Chapter 2
Bio-microelectromechanical Systems (BioMEMS) in Bio-sensing Applications-Colorimetric Detection Strategies

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2.1 Introduction

One of the most well-known analytic methods of detection is based on quantifying the UV–vis absorption or reflection, also known as colorimetric (Li et al. 2019a). Colorimetric biosensors are simple, rapid, disposable, and low-cost (Zhang et al. 2006; Li et al. 2019b). Additionally, they enable the signal readout with color changes or even directly judged by the naked eye (Li et al. 2019b). For MEMS, specially BioMEMS, these qualities enable medical exams to become more accessible and user friendly. Likewise, biological processes can be carefully assessed due to the concentration of the color which is commonly directly proportional to the concentration of the analyte of interest (Aldewachi et al. 2018). In this chapter, we review the latest advancement of paper-based, microfluidics and alternative BioMEMS that operate based upon the principles of colorimetric detection. A comprehensive review of these devices is presented and summarized in Table 2.1.

2.2 Colorimetric Detection Strategy

The detection method known as colorimetric determines the presence and concentration of an analyte through comparing the color changes of a solution. This method is based on the quantification of the change in the absorbance of the solution due to the concentration of the analyte of interest. This relationship can be described using the Beer-Lambert equation, which relates the concentration to the absorption, with the light path remaining constant (Wilson 2013). When white light passes through a
| BioMEMS platform | Main components | Fabrication strategy | Mechanisms of operation | Detected analyte | Specifics | References |
|------------------|-----------------|----------------------|-------------------------|------------------|----------|------------|
| PAD DNA sensor based on acpcPNA-induced nanoparticle aggregation | • AgNP  
• acpcPN probe  
• PAD Multiplex DNA  
• Sensor | The PAD was made with a wax-printing technique and the sensor by folding two-layer halves while a PDMS lid held it together | The sample reservoir was punched through the whole device. The acpcPNA probe and AgNPs solution were added onto the detection and control zones. Finally, the sample solution was added onto the sample reservoir and flow through the channels to wet the colorimetric detection zones | • MERS  
• TB  
• MERS-CoV  
• HPV | This colorimetric DNA sensor exhibited high selectivity and has the potential to be a low-cost and disposable alternative tool for rapid and selective screening and detecting in infectious diseases | Teengam et al. (2017) |

(continued)
| BioMEMS platform | Main components | Fabrication strategy | Mechanisms of operation | Detected analyte | Specifics | References |
|------------------|----------------|----------------------|-------------------------|-----------------|----------|------------|
| PAD ELISA platform | • Wax-printed 96-microzone paper plate  
• Image interpretation device  
• AuNPs | The AuNPs were synthesized through citrate-reduction techniques, later joined with PEG and were added to the IgG antibodies. A wax printing method was used to create the pattern on the paper designed. The dimensions were according to the standard Costar 96-well microtiter plate | A direct ELISA protocol was performed in the device to detect the IgG with a colorimetric substrate. Multiple P-ELISAs were conducted in parallel, allowing the use of common microplate processing techniques with the P-ELISA format | Neuropeptide Y | This device provides an inexpensive platform to carry out highly sensitive (picomolar level) biomolecular assays at very low sample and reagent volumes | Murdock et al. (2013) |

(continued)
| BioMEMS platform | Main components | Fabrication strategy | Mechanisms of operation | Detected analyte | Specifics | References |
|------------------|-----------------|----------------------|-------------------------|------------------|----------|------------|
| Integrated PAD biosensor incorporating nucleic acid extraction | • Silica microbead channels  
• Siphon channels  
• Glass fiber  
• Absorbent pads  
• Rotary machine  
• Heating block | The device is composed of three major microfluidic layers joined by double sided adhesive film. The micropatterns of the first and third layers were fabricated on polycarbonate sheets, and the microstructures on the second layer were fabricated on a PMMA sheet using a CNC milling machine. The lateral flow strips were inserted between the second and the third layer | The lysate mixture was introduced into the inlet, which was filled in a channel by capillary forces. The lysate debris in the microbead-bed channel flowed out into the waste chamber, followed by the washing buffer eruption. The solution passed through the microbeads to carry the adsorbed DNA on the microbeads into the LAMP chamber and the LAMP reaction mixture was also transferred into the LAMP chamber. Finally, the LAMP products and the running buffer solution were loaded onto the lateral flow strip via the connecting channels | • Streptococcus pneumonia  
• E. coli | This device can perform simple nucleic acid extraction, amplification and colorimetric detection by the naked eye in about an hour, thus making it a high performance microdevice | Choi (2016) |
| BioMEMS platform                     | Main components                                                                 |
|-------------------------------------|----------------------------------------------------------------------------------|
| **PAD smartphone accessory for biomarkers** | • Smartphone application  
• Test strips  
• Smartphone case with inlet slot  
• Smartphone |
| Fabrication strategy                | The indicator strip consisted of a cutout of a pHydron Spectral plastic pH indicator strip for sweat testing and a strip for saliva testing. The reference strip was made of white plastic material. The flash diffuser consists of a PDMS membrane. The smartphone case was 3D printed in order to isolate the test strip from external light |
| Mechanisms of operation             | The app was loaded, and the strip was selected for the readout. The calibration data updated while the sample was inserted |
| Detected analyte                    | pH in saliva and sweat |
| Specifics                           | This device allowed noninvasive real-time analysis by means of disposable test strips |
| References                          | Oncescu et al. (2013)                |

(continued)
| BioMEMS platform                  | Main components                                                                 | Fabrication strategy                                                                 | Mechanisms of operation                                                                 | Detected analyte       | Specifics                                                                 | References                  |
|----------------------------------|---------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------|------------------------|---------------------------------------------------------------------------|-----------------------------|
| Glass nanofibers colorimetric biosensor | • Detection ribbon made from hardened PVC  
• Detection fabric with immobilized and stabilized AChE  
• Cellulose paper strip impregnated with ATChI and Ellman’s reagent | The biosensor was a plastic strip with an indication fabric and the carrier impregnated with a substrate and an indicator. The Ellman’s reagent is used as a color indicator, joined with the substrate on the cellulose filter paper. To enhance the color, glass nanofibers were used as the substrate | The moistened detection fabric was exposed to air, contaminated water or pressing a wet detection zone to the test surface resulting in color change. Yellow was the indicator that no inhibitors exist while white demonstrated the presence of inhibitors | Cholinesterase inhibitors | The paper made of glass nanofibers provided a better color effect than the standard cellulose paper within the same 2 min | Matějovský and Pitschmann (2018) |

(continued)
### Table 2.1 (continued)

| BioMEMS platform                       | Main components               | Fabrication strategy                                                                 | Mechanisms of operation                                                                 | Detected analyte | Specifics                                                                 | References           |
|----------------------------------------|-------------------------------|--------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------|------------------|---------------------------------------------------------------------------|----------------------|
| Paper/polymer hybrid µPAD microplate   | • PMMA                        | Micro-machining using laser ablation was used. Pieces of chromatography paper were cut using a laser cutter and placed inside the device. To assemble the device, different PMMA layers were clamped together | Assay reagents were loaded in the top PMMA layer. Then, the reagents arrived to the chromatography paper in each microwell by capillary forces and allowed the excess of it flow to the third layer for removal | • HBsAg         | The use of paper-based technology resulted in rapid immobilization of antibody/antigen and avoided complicated surface modifications. The device was simple, portable, rapid and highly sensitive | Sanjay et al. (2016) |
|                                        | • Chromatography paper disks  |                                                                                      |                                                                                           | • IgG            |                                                                           |                      |
|                                        | • Microwells                  |                                                                                      |                                                                                           |                  |                                                                           |                      |
| Double-layered µPAD                    | • Nylon mesh                  | The 3D microfluidic channels were made through traditional wax-screen printing technology | A blood sample was dispensed on sampling zone, and passed through the hydrophobic channels in order to react with the reactants. The color intensity was recorded by an Image J software | • Glucose        | This device allowed the detection of four biomolecules simultaneously, and improved colorimetric performance, sensitivity, and detection range | Li et al. (2018)    |
|                                        | • Whatman paper               |                                                                                      |                                                                                           | • Uric acid      |                                                                           |                      |
|                                        |                               |                                                                                      |                                                                                           | • Lactate        |                                                                           |                      |
|                                        |                               |                                                                                      |                                                                                           | • Choline        |                                                                           |                      |

(continued)
| BioMEMS platform                  | Main components                  | Fabrication strategy                                                                                                                                                                                                                                                                                                                                 | Mechanisms of operation                                                                                                                                                                                                 | Detected analyte | Specifics                                                                                                                                                                                                 | References            |
|----------------------------------|----------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Multilayer μPAD coupled with filtration | • Chromatography paper  
• Filter paper | Channels were printed using a wax printer. The bottom layer was made of Whatman paper. All the layers were inserted in a hydrophobic wax paper holder.                                                                                                                                  | Colorant reagents were applied to the channels to form metal hydroxides. These ions captured the antibiotics and created metal complexes which could not go through the filter paper. The rest of the liquid was absorbed for colored complexes to be seen. | • Oxytetracycline  
• Norfloxacin | With a wider range of detection agents, greater recovery rate, and quicker assembly and testing, this device and method was found to be a valuable platform for food safety surveillance | Nilghaz and Lu (2019) |
| Colorimetric μPAD Biosensor      | • Paper substrates  
• Wax printer  
• Adhesive tape | The biosensor was wax-printed on paper platforms and modified with chitosan as explained previously.                                                                                                                                         | Tears were placed on the inlet area and pulled up to the detection zone by capillary forces. The color changes were detected by an office scanner and converted to Red–Green–Blue scale. | GLU | The device was proposed as a noninvasive alternative capable of distinguishing different concentration levels in a sample to control glucose for diabetic patients | Gabriel et al. (2017) |
| BioMEMS platform | Main components | Fabrication strategy | Mechanisms of operation | Detected analyte | Specifics | References |
|------------------|-----------------|----------------------|-------------------------|------------------|----------|------------|
| Compact embeddable μCAD | • Cotton fabrics/cloth   <br> • Dual inlet | Wax patterning technique was utilized to pattern microfluidic channels on scoured-cotton cloth fabric and the 3D colorimetric microfluidic device was made by folding a 2D pattern | Solutions of the respective colorimetric reagents were pipetted into the detection zones. The inlet point was immersed into the urine. The sample analyte was introduced through an inlet point to react with the reagents | • GLU <br> • Nitrite <br> • Protein | The easy to pattern, inexpensive and environmentally friendly device was stable for a week proving useful for applications in underdeveloped areas | Nilghaz et al. (2015) |
| C-μPAD | Chromatography paper | The hydrophobic barriers were made by CVD of TCS on a chromatography paper | A pattern was cut out onto a reactive surface that was glued to paper surface and placed inside the CD chamber. A colorimetric reagent was immobilized for a stationary and uniform reaction through thermal condensation coupling method | • GLU <br> • Metals | More complex detection chambers and higher degree of reliability resulted from this device by controlling temperature, pattern size, and CVD duration | Lam et al. (2017) |
Table 2.1 (continued)

| BioMEMS platform                | Main components                                                                 | Fabrication strategy                                                                 | Mechanisms of operation                                                                 | Detected analyte  | Specifics                                                                 | References       |
|--------------------------------|--------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------|-------------------|--------------------------------------------------------------------------|------------------|
| Microfluidic colorimetric biosensor | • 8 microchannels • CuFe$_2$O$_4$/GQDs MNPs • Microfluidic chip            | The microfluidic chip was made by traditional soft lithography technique. The colorimetric assay was conducted with peroxidase-like CuFe$_2$O$_4$/GQDs MNPs | Analytes of different concentrations were injected into the microchannel from two entrances simultaneously, and converged at the intersection point. ACh and NaH$_2$PO$_4$ buffer solutions were injected to other two entrances and were directed to the color area | • Chlorpyrifos inhibitors | The GQDs MNPs were added to enhance the colorimetric result. The device was compact and precise using small sample volume, lowering the limit of detection | Mao et al. (2017) |
| BioMEMS platform                                                                 | Main components                                                                 | Fabrication strategy                                                                 | Mechanisms of operation                                                                 | Detected analyte | Specifics                                                                                                                                                                                                 | References |
|---------------------------------------------------------------------------------|----------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------|-----------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| Microfluidic colorimetric biosensor using AuNPs aggregation and smartphone imaging | • AuNPs                                                                         | Solidworks was used to create the design of the chip. It was 3D printed and underwent surface plasma bonding. Surplus support material was removed while the PDMS prepolymer attached to it. The PDMS replica was peeled off and joined to the glass slide | The MNPs and the PSs reacted with the target bacteria in the first mixing channel. In the second step, AuNPs and the crosslinking agents reacted with the catalysate. This interaction was responsible for the color change | • E. coli O157:H7 | The addition of AuNPs effectively increased the signal indication. Also, the chip’s mixing productivity impacted the detection time and sensitivity for foodborne pathogens                                                                 | Zheng et al. (2019) |
| BioMEMS platform                      | Main components       | Fabrication strategy                                                                 | Mechanisms of operation                                                                 | Detected analyte | Specifics                                                                 | References          |
|---------------------------------------|-----------------------|--------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------|-----------------|--------------------------------------------------------------------------|---------------------|
| Smartphone-assisted microfluidic chemistry analyzer | • Microfluidic chip  
• Smartphone  
• Step motor  
• Microcontroller  
• Bluetooth module | The main structure was made by plastic injection molding and two PSA layers were added on either side. It included four reaction chambers for the multiplex measurements. | Reaction mixture were pipetted into each detection chamber and allowed to dry, leaving one chamber empty for quality control. The LED emitted and transmitted by the reagent. The step motor rotated the camera through all four chambers and were then processed by the microcontroller. The color changes were measured by a custom analyzer and built-in optical system | • GLU  
• TG  
• TC | Multi-index monitoring was done with a high accuracy and low cost, while maintaining excellent consistency with conventional chemistry analyzers | Li et al. (2019c) |
Table 2.1 (continued)

| BioMEMS platform                                                                 | Main components               | Fabrication strategy                                                                 | Mechanisms of operation                                                                 | Detected analyte | Specifics                                                                 | References               |
|--------------------------------------------------------------------------------|-------------------------------|--------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------|------------------|--------------------------------------------------------------------------|--------------------------|
| Molecular diagnostic quantitative pregnancy test on a DVD                        | • DVD                         | The DVD’s PS surface underwent activation by UV irradiation and treatment with EDC and NHS. A PDMS plate was added with microchannels. Anti-hCG α were immobilized on the surface, streptavidin nanogold conjugates were bound and a silver staining treatment was performed | Samples were loaded into the PDMS microfluidic channels on the DVD. The DVD was spun, and the samples create a different radial distance depending on the concentration of the analyte. The assay was tested with a diagnostic software and evaluated based on radial distance and optical darkness ratio | • hCG             | Th strategy provided a low-cost POC tool for worldwide testing and comparable results to common immunoassays and ELISA | Li et al. (2014)          |

(continued)
| BioMEMS platform                          | Main components                                    | Fabrication strategy                                                                 | Mechanisms of operation                                                                 | Detected analyte                      | Specifics                                                                 | References            |
|------------------------------------------|----------------------------------------------------|--------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------|---------------------------------------|--------------------------------------------------------------------------|-----------------------|
| LAMP Integrated rotary microfluidic system| • Centrifugal microfluidic platform<br>• Glass microbeads solid phase matrix base | Multiple microfluidic layers were created using a CNC milling machine. To assemble the three layers, a PSA film was used after cutting the micropattern by a plotter. All layers were aligned and bonded by a hot press | This microdevice incorporates the whole procedure for nucleic acid-based test, including the solid-phase nucleic acid extraction, the LAMP reaction, and the lateral flow strip based colorimetric detection | • Salmonella Typhimurium<br>• Salmonella Typhimurium<br>• Vibrio parahaemolyticus | High performances of the microdevice provided great potential as a user-friendly POC analyzer that can be applicable to resource limited environments | Park et al. (2016)    |
| Smartphone spectrometer                  | • Smartphone<br>• Grated CD<br>• Built-in LED<br>• CMOS camera | The grating tracks of the CD were tilted with respect to the LED but normal to the incident light so the light may be refracted from the CD on the camera. The flashlight was emitted through a pinhole in front of the LED | A bi-enzymatic cascade assay and peptide-functionalized AuNPs were used to detect glucose and troponin I respectively | • GLU<br>• Troponin I | This device allows real-time measurements with LOD of approx. 50 ng mL⁻¹. It presented similar results to commercial devices while offering a compact cost-effective tool | Wang et al. (2016)    |
| BioMEMS platform                              | Main components                        | Fabrication strategy                                                                 | Mechanisms of operation                                                                 | Detected analyte | Specifics | References |
|----------------------------------------------|----------------------------------------|--------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------|------------------|----------|------------|
| Electronic-based ELISA                       | • Circuit board<br>• TFT touch screen<br>• Au surface<br>• Commercially available IFNγ | Using heat and adhesive properties, Cu foil was laminated on FR4 PCB substrate and a gold layer was plated on top. The PMMA wells were fixed on the PCB surface | TMB was used as the reporter reagent. Amperometric detection was performed by a second generation amperometry | • IFNγ           | This device facilitated colorimetric and amperometric assays in an inexpensive and portable manner | Evans (2017)    |
| Centrifugal microfluidic device integrated with LAMP | • PMMA layers<br>• PSA layer<br>• Loading and mixing chambers<br>• Smartphone | The device was made of two PMMA layers and a middle PSA layer press-bounded together. A square-wave microchannel, metering chambers, and RPM control allowed the process to flow | The LAMP reagents and primers were injected to the loading chambers while the sealing material was loaded into the sealing chambers and the wax into the wax valve and the DNA samples were injected into the amplification chambers | • E. coli<br>• Vibrio cholerae | This device allowed 30 simultaneous genetic analyses of three different foodborne pathogens. This process was performed in approx. an hour and presented a considerably low LOD | Sayad et al. (2017) |
| BioMEMS platform | Main components | Fabrication strategy | Mechanisms of operation | Detected analyte | Specifics | References |
|------------------|-----------------|----------------------|-------------------------|-----------------|----------|------------|
| RCA-based biosensor for attomolar detection | • Polymerase  
• MB amplification template  
• MB/GDNA probe  
• Spectrophotometer  
• Electrophoresis analyzer | N/A | miRNA triggered MB mediated strand displacement to release nicking triggers cyclically, leading to a TIRCA. This result was vast production of GDNA, which joining with hemin, form an HRP mimic, has catalyzed the colorimetric reaction | The machine united the advantages of enzymatic signal amplification and TIRCA. It allowed a decrease in background noise and improvement in stringent target recognition as well as sensitivity | Li (2016) |
2 Bio-microelectromechanical Systems (BioMEMS) …

colored substance, light wavelengths are absorbed in different proportions (Fuwa and Vallee 1963). The intensity of the resultant color will be proportional to the concentration of the measured analyte and the amount of absorbed light will be proportional to the intensity of the color (Ricci et al. 1994).

Colorimetric analysis is applicable to detect the presence of organic and inorganic compounds, making it a suitable option for biosensors. Some applications for colorimetric detection devices include hand-held bio-diagnostics, point-of-care diagnostics, and naked-eye detection. Current chapter focuses on the fabrication of microfluidic, paper-based, or polymer-based platforms based on this common detection strategy.

2.3 Recent Advances of Colorimetric Detection in Paper-Based BioMEMS

One of the major problems in healthcare nowadays is accessibility. Many people worldwide have limited to no access to laboratories or hospitals, hence, in order to reach these communities, smaller portable devices with the same accuracy are needed. Among these developments, the paper-based analytical devices (PAD) attract a great deal of attention. PAD devices have proven to be the inexpensive, simple, portable, and disposable. Likewise, they are easy to use, make complicated readout equipment unnecessary, and produce semi quantitative results (Teengam et al. 2017) in a short amount of time (Murdock et al. 2013). For that reason, PADs are being used to diagnose diseases via DNA and/or RNA recognition (Teengam et al. 2017), monitor human activity (Murdock et al. 2013), detect nucleic acids (Choi 2016), sense pH in sweat and/or saliva (Oncescu et al. 2013), and recognize cholinesterase inhibitors (Matějovský and Pitschmann 2018), thus, making such devices favorable for a wide range of applications including medical (Teengam et al. 2017), military (Murdock et al. 2013), nutrition (Choi 2016), biochemical (Oncescu et al. 2013), and nerve chemical warfare (Matějovský and Pitschmann 2018). Below, several examples of PADs are presented with a specific focus on biosensing application.

In addition to the previously stated benefits of paper-based devices, μPADs enhance point of care for detecting diseases (Sanjay et al. 2016), biomolecules (Li et al. 2018; Gabriel et al. 2017), and antibiotics (Nilghaz and Lu 2019). They offer inexpensive, simple, eco-friendly, portable and quick bioanalysis (Nilghaz and Lu 2019). Additionally, due to their small size, these devices provide a greater surface to volume ratio, improving the immobilization of proteins through processes such as enzymelinked immunosorbent assay (ELISA) integrated into this platform, and other biological agents (Sanjay et al. 2016). Many variations and additions can be made to μPADs in order to enhance its properties. For example, cotton, being a similar material to paper and providing the same advantages as well as being stronger and more durable, becomes an option for embedding into daily wearable products (Nilghaz et al. 2015). Also, by using chemical vapor deposition (CVD) instead of
wax printing, more complex detection chambers and a higher degree of reliability for colorimetric detection result can be achieved (Lam et al. 2017). Some of the latest examples of the paper-based BioMEMS used for colorimetric detection are provided here.

Teengam et al. (2017) produced a paper-based colorimetric assay for DNA detection based on pyrrolidinyl peptide nucleic acid-induced nanoparticle aggregation in order to have a simple and quantitative means of detecting diseases such as Middle East Respiratory Syndrome (MERS), Tuberculosis (TB), Middle East Respiratory Syndrome coronavirus (MERS-CoV), and Human Papillomavirus (HPV). To create the device, a multiplex colorimetric PAD with a derived backbone from D-proline/2-aminocyclopentanecarboxylic acid (acpcPNA), silver nanoparticles (AgNPs) and a paper-based multiplex DNA sensor were used. The actual PAD was made through a wax-printing technique, and the sensor was based on an origami concept made of two layers, as can be seen in Fig. 2.1. The base consisted of four wax-defined channels extending outward from the sample reservoir (6 mm i.d.) and the top layer which had four detection and control zones (4 mm i.d.). The sample reservoir at the top was fully punched to the bottom layer, and the top was folded over. Together with a polydimethylsiloxane (PDMS) lid and a 6 mm diameter hole over the reservoir, they were held together. Eight 4 mm holes and control zones were aligned to maintain a constant pressure across the surface where the acpcPNA probe and AgNPs solution were included. The sample solution was added to the sample reservoir where it flows through the channels to wet the colorimetric detection zones. These zones were obtained by placing 10 μL of AgNPs in 0.1 M phosphate buffer saline (PBS)

![Fig. 2.1 Design and setup of paper-based multiplex DNA sensor (Teengam et al. 2017)](image-url)
pH 7.4 with a ratio of 5:1 (AgNPs:PBS). For the colorimetry, special acpcPNA probes were designed and fabricated to detect synthetic oligonucleotide targets with sequences in MERS-CoV, MTB, and HPV (DNA_{com}). The intensity of the color was compared to a single-base mismatch (DNA_{m1}), two-base mismatch (DNA_{m2}), and DNA_{nc} sequences. In the presence of the DNA_{com} the intensity decreased and was unaffected by the mismatched and noncomplementary targets. There was a high selectivity to single-base mismatch, two-base mismatch, and noncomplementary target DNA.

Paper-based enzyme-linked immunosorbent assays (P-ELISAs) were created by Murdock et al. (2013) in order to measure biomolecules concentrations. In comparison to regular ELISAs, P-ELISAs are faster to make and obtain results. Neuropeptide Y was the point of interest as it is related to regulating stress, anxiety, fear, and overall sympathetic nervous system activity (Eaton et al. 2007; Heilig 2004). The target of this study was diagnosing Post Traumatic Syndrome and identifying the difference between more exposed soldiers from beginners. The platform of the P-ELISA consisted of a wax-printed 96 (12 by 8 arrays of circular test zones)-microzone paper plate, designed on Office PowerPoint according to the standard Costar 96-well microtiter plate (Murdock et al. 2013). The wax covered the areas between wells, leaving the 5.56 mm diameter wells hollowed. 3 μL of target solution was inserted into each well, followed by blocking and addition of antibody and incubation. Each test zone was 3 mm in diameter and needed 1.5 μL to be damp. Gold nanoparticles (AuNPs) of 16 nm were added to poly(ethylene glycol) (PEG) and cleaned by centrifugation and buffer exchanges. Anti-rabbit IgG antibodies were linked to the carboxylic end of the PEG. For the P-ELISA, the same process was followed except for the AuNP-IgG which was included instead of the conventional antibody through a silver enhancement kit (Ted Pella/BBInternational). The operation was tested by means of a standard 96-well plate-based ELISA procedure detecting rabbit IgG with a colorimetric substrate (Fig. 2.2). The reason behind using wax-printed paper-based was to create an inexpensive method for carrying out biomolecular assays in small volumes. The device allowed the limit of detection (LOD) to be reduced from nano to picomolar scale. Additionally, it permitted a broader range of colorimetric substances be used since the dynamic imaging ranges through conversion to grayscale. Hence, a portable device camera can be used instead of laboratory equipment to carry out the read out. The device increased the number of samples analyzed per dollar unit typically spent on diagnosis while equally increased the number of patients helped. This device holds great promises for its application in remote or resource-limited areas.

Choi et al. (2016) developed an integrated paper-based sample-to-answer biosensor for nucleic acid extraction and amplification at the POC. This provided a new view to the operation of paper-based devices as the readout could be done through visual detection or quantification using a smartphone. The device was credited as a high performance microdevice since the colorimetric detection by the naked eye could be performed within an hour. Following this strategy, a battery-powered heating device was introduced to amplify the nucleic acid in POC, which, coupled with the assay, offered a rapid target detection. A Fast Technology Analysis (FTA)
card and glass fiber were added to a lateral flow strip for nucleic acid extraction and amplification. First, the paper matrices were separated by hydrophobic polyvinyl chloride (PVC) layers, or valves, shown in Fig. 2.3. These valves controlled the flow from the nucleic acid extraction to the amplification zone and lateral flow strip. The device had three microfluidic layers in total: first layer for the injection holes and microfluidic channels for reagent transportation, second held the micropatterns for DNA extraction and amplification, and third incorporated a lateral flow strip for the colorimetric detection. The micropatterns were obtained from polycarbonate (PC) sheets and the microstructures from a poly(methyl methacrylate) (PMMA) sheet using a CNC milling machine. A double-sided adhesive film was used to assemble these layers. Lastly, the lateral flow strips were placed between the last two layers. The heating device was included to the integrated biosensor for very sensitive and specific loop-mediated isothermal amplification (LAMP). The bacteria were lysed outside of the device before being introduced to the inlet. This was filled in a channel by capillary forces and the debris in the microbead-bed channel flashed out to the waste chamber. Subsequently, the washing buffer erupt, forcing the solution to go through the microbeads and carry the adsorbed DNA and reaction mix into the LAMP chamber. The reaction is performed at 66 °C for 50 min and later loaded onto the lateral flow strip through the connecting channels. The developed biosensor in this study detected Escherichia coli (E. coli) in several food types with LOD as low as 10

Fig. 2.2  a RGB versus IgG concentration using Au NP-silver enhancement procedure. b Enzyme-free P-ELISA assay (Murdock et al. 2013)
Fig. 2.3  
a Integrated paper-based biosensor, b experimental procedure, c Biosensor during (i) extraction, (ii) amplification and (iii) lateral flow detection, d Handheld heating device (Choi 2016)

to 1000 CFU mL\(^{-1}\) and Streptococcus pneumonia in blood samples. Hence, proving the potentials in medical, food safety and environmental applications.

Due to the worldwide use of smartphones, Oncescu et al. (2013) created a health accessory for colorimetric detection of biomarkers in sweat and saliva based on the fact that the pH in saliva can be used to point out enamel decalcification and the pH in sweat helps indicate dehydration. The device is a noninvasive real-time analysis with disposable test strips that is connected to the phone. The pH sensing system consisted of a smartphone case, application, and test strips. The case has a slot where the strips could be inserted to be analyzed and was 3D printed from opaque Vera black material to isolate the strip form variable external light. The colorimetric analysis took place with the help of the phone’s camera and a storage compartment for up to six strips. The strips were 3D printed to include an indicator strip, reference strip, and a flash diffuser. The first strip was 9 × 4 mm and was cut out from a pHydron Spectral 5.0–9.0 plastic pH indicator strip for sweat testing and a 1.0–14.0 strip for saliva. The second strip was made of white plastic material and its purpose was to detect changes in white balance on the camera by the different light conditions or user error. The latter strip was a 2 mm thick membrane of PDMS used to minimize variations in the reading for different lighting conditions, allowing light from the camera’s flash to diffuse and illuminate the posterior part of the test strip equally. Additional to the hardware, a software app was made for image acquisition and processing, and data storage and manipulation. The system as a whole, shown in Fig. 2.4, worked by first loading the app and selecting the test strip of different biomarker tests. Once the app
loads the calibration data and user interface, the test strip was to be inserted into the case and touch “Analyze” on the screen. The app takes a flashed image of the strip and then is categorized by color.

Matějovský and Pitschmann have created an addition from glass nanofibers to the Detehit biosensor (Pitschmann et al. 2018) for cholinesterase inhibitors (Matějovský and Pitschmann 2018). These inhibitors interfere with the nerve impulses cholinergic transfer mechanism. The biosensor was made based on the cholinesterase reaction based on enzymatic degradation of the substrate to obtain the appropriate acid or thiocholine. The Detehit biosensor was a detection ribbon based on hardened PVC and contains a detection fabric with immobilized and stabilized acetylcholinesterase (AChE), and a cellulose paper strip with acetylthiocholine iodide (ATChI) and Ellman’s reagent, shown in Fig. 2.5. For its operation, the detection fabric ought to be moist and exposed to air, by placing the fabric in contaminated water or pressing a wet detection zone against the test surface. The cellulose strip was squeezed with the exposed detection fabric, making the color change visible to be analyzed. If the white detection fabric changed to yellow, there were no inhibitors, however, if the color did not change, there were inhibitors present. This is depicted in Fig. 2.6a. However, because the color differentiation was sometimes difficult, a new substrate carrier made of glass nanofibers and using a chromogenic reagent (Ellman’s reagent) was developed to increase the intensity of the yellow color. The two were compared by having Ellman’s reagent combined with the ATChI and an alternate butyrylthiocholine iodide (BuTChI) and testing the new device with AChE and butyrylcholinesterase (BuChE). It was a 10 cm long by 1 cm wide plastic strip, having an indication fabric on one end with an immobilized enzyme covering about 1 cm², while the carrier was at the other end impregnated with a substrate and an indicator, also measuring 1 cm², as seen in Fig. 2.6b. The detection fabric was made by impregnating a white cellulose fabric with a solution containing the enzyme (AChE tissue, AChE, or BuChE) with total activity of 21 nkat/mL, 5% of dextran, and 2%
of anionic tenside, in a phosphate buffer solution with a 7.6 pH to be later dried at 25 °C for 24 h. The glass and cellulose papers were impregnated with a 4.3 mmol/L solution of Ellman’s reagent and with 6 mmol/L of ATChI or BuTChI in ethanol. The indicator paper was dried for 6 h at 25 °C. A blank test was performed to compare, and it showed that the glass nanofibers provided an augmented color effect, as is shown in Fig. 2.7.

Fig. 2.5  Schematic of reaction in the Detehit biosensor (Matějovský and Pitschmann 2018)

Fig. 2.6  a(I) Detehit biosensor before and after the test with a negative result, a(II) connection of opposite zones after incubation of Detehit biosensor. b Diagram of the biosensor, b(I) detection fabric; b(II) plastic strip; b(III) carrier of substrate and indic (Matějovský and Pitschmann 2018)
Recent examples of micro paper-based devices have been composed of 96 microfluidic wells (Sun et al. 2010; Sapsford 2009; Kai 2012). Sanjay et al. (2016) created a 56-microwell paper/PMMA hybrid microfluidic microplate for detection of infectious diseases and other bioanalytes, such as Immunoglobulin G (IgG) and Hepatitis B surface Antigen (HBsAg). The chip was laser cut based on the Adobe Illustrator design. In the mask-less laser ablation, the PMMA substrate was placed on a stage. The choice of using porous paper for the flow-through microwells in the PAD allowed the antibodies and antigens to be quickly immobilized, washed effectively, and avoid complicated surface modifications. The microfluidic microplate was composed of three PMMA layers, as seen in Fig. 2.8a. The first layer (Fig. 2.8bI) consists of an inlet reservoir (Fig. 2.8b1) and fluid distribution channel (Fig. 2.8b3) which was used for fluid delivery. It delivered the assay reagents to multiple microwells, which avoids manual pipetting and costly machinery, and forms the cover for the microwells in the following layer. Every channel in the top layer was connected to different inlet reservoirs and delivered the reagents to 7 microwells to the next layer. The second layer (Fig. 2.8bII) was for incubation and made up of 56 2 × 0.3 mm funnel-shaped microwells (Fig. 2.8c), with an upper microwell (Fig. 2.8b4) and lower microwell (Fig. 2.8b6). Paper disks (Fig. 2.8b5) were placed in between the two parts of the microwells. The microwells were created within a few minutes with a simple laser ablation method. This method offers a quick prototyping for developing microfluidic devices by means of high intensity laser beams that evaporate polymers at the focal point. Varying the intensity results in microstructures with different depths. The paper was held in place and prevented backflow of reagents as it is where the antigen or antibody were immobilized. The bottom layer (Fig. 2.8bIII) was fluid removal by means of the outlet channel (Fig. 2.8b7) leading to a common outlet reservoir (Fig. 2.8b8). Each channel was connected to a single outlet microwell to act as an outlet reservoir with a negative pressure. For the color change in HBsAg,
Fig. 2.8  a Schematic of the hybrid device, b cross-section of the device, c 3DFunnel-shaped microwell, d assembled device with different dyed water, e colorimetric representation of different concentrations of IgG (Sanjay et al. 2016)
the antigen was immobilized on the paper surface of the microfluidic microplate, reacting with the primary antibody conjugated with alkaline phosphatase (ALP). The enzymatic reaction between ALP and the colorimetric substrate BCIP/NBT was what produces the purple color. The colorimetric result could be observed by the naked eye within an hour or could be alternatively scanned by an office scanner for quantitative analysis. Figure 2.8e shows the variety of purple shades that corresponded to the concentrations inserted; the highest IgG concentrations resulted in a darker shade and as that quantity of IgG was decreased, so did the color intensity as is shown from left to right in the image.

Li et al. (2018) developed a double-layered microfluidic paper-based device with multiple colorimetric indicators for simultaneous detection of glucose, uric acid, lactate and choline. Linear calibration curves were obtained to identify these biomolecules. These values found from the experiments showed great sensibility (Fig. 2.9a) by exhibiting very wide linear ranges over two to three orders of magnitude: glucose (0.01–10.0 mmol/L), uric acid (0.01–5.0 mmol/L), lactate (0.04–10.0 mmol/L), and choline (0.04–24.0 mmol/L). The double-layered μPAD was first designed in AutoCAD. Different patterns were needed as the top layer was dedicated to detection and the bottom was auxiliary to construct 3D microfluidic channels. For detection, a 10 mm central sampling zone surrounded by eight $3 \times 8$ mm microfluidic channels and eight 6 mm detection zones were created. These were modified with colorimetric reagents, different kinds of oxidase and HRP, which

![Calibration curves of glucose, uric acid, choline and lactate. Fabrication of double layer μPAD Li et al. (2018)](image)
can be observed in Fig. 2.9b. The immobilized chromogenic reagents, once oxidized by the H$_2$O$_2$ from enzymatic reactions between the oxidases and the corresponding substrates, resulted in the color change with co-immobilized HRP as catalyst. The auxiliary layer was made of one 10 mm central sampling zone and eight 3 × 10 mm microfluidic channels connected with eight 6 mm sampling zones. Also, it provided a solution connection by 3D microfluidic channels resulting from overlapping the microfluidic channels and detection zones from the top layer. A traditional wax-screen-printing technique was used to produce the hydrophilic microchannels and hydrophobic barrier on the detection and auxiliary layers. In order to prove the use, a blood sample was introduced into the sampling zone. It was then passed through the hydrophobic channels in order to react with the reactants, thus producing the color, which the Image J software could read. As can also be seen in Fig. 2.9b, two kinds of colorimetric indicators were used for each biomolecule in order to widen the detection range. This new bilayer microfluidic PAD proved to have a strong colorimetric performance, enhanced sensitivity and extended detection range.

Nilghaz et al. (2019) incorporated metal complexation to a μPAD in order to identify antibiotic residues such as oxytetracycline and norfloxacin in pork. This was done by employing the filtration quality of paper combined with aggregation and precipitation of chemical reagents. Ultimately, these processes allowed a LOD and easy result interpretation. For antibiotic residue detection, three layers of filter paper were inserted into a hydrophobic wax paper holder. The topmost layer was made from chromatography paper to serve as the detection zone. In order to detect antibiotic residues, a base substrate made from Whatman #1 and #4 chromatography paper with printed letter channels of both substances from hydrophobic wax paper, was functionalized with copper sulfate pentahydrate in 0.5 M sodium hydroxide and iron nitrate nanohydrate (colorant reagent for oxytetracycline) in a 5 mM ammonia solution (colorant reagent for norfloxacin). A transition metal hydroxide formed when a reaction occurred, allowing the residues to bind to the metal ions through coordination chemistry. In Fig. 2.10a, a schematic of the individual devices for each antibiotic residue detection can be observed. This complex coupling could result on the filter paper and provided a visible color change as the concentration increased: oxytetracycline was detected with a blue to green color change, while norfloxacin with brown to orange, as can be seen in Fig. 2.10b. The other two layers were of Whatman #4 filter paper as they were absorbance layers, meant to remove the residual liquid under the base substrate. It is important to note that the colorimetric reagents from the first layer could not diffuse into the bottom layer. The LOD for either was 1 ppm and the recovery rate for oxytetracycline was approximately 88.6% while for norfloxacin recorded to be 111.3%. The whole process of assembly and testing required less than an hour, resulting in a sensitive and rapid method to detect antibiotic residues in food samples. Since the reactions were not interfered by other antibiotics, this device can be implemented to detect other antibiotics from the same families including tetracycline and floxacin. Furthermore, The device has proven to be valuable to food safety surveillance and suitable for large-scale production.

As a common biomolecule for detection, glucose was measured from tear samples in the μPAD biosensor Moreira et al. (Gabriel et al. 2017) designed. The chromogenic
reagent used for the samples was 3,3′,5,5′-tetramethylbenzidine (TMB). The device resulted in a linear behavior between 0.1 and 1.0 mM, as seen in Fig. 2.11a, analytical sensitivity of 84 AU/mM and LOD of 50 μM. This provides an alternative for diabetic patients pricking their fingers with a lower potential interference, non-invasive, and pain-free sample (Cha et al. 2014). In order to detect glucose from tears, the desired geometry of the device was designed on Corel DrawTM graphical software and, like other μPADs, was printed on paper substrates by a wax printer (Gabriel et al. 2017). Effective hydrophobic barriers were fabricated by melting the printer wax while one side of the device was covered with adhesive tape to prevent leaking of the samples. With the basic structure, two 5 mm circular zones, identified as the control and detection zones, and a square region as the sample inlet were defined. The control zone’s purpose was to detect potential interferant compounds and to minimize the matrix effect. The sample inlet is evidently where the tears are places to be pulled up to the detection zone by capillary forces. All three zones are connected by a 14 × 2 mm microfluidic channel, whereas the entire device is 24 × 10 mm. The paper
surface was modified with chitosan in order to enhance the surface attachment of enzymes. The chitosan was first prepared in 2% (v/v) acid acetic, subsequently 2 μL of the solution was introduced to the control and detection zones and allowed to dry. The detection zone was spotted with a chromogenic solution of 15 mM of TMB and 120 U mL⁻¹ of an enzymatic mixture of GOx and 30 U mL⁻¹ HRP. The control zone was only spotted with the enzymatic solution. 5 μL of sample aliquots were introduced to the sample inlet and left to reach the detection zone under lateral flow. This can be visualized in Fig. 2.11b. The actual colorimetric detection was done with an office HP scanner with a 600-dpi resolution. Images were taken 15 min after sample addition and were converted to Red–Green–Blue scale for simpler analysis within the Corel Photo-Paint™ software. The color intensity was directly proportional to the concentration of glucose, however, most importantly, it was compared to a personal glucometer, and no statistical difference existed with a confidence level of 95%.

Among different μPADs, Nilghaz et al. (2015) created a compact embeddable microfluidic cloth-based analytical device (μCAD) in order to detect glucose, nitrite and proteins with the naked eye and with concentrations as low as 0.5 mM, 30 μM,
and 0.8 mg/dL, respectively. The device proved to be mechanically durable, robust, and flexible (Parikesit 2012). Cotton was chosen as the raw material for the cloth-based analytical device as it is mechanically robust, deliverable to the end user (Nilghaz et al. 2011), provides an excellent immobilization matrix for biomolecules (Malon et al. 2014), and a better uniform mixing of reagents and analyte through detection zones (Ballerini et al. 2011; Reches et al. 2010). Additionally, it can be easily patterned with adhesive wax to create the hydrophobic-wall microfluidic channels. Both wax and cloth are inexpensive Bhandari et al. (2011) and environmentally friendly structural material for disposable diagnostic assays (Park et al. 2004). Also, cloth-based microfluidic channels can be stable for one week at ambient temperature, making it an optimal factor for application and use in underdeveloped areas (Nilghaz et al. 2015). Overall, the instrument is a one wax-patterned cloth layer double-inlet device that includes 11 sections among the inlet points, stock zones, detection zones and isolator layers (Nilghaz et al. 2015). In order to create the 3D colorimetric microfluidic device, the 2D pattern was folded along certain predefined lines. The stock and detection zones were placed in the middle layers and separated by wax-impregnated cloth as isolators. Between 0.1 and 0.5 μL of a solution with colorimetric reagents for glucose, nitrite and protein assays were poured into multiple detection zones by a micropipette, while the detection zone held the reagents for the assay. The traditional wax patterning technique was used to pattern the microfluidic channels on scoured cotton cloth fabric. Furthermore, the ability of wax-patterned cloth fabric with hydrophilic or hydrophobic sections in order to have various designs for multiple bioassays was explored. By stacking layers of individual assay within a small surface area, and separating them by wax-impregnated fabric, multiple assays were able to be conducted. Further improvement was attempted by having an on-chip colorimetric calibration by having predefined serially diluted samples next to the detection zones.

Additionally, Lam et al. (2017) developed a chemically patterned μPAD (C-μPAD) by forming hydrophobic barriers using CVD of trichlorosilane (TCS) on chromatography paper. This C-μPAD allowed the measurement of glucose, tumor necrosis factor alpha (TNFα), and heavy metal nickel for point of care diagnostics. To create the structure of the C-μPAD, the desired fluidic pattern was designed in AutoCAD and cut out onto a vinyl tape. This tape was transferred to a 4.5 × 5 cm chromatography paper. In order to silanize the chromatography paper for the hydrophobic barriers, a low-pressure chamber and heat block were required. The vaporized TCS molecules penetrated the paper to bond covalently with hydroxyl groups on cellulose fibers creating an extremely stable and highly reproducible hydrophobic barriers, shown in Fig. 2.12a. The deposition of these TCS molecules depended on pressure, CVD duration, temperature, volume of TCS, and the mobility of the molecules. By controlling these variables, the chemicals traveled through the paper and uniformly immobilized throughout the paper. The patterned paper was placed on a hotplate to remove the vinyl tape to leave the hydrophilic area while other parts remained hydrophobic. This chemically patterned chromatography paper was then evaluated with color dyes, as seen in Fig. 2.12b, c. For glucose, the LOD was 13 mg/dL, which is that of a commercial glucose sensor. The LOD of TNFα was found to be
3 ng/dL which again presented similar results as those of the commercial platforms. However, for nickel, a colorimetric agent was immobilized to obtain a stationary and uniform reaction through thermal condensation coupling method. This resulted in the detection of nickel with a LOD as low as 150 μg/dL. These LODs provided high expandability and adaptability for the device. With these results, this C-μPAD produced simple, quick, and cost-effective bioassays for environmental monitoring.

Fig. 2.12  a Schematic of fabrication process, b and c positive and negative features of 2D channels of C-μPAD (Lam et al. 2017)
2.4 Recent Advances of Colorimetric Detection in Microfluidic BioMEMS

2.4.1 Recent Advances of Colorimetric Detection in Lab-On-Chip (LOC) Devices

Microfluidic technology has raised an increasing interest in POC diagnostics as it requires small reagent consumption, and offers fast analysis and portability. Capillary and centrifugal forces are the driving forces in these devices that have proven great candidates for integrated genetic analysis due to the versatility of fluidic control without intricate microvalves and tube lines and easy integration of the functional units (Park et al. 2016). Additionally, expensive and large laboratory set ups may be replaced by smartphones for detection analysis (Wang et al. 2016). Centrifugal microdevices usually take on the shape of a compact disc (CD) and involve a combination of microfluidic unit operations such as liquid mixing, metering, or valving which are controlled by the rotational speed of the device. Due to this versatility, many applications have forth come such as molecular diagnostics and immunoassay analysis (Sayad et al. 2017). Among the developed tests for lab-on-chip platforms, some have found worldwide applications including pregnancy tests (Li et al. 2014). Moreover, microfluidic platforms are reported for detection of Tuberculosis (Evans 2017). Furthermore, new devices are being developed to detect various pathogens for detection of foodborne diseases (Sayad et al. 2017). Some of the latest examples of the microfluidic BioMEMS used for colorimetric detection are as provided here.

Mao et al. (2017) designed a microfluidic chip with eight microchannels in order to determine chlorpyrifos based on peroxidase-like CuFe$_2$O$_4$/Graphene Quantum Dots magnetic nanoparticles (GQDs MNPs). The nanoparticles were included to amplify the color signal as peroxidase mimetic using a one-step hydrothermal method with electrostatic adsorption. The chlorpyrifos device was made up of a microfluidic chip with an enzyme inhibition reaction, color reaction, and UV spectrophotometric detection areas. The graphene quantum dots were synthesized from a carbonization during the pyrolysis of citric acid. A traditional soft lithography technique was used to create the microfluidic chip where a 50 μm SU-8 photoresist was spun on the silicon wafer. Subsequently, the pattern of the chip was printed on a clear film by 2880 dpi resolution ratio. The male mold of the photoresist was obtained by an ultraviolet exposure for 70 s followed by development. It was mixed with the PDMS prepolymer with a 1:10 ratio and later had air bubbles removed. The mixture was cured for 3 h under 60 °C. Later, the inlets and outlets were created on the curing PDMS substance with the microchannel structure by a puncher. Lastly, the PDMS chip was formed by plasma treatment and slide bonding. For testing, 100 μL of chlorpyrifos were injected from the first two entrances to converge at the same point and time. 100 μL of acetylcholine (ACh) and 200 μL NaH$_2$PO$_4$ buffer solutions were added to the second two entrances where the mix flew to the color area. The TMB oxidation produced the color variation and was affected by the H$_2$O$_2$ concentration with the
CuFe₂O₄/GQDs (Fig. 2.13). ACh was inhibited as the organophosphate pesticides (OPs) concentration increased, hence reducing the production of H₂O₂ and thus, provoking a weak color reaction and absorbance. Therefore, it was concluded that the absorbance is inversely proportional to the concentration of OPs.

Another example is a novel biosensor that uses gold nanoparticles to detect the concentration of E. coli O157:H7 equipped with an app for color monitoring (Zheng et al. 2019). The microfluidic chips have proven to detect foodborne pathogens rapidly due to its precise control of the fluids, few sampling, and decreased detection time. For the E. coli-detecting biosensor, shown in Fig. 2.14, the 3D printed, and surface plasma-bonded microfluidic chip was the most important piece. The mold of the chip was placed in 5% NaOH for half an hour and later mixed with curing agent at a ratio of 10:1. It was placed into the mold for 12 h at 65 °C and once peeled, it was united with the glass slide through surface plasmon treatment. It had two 600 × 100 μm serpentine mixing channels, where one is to mix the bacterial sample with the MNPs and polystyrene (PS) microspheres, and the other for mixing catalysate with the AuNPs and cross-linking agents. The COMSOL platform was used in these channels to stimulate them based on free triangular grid and finite volume. The chip
also incorporated a $14 \times 14 \times 1$ mm chamber that separated the MNP-bacteria-PS complexes and catalyzed hydrogen peroxide. The last part was a $14 \times 14 \times 2$ mm detection chamber where the AuNPs color modifications were observed. The MNPs modified with the capture antibodies and the PSs modified with the detection antibodies were used to interact with the target bacteria in the first mixing channel. AuNP were added for signal indication, Hue-Saturation-Lightness (HSL)-based smartphone imaging correctly detected changes in color, and the microfluidic chip was created for on-chip bioreaction. The results showed that the LOD was 50 CFU/mL for E. coli O157:H7 and the mean recovery was $-96.8\%$.

An analyzer was created by Li et al. for multi-index monitoring of diabetes and hyperlipidemia from a patient’s blood Li et al. (2019c) The indexes for monitoring involves glucose (GLU), triglyceride (TG), and total cholesterol (TC). The color changes originated from the peroxidase-$H_2O_2$ enzymatic reactions and were taken with a smartphone analyzer that contained a LED light and a charge-coupled device (CCD) camera. The smartphone-assisted microfluidic analyzer contained a 2 mm thick structural layer made from plastic injection molding with pressure sensitive adhesive (PSA) layers on either side. The most important piece was the fan-shaped body of the device with 3.25 cm radius, a buffer pool, four reaction chambers with vent holes, and two positioning holes, each with three capillary stop valves (Fig. 2.15). The analyzer was an optical detection system based on step motor, microcontroller, and Bluetooth module. The detection zone included a white LED, macro lens and a CCD camera. To build the microchip, the plastic was first attached to only the bottom layer of adhesive. Subsequently, 13 $\mu$L of each detection reagent was input to each of three chambers, leaving one empty to serve as control. The reagents were left there during incubation for 4 h at 37 °C and then the top adhesive was added.
The chip could then be sealed into a vacuum pouch and stored for 6 months at 4 °C. In order to test the system, 10 µL of serum was mixed with 190 µL of Tris–HCl and 4-Aminoantipyrine in an Eppendorf tube. 95 µL of the mixture was input to the inlet hole under the pressure of the pipette and the chip went into the Smartphone-assisted microfluidic chemistry analyzer for incubation for 15 min. The detection was done with the phone by means of the light provided by the LED and transmitted by the reagent. It was then collected by the macro lens and hence, detected by the camera as a step motor rotated the chip so all four chambers could be recorded. The images were processed by the microcontroller and sent via Bluetooth to a smartphone for the final analysis. To improve the system, the reagent addition steps could be automated to reduce the manual operation. Additionally, a cost-effective and reliable detector must be put in place to obtain such quantitative results as in Fig. 2.16. In this device, the detection reagents were mutarotase, glucose oxidase, peroxidase and 2-hydroxy-3,5-dichlorobenzenesulfonic acid (DHBS) for glucose; cholesterol esterase, cholesterol oxidase, peroxidase and DHBS for cholesterol; and lipoprotein lipase, glycerokinase, glycerol-3-phosphate oxidase, peroxidase and DHBS for triglyceride.

An Electronics-based ELISA (e-ELISA) using a Lab-on-a-Printed Circuit Board (LoPCB) device for Point of Care (POC) of Tuberculosis was developed by Evans et al. (2017). The device was a modified ELISA that operated with 10 µL volume and included PMMA wells, gold surface, TMB as the reporter reagent (Fig. 2.17a) and Interferon Gamma (IFNγ), a pro-inflammatory cytokine key in innate and acquired immunity, as the assay target. Copper (Cu) foil was laminated to the FR4 PCB substrate through thermal adhesion. Subsequently, tracks made from electrical connections, and electrode pads were patterned by etching the Cu layer. A gold layer was plated on top of the copper one and was fixed by the copper primer which determined the final distribution of gold. The fluid wells were cut from PMMA and fixed onto the surface of the circuit board. The board (Fig. 2.18a) included reference electrode circuitry, working electrodes with amplification circuitry, voltage input Analogue-to-Digital Converters (ADCs), processing unit and the user interface consisted of an embedded on-board TFT touch screen USB port (Fig. 2.18b). Both amperometric and colorimetric signals were measured by the device. The first was measured by second generation amperometry where it detected charge carrier concentration through the measurement of total current magnitude charge carriage.
To accomplish this, a reporter molecule was needed that had a relatively low conductivity. For colorimetric readout, capture antibody $\alpha$ IFN$\gamma$ Fab‘-3Cys-6His) was immobilized on the Au sensor chip surface and with the addition of cysteine residue. Covalent bonding was achieved, hence capturing and immobilizing antibody fragments by Cysteine (thiol) linkage. The color change was generated from the resulting color of TMB which was originally a colorless liquid that turned bright blue after the reaction (Fig. 2.17b). The outcome of this study was a lightweight, low-cost, amperometric and colorimetric detection unit made according to standard commercial processes which embedded microfluidics and multi-channel amperometric sensing.
Fig. 2.17  a Schematic of fabricated PCB-based biosensor, b representation of HRP-catalyzed oxidation of TMB (Evans 2017)

Fig. 2.18  a Schematic of custom-made electronic board, b individual sections of the electronic board and location on (a) (Evans 2017)
2.4.2 Recent Advances of Colorimetric Detection in Lab-On-Compact Disk (LOCD) Devices

A digital optical disc (DVD) was used by Li et al. (2014) as the platform for a molecular diagnostic and quantitative pregnancy test. The analytes of interest were human Chorionic Gonadotropin (hCG) identified from urine samples. A standard DVD was prepared for signal readout. The polycarbonate surface was first activated by UV irradiation and then treated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS). Afterwards, a PDMS plate with six embedded microfluidic channels was placed inside the DVD. Once activated, anti-human Chorionic Gonadotropin Gα (anti-hCGα) monoclonal antibodies were immobilized on the surface (Fig. 2.19a) by an amide-coupling reaction. A streptavidin nanogold conjugate was added to the surface via biotin-streptavidin interaction following a silver staining treatment, which ultimately resulted in an enhanced signal in order to obtain a significant disruption of the laser readout in the optical drive. A standard, unmodified optical drive was used for the assay readout, and free disc-quality analysis software for the processing of the obtained data. To use the device, samples were initially loaded into the PDMS microfluidic channels, subsequently, the DVD was spun within the optical drive, undergoing centrifugal forces that created a different radial distance according to the analyte concentration. The assay was tested.

![Fig. 2.19](image)

**Fig. 2.19** a Schematic of DVD assay design and principle of signal reading. b(I) Quantitation of urine hCG level of seven pregnant women during different points of pregnancy. b(II) Quantitation of urine hCG levels of one woman during different days of pregnancy by ELISA and parity inner fails (PIF) methods (Li et al. 2014)
with a DVD diagnostic software and the readings were interpreted according to the radial distance and optical darkness ratio. The results showed comparable sensitivity and selectivity to well-established colorimetric methods and ELISA (Fig. 2.19b). Additionally, it is an inexpensive, easy to use, multiplex, POC diagnostic instrument for prompt response used in remote and/or rural areas.

Park et al. (2016) developed an integrated rotary microfluidic system (Fig. 2.20a) with DNA extraction unit [Fig. 2.20c(I)], LAMP [Fig. 2.20c(II)], and lateral flow strip [Fig. 2.20c(III)]. The device was used for detection of the food-borne bacterial pathogen, monoplex Salmonella Typhimurium and multiplex Salmonella Typhimurium as well as Vibrio parahaemolyticus. The LAMP reaction replaced...
polymerase chain reaction (PCR), allowing higher specificity and sensitivity, and eliminating the bulky thermocycler. A glass microbead-based centrifugal nucleic acid extraction was used as a solid phase matrix where the genomic DNA could be purified from the lysate sample. Lastly, the colorimetric based lateral flow strip provided a cost-effective and equipment-free detection method. The device integrated these three techniques for detecting in a sequential manner with an optimized microfluidic design and rotational speed control. The microdevice was a five-layer stacked disc with three identical units. Each unit consisted of three functional parts: solid phase DNA extraction, LAMP reaction, and a lateral flow strip. The main three microfluidic layers were created by a CNC milling machine and PSA film was used once the micropattern was cut by a plotter, following a hot press bonding all layers together. The first layer had injection holes and microfluidic channels to transport a LAMP product and a running buffer from the second layer into the lateral flow strip on the third layer. The second layer contained the micropatterns for DNA extraction and amplification. The third layer was for embedding a lateral flow strip for the colorimetric detection (Fig. 2.20b). The detection could be made with the naked eye due to the LAMP product. In order to do so, a lateral flow strip containing a buffer loading pad for introducing a running buffer, a conjugate pad (including streptavidin coated AuNPs for the conjugation with the LAMP products), a detection zone (where anti-Digoxigenin, anti-Texas Red and biotin were immobilized in the test line 1, test line 2, and control line, respectively), and an absorbent pad for liquid wicking were incorporated within the device. Thermal guards were patterned around the lateral flow strips to prevent the heat from influencing the anti-hapten on the detection zone of the lateral flow strips. The resulting microdevice presented great potentials as a user-friendly POC analyzer for application in resource-limited setups. The design allowed efficient fluid transfer without sample loss from sample pretreatment to strip detection. The automatic and integrated genetic analysis could then be successfully performed by controlling the rotational speed without the use of expensive equipment.

A cheap and portable smartphone spectrometer for monitoring optical changes as they occur was created by Wang et al. (2016). The device was aimed at detecting glucose and troponin I, a myocardial infarction biomarker by means of a smartphone with a built-in LED and complementary metal oxide semiconductor (CMOS) camera to use as the light source and the detector, respectively. No external light source, lens, or filter were required. As the dispersive unit, a CD with grating was used. For human cardiac troponin I detection, peptide functionalized AuNPs were taken as the reporters. For the detection of glucose, a solution of 2,2′-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS), HRP, and glucose oxidase (GOx) was utilized. A bi-enzymatic cascade assay was used where the glucose was catalytically converted into hydrogen peroxide, which then converts ABTS by HRP into oxidized form. Once oxidized, a blue color appeared and was read from the color band with the spectrometer bases on the change of intensity. The spectrometer relied on a sample cell with an integrated grating substrate, and the phone’s LED flash and camera. The CD was placed 50 mm away from the LED and tilted 5º so that the flashlight passed through a 1 mm diameter pinhole. The grating tracks were aligned to the
incident light, and the light was refracted from the CD onto the camera (Fig. 2.21). This allowed a real-time measurement and resulted in a LOD of 50 ng mL. The biosensing system coupled with a smartphone platform offered a promising method for the phone to detect, interpret, and communicate targeted biological information. Likewise, a higher sensitivity, speed, and simultaneous monitoring was possible. Although it had a comparable performance to commercial devices, it was more compact, cost-effective, and portable.

Sayad et al. (2017) created a 165 mm diameter centrifugal microfluidic device platform integrated with LAMP technique (Fig. 2.22a, c) for quick, monoplex and colorimetric detection of foodborne pathogens. Three main pathogens were studied: Salmonella spp, Es. coli and Vibrio cholerae. 24 strains of these pathogenic bacteria with eight strains of each bacterium were tested and DNA amplification on the microfluidic CD was performed for 60 min. The device consisted of three layers: PMMA top and bottom layers, and a PSA middle layer as shown in Fig. 2.22d. The layers were aligned and press-bounded together. The top layer contained the venting and loading holes for liquid insertion and wax plug. For the microfluidic structures in the bottom layer, six identical units (Fig. 2.22b) were designed to be able to perform 30 genetic analyses of the three pathogens. One of the units was the loading chamber which loaded the LAMP reagents and primers. Another was the mixing channel and chamber that aliquot the LAMP assay into equal volumes. The sealing chambers contained the sealing material used to seal the connection
channel between the metering and amplification chambers to prohibit liquid evaporation. Lastly, the amplification chamber was designed for the amplification and detection of DNA. An optimized square-wave microchannel, metering chambers, and revulsion per minute (RPM) control were utilized to constantly load, mix, and aliquot the LAMP primers/reagents as well as DNA samples. The LAMP reaction amplicons were detected by the calcein dye colorimetric method and analyzed with the developed electronic endpoint detection system (Fig. 2.23a) including the Bluetooth interface to send the results to a smartphone (Fig. 2.23b). Calcein is a synthetic fluorescein that emits a bright fluorescence creating a visual color change. A positive sample changes from yellow to green while a negative readout remains light orange. The entire process in only one CD lasted about 65 min and presented a LOD of $3 \times 10^{-5}$ ng $\mu$L$^{-1}$. 

Fig. 2.22  a Schematic of centrifugal LAMP microdevice, b top view of one unit of the microdevice, c photograph of the microdevice, d schematic top view of the device’s layers (Sayad et al. 2017)
2.5 Alternative BioMEMS for Colorimetric Detection

In order to improve the colorimetric biosensing strategy, different amplification methods have been developed. Among these alternative strategies, exonuclease (Exo)-assisted signal amplification, strand displacement amplification (SDA), and rolling circle amplification (RCA) are promising methods. The latter has proven to result in ultrasensitive biosensors due to its excellent properties in signal amplification. However, it creates nonspecific amplification due to the impurity of the circular template, and generation of large fragments of single-stranded DNA (ssDNA) which may decrease the solubility. To counteract this disadvantage, an improved alternative was developed (Li 2016).

Li et al. (2016) established an RCA-based colorimetric biosensor with an enhanced nucleic acid-based amplification machine to detect attomolar microRNA (miRNA). The machine was composed of a complex of trigger template and cytosine-rich
DNA co-modified molecular beacon (MB) and guanine-rich DNA (GDNA) as a probe. This was made by mixing MB and GDNA at a 1.2:1 ratio incubated for an hour at room temperature. Seal probe was prepared by self-templated ligation of 5'-phosphorylated dumbbell-shaped DNA sequence using T4 DNA ligase. The machine also required polymerase and nicking enzyme, and a dumbbell-shaped amplification template. The MB template was composed of four sections: miRNA-recognition domain (Fig. 2.24a), GDNA hybridization domain (Fig. 2.24b), amplification domain for producing the nickel triggers (Fig. 2.24c), and a nicking domain for Nb.BbvCl recognition. The target miRNA triggered MB mediated strand displacement to cyclically release nicking triggers, leading to a toehold-initiated RCA (TIRCA) to produce large amounts of GDNA (Fig. 2.24). These can stack with hemin to form G-quadruplex/hemin DNAzyme, an HRP mimic, in order to produce a colorimetric reaction. The modified MB decreased the background signal and improved the stringent target recognition. A DYY-6C electrophoresis analyzer was used to perform gel electrophoresis for the seal probe and a Bio-rad ChemDoc XRS for imaging. A NanoDrop 100 spectrophotometer, a UV–visible spectrophotometer, collected the signal. The outcome was a simple, label-free ultrasensitive visual colorimetric biosensor (down to a LOD of 5aM, a detection range of nine orders of magnitude for practical sample analysis). The sensitivity was due to the reduction of steric hindrance and facilitated solution of TIRCA products. The entirety of the process was completed.

Fig. 2.24  Schematic of nucleic acid-based amplification machine (Li 2016)
within 90 min. The machine itself offered a combination of advantages provided by enzymatic signal amplification and toehold-initiated RCA.

## 2.6 Summary

Colorimetric detection can be done visually, hence no expensive equipment or much time is needed. Paper-based devices that operate based on colorimetric detection strategy have improved the accessibility, speed, and accuracy of tests while offering considerable cost effectiveness. Smartphones and tablets opened yet another window of opportunity to easy and onsite analysis of the readout results. By combining microfluidics with the \( \mu \)PADs, the advantages of these devices are as \( \mu \)PAD with colorimetric results attract even more attention due to its simplicity, versatility, straightforward detection results and applicability, especially in point of care analysis without advanced instruments (Li et al. 2018). Microfluidic colorimetric biosensors offer small size, high precision with small sample size, simple operation, and low cost (Mao et al. 2017). Overall, colorimetric-based enzymatic assays are fast, adaptable, and cost-effective while allowing the color change to be seen by the naked eye or by digital sensors (Li et al. 2019c). Centrifugal microfluidic devices present excellent opportunity to detect a variety of biomolecules for different applications. While the literature has witnessed a great deal of advancements in fabrication and application of BioMEMS for colorimetric biosensing, further optimization of these devices for high throughput detection present an opportunity for further improvement.

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