchannels, in a controlled manner.

Polydimethylsiloxane (PDMS): a non-toxic, optically clear, silicon-based organic polymeric compound with hydrophobic properties, commonly used in the fabrication of microfluidics and medical devices.

Point-of-care (POC): an on-site diagnostic test performed next to the patient or by the patient with minimal assistance.

Recognition elements: composed of nucleotides or peptides for specific interaction with target analyte.

Sample-to-answer: an automated performance with minimal or no user interaction from the time the raw sample is inserted until the result as answer.

Transducers: processes the signal from recognition element and gives out a measureable datum as output.

Table 1. Summary of 2D to 4D microfluidic printing methods

Dimension	Method	Example of Printing Materials	Principle	Advantages	Limitations	Complexity (1: lowest; 5: highest)	Cost (1: lowest; 5: highest)	Ref.
2D	Lateral flow	Nitrocellulose	Spraying and stacking	Capillary action for fluid actuation	Large sample volume is needed	1	1	1
	Hydrophobic printing	Wax, polystyrene, poly(styrene-co-acrylic acid), methylsilsesquioxane (MSQ), silicone resin	Inkjet printing	High planar resolution; Easy printing of various structures (e.g. micro-rings for spot assays, pillars as delay barriers); Resistant to surfactants (e.g. SDS, CTAB, Triton X-100); Resistant to organic solvents (e.g. toluene and DMSO)	Precise control in volume and flow velocity	2	1	6, 7, 9
	Flexographic printing	PDMS	Roll and mask printing	Mass production; Resistant to organic solvents (e.g. methanol)	Flow velocity control	2	3	16
3D	Pseudo-3D stacking	Cellulose fiber, Polymer (Polyester, PMMA)	Lamination/stacking of multiple layers of 2D-printed microfluidics	Spatial dimension available to increase assay throughput	Low spatial resolution	3	2	12, 20-22 64,70
	Fused deposit modeling (FDM)	Polymer (ABS, PLA, PDMS), wax, epoxy	Extrusion of heated polymer on a surface with vertical, i.e. z-axis movement of printer head	High spatial resolution	External pump required for fluid actuation	4	2	23-30
	Stereolithography (SL)	Light-sensitive resin, light sensitive PDMS	Focused optical beam on photo-sensitive liquid substrate	Printing precise and complicated structures	Printed materials with a low melting point only	5	4	3, 37-39

	Selective laser sintering (SLS)	Resin, nylon metal particle	Laser sintering on solid powder	Printing rigid product	Laser hazard, rough surface	5	5	35, 36
4D	Various (Similar to 3D)	Soft responsive polymers	3D printing with responsive printing materials	External trigger for microfluidic control	Limited choice of printed materials	5	5	40, 41

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Table 2. Examples of different types of integrated printed microfluidic biosensors in healthcare and food safety applications.

Application	Microfluidics	Target	Recognition element	Transducer / signal detection	Detection limit	Ref.
Small molecule biosensing	2D-printed paper	Potassium ion	Ionophore I (valinomycin)	Optical (colorimetric)	0.1 mM (in 3 µL buffer)	62
	2D-printed paper	Glucose	Oxidase enzymes	Electrochemical	2.8 mM (in 4.5 µL buffer)	63
	3D-printed FDM	ATP	Luciferase	Optical (silicon photomultipliers)	8 nM (in 100 µL *E. coli* cell lysate)	4
Metabolic profile analysis	3D-printed FDM	Pyruvate, lactate; Overall conversion rate (metabolic flux)	Radioactive ^{13}Carbon	Magnetic resonance (hyperpolarized micromagnetic resonance spectrometer (HMRS))	10^4 cells (K562 and Jurkat cells)	58
Vaccination antibody screening	2D-printed polymer and 3D-printed FDM	Antibody IgG (	ELISA	Optical (colorimetric)	0.14 mIU/mL (measles IgG); 0.15 IU/mL (rubella IgG) (in 100 µL human blood)	3
Cancer diagnosis	2D-printed paper	Cancer overexpressed biomarkers (e.g. carcinoembryonic antigen (CEA), alpha-	Nano-liposomal amplification	Electrochemical (Impedance spectroscopy)	0.01 ng/mL (CEA); 0.01 ng/mL (AFP); 0.05 ng/mL (CA125); 0.05 ng/mL (CA153)	66

		fetoprotein (AFP), cancer antigen 125 (CA125), carbohydrate antigen 153 (CA153))			(in 2 µL buffer)	
	2D-printed polymer	EGFR and VEGF	Amplification-by-polymerization	Electrochemical	0.01 pg/mL (EGFR); 0.005 pg/mL (VEGF) (in 50 µL human serum)	67
Viral infection screening	2D-printed paper	hepatitis C virus genome HCV-1 DNA	RCA with Peroxidase-mimicking DNAzyme PW17	Optical (colourimetic)	10 pM (in 15 µL buffer)	44
	2D-printed paper	Zika viral gene markers	LAMP	Optical (smartphone imaging)	1 copy/µL (in 50 µL water)	54
	3D-printed FDM	Zika viral gene markers of different strains	CRISPR/Cas9 synthetic gene network	Optical (luminosity)	1 fM; Single-base discrimination (in 30 µL 7% human serum)	46
Determination of HIV antiretroviral therapy initiation	3D-printed FDM	CD4+ Cell-counting	APC-αCD3 (stains all T-lymphocytes); PerCP-αCD4 (stains the CD4+ subpopulation),	Optical (microscopic imaging)	< 200 / µL (in whole blood)	73
Toxin contamination	2D-printed paper	Alfatoxin B1 (in corn)	Anti-Alfatoxin B1 antibody	Optical (luminance)	< 5 ppb (in spiked corn sample)	55

1

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Integrated printed microfluidic biosensors

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