# Strategies for Multiplexed Electrochemical Sensor Development

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## Abstract

Detection of multiple biomarkers for disease diagnosis or treatment monitoring has received a lot of attention due to their potential impact on clinical decision making. Electrochemical biosensors have become one of the preferred detection approaches, due to the simplicity of the accompanying instrumentation. This chapter will explore how electrochemical sensors can be utilized for detection of multiple analytes by integration of sensors into microfluidic microsystems. Some key fabrication technologies for such devices will be presented utilizing polymer microfabrication, paper-based approaches, and the use of printed circuit boards. Next, the use of electrode arrays will be presented along with some commercial platforms, outlining plausible paths towards a successful electrochemical multiplexed sensor. Novel approaches based on microbeads and various labels will then be introduced along with various strategies and technologies utilized to achieve ultrasensitive multiplexed detection.

Keywords: electrochemical biosensors, multiplex immunoassays, microfluidics integration, printed circuit boards, electrode arrays.

# 1 Introduction

Electrochemical sensors for ultrasensitive multiplexed diagnostics of biologically relevant health and environmental markers are an important area of scientific interest. The importance of developing such devices has never been more topical than now, with global disease prevalence increasing, associated with a growing global population and worsening environmental pollution levels. Thus, the next generation of medical and environmental diagnostic and monitoring technologies needs to be robust enough to address current and future unmet needs. To develop robust and truly integrated electrochemical sensors for multiplexed and ultrasensitive devices, a number of strategies have been developed and utilized.

This chapter discusses the importance of microfluidics and fabrication techniques associated with the development of electrochemical sensor devices. It looks at the various techniques implemented and how they may be applied to the fabrication of important components for sample transport, preparation and analysis. It takes a specific look at polymer-based, paper-based and printed circuit board (PCB)-based devices for this effort of developing truly integrated microsystems.

This chapter has further been divided into the three main routes for multiplexed device development including: microfluidic microsystems, multi-electrode arrays and the use of microbeads and labels. Microfluidic microsystems are discussed encompassing the use of fabricated and polymer-based systems as mentioned above. The Multi-electrode arrays section explores the use of sequencing and detection of nucleic acids using electrochemical devices alongside detection of proteins, whole cell pathogens and small molecules. Finally, we discuss the important role of microbeads and labels for multiplexing. In this section we cover the many ways in which this approach can be implemented such as microbeads, enzyme labels, using multiple labels on single transducer surfaces (barcoding) and charged nanoparticles. Throughout this chapter specific examples are detailed of devices and technologies that have been developed

using these various approaches. Also discussed are some of the key commercial devices that have made it to market using these techniques and strategies.

## 2 Microfluidic Microsystems for Multiplexed Analysis

Microfluidics enable spatial separation of multiple biosensing reactions or analyte recognition sites, allowing detection of multiple analytes in a single system. A multitude of techniques have been developed for the fabrication of channels, chambers, mixers and filters ranging from the macro- to the nanoscale. Using these techniques to fabricate such miniaturized structures allows scientists and engineers to develop integrated devices. Integrated devices can include sensing electrodes, fluidic channels, sample chambers, micromixers, pumps and filters. All of these components can be fabricated using such materials as glass, silicone, metals, quartz, hydrogels, paper alongside a multitude of polymers [1]. All built into one, this allows for the realization of a truly portable ‘lab-on-chip’ (LoC) device. The first LoC device was created by S.C. Terry at Stanford University in 1979 for gas chromatography analysis [2]. Lab-on-Chip devices have now gained much interest since the early 1990’s for the integration of multiple laboratory-based procedures onto miniaturized platforms [3].

Several fabrication techniques for sensing microsystems have been established. Some techniques for construction of signal transduction elements *e.g.* electrodes include: physical vapor deposition (PVD) (*i.e.* e-beam, resistive thermal evaporation and sputtering), etching, inkjet printing, screen-printing and electrodeposition. Each approach comes with a multitude of advantages for various applications, some of which are described throughout this chapter. For fabrication of microfluidic components *e.g.* channels, mixers, filters etc. there exist various polymer-based techniques such as: photolithography, soft lithography, PDMS casting/molding, superimposed acrylic layers, 3D resin printing and injection molding.

## 2.1 Microfabricated Microsystems with Polymer-Based Microfluidics

Microfabrication of transducer electrodes in combination with polymer chemistry techniques are commonly used for construction of diagnostic microsystems. After significant growth of the field, it has become clear that microfabrication is vital as an enabler for the growth of already existing technologies [4]. One way in which microfabrication has enabled existing diagnostic approaches is the miniaturization of electrochemical sensors. This has enabled electrochemical sensing devices capable of rapid detection, reduced reagent cost, high sensitivity and high-throughput capability [5].

For example, Hwang *et al.* utilized several microfabrication techniques for construction of electrodes and microchannels using polymer materials to fabricate an integrated biosensor chip detecting matrix metalloproteinases (MMPs) for cancer diagnostics [6]. Electrodes were fabricated by firstly spin coating positive photoresist onto glass wafers and patterned using photolithography. This was followed by separately evaporating Au and Pt using E-beam PVD and then wet etching to reveal the electrode designs (see Figure 1d). The microchannels were then created through spin coating and photolithography of negative photoresist (SU-8) for chamber I, II and III (see Figure 1a). The final top layer consists of three gates and a pectinate micromixer (see Figure 1b) fabricated by patterning PDMS over an SU-8 mold. This was then bonded to the chip through oxygen plasma treatment heated in an oven at 100°c for an hour.

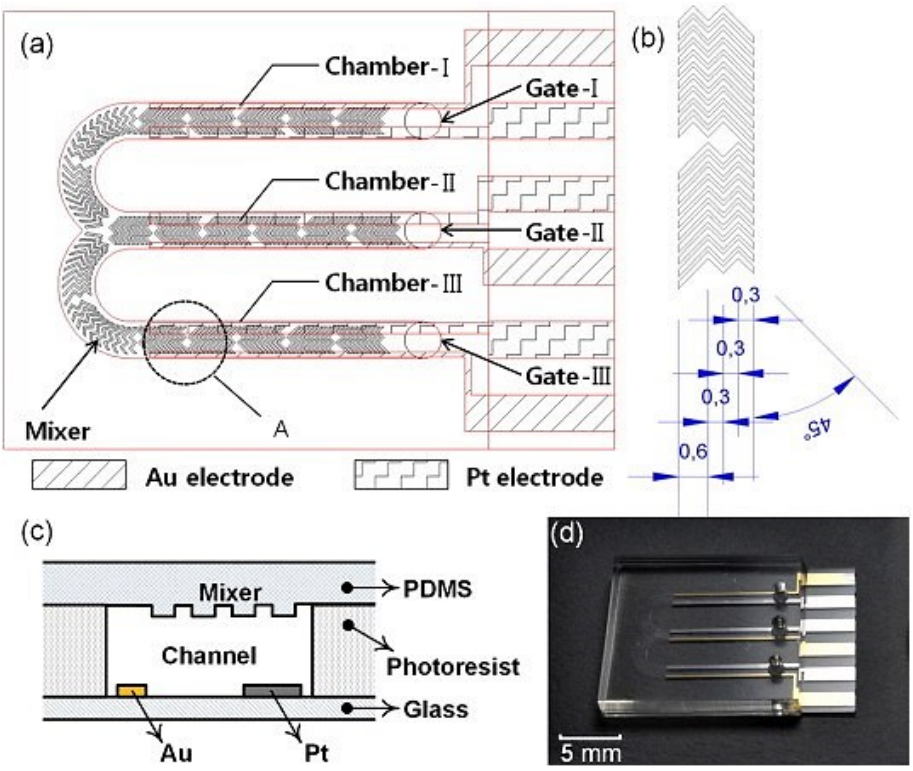


Figure 1. Fabricated multiplex electrochemical biochip. Shown are the 2-D drawings of (a) the design, (b) the chaotic passive mixer (in mm) and (c) a schematic sectional view of 'A'. Also shown is (d) a photograph of the fabricated microchip on glass substrate. Reprinted from [6], with permission from Elsevier.

A similarly fabricated device by Lee et al. showed the high-detail extent to which these kind of fabrication techniques can be used [7]. In the developed device, a three-layered PDMS microfluidic system was developed, superimposed over evaporated Au electrodes on a glass wafer. The bottom layer of the PDMS systems consisted of the microchannels and chambers for reagent transport and reactions. The middle layer of PDMS acted as a membrane layer and finally the top layer of PDMS hosted numerous pneumatic valves, three inlets and fourteen outlets (see Figure 2).

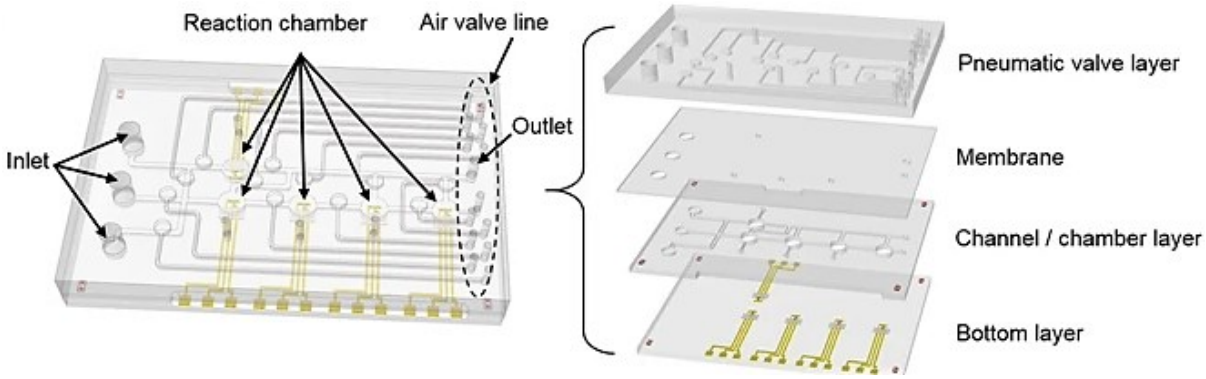


Figure 2. Schematic illustration of the microfluidic system for electrochemical analysis in a single device. Shown above (left) is the schematic description of the microfluidic system integrated electrochemical sensor. Also shown (right) is an exploded model of the microfluidic electrochemical biosensor

composed of a glass bottom layer with electrodes, PDMS channel/chamber layer, PDMS membrane, and PDMS pneumatic valve layer Reprinted from [7], with permission from Springer Nature.

A study in 2017 shows the simple use of two double-sided tape layers with various laser cut designs followed by a third laser cut acrylic polymer layer [8]. Figure 3 shows the design of a multiplexed electrode layer superimposed by a three-layer microfluidic system for simple sample flow over separated channel wells for each set of three-electrode sensors. First, a positive photoresist layer was photopatterned onto a $SiO_2$ wafer, followed by thermal evaporation of Au and wet etching to create the electrode layer (see Figure 3A). Second, two layers of double-sided tape were laser cut. The bottom layer consisting of a 20x array of separated wells to cover each set of electrodes, while the middle layer acted as a chamber spacer between the bottom and top layers. Finally, a plastic layer was laser cut with two holes either end of the sensor array, acting as an inlet and outlet fit with microfluidic tubing for sample flow (see Figure 3B). The developed sensor utilized DNA nucleic acids for detection of bladder cancer-specific DNA samples down to a sensitive detection limit of 250 fM within only 20 minutes. The developed sensor demonstrates the vast potential of simplistically designed microsystems for the detection of clinically relevant analytes.

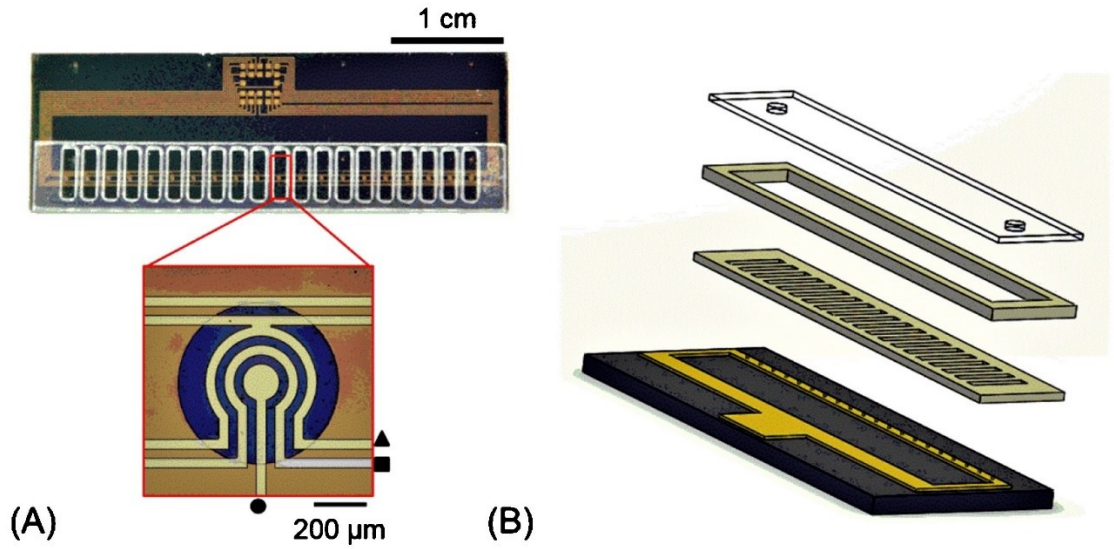


Figure 3. Simplistic design of the microfluidic microsystem for detection of biological samples. Shown above is the photograph of (A) the device used with an array of 20 sensors including a magnified image of an individual sensor with working electrode (scale bars indicate the length scale). Also shown is the exploded 3D model for (B) the schematic of the electrode device and the microfluidic channel design including assembly parts Reprinted from [8], with permission from Elsevier.

An example of pathogen identification from whole blood with minimal sample pre-treatment and no amplification steps has been demonstrated by GeneFluidics Inc.

Their detection chip was based on plastic substrate and sputtered gold electrodes. In total 16 sets of working, reference and counter electrodes were presented on the chip where each working electrode can be functionalized by individual thiolated DNA capturing probes, see Figure 4.

GeneFluidics are focusing on detection of urinary tract infections along with antibiotic susceptibility testing. Pathogen identification is achieved by pathogen specific probes for 16S rRNA, immobilized on the chip. Sample is enzymatically lysed and hybridization of DNA probe to rRNA is facilitated using electrokinetic hybridization based on AC current generating localized heating. The assay is completed by a detection probe-HRP conjugate and incubation with TMB [9, 10]. The assay takes 50 min to complete and GeneFluidics have used it to commercialize urinary tract infection diagnostic system UtiMax™. However, to perform an antibiotic susceptibility test, a sample first needs to be incubated in increasing antibiotic concentrations before analysis. 2.5 h incubation is sufficient, but this increases the total analysis time. One of the advantages of 16S rRNA detection is that multiple copies of rRNA are present in a single pathogen, hence amplification steps are not required when a sensitive sensor is utilized [11].

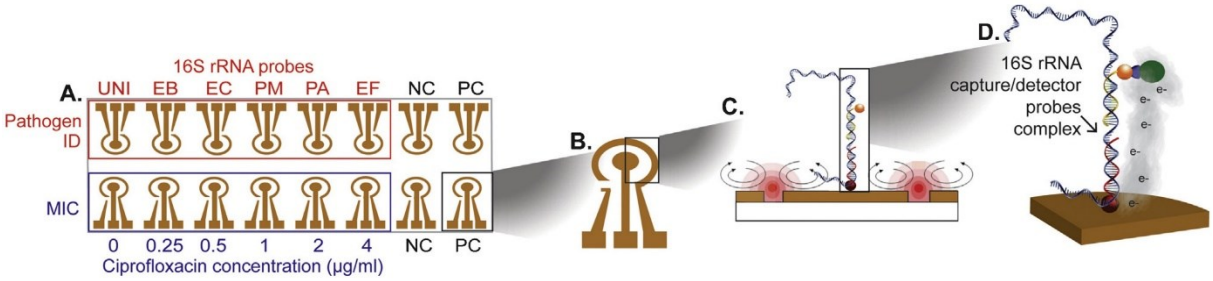


Figure 4: a) The microchip presented by GeneFluidics Inc. 16 individually addressable electrodes can be functionalized (top row; universal bacterial probe marked as UNI, Enterobacteriaceae is EB, Escherichia coli is EC etc. while sample pre-incubated in increasing antibiotic concentration can be introduced to electrodes in the bottom row). b) Individual electrode set comprising of standard 3 electrode cell. c) Hybridization efficiency is increased with AC current mediated localized heating. d) A sandwich ELISA assay format with DNA probes is utilized to bring HRP closer to the surface for amperometric detection. Reprinted from [11], with permission from Elsevier.

More examples of microfluidic devices fabricated using lithographic technologies are presented in Table 1.

Table 1. Examples of electrochemical biosensor microsystems utilizing microfabrication techniques and various polymer materials.

| Electrode type | Microfluidic components | Analyte | Bio-recognition element | Detection technique | Ref. |
|---|---|---|---|---|---|
| **Au & Ag thermally evaporated electrodes** | PDMS microfluidic layer | Cocaine, ATP | Aptamers | CV, eSPR & CC | [12] |
| **Au microneedles sputtered after MRDL on flexi-PCB** | In-house fabricated multi-channel portable electrochemical analyzer | Glucose, uric acid & cholesterol | GOx, uricase & CHOx | CV & CA | [13] |
| **Inserted Ag/AgCl macro electrodes** | PDMS capture chamber & two sensing channels using double layer SU-8 mold | Human ferritin, mouse anti-rabbit IgG | Antibody-functionalized polystyrene NPs | Resistive pulse sensing | [14] |
| **Au & Pt electrodes using E-beam evaporation and photolithography patterning** | One layer of negative photoresist, superimposed by PDMS layer | Matrix metalloprotease (MMP) peptides | Peptides | CV & EIS | [6] |
| **Au & Ag electrodes using E-beam and stencil lithography utilizing shadow mask** | Three-layer PDMS microfluidic layers containing; channels, chambers, membranes and pneumatic valves | Fibrinogen, adiponectin, low-density lipoprotein, 8-isoprostane | Antibodies | CV & SWV | [7] |

Although such devices can be fabricated with high precision, the manufacturing process requires cleanrooms adding to the cost of the final LoC device. Furthermore, multi-step assays are usually based on pressure or vacuum driven microfluidic flow requiring a pump for the chip to be operational. This increases the cost of the instrumentation needed, which makes such devices best suitable for applications where accuracy and precision are the main requirements. On the other hand, low cost

devices with the need for minimal or no external instrumentation are best utilized using paper-based systems, which are presented in the next section.

## 2.2 Paper Based Systems

Since its invention, paper has provided humanity with a simple, multipurpose substrate that has proven useful in countless applications. In sensing, paper is most well-known for its use in lateral flow pregnancy tests; however, it has also been particularly helpful in the fabrication of microfluidic paper-based analytical devices (μPADs) – microfluidic sensors that substitute traditional substrates (e.g. plastic, glass) for paper alternatives. Paper offers several significant advantages given its low cost, lightweight nature, flexibility, biodegradability, and chemical compatibility. Most importantly, the fibrous nature of paper allows liquid to flow via capillary action, with individual fibers acting as capillaries, without the need for external driving forces [15]. The elimination of an external pump not only saves space and energy, but also makes μPADs more portable and easier to implement in non-laboratory environments.

The basic μPAD design consists of hydrophilic channels that are isolated through either precision cutting of the paper or the creation of hydrophobic boundaries. Hydrophobic regions are created through various methods including photolithography, inkjet printing, wax printing, and screen printing [16]. There are several types of μPADs, classified by the detection technique they use; here, we focus specifically on electrochemical paper-based analytical devices (ePADs) – devices that employ electrochemical techniques to detect and quantify analytes. Electrochemistry offers several advantages in microfluidic systems as electrodes are easily miniaturized, potentiostats can be small and portable, and previously developed sensors have shown both high sensitivity and selectivity [17].

In recent years, there has been increased interest in the development of ePADs capable of simultaneously detecting multiple analytes. These multiplexed sensors offer a new level of complexity, without necessarily increasing the device size or cost. Several methods have been employed to construct multiplexed ePADs, which typically depend on both the nature of the analyte/biomarker and the number of electrodes (one or multiple) in the device [18].

When analytes are not redox active, or their redox activity is irrelevant to the mechanism of detection, steps must be taken to ensure the analyte is detectable at the electrode interface. This can be achieved through the addition of biorecognition elements (e.g. antibodies, aptamers, enzymes) to the electrode. Wang *et al.* reported an ePAD for the simultaneous detection of two cancer biomarkers – carcinoembryonic antigen (CEA) and neuron-specific enolase (NSE) – using nanocomposite gold nanoparticle-bound aptamers on separate working electrodes [19]. With the aid of the nanocomposite particles to enhance electron transfer, the ePAD achieved limits of detection (LODs) of 2 pg/mL (CEA) and 10 pg/mL (NSE). To achieve multiplexed detection without the need for multiple working electrodes, it is also possible to add distinguishable labels so that one electrode is sufficient to measure multiple analytes. Li *et al.* developed a foldable origami ePAD with a nanoporous silver (NPS)-modified electrode for the simultaneous detection of CEA and alpha-fetoprotein (AFP) [20]. By printing the working electrode and reference/counter electrodes on separate sides of paper, the device was easily folded to bring all electrodes together for sampling (Figure 5). As the biorecognition element, antibodies specific to CEA and AFP were bound to the electrode. After exposure to the analytes, CEA and AFP were sandwiched with a second antibody bound to nanoporous gold-chitosan, complexed with either $Cu^{2+}$ or $Pb^{2+}$, respectively. Given the disparity in redox potentials between these ions, voltammetry was able to measure both simultaneously and obtain LODs of 0.06 pg/mL (CEA) and 0.08 pg/mL (AFP).

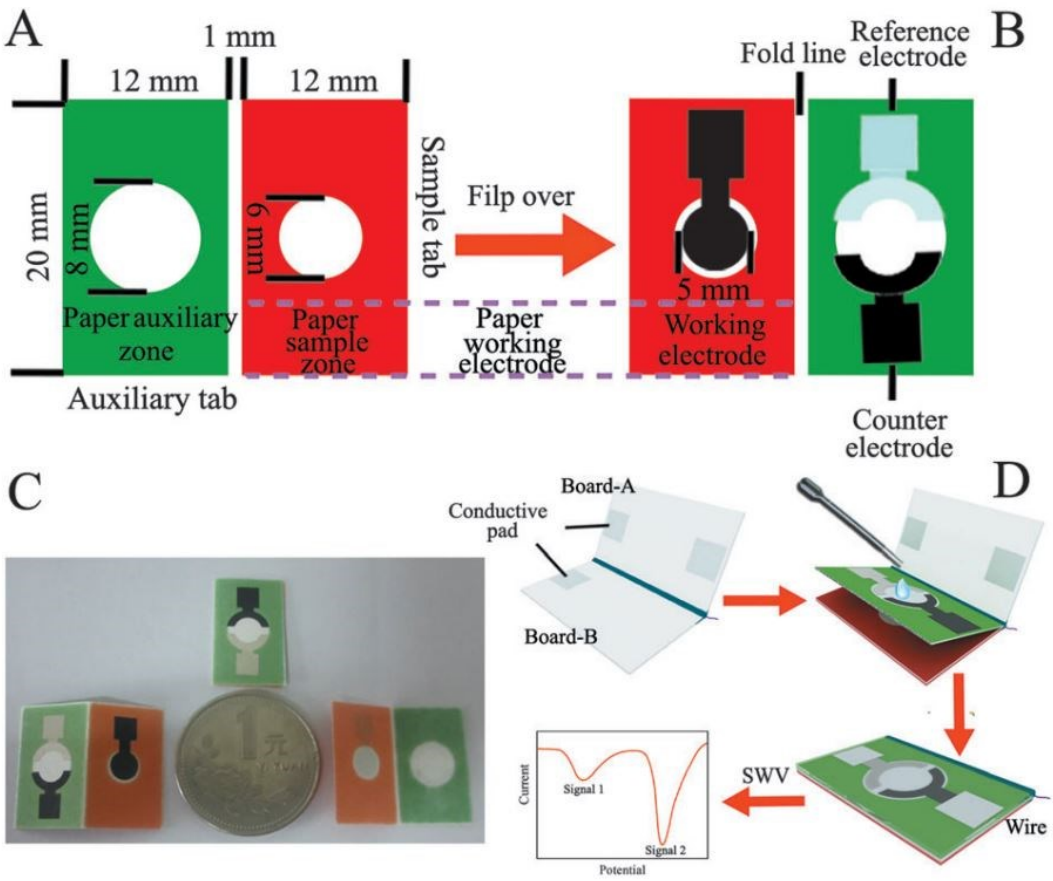


Figure 5: Foldable ePAD constructed using origami paper showing (A) the back of the sample and auxiliary tabs of the device (B) the front of the tabs with the working electrode (sample tab) and reference and counter electrodes (auxiliary tab) (C) several views of the device relative to a yuan coin (D) the insertion of the device into a transparent device holder, with subsequent sample addition and electrochemical measurement. Reprinted from [20], with permission from the Royal Society of Chemistry.

When the analytes of interest are redox active, and have sufficiently different oxidation/reduction potentials, then multiplexed detection can be carried out like the above $Cu^{2+}$/$Pb^{2+}$ label example. However, analytes with similar redox potentials, and thus overlapping curves, require either additional electrode modification, to alter the kinetics of the redox reaction, or separate quantification on different electrodes [18]. Separate working electrodes can also be used to maintain unique chemical environments that are optimal for detection of specific analytes. Janus ePADs, aptly named after the two-faced roman god of duality, store differing reagents in paper near the working electrodes to tailor the pH for each target [21]. Nantaphol *et al.* describe a Janus ePAD for the simultaneous detection of two neurotransmitters, norepinephrine (NE) and serotonin (5-HT), whose electrochemical behavior is ideal under different pH conditions. By impregnating the paper with $H_3PO_4$ and NaOH, the solution reaching

the working electrodes measured pH 6 and pH 8, respectively. Under their optimal pH, NE and 5-HT were measured with LODs of 0.71 µM and 0.38 µM, respectively.

While there are many benefits to using paper in place of other materials, there are still challenges that have yet to be overcome. The largest issue with µPADs is the lack of flow control within the capillary-driven system; the wicking of water into all available fibers can disrupt the desired flow path and lead to inconsistencies between trials [15]. Also, the current scarcity of out-of-lab, real-world µPAD studies makes development and eventual commercialization difficult [16]. An alternative approach to low-cost LoC devices for multiplexed analysis is the use of readily available printed circuit board (PCB) technology, which will be presented next.

## 2.3 Printed Circuit Board Technology

In the effort to integrate the necessary components of LoC devices with electronic readers, printed circuit boards are commonly used as a chip-to-world interfaces. In 1997, Jobst *et al.* presented an idea to incorporate LoC components within the PCB by constructing a PCB interface, with outlined microchannel and a counter electrode, where a microfabricated glass chip bearing working and reference electrode can be inserted and used for detection of glucose and lactate [22]. Merkel *et al.* demonstrated PCB integrated microfluidics with the use of photopatterned copper traces as channel borders [23]. Copper traces were covered with thin layer (4 µm) of epoxy resin to isolate copper from the fluid, preventing corrosion and enable bonding with a cover board, closing the microfluidic channels using a hot press. Using this strategy they also report heating elements that can be used as temperature sensors and microfluidic valves and actuators using flexible Kapton® foil. The advantages of this approach lie in the accessibility of PCB manufacturing, which has been extensively used in the last decades to fulfil the need of booming consumer electronics market. Materials used in PCB construction have excellent thermal and mechanical stability, excellent dielectric properties and are resilient to organic solvents and acid/base solutions, with available recycling strategies already standardized and established.

One strategy for multiplexed detection was demonstrated by Kling *et al.* where a dry photoresist was used to fabricate a device with microfluidic channels and platinum

electrodes were evaporated by physical vapor deposition [24]. Microchannels were pre-treated with anti-fluorescein antibodies so any DNA sequence labelled with fluorescein can be used as a capturing probe. Detection of tetracycline and pristinamycin was achieved using tetO or Pir operator fluorescein tagged DNA sequences, specific to TetR or PIP repressor proteins, which were biotinylated to enable labelling with streptavidin-glucose oxidase (GOx) conjugate. Eight separate channels were constructed and although reference and counter electrode were short circuited to connect in two individual pads, it was the integration of microfluidics that enabled multiplexed detection, see Figure 6.

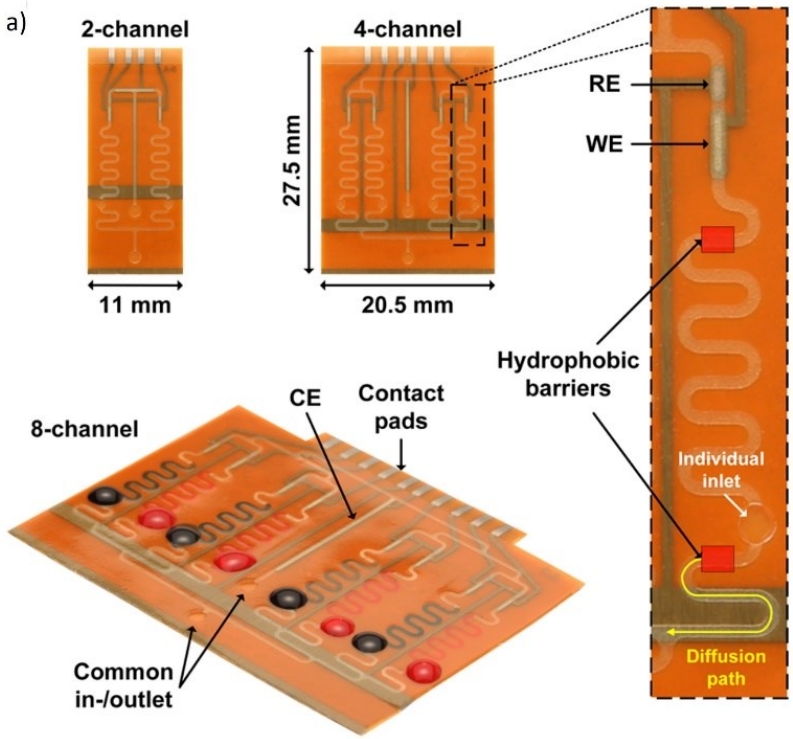


Figure 6: Tetracycline and pristinamycin detection based on dry photoresist technology. 2-,4- and 8-channal system is presented with 8 individual measuring chambers incorporating reference and working electrode, while counter electrode is positioned downstream form the measuring chamber. Reprinted from [24], with permission from American Chemical Society.

Panneer Selvam & Prasad demonstrated a label free multiplexed detection of procalcitonin, lipoteichoic acid and lipopolysaccharide on PCB electrodes using non-faradaic electrochemical impedance spectroscopy [25]. The PCB electrodes were covered with microporous membrane and encapsulated with PDMS to achieve a nanoconfinement stimulating macromolecular crowding, contributing to increased

binding affinity of the analytes. Spiked human serum samples were tested and clinically relevant dynamic ranges were achieved. PCBs have also been used for detection of multiple mRNAs [26], but in a microarray based approach, described in the following section.

The potential of integrating all necessary lab-on-chip components in a PCB gave rise to the Lab-on-PCB approach [27, 28]. Overall, PCB technology offers extensive commercial opportunities. The materials used are inexpensive leading to potentially extremely low-cost devices, especially when manufactured at scale. As the manufacturing facilities are widely available, product upscaling can be performed with ease and at minimal costs. LoC components can be standardized, in a similar way compared to electronics components PCB footprints, to promote system level integration.

To date, most of the PCB based multiplexed biosensors presented are not compatible with current PCB manufacturing techniques and workflows putting pressure on the PCB manufacturing community to uptake new processes. Limited research has been performed using commercially fabricated LoC devices with few examples from Moschou *et al.* demonstrating PCB based Ag/AgCl reference electrodes and biosensors for cytokine IFN-γ, DNA, glucose and pathogens [29-31]; however, more work is needed to achieve commercial PCB based multiplexed devises.

In a different approach, GenMark used PCBs to develop a cartridge that can perform automated sample purification, concentration and PCR amplification integrated with electrochemical detection for rapid pathogen identification. The single use cartridge is composed of a printed circuit board with hydrophobic cover, allowing for electrowetting-on-dielectric (EWOD) technology for sample pre-treatment. Droplets can be moved in 2D by sequential voltage application in neighboring electrodes, which modifies the surface tension in the drop and the droplet aligns with the activated electrode. In this way, the blood sample is purified, and nucleic acids are concentrated with the use of paramagnetic beads, magnetically immobilized in dedicated areas. PCR is performed by moving the droplet through the heated zones, for amplification. Afterwards, the exonuclease digestion of DNA is performed leaving single stranded amplicons, which are then hybridized with a ferrocene (Fc) labelled probe. This mixture is then exposed to PCB electrodes with individually immobilized DNA capturing probes

and when a target is recognized, a potential sweep is performed and an Fc oxidation peak can be observed, identifying the presence of a pathogen (see Figure 7) [32-35].

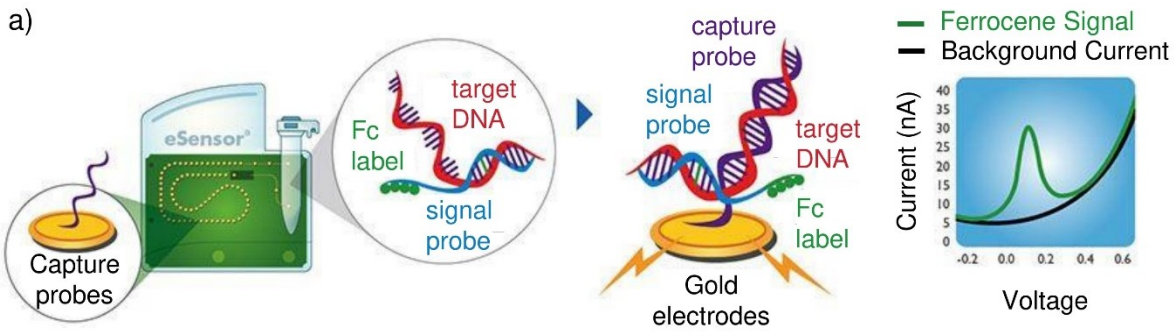


Figure 7: a) GenMark's first version of eSensor technology, employing PCB gold electrode with immobilized capture DNA binding to target DAN, which is labelled with ferrocene conjugated signal DNA strand enabling electrochemical detection. Figure modified from [35], with permission from John Wiley and Sons.

In this example, multiple electrodes are individually addressed with specific probes. Although microfluidic channel delivers the sample to the electrodes, it is the array of electrodes that enables multiplexed detection. Electrode arrays and it uses will be covered in more detail in the next section.

# 3 Electrode Arrays

Microarrays on planar surfaces are a popular method for multiplexed analysis enabled by precise inkjet printing of microspots with volumes as low as picoliters [36]. Microarrays are mostly used in combination with optical detection methods, but here we will focus solely on electrochemical approaches. This includes arrays of individually addressable electrodes as well as arrays of ion-sensitive field-effect transistors (ISFETs) and nanopores used for electrochemical sequencing of nucleic acids.

## 3.1 Electrochemical Sequencing and Detection of Nucleic Acids

Nucleic acids comprise an important group of macromolecules that are highly specific, ubiquitous to all life, and can be amplified to detectable quantities. For these reasons, nucleic acids are often the target of sensors, both for the sequencing of genetic

material and the identification of known sequences. Given the immense diversity within many nucleic acid samples, multiplexed devices that can simultaneously measure multiple sequences are of great interest to scientists targeting DNA and RNA. To achieve this level of detection, devices commonly employ arrays – networks of repeating units, either identical or variable – which can provide thousands of individual recognition sites. Some current array technologies include microwell- and nanopore-based sequencing, as well as diagnostics that employ DNA/RNA microarrays [37]. Though these methods differ in their approach to nucleic acid analysis, all allow for the simultaneous measurement of complex mixtures of sequences.

Since the development of Sanger sequencing in 1977, there has been substantial efforts to improve the speed and ease with which genetic code is sequenced. Next-generation sequencing (NGS) methods aim to process genetic material in a massively parallel manner, allowing thousands to billions of sequences to be read simultaneously [38]. While there are many types of NGS available, we will focus on two specific methods employing electrochemical detection. Ion semiconductor and nanopore sequencing both provide rapid, simultaneous sequencing, while also eliminating the optical component found in many other NGS approaches [39].

Ion semiconductor sequencing is a relatively new and rapid method of DNA sequencing that exploits natural nucleotide chemistry to identify DNA bases. This technology, first released as the Ion Torrent™ line by Life Technologies and later acquired by ThermoFisher, consists of millions of microwells, each of which contain an ion-sensitive field-effect transistor, embedded in a semiconductor chip (Figure 8) [40]. In preparation for sequencing, carrier beads, each bound with clones of a single template, are deposited in the microwells such that each well contains a single bead. Sequencing is achieved through the repeated sequential addition of the four nucleotides (A, C, G, T); when the next complementary nucleotide is added, DNA polymerase catalyzes the addition, and hydrogen ions ($H^+$) are released as a byproduct of the reaction. This decreases the pH in the well which is detected by the ISFET in real time. If consecutive identical bases are present in the sequence, the pH lowers accordingly. Sequential flow of reagents with different nucleotides causes proportional changes in the electrical signal. As of 2020, the Ion GeneStudio™ S5 Prime System is capable of sequencing 100-130 million reads (200bp per read) in just 8.5 hours [41].

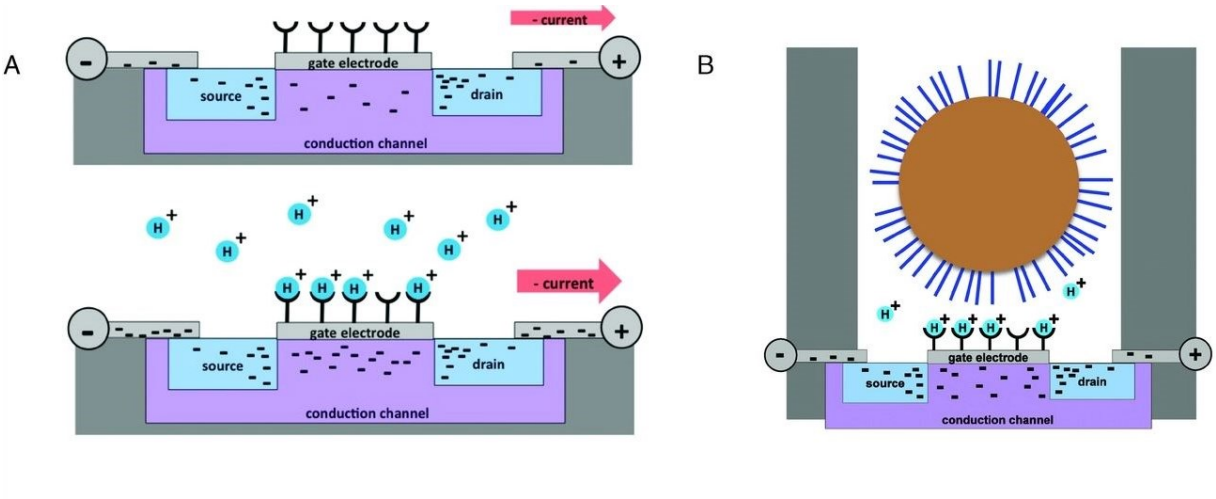


Figure 8; Ion Torrent sequencing setup showing (A) the field effect transistor-based sensor with electrons (-) flowing from source to drain through the conduction channel (top) and increased electron flow upon collection of hydrogen ions in proximity of the gate. (B) A carrier bead bound with template clones inside of a well. Reprinted from , with permission from John Wiley and Sons.

A different approach is based on nanopore sequencing, an NGS technique that determines nucleotide identity as DNA or RNA is transported through pores in a membrane. Nanopore sensors consist of large arrays of nanopores, inserted into membranes either through the incorporation of transmembrane proteins (biological pores) [42] or, more recently, the fabrication of synthetic nanopores (solid-state nanopores) [43]. MspA and α-hemolysin are two bacterial pore-forming proteins commonly used to create biological nanopores, owing partly to their optimal channel diameters [42]. Figure 10 demonstrates how a single strand of DNA, moving from the cis side of the membrane to the trans side, leads to changes in current across an MspA pore. These alterations in current are unique for each respective nucleotide, and thus allow for accurate sequencing of the entire strand. The pore depicted in Figure 9also contains an assistive enzyme, whose helicase activity helps to both separate the DNA strand and control the speed of strand passage. Oxford Nanopore's MinION™, released in 2014, is one example of a successful commercial nanopore-based sequencer [43]. As of 2020, the MinION™ Mk1C can read up to 30 Gbp in less than 48 hours with a single flow cell [44].

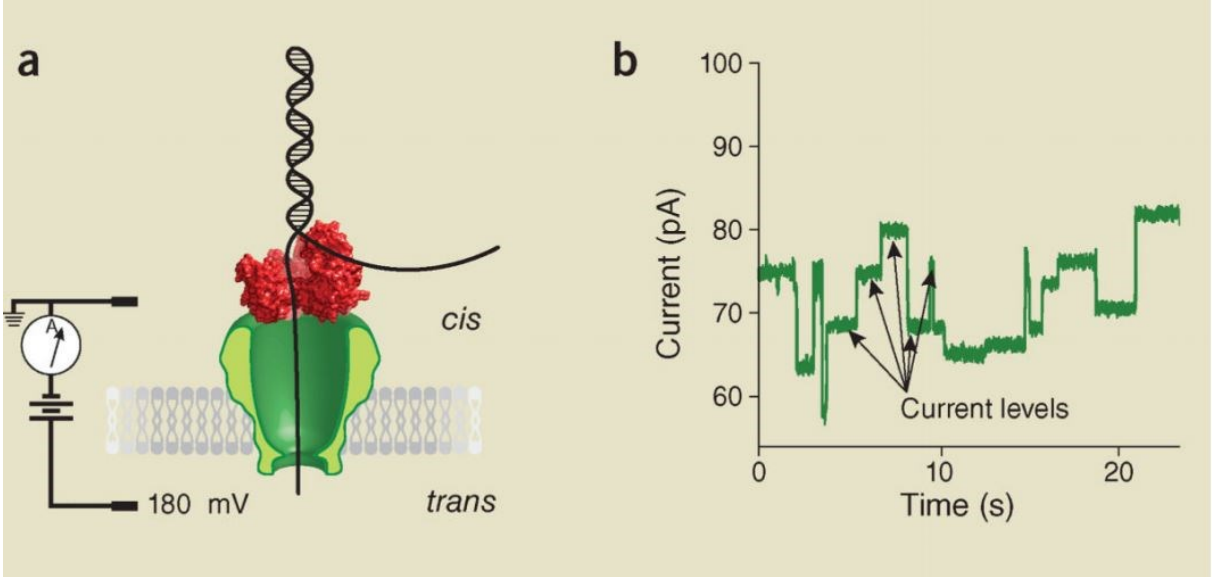


Figure 9; Nanopore sequencing setup showing (A) a helicase enzyme (red) unwinding double stranded DNA as the single strand passes through the MspA nanopore (green) in the membrane (B) changes in current that are observed across the nanopore as different nucleotides pass through the channel. Reprinted from [42], with permission from Springer Nature.

When arrays of electrodes can be individually addressed with specific probes, they can be used for detection of multiple analytes simultaneously. An example of a PCB based electrode array was presented by Sánchez *et al.* who demonstrated the detection of seven breast cancer microRNA sequences using an asymmetric multiplex ligation-dependent probe amplification and electrochemical detection using an HRP label. The authors used precipitating TMB to detect hybridization leading to minimal cross-reactivity [45].

A similar setup was presented by GenScript® utilizing a CMOS chip named the CustomArray 12K™, containing 12,000 individually addressable microelectrodes, each of which can capture a different oligonucleotide sequence simultaneously [46]. Once functionalized, these microarrays bind sample DNA or RNA and are processed with the ElectraSense® Reader – a device capable of electrochemically measuring up to 12,000 probes in less than 45 seconds. This device has been employed for numerous applications, including influenza A subtype analysis, where it exhibited 100% specificity and 95.2% sensitivity [47]. The use of electrode arrays is by far not limited to DNA and RNA based applications but can be used with varying analytes from proteins to pathogens and small molecules, described in the next section.

## 3.2 Detection of Proteins, Pathogens and Small Molecules

Detection of multiple analytes can be performed in parallel with a multi-channel potentiostat or using sequential measurements. Due to the ability of detecting a large number of analytes, electrode arrays can be cost-effective in terms of cost per single test and also require low sample volumes for multiplexed detection due to the miniaturization achieved via microfabrication [48]. Rapid, high-throughput, reproducible, stable and sensitive biosensors, exhibiting wide dynamic ranges have been demonstrated using multi-electrode arrays and is presented in this section [49-52].

Similar to a three-electrode cell, multiple electrode array systems can be used with various electrochemical detection techniques. Linear sweep voltammetry (LSV), differential pulse voltammetry (DPV), square wave voltammetry (SWV) as well as electrochemical impedance spectroscopy (EIS) have been used most extensively in various labeled or label-free set-ups [51-53].

Eissa *et al.* demonstrated multiplex detection of survival motor neutron 1 (SMN1), cystic fibrosis transmembrane conductance regulator (CFTR) and Duchenne muscular dystrophy protein (DMD) on a carbon screen printed electrode array [54]. The electrode system was composed of 8 working electrodes, a round-shaped central Ag/AgCl reference electrode and a ring-shaped carbon counter electrode. Working electrodes were functionalized with carbon nanofibers, to increase electron transfer efficiency, and with respective antibodies for assay construction. Label-free detection of protein biomarkers was achieved using SWV measurements in ferro/ferricyanide solution with impressive detection limits (SMN1: 0.74 pg/mL, CFTR: 0.9, DMD: 0.7 pg/mL). In another work, the authors report detection of dedicator of cytokinesis 8, phosphoglucomutase 3 and signal transducer and activator of transcription 3 proteins [55]. This time, AuNPs were used to improve surface properties, achieving both a higher surface area and enhancing the electron transfer. Cysteamine self-assembled monolayers (SAM) was employed for a label-free detection using SWV with detection limits of 3.1 pg/mL, 2.2 pg/mL and 3.5 pg/mL respectively. Label-free detection has multiple advantages over labeled techniques such as simplicity of the measurement steps needed to obtain the result. However, label free techniques rely on the intrinsic properties of the analytes. In case of analytes with small molecular weight or zero net

charge, these techniques are hard to implement. The same group presented a competitive multiplex immunosensor for the detection of three small metabolites, morphine, tetrahydrocannabinol, and benzoylecgonine, depicted in Figure 10. After AuNP functionalization, cysteamine and glutaraldehyde linkers were used to link monoclonal antibodies (mAbs) to the surface. For competitive detection to occur, drug-BSA conjugates competed with unbound drugs to attach to the binding sites available on respective mAbs. Ultrasensitive detection limits were achieved at 1.2 pg/mL, 7.0 pg/mL and 8.0 pg/mL, respectively. When using spiked urine sample to test for the three small molecules, recovery percentages averaged between 88 to 115%, demonstrating the potential to be used for drug detection in biological samples [56].

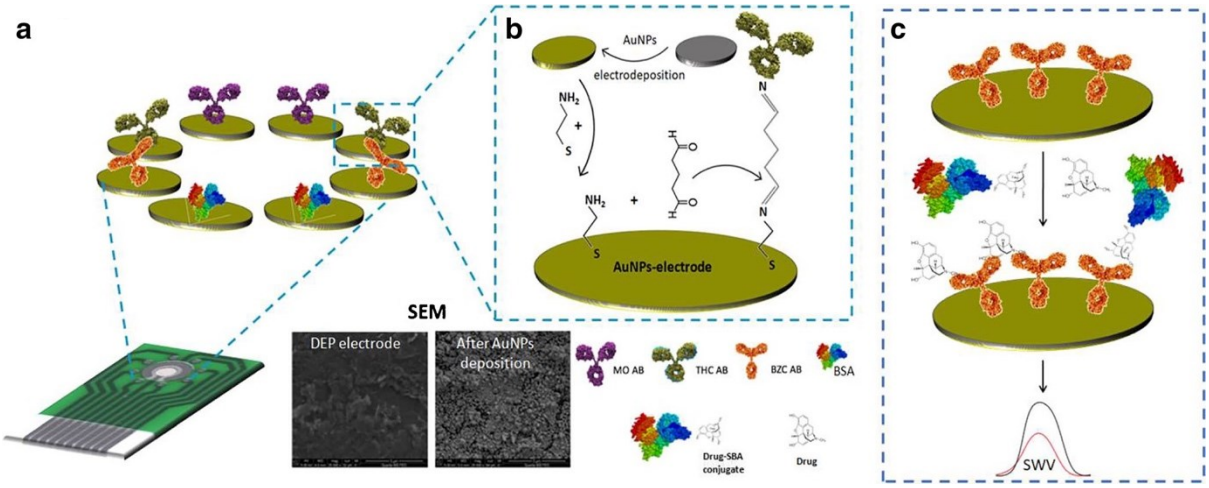


Figure 10: Competitive label-free multiplexed antibody based immunosensor for the detection of morphine (MO), tetrahydrocannabinol (THC), and benzoylecgonine (BZC). (B) The working electrodes are functionalised with AuNPs by using electrodeposition. (C) During SWV detection, drug-BSA conjugates compete with unbound drugs for antibody binding sites within the competitive assay . Reprinted from [56], with permission from Springer Nature.

Another miniaturized electrode array for protein detection was demonstrated by Gupta et al. with a nanoelectrode 3x3 array device that could detect C-reactive protein, cardiac troponin-I and myoglobin in a label-free set-up [57]. To ease the simultaneous detection of the cardiac biomarkers and to avoid the use of a complex microfluidic channel, a hydrophobic resist layer was coated and etched onto the electrodes, eliminating cross talk. Antibodies were attached to the carboxylated vertically aligned carbon nanofibers immobilized on the working electrode, using EDC/NHS chemistry and DPV was used for detection analysis. Tang et al., reported a sensitive electrochemical immunoassay array, integrated with a microfluidic system, for simultaneous detection of four prostate cancer biomarkers [58]. They described a 32-individually addressable microarray that could be multiplexed into a system with 256

working electrodes. To achieve high sensitivity, the detection antibodies were modified with magnetic nanoparticles and linked to HRP. DPV was utilized for detection in diluted calf serum solutions. The limit of detections reported for prostate specific antigen, prostate specific membrane antigen, interleukin-6 and platelet factor-4, diluted in calf serum, were 2, 0.15, 0.05 and 0.1 pg/mL, respectively. Overall, this is a high-throughput and sensitive approach that can provide results within one hour, at a low-cost.

In general, the use of signal amplification labels increases the possibility of cross talk and electrodes need to be placed further apart or electrode antifouling surface chemistry needs to be carefully controlled. The beforementioned CustomArray 12K™ has also been employed for electrochemical detection of pathogens and endotoxins [59]. *Yersinia pestis*, *Bacillus anthracis*, and the bacterial enterotoxin B were detected in this platform that was adopted for electrochemical EILSA with the total assay time of 3.5 hours. The authors reported an avidin-biotin system utilizing tertiary labelling step creating scaffolds of HRP, further enhancing signal amplification.

An example of a commercially available multiplexed device is Abbott's i-STAT. The technology is based on microfabricated silicon chip incorporated in a plastic cartridge, needed for microfluidic sample delivery. Mass manufacturing of the silicon sensors enables reasonable cost of the final cartridge, which is prefilled with calibration solution to perform self-calibration before every individual test. Upon sample addition, the calibration solution is released, and the sensor chip is wetted and calibrated. Next, the sample is flown through the sensors array, which consists of multiple electrodes, functionalized with enzymes or ion-selective membranes. Potentiometric detection is performed for analytes such as sodium, potassium, chloride, and pH, where the concentration of the analyte changes the potential developed on the electrode. Since potentiometric sensors are temperature dependent, calibration is especially important for such analysis. In parallel to potentiometric detection, amperometric reading of glucose is based on glucose oxidase while Clark type electrode with gas permeable membrane is utilized for amperometric detection of oxygen. Hematocrit levels are determined using conductivity measurement between two electrodes, using alternating current passing the two-electrode cell [60]. The combination of multiple electrochemical detection techniques integrated in a single platform demonstrates the potential of electrochemical sensors for multiplexed detection of variable analytes.

Although substantial multiplexing capability has been demonstrated by i-STAT, incorporation of immunoassays into the platform is not straightforward. Cardiac protein biomarkers assays for creatine kinase MB, cardiac troponin I and B-type natriuretic peptide have been incorporated into i-STAT single use cartridges, but are currently only available as single biomarker tests, demonstrating the challenges in multiplexed analysis [61].

Another approach to further increase multiplexing capability is to capture multiple analytes on a single electrode and achieve multiplexing through labels with different properties. Such approaches will be reviewed next.

# 4 Label-Assisted Multiplexing Techniques

Many recent advances in the development of multiplexed system have been demonstrated with the use of different labels including enzymes, redox markers, magnetic beads, nanoparticles, or quantum dots [62-64]. These strategies are mostly used in immunoassays, where labels are conjugated to secondary antibodies, completing the sandwich assay [65]. This allows ultrasensitive detection, minimizing the effect of non-specific binding and bio-fouling [66].

This section discusses microbeads, enzyme labels, redox active labeling molecules and nanoparticles in more detail.

## 4.1 Use of Microbeads in Multiplexed Biosensing

Magnetic beads are regularly used for sample pre-processing, where they act as capturing agent which can be easily extracted from the sample by a magnet allowing analyte separation or enrichment. Immobilization of capturing probes on microbeads can reduce assay time and increase the dynamic range of the sensor, due to increased immobilization surface presented on the beads. Additionally, biological fouling on the sensing electrodes can be eliminated, as the sample does not have to get in contact with the sensing element [67].

One strategy for bead-based multiplexed electrochemical detection is separating microbeads-capturing probe conjugate into separate sections where detection is performed. This was demonstrated by Ko *et al*. who presented a biosensor for alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), and prostate-specific antigen (PSA) based on microbeads in a PDMS based microsystem on a glass surface [68]. Microbeads were conjugated to specific antibodies and flown to the measurement chamber, which consisted of microfabricated interdigitated electrodes upstream of an array of PDMS micropillars, acting as a filter for capturing of microbeads (see Figure 11a). Once the microbeads were in place, the sample was flown through the channel and antigen-antibody binding occurred, followed by gold-conjugated labelling antibody and a silver enhancer, which reduced to metallic silver in the presence of gold nanoparticles, catalyzing the reaction. Precipitated metallic silver then causes changes in resistivity between the interdigitated electrodes. Multiplexing was achieved by constructing multiple parallel sensing chambers and channels.

A second approach for multiplexed detection is based on the use of multiple beads with varied properties like size or shape. Han *et al*. demonstrated multiplexed detection of biomarkers exploiting immunoaggregation with magnetic microbeads [14]. The sensing mechanism is based on sub-micrometre pore and a resistive pulse sensor (RPS), measuring resistivity through the pore, which changes when an immunoaggregate passes through. Microbead size can be distinguished by the sensor, hence different sized microbeads can be used simultaneously for parallel multiplexed detection. An alternative approach is based on the versatile properties of the microbeads e.g. the use of magnetic and non-magnetic microbeads. In the first step, a sample is mixed with antibody functionalized microbead mixture, which induce immunoaggregation (see Figure 11b). In a two-stage sensor, the authors demonstrated RPS sensor can quantify two biomarkers by obtaining a total concentration of both biomarkers, then the magnetic beads are removed with a magnet and non-magnetic beads are quantified again in a second RPS sensor. The concentration of biomarker targeted by magnetic beads can then be deduced. Detection of anti-rabbit IgG and human ferritin were demonstrated in this work but up to four biomarkers can be quantified by this device when utilizing microbeads versatile in size and magnetic properties.

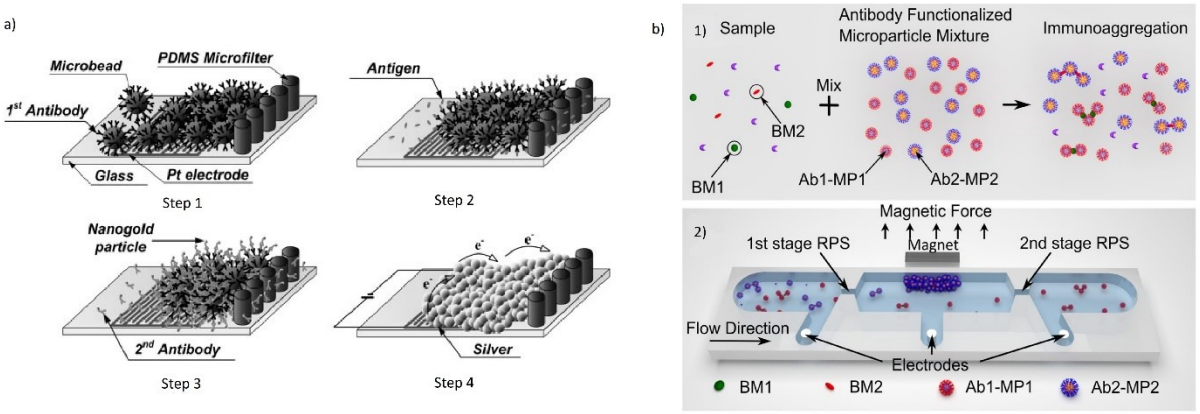


Figure 11: a) Microbead based detection strategy. Microbeads conjugated to mAbs are first flown through the channel and captured by micropillar filter, then sample is introduced followed by a AuNP-conjugated labelling mAb and silver enhancer. Reprinted from [68], with permission from John Wiley and Sons. b) Microbead based resistive pulse sensor. In the first step sample is mixed with mAb-conjugated microbead mixture and the solution is flown through RPS. In the middle chamber, magnetic beads are captured while non-magnetic beads are counted again with a second RPS. Reprinted from [14], with permission from AIP Publishing.

A third and most utilized approach explores magnetic microbeads as carriers of capturing probes, which are then labelled using varied labelling strategies including enzymes, redox tags, quantum dots or metal ions. When such approach is integrated in microfluidic systems there is no need for immobilization of capturing probes within microfluidic channels, simplifying the final production process for such devices. There are two main procedures of transferring the signal from analyte recognition site (on the magnetic microbead) to the working electrode. First procedure is based on enzymatic 12reactions taken place on the magnetic beads and the concentration of the produced product is then analyzed separately, by moving the solution from a reaction chamber to measurement chamber [69] or, as an alternative procedure, a magnet is used to guide magnetic beads to the electrode surface, where the signal is read [70].

In both procedures, there is clear need for labelling strategy, which can also determine the level of multiplexed detection. Various labelling strategies will be covered in the next section.

## 4.2 Barcoding with Redox Probes

A method of labelling that has been widely employed for multiplexing is barcoding. Barcoding is the concept of utilizing multiple labelling molecules on a single sensor electrode platform with a variety of probes for the purpose of multiplexing. This is done

by labelling with various ligands that contain different electroactive signal properties. These properties may be redox based *e.g.* methylene blue and ferri/ferrocyanide, or they may give varying potentials upon anodic stripping of the electrode using stripping techniques such as in the case of dissolved metal-based probes [71]. By doing so, a simple setup can be fabricated in which multiple unique labels are attached to targets that bind to separate probes simultaneously giving signals that are easily distinguishable [72]. A distinct advantage of the barcoding technique is that signals may can be easily obtained during a single voltammetry/amperometric scan. However, it has been shown in some cases that if the electroactive potentials are similar 'cross-talk' may be observed [73].

The labelling of target molecules with redox-active species is a frequently used technique for detection using electrochemical biosensors, enabling ultrasensitive detection at varying redox potentials. Redox labels have the capability of providing increased sensitivity for detection due to excellent electron transfer ability that non-labelled alternatives may not provide [74]. For example, one study used labelled gold nanoparticles (AuNPs) with either ferrocene (Fc)-based or mercaptohexanol (MCH) spacing molecules co-immobilized with aptamers for prostate cancer detection to enable both impedimetric and amperometric signal acquisition [75]. Other examples of redox-active species used for labelling include Methylene Blue (MB) [76-78], erythrosine, anthraquinones/hydroquinones [79], hemin, thionene and ruthenium [80, 81]. Redox-active species are those molecules that are capable of both donating and accepting electrons at specific potentials to provide reduction/oxidation (redox) signals. Different species undergo reduction and oxidation at significantly different potentials. This enables multiplex measurements of various targets in samples by labelling each target with different redox-active species.

A study in 2015 utilized two redox-active labels, hemin and ruthenium (Ru), for multiplex detection of both alpha-fetoprotein (AFP) and carcinoembryonic antigen (CEA) tumor markers within serum samples [80]. Antigen-specific antibodies were first immobilized onto glassy carbon electrodes. The functionalized sensor was then exposed to antigen samples and labels to form a sandwich-type immunoassay with either hemin or Ru labelled antibodies. Both redox-active species were also attached to nanotags for CV and electrogenerated chemiluminescence (ECL) detection techniques (see Figure 12). Utilizing this assay, the researchers were able to reach

ultrasensitive LODs of 1 and 0.5 pg $mL^{-1}$ within serum samples for AFP and CEA respectively.

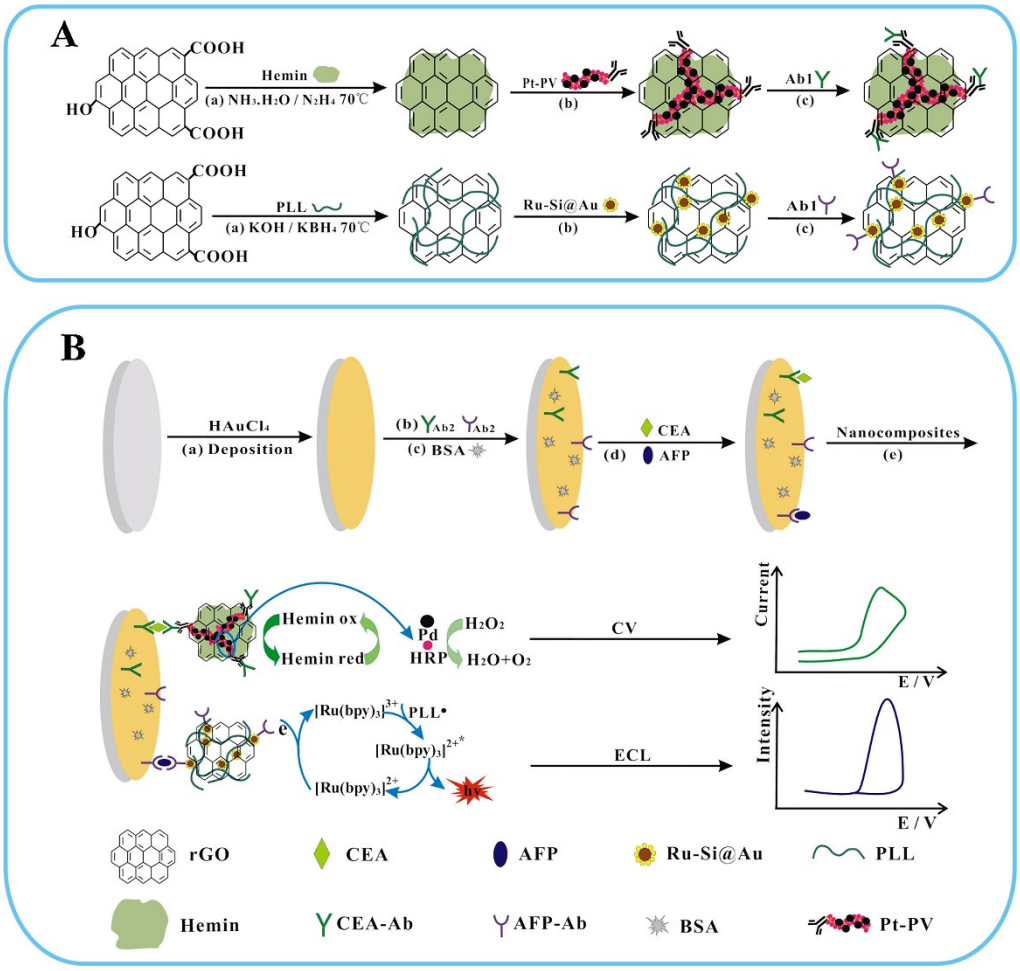


Figure 12. Schematic representation of; (A) the preparation protocol for CV and ECL nanotags. (B) Immunosensor fabrication process and the signal generation mechanism. Reprinted from [80], with permission from Elsevier.

Another study looked at the multiplexed detection of ochratoxin A (OTA) and fumonisin $B_1$ (FB1) toxins commonly found during the beer production process. In order to achieve multiplex detection on Au electrodes, the authors implemented the use of a Y-shaped aptamer probe labelled with thionene and Fc (see Figure 13). Firstly, thiolated cDNA, which is half complementary to each aptamer, is passively immobilized on the Au electrode surface. The label-functionalized aptamers are then bound to cDNA to form the Y-shaped double aptamer probe structure and DPV or EIS signal is measured. Upon binding of OTA and FB1, conformational change of the aptamers causes them to detach leading to a decrease in both DPV peaks and EIS signal. Implementing this method of multilabel multiplexing the authors were able to achieve LODs of 0.47 and 0.26 pg $mL^{-1}$ for OTA and FB1 respectively [81].

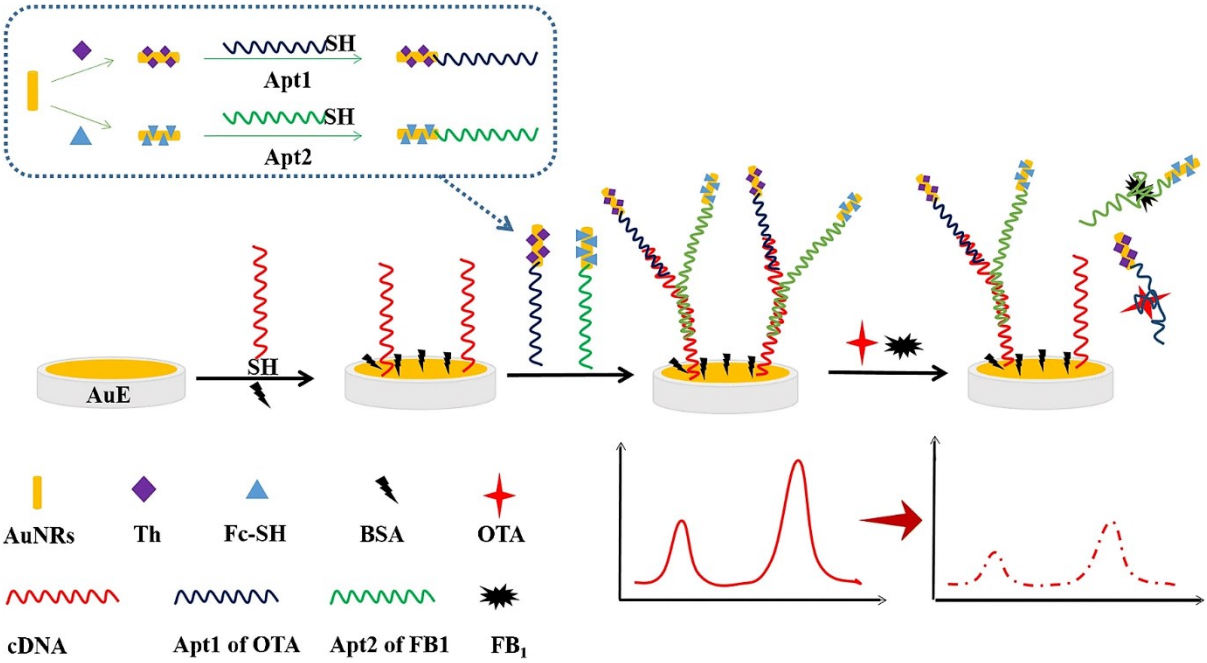


Figure 13. Schematic representation of OTA and FB1 detection based on Aptamer 2-AuNRs-Fc and Aptamer 1-AuNRs-Th/cDNA/AuE assay using DPV. Reprinted from [81], with permission from Springer Nature.

## 4.3 Enzymes

Enzymes can be used in multiplexed sensors as biological recognition elements, providing sensor specificity or to amplify the signal in an affinity-based sensor based on immunoassays. Stable and reproducible electrochemical signals can be obtained using an enzyme label by measuring the electroactive product of its respective substrate [71, 82-84]. Most used enzyme labels include alkaline phosphatase (ALP), glucose oxidase (GOx) and horseradish peroxidase (HRP). The use of enzyme-labels can require the presence of a mediator to aid with the electron transfer during the enzymatic reaction [64]. For instance, the oxidation of hydroquinone is catalyzed by HRP in the presence of hydrogen peroxide ($H_2O_2$), a substrate [64, 85].

When an analyte of interest can be catalyzed with an enzyme, multiplexed detection can be achieved by immobilization of enzyme to an individual electrode in a multiple electrode array set-up. This was demonstrated by Kucherenko *et al.* who reported the detection of six analytes with detection limits of 1 µM for glutamate, 1 µM for glucose, 2 µM for choline, 3 µM for acetylcholine, 2 µM for lactate and 5 µM for pyruvate [86]. Such sensors have been successfully used as point-of-care (POC) diagnostics. An

example is a commercially available device from Siemens named ePOC system. Amperometric detection of glucose, lactate and creatinine is demonstrated using multi-level sensor construction. In a lactate sensor, gold electrode is covered with a layer including lactate oxidase, HRP and ABTS substrate (2,2'-azino-bis3-ethylbenzothiazoline-6-sulfonic acid diammonium salt). A diffusion barrier layer encapsulates the reagents and when lactate is present, its oxidation produces hydrogen peroxide, which enables the HRP modulated reaction. The creatinine sensor is based on three-layer multi-enzymatic reaction including creatinine amidohydrolase catalysis of creatinine to creatine, creatine amidinohydrolase mediated hydrolysis of creatine to sarcosine and urea and finally sarcosine oxidase needed to produce hydrogen peroxide which enables HRP mediated reaction [87].

The use of enzymatic labels in electrochemical immunoassays has also been demonstrated commercially (*e.g.* i-STAT), however, these usually rely on individually addressable electrodes. Jia *et al.* presented a strategy where two capture antibodies can be immobilized on a single electrode and multiplexing is achieved with a label [88]. Secondary antibodies for carcinoembryonic antigen and alpha-fetoprotein were labelled either with thionine or ferocenne and conjugated to a graphene oxide nanosheet which also carried platinum nanoparticles, GOx and HRP. Thionine or ferocenne acted as electron mediators yielding a separate voltammetric peak, enabling multiplexed detection of the sandwich immunoassay. Using square wave voltammetry (SWV), two distinguishable redox peaks were observed at -0.15 V for thionine and +0.35 V for ferocenne. The detection limit was 1.33 pg/mL and 1.64 pg/mL, respectively.

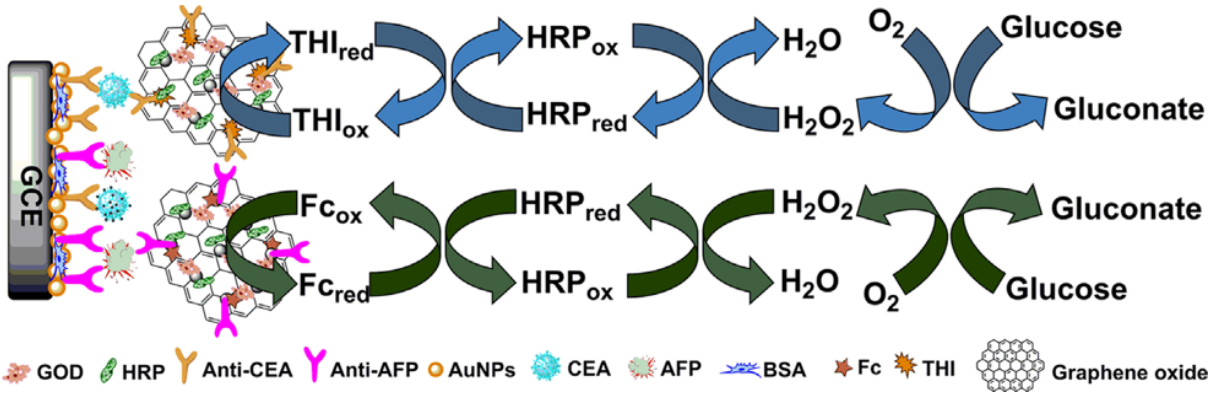


Figure 14: AuNPs modified working electrode is immobilized with the capture antibodies. Graphene oxide nanosheet equipped with labelling antibodies, thionine or ferocenne electron mediators, platinum nanoparticles and HRP with GOx. Reprinted from [88], with permission from Elsevier.

Combining different assay configurations such as enzymatic sensors and affinity sensors in a single platform for simultaneous detection was demonstrated by Vargas *et al.*, who devised a dual-marker biosensor for diabetic markers glucose and insulin [89]. Glucose sensor was based on glucose oxidase and tetrathiafulvalene mediator incorporated into a chitosan biopolymer layer. On a separate electrode, a sandwich immunoassay was constructed using specific anti-insulin capture antibodies and HRP labelled detection antibodies. Both sensors employed amperometric detection, and due to different modes of operation, glucose was quantified while the insulin sensor was exposed to the sample, hence the labelling step didn't interfere with the glucose measurements. Total analysis time was under 30 minutes, with a sample volume of 10 µL.

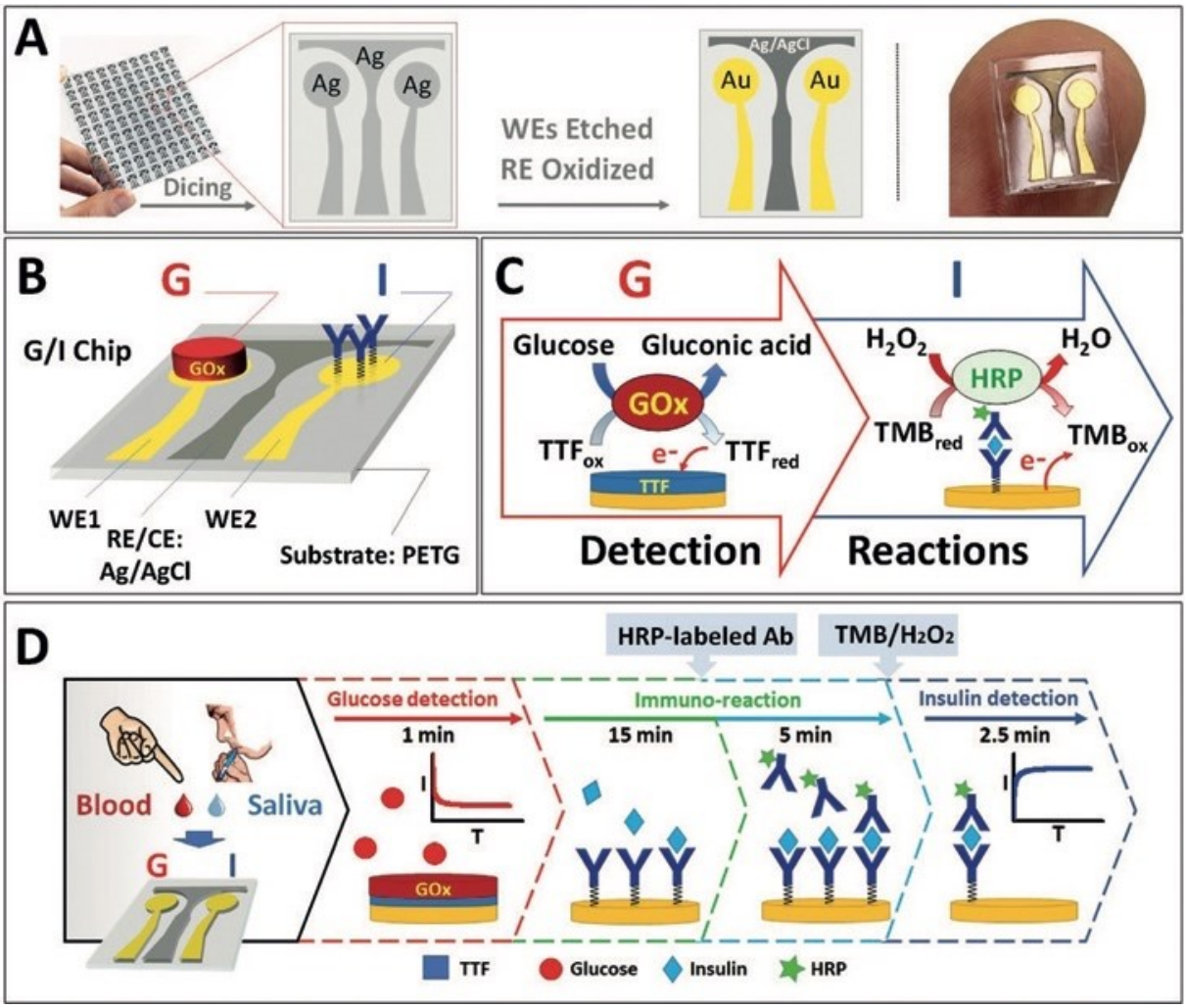


Figure 15: (A) Array fabrication on the plastic PETG substrate in the form of a two-electrode cell with two working electrodes. (B) Illustration of a single biosensor chip in which the glucose biosensor involves the formation of chitosan film immobilized with GOx, whereas the insulin sensor involves the use of an insulin capture antibody. (C) Schematic of the amperometric detection principle of glucose and insulin sensors. D) Detection steps required for the measurement of glucose and insulin via whole blood and human saliva samples. Reprinted from [89], with permission from John Wiley and Sons.

Overall advantages of operating multiplexed electrochemical systems with enzyme labels include higher biocatalytic activity with increased specificity and sensitivity [90, 91]. However, enzymes are known to lose their activity over time and are usually required to be kept at low temperature to maintain functionality. Thus, other labelling strategies will be discussed further in this section.

## 4.4 Nanoparticles

Nanoparticles are any type of particulate matter that has an individual diameter in the nanometer range (typically 1-100 nm). Given their extremely small size, diverse

compositions, and ability to be functionalized with a wide range of molecules, nanoparticles have led to significant advances in $CO_2$ capture, drug delivery, and disease diagnostics [92]. Nanoparticle-based labelling has also provided several unique methods of achieving multiplexed molecular detection – some of which show great potential for developing comprehensive diagnostic tests in the future. The two main types of nanoparticles commonly employed as multiplex labels include quantum dots and metal nanoparticles [93]. Quantum dots are among the smallest of nanoparticles, with diameters as low as 2 nm, and are composed of semiconducting materials (e.g. ZnO). Because of their size, quantum dots display unique optical and electrochemical properties that make them well-suited for distinctive labelling. Metal nanoparticles are typically larger than quantum dots and composed of a pure metal or compound. Some commonly used metal nanoparticles include silver and gold nanoparticles, which have been employed in a variety of applications.

While quantum dots are often exploited for their unique optical properties, they can also be used for their electrochemical characteristics. Since quantum dots are composed of a wide range of compounds that have significantly different redox potentials, different quantum dot labels can enable multiplexed detection using a single working electrode [93]. Vijian *et al.* developed a genosensor for the multiplexed detection of pathogen RNA that utilized metal sulfide quantum dots as unique electrochemical labels for SWV (Figure 16) [94]. Using PbS, CdS, and ZnS particles conjugated with DNA reporter probes, the device detected RNA for *V*. *cholerae*, *Salmonella* sp., and *Shigella* sp. with LODs of 51 aM, 53 aM, and 38 aM, respectively. Similarly, Kong *et al.* employed CdS and PbS quantum dots coupled to secondary antibodies to simultaneously detect both CEA and AFP biomarkers with LODs of 3.3 pg/mL and 7.8 pg/mL, respectively [95].

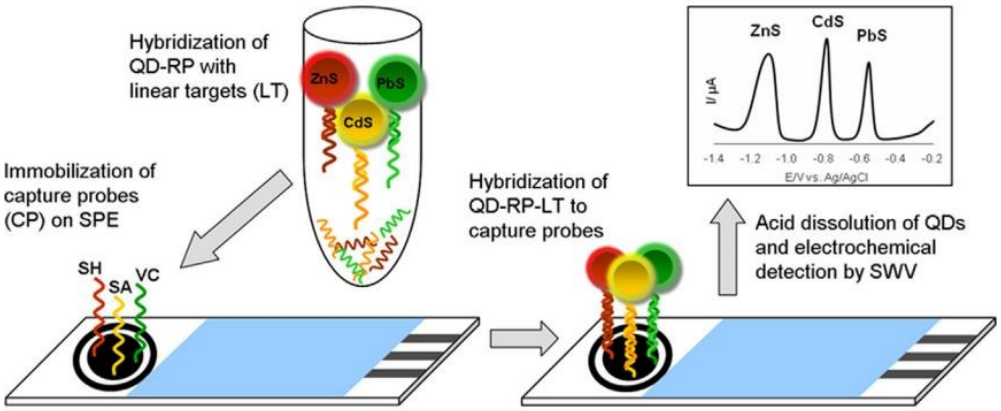


Figure 16: Addition of quantum dot (QD)-labelled reporter probes (RP) and subsequent binding to linear targets (LT) of *V. cholerae* (VC), *Salmonella* sp. (SA), and *Shigella* sp. (SH) pathogens. Nitric acid was added to encourage dissolution of quantum dots prior to SWV to increase signal readout. Reprinted from [94], with permission from Elsevier .

Metal nanoparticles have been used to achieve multiplexed detection in several different manners. Wan *et al.* employed Cu, Ag, and Pd nanoparticles conjugated with antibodies and aptamers to simultaneously detect three different cancer cell lines (MDA-MB-231, SK-BR-3, VCaP) with an LOD of 2 cells per sensor [96]. Since the metals had adequately separated redox peaks, linear sweep voltammetry was able to resolve all three nanoparticles on a single electrode. In addition to redox labelling, metal nanoparticles may also function as catalytic labels, enabling the production of redox active material. The sensor published by Lai *et al.* utilized secondary antibody-conjugated gold nanoparticles as labels to detect CEA and AFP biomarkers [52]. After labelling, a silver enhancer solution was added to the electrodes, which enabled the gold-catalyzed deposition of silver nanoparticles onto the sensor. Subsequent anodic stripping analysis quantified the amount of deposited silver with LODs of 3.5 pg/mL and 3.9 pg/mL for CEA and AFP, respectively.

Another approach of using nanoparticles mainly in immunoassays is to conjugate them to a labeling antibody and an enzyme, allowing a high number of molecules to attach to a single conjugate. This allows a single binding event to be labeled with a high number of labeling molecules, increasing signal generation and lowering detection limits [84, 85]. An example was demonstrated by Krause *et al.*, as they also used a conjugate of antibodies, magnetic nanoparticles and HRP to obtain a label with magnetic properties allowing ultrasensitive detection of four oral cancer biomarkers in a microfluidic immunoarray setup [97]. The limits of detection were 10 fg/mL, 18 fg/mL, 40 fg/mL and 15 fg/mL for tumor necrosis factor (TNF-α), interleukin-6 (IL-6),

interleukin-1β (IL-1β), and C-reactive protein (CRP) in diluted calf serum, respectively. Only 5 μL was required to achieve ultrasensitive detection in 30 minutes. In comparison, the authors used the conventional ELISA method, which required 400 μL of the biological sample with a total analysis time of about 12 hours.

# 5 Conclusion

Electrochemical biosensors with multiplexing capability offer several advantages over single test devices such as decreased cost per single test and high assay throughput. We expect multiplexed sensors will become frequently used in applications such as cancer biomarker screening, where new biomarkers are still being proposed and there is no ideal single biomarker. Multiple strategies for construction of such sensors for multiplexed analysis have been presented with the focus on the efforts towards ultrasensitive sensors. Detection of multiple analytes can be achieved mainly by spatial separation of detection chambers with the use of microfluidics, where we examined microfabricated devices offering high precision and reliability and are therefore best suited for applications with such needs. Low cost devices can be fabricated using paper technologies or printed circuit boards, which could enable greater accessibility of such sensors and improve the social impact. To date, commercially most successful platforms have been based on electrode arrays, which offer great scalability due to miniaturization and simple electrode fabrication and functionalization. In the effort to further miniatures such devices, strategies of combining multiple probes on a single electrode and achieve multiplexing using labels with different electrochemical properties have emerged. Novel approaches are continuously being developed with primary focus on the use of nanomaterials for increasing the rate of electron transfer at the measuring electrode or differential redox activity of electroactive labels. Such developments hold great promise but need more work. A limited number of electroactive species with minimal cross-reactivity is currently available, limiting multiplexing capability. Sensors with capability to detect more than two analytes in this format are rare. Despite all recent advances, incorporating high number of immunoassays into a single platform remains challenging, with rare examples detecting more than four analytes. However, there is

a lot of potential for electrochemical sensors to be in the forward seat in the innovation in this field and someday achieving reliable, low cost and portable devices that can have a major impact on clinical decision making and consequently public health.

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