Chapter 15
DNA-Based Sensors

Geeta Bhatt and Shantanu Bhattacharya

Abstract Sensing and detection for clinical diagnostic can be accomplished through various routes. Additionally what and how of sensing is critically optimized to meet individual needs. Diagnostics is carried out with various types of sensors out of which the electrochemical sensors are most used due to their unique ability to couple seamlessly with electronic circuitry. The DNA sensor is one of the most common types of sensors which is majorly deployed to perform expression monitoring, transcription profiling, etc., for example, the products developed by Affymetrix and Nanogen. This chapter is a consolidated review of the various aspects of DNA sensors, like the principle of detection, various ways of sensing and detection, applications of such DNA-based sensing. It looks at the various principles that are utilized for gene mapping like dielectrophoresis, polymerase chain reaction (PCR), real-time PCR or quantitative PCR (better known as q-PCR), hybridization, solid-phase PCR, droplet-based PCR, etc. It also reviews various sensing/detection strategies for sensing DNA like electrophoresis, impedance spectroscopy, colorimetric sensing, optical sensing and inertial sensing. The chapter provides a state-of-the-art review of basic techniques, sensing methodologies and applications for DNA-based diagnostics as carried out by industry.

Keywords DNA-based sensing · Fluorescence · Impedance spectroscopy PCR · q-PCR · Electrophoresis · Dielectrophoresis · Microcantilevers Electrochemical sensing

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1 Introduction

The field of biosensing deals with the development of a sensory platform (generally equipped electrodes) to sense multiple analytes in various samples (e.g., deoxyribonucleic acid (DNA), ribonucleic acid (RNA), bacteria/microorganisms, chemical analytes) to pick up specific targets in those samples which would give important diagnostic information. The sensing is carried out by generating an equivalent signal of various kinds (optical, colorimetric, fluorescence, impedance, etc.) generated differentially from the different chemical arrangements of bases on a DNA which may furnish specific molecular address for the recognition of biological entities. DNA-based sensors are very prominently used in diagnostics/detection purpose, be it disease detection, expression monitoring, transcription profiling, or DNA sequencing. In almost all DNA-based sensors, the detection is carried out through a mediating known sequence of a DNA molecule which detects target DNA that is otherwise present in the sample. There are various principles associated with such detection which may either involve the selective bonding of a base pair sequence of a DNA or an RNA to another similar molecule or molecules (better known as hybridization) or simple copying of the DNA or RNA molecules through an in vitro copying process similar to in vivo processes which keeps happening as cells grow and divide within living systems (better known as PCR). Detection can be carried out further by immobilizing concentrated molecules acting as probes over a small region or by simply trapping the biological constituents in one place from the bulk solution and generating an enhanced signal from this concentrated or trapped sample to enable sensitive detection. There are several techniques developed by researchers which will aid one or more steps of detection as briefed earlier as follows:

1. Dielectrophoretic capture of biological entities containing DNA molecules or molecules themselves.
2. PCR where copying of a specific sequence or sequences is carried out in vivo and detection is carried out simultaneously as the sample gets copied multiple times. Further, the types of phases where this can be carried out are the following:
   (a) Solid-phase PCR
   (b) Liquid-phase PCR with or without the real-time detection capabilities of the copying process
   (c) Droplet-based PCR, etc.
3. Electrophoresis wherein separation of various lengths is achieved by sieving molecules through gelatinous substances with highly porous networks.
4. Immobilization of molecules in a concentrated manner on a substrate which will perform capture and localization followed by tagging of a fluorophore molecule which may result in enabling DNA detection.

5. Sensing of the electrochemical impedance by using the strategy mentioned in step 4 above with a change of the optical label/tag to electrochemical label (e.g., Ferro-cyanide label)
6. Sensing the whole DNA molecule as such by looking at the impedance signal of the solution which contains DNA, etc.

Dielectrophoretic capture deals with manipulation of neutral particles while these particles are kept in varying field intensity. An electrical force is exerted on neutral particles of a certain dielectric constant immersed in a medium of a different dielectric constant particularly as the particle is guided into a non-uniform electric field. This concept can be used to pre-concentrate at a certain place neutrally charged particles and can be used to trap, separate, count, etc., while PCR deals with amplification of DNA to make it detectable. A lot of variants to the conventional well-based PCR system are developed for enhancing the processing rapidity and accuracy. The variants are developed by looking at microchip-based solutions for conducting PCR/real-time PCR (RT-PCR), solid-phase PCR and droplet-based PCR. This is mainly done to miniaturize sample volume so that the overall reaction becomes inexpensive while the limit of detection can be reduced and sensitivity of the system can be greatly improvised. Apart from these techniques, the basic hybridization techniques have been included in sensing/detection technique section. Among the various sensing techniques, the following are discussed in detail in this chapter:

(i) Electrophoresis.
(ii) Electrochemical impedance spectroscopy (EIS).
(iii) Colorimetric sensing.
(iv) Optical sensing.
(v) Inertial sensing.

Electrophoresis is a standard technique for sieving the molecules under the effect of electric field according to their size. The particles get arranged as per their size (larger molecules at the top and so) while the solution travels through the agarose gel which is connected to uniform electric field. This is also used as a confirmation test for conventional PCR. It is the most basic technique which uses dye to visualize the location. The colorimetric sensing and optical sensing can also be considered to be very conventional in nature. In the colorimetric sensing technique, a change in the wavelength is used for detection. In optical sensing technique, either fluorescence or overall sample absorption is used to perform detection. As colorimetric detection and optical detection involve expensive instrumentation, there is an emergent trend in utilization of some other non-optical/non-colorimetric routes like electrochemical impedance-based detection (popularly known as EIS) and inertial/mass-based detection. In impedance sensing route, the impedance (a combination of resistance/capacitance/inductance under alternating electric field) change of the solution containing the sample is observed so that the analyte can be detected. Both the electrical techniques have a niche among the contemporary processes as they are compatible with electronic systems and are easily integrable with

microelectromechanical systems. In inertial sensing approach as developed initially by IBM (Lang et al. 1999), a small array of microcantilevers are used with reference and signal measurements. The signal comes from deflection of the cantilevers owing to small surface stresses due to binding. There are other techniques apart from EIS techniques which are also deployed although not so frequently to perform biological detection. These include amperometric (change in current) and conductometric (change in conductivity) detection which are generally carried out in order to detect traces of chemical species, e.g., nickel oxide-based glucose sensor (Mu et al. 2011) and monitoring the condition of lubricating oil (Latif and Dickert 2011), etc., and generally may not be a good proposition for doing standalone DNA detection.

2 Principles of DNA-Based Biosensors

DNA-based biosensors deal with sensing of different biological analytes by carrying out identification of these analytes through the genetic route. A variety of analytes that are normally detected through these sensors can be purified DNA, RNA samples, other biological entities (e.g., viruses and microorganisms or spores), various drugs, various organic and inorganic particles, chemical elements, etc. The concept of detection in these sensors remains more or less same; the analyte is taken close to the sensory surface, and a chemical/biochemical change is initiated which results in a transduced signal which may be able to reveal information about the basic molecular DNA/RNA, etc. Interaction of the analyte with the sensory surface can be carried out through direct immobilization, adhesion, or partial hybridization to the surface. One of the easiest routes of direct detection of analytes like DNA/drug molecules or organic/inorganic nanoparticles happens through direct adhesion or capturing of the analyte on the substrate. Sometimes, it is even preferable to deploy a technique that may concentrate and accumulate the analyte over the sensory surface. A very effective technique that was developed by Pohl (1951) exists which is popularly called dielectrophoresis (DEP). The other way of accumulating a target sequence of DNA over a surface is to copy the target sequence in vitro through the PCR route while keeping the overall volumes for the PCR very less and enhancing the sensitivity of the system multifolds.

2.1 Dielectrophoretic (DEP) Capture

DEP is a particle manipulation technique in which an external force is exerted on otherwise neutrally charged particles as they are influenced by a of non-uniform electric field. The external force generated tends to alter the motion of these particles, and depending on the relative polarizability of these particles in reference to the medium in which they are immersed, they show different characteristic

behaviours. The limit to which particles are polarized depends on various factors like electrical field strength, frequency of the input voltage, the shape and size of particles, and the medium/particle/free space permittivity. The concept of DEP was shown long back (Pohl 1951) by Pohl and was computed from the Maxwell equation by the famous Clausius–Mossotti projections (Morgan et al. 2007). The magnitude of the DEP force that is felt by a particle in an electric field is given by Eq. 1,

$$F_{\mathrm{DEP}} = 2\pi r^3 \varepsilon_m \mathrm{Re}\left\{\frac{\varepsilon_p^* - \varepsilon_m^*}{\varepsilon_p^* + 2\varepsilon_m^*}\right\} \nabla |E_{\mathrm{rms}}|^2; \quad \varepsilon_i^* = \varepsilon_i - \frac{j\sigma_i}{2\pi f} \quad i = p, m \tag{1}$$

where r is the radius of neutral particle which experiences the DEP force; ε_p is the electrical permittivity of suspended particle; ε_m is the electrical permittivity of the medium in which the particle is suspended; ε_p^* is the complex permittivity of particle; ε_m^* is the complex permittivity of medium; $\left\{\frac{\varepsilon_p^* - \varepsilon_m^*}{\varepsilon_p^* + 2\varepsilon_m^*}\right\}$ is better known as the Clausius–Mossotti factor; E_{rms} is the root-mean-square value of electric field; σ_p is the conductivity of the particle; σ_m is the conductivity of the medium; and f is the frequency of the electric field. Clausius–Mossotti factor is a measure of the polarizability of particles and depends on particle permittivity and medium permittivity. DEP can be applied to a variety of purposes like particle capture, particle manipulation, particle sorting (depending on the size/type of the particles), etc. Depending upon the extent of polarizability, the particles are captured at different instances at different positions during their traverse across microfluidic chips.

Depending on the nature of influence of the external electric fields upon the capture behaviour of particles, DEP can be categorized into two different forms, viz., positive DEP and negative DEP. If the particles are captured in the direction of increasing field strength, it is regarded as positive DEP, and if particles are captured in the direction of diminuting electric fields, it is regarded as negative DEP. Figure 1 explains the effect of uniform and non-uniform external electric field on neutral particles crossing such field. Figure 1a depicts no movement behaviour of neutral particle under the effect of uniform field, while its position is altered in case of non-uniform field (Fig. 1b). Figure 1b shows the concept of negative and positive DEP effects. Since biological cells are mostly neutral, DEP finds wide application in manipulating cells that can be very useful for medical diagnostics, drug delivery, cell therapeutics, etc. DEP can also be classified based on the type of electrode that is deployed. It can be performed through metal electrodes (most common type of electrodes), insulating structures working as field splitters, liquid electrodes (Demierre et al. 2007), carbon electrodes (Jaramillo et al. 2010), light-induced electrodes (Hoeb et al. 2007), etc.

DEP has proved very effective in manipulating cells. In this light, use of DEP using pin and plate electrodes for separating dead and live yeast cells (Pohl and Hawk 1966) has been observed in great detail and it has further been shown that live cells get concentrated on the pin electrode while dead cells remain in the

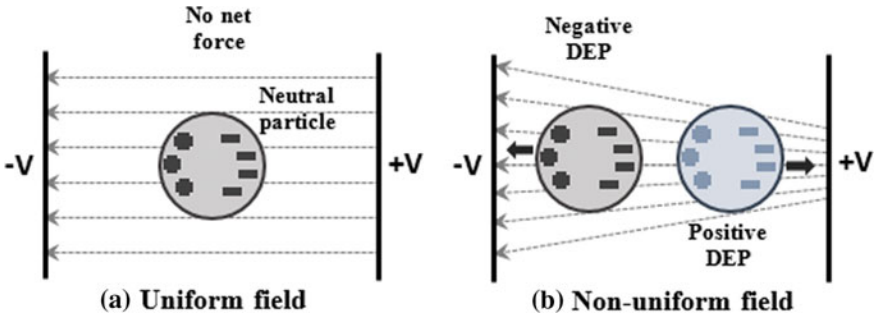

Fig. 1 Behaviour of particle under **a** uniform field; **b** non-uniform field: concept of DEP

solution. Manipulation of bacteria (*Bacillus globigii spores* and *Erwinia herbicola* bacteria) for concentrating and purifying samples (Miles et al. 1999) reduces the level of cross-contamination among samples that may otherwise induce through manual handling processes particularly within microfluidic chips. As compared to electrode-based DEP, electrodeless DEP also utilizes a relatively higher voltage to show capture/manipulation as two electrodes are placed at two extreme ends having symmetric/asymmetric insulator array in between. DEP-based manipulation/separation of cells and polystyrene beads has also been carried out (Saucedo-Espinosa et al. 2016) using insulating posts. The research has also elaborated how surface topology plays a significant role in the process of bacteria (*Escherichia coli*) capture on printed circuit board (PCB) with non-uniform FR4 zone in interelectrode gap (IEG) of copper interdigitated electrodes (IDEs), and it is shown that as compared to the planar surface, rough surface enhances capture efficiency as it adds advantage of both metal electrode-based DEP and insulating DEP (Bhatt et al. 2017).

DEP has also proven its worth in processing direct DNA apart from cells and bacteria. Even though DNA carries overall negative charge and possesses permanent dipole, it is observed that the DNA gets polarized by getting rotated around the major axis (Takashima 1966). DNA polarization is also specific to frequency range and is majorly dependent on the size of the strand. DNA has been shown to be rapidly concentrated over IDEs (Miles et al. 1999). While increasing the degree of miniaturization among the tool providing the non-homogenous electric fields, the input voltage requirement reduces substantially as a very high value of electric field can be achieved through a smaller voltage operating over electrodes spaced through smaller distances. This advantage has been used well while performing DEP capture on carbon nanotube electrodes (Tuukkanen et al. 2006). It has also been observed that carbon nanotube-based platforms are more effective than the lithographically prepared metallic electrodes with nanometric length scales. DNA with very low overall size has been successfully captured over carbon nanotube electrodes. Microarrays of platinum electrodes have been used for separating DNA and delivering drug particles with nanometric dimensions (Sonnenberg et al. 2012) from

blood cells in whole blood samples. It has been observed that both DNA molecules and nanoparticles undergo positive DEP and hence get accumulated in high electric fields while blood cells undergo negative DEP and get collected in the low field zones. A simple wash step introduced leads to the collection of DNA and nanoparticles in the above work from blood cells. In yet another work the DEP capture of DNA copied through a PCR process and labelled with microbeads is observed on castle-walled interdigitated microelectrodes (Kasahara et al. 2015). This paper has also studied well the concept of crossover frequency (frequency over which DEP behaviour changes) in manipulating single DNA molecules, and it is observed that positive DEP (capture in IEG) occurs below crossover frequencies while negative DEP (capture at electrodes) occurs above crossover frequency. The crossover frequency is further seen depending on the length of DNA molecules.

Various other microstructures are also used for capturing DNA. One of them is a nanostructured tip (Yeo et al. 2009). It was observed in this work that initially the DNA and other biological entities were attracted towards the nanotip with DEP and subsequently a size-specific capture was facilitated using capillary action to complete the whole specific capture process. Electrodeless traps are also used to trap and hence concentrate single- as well as double-stranded DNA molecules (Chou et al. 2002). It was observed that DEP force increases rapidly with increase in the size of the DNA strand and additionally DNA is seen to respond well in audio frequency range.

2.2 Polymerase Chain Reaction

PCR is DNA copying/amplification technique in which multiplication of DNA takes place in a particular environment. PCR is carried out in solution phase which contains DNA that is copied/multiplied through PCR technique (using a mix which comprises of various deoxy nucleotide triphosphates (dNTPs) like adenine (A), thymine (T), cytosine (C) and guanine (G); buffer solution; Mg^{2+} ions and *Taq* polymerase) and primers. The mix is thermally cycled across three different temperatures so that the target DNA can copied. The various steps of the thermal cycle include a 94–96 °C heating step for a relatively long time (5 min or so) for activating the *Taq* polymerase enzyme followed by a denaturation step (carried out by heating the mix at 95 °C for 30 s), in which double-stranded DNA (dsDNA) gets unzipped into two single-stranded DNA molecules (ssDNA). This is followed by another thermal step better known as annealing where the sample is cooled and maintained at 55 °C for 45 s, where binding of the primer from the solution to the 3' end of the ssDNA takes place. The primer starts binding from alternate ends of both single strands obtained in the last step. Thus, the primers are really complementary to the end sequences of the molecular sequence being identified. The identified sequence is a part of whole DNA molecule and corresponds to defining the molecular address of a biological entity where it is uniquely present, and such a sequence may not be present is another biological entity. In fact, such sequences are

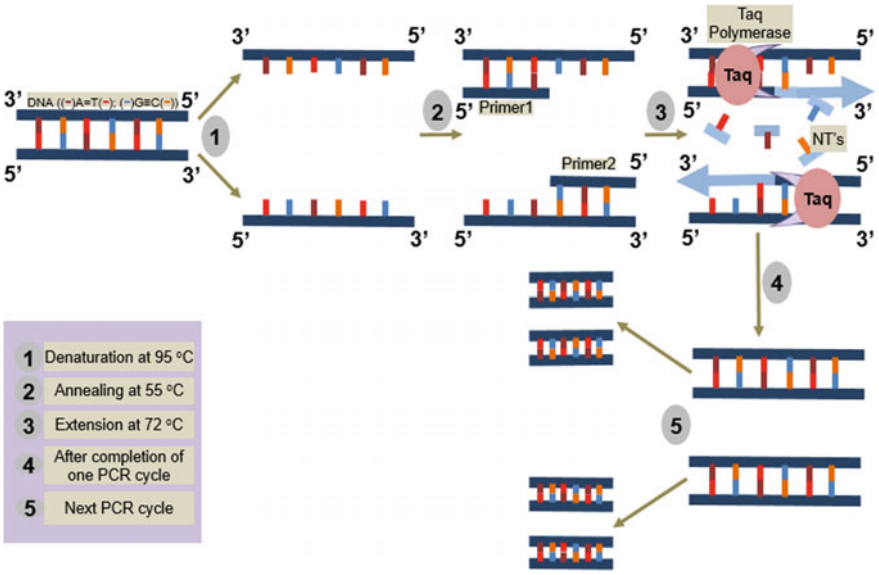

Fig. 2 PCR steps

recognized from the fact that each DNA will be responsible within the host cell to transcribe and translate protein structures and proteins provide functionality and response to a large extent to the living cells and there may a functionality or response associated uniquely with each cell. In this way, PCR continues from 5′ to 3′ direction. Then, the another third step is followed in sequence where the mix is reheated back to 72 °C and held for 90 s corresponding to which an extension in the primer hybridized DNA takes place through action of the initiated *Taq* enzyme. The *Taq* enzyme is highly efficacious and performs the copying action with high accuracy making one mistake in about 10^9 bases that are being sutured to the growing strands of the DNA molecule. The PCR process in this way is a highly stringent process and relatively error-free. Finally, the mix after repeating many thermal cycles as detailed above is cooled at 4 °C for some time and the whole process is evaluated using a separate step of gel electrophoresis. Figure 2 shows the major steps involved in carrying out the thermal cycling related to the PCR process (NT's denotes nucleotides in Fig. 2). The number of copies of the target sequence of the template DNA at the end of number of such thermal cycles is estimated by a product of the initial concentration of the template DNA with 2^n, (where n is the number of cycles). If RNA is subjected to the same process, the RNA template first undergoes a reverse transcription to get the corresponding double-stranded DNA sequence and then the usual thermal cycle is applied to copy the dsDNA obtained from the RNA (Gouvea et al. 1990). As such this process is better known as reverse transcriptase PCR. The RNA/DNA sample of a particular gene can also be extracted directly from the analyte sample for carrying out PCR. The PCR although is a liquid-state reaction carrying out the molecular templating; an copying process is by

itself unable to provide an update on the amplification and thus needs to have a coupled step to generate a transduced signal as the process goes on so that a readable signature generated through the transduced signal can give a fair idea for successfully carrying out the amplification step. The way of looking at the success of the amplification process is to resolve the amplified product using a gelatinous material (process better known as electrophoresis) which may have achieved a certain peak concentration and which may have been cleaved and sized during the PCR process to a unique size as determined by the primer sequences (both forward and reverse primers).

Thus, the normal PCR process is not self-sufficient if operated standalone to perform identification and detection. One way of identifying the sequence DNA is to design a known sequence of a primer assuming that the primer molecules will bind to the target sequence if the target is really present in the sample. So, if we know in advance the sequence that we may have to look for as we are looking for a specific molecular address related to a biological entity (remember each entity has its own unique molecular address), then we may design the primers in accordance and the success of the amplification process determines that the target was present. The different ways of knowing the success of the amplification process are through electrophoresis, q-PCR (using TAQ man assays, molecular beacons or fluorescent dyes).

For carrying out the entire PCR process, thermal cyclers are commercially available in markets, where amplification is performed over many different wells using multiple samples. Conventional cyclers though high throughput have additional complexity in terms of poor heat transfer and extended ramping up/down times, and therefore, if the same were to be done over a thin film orientation would prove out to be a much rapid alternative. Additionally, the time taken to complete the whole process is high as well as the final confirmation is done through gel electrophoresis at the end only which is a cumbersome task in itself. So keeping all this in mind, the shortcomings are eliminated by producing extremely thin chambers (amounting to a few hundred microns width) and carrying out rapid thermal cycling of a lesser amount of thermal mass having lower thermal inertia. In this case, generally fluorescence-based detection is implemented in PCR for evaluating the progress of PCR in real time and the process is better known as RT-PCR. RT-PCR focuses on studying fluorescence level of PCR sample after every amplification cycle, mainly in case when amplification is done on chip rather than well. Microchips with characteristics of either confining the PCR mix or continuously flowing it have been designed, fabricated (using silicon technology) and tested. Continuous flow systems are particularly fast due to their sufficiency to perform thermal cycling without any ramping up or down of the temperature. Such systems are based on flowing of solution drops over three differentially heated zones maintained at different temperatures and as such the rapidity of oscillations of the droplet over the varied thermal zones determines the rapidity of the amplification process. PCR/RT-PCR is performed on large scale involving miniscule volumes of reagents so that huge reaction data can be collected in real time in step-by-step manner and recorded to maintain the reaction statistics of several samples consistently.

Various in-house manufacturing techniques have been utilized to produce inexpensive microfluidic chips for performing DNA amplification (Bhattacharya et al. 2007). A silicon base with printed platinum heaters is utilized to carry out PCR over an intermediary spin- on glass-coated layer irreversibly bonded to another replicated polydimethylsiloxane (PDMS) chamber with inlet and outlet channels. A top-mounted thermocouple is used to acquire thermal data of the chamber, and an electronic instrumentation developed is utilized to rapid heat/cool the chamber. Figure 3 shows the assembled chip for carrying out PCR. The chip has further shown little or no non-specific adhesion of DNA strands during the PCR process enabling the microchip to perform amplification in very lean template mixtures going up to a few femtograms per microlitres. The PCR products extracted at the end of the thermal cycling process are further run through gel for electrophoresis-based identification of the molecular length of amplified sample [details of electrophoresis given later].

The chip fabrication process is further extended towards developing on chip systems for carrying out capture of bacteria (*Listeria monocytogenes* V7) and subsequent PCR using silicon-glass platform having channels, chamber and electrodes with the whole assay mounted over a printed circuit board (PCB) (Bhattacharya et al. 2008). The PCB is enabled with a set of embedded heaters and the instrumentation is realized by assembling a set of programmable power supplies and digital millimetres which can sense the temperature varying resistivity of a thin platinum film printed over the silicon substrate in the microchip. Both DEP-based pre-concentration of the inflowing microorganisms and the PCR process are performed in the same microchip to achieve a higher detection efficiency/detection limit.

Figure 4 shows the basic schematic of the designed chip. This chip is designed in a way to comprise of two chambers, one for DEP-based diversion and second for carrying out PCR by rapid thermal cycling. This system detects the amplification through a mapping of the products of the PCR by means of fluorescent labels which

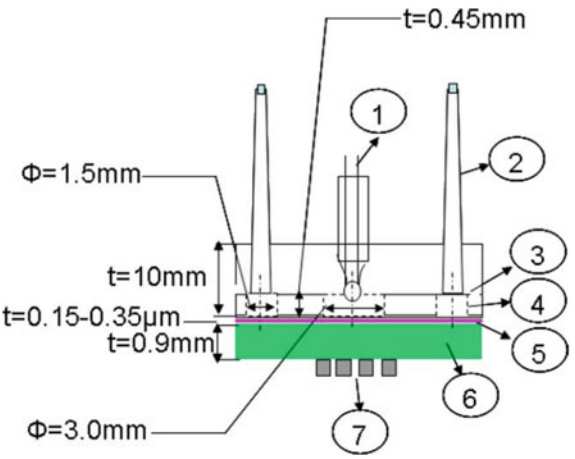

Fig. 3 Schematic of the silicon PDMS cassette. (1) glass-housed thermocouple, (2) epoxied inlet/outlet ports, (3) PDMS channels, (4) inlet/outlet reservoirs, (5) SOG layer, (6) thermally oxidized silicon wafer, (7) heaters. Reprinted with permission from Bhattacharya et al. (2007)

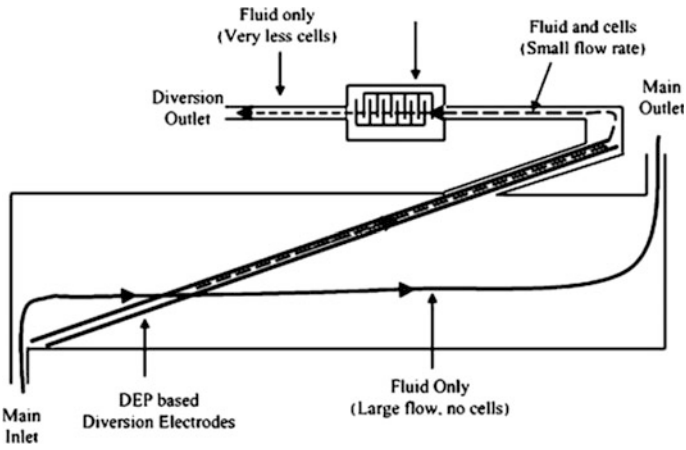

Fig. 4 Principle of operation of the DEP-based diversion and capture of cells. Reprinted with permission from Bhattacharya et al. (2008)

are intercalated into the DNA structure as the amplification is carried out. The dye SYBR green is utilized for this intercalation. The fluorescent signal is investigated through an epi-fluorescence microscope with a solid-state photomultiplier tube (PMT) and through acquired data coming out of the PMT module. Fluorescence increases first linearly due to the diffusional limitations towards the beginning of the thermal cycle and then exponentially as the molecules are able to migrate into every corner of the chamber, thus making the mix homogenous followed by a plateaued growth due to the fall in concentrations of the primer. The low limit of detection is also achieved through simultaneously sorting, concentrating and replicating lean samples of microorganisms (Nayak et al. 2013). Figure 5 presents the plots depicting fluorescence increase during the RT-PCR of normal cells at different number of cycles corresponding to different starting microorganism concentrations in multiple runs in this study. The high sensitivity is further assured through another antibody-based recognition step in a separate work where the DEP is carried out on antibody-/nanoparticle-mediated capture process from the solution phase prior to PCR.

In order to increase the sensitivity of the detection process, DEP and PCR combination is again used for first removing PCR inhibitors and carrying out PCR at higher efficiency (Perch-Nielsen et al. 2003). PCR inhibitors can be removed in various conventional ways like filtration and centrifugation, but with DEP, the main advantage is miniscule manual intervention which helps prevent sample cross-contamination. Separation of haemoglobin and heparin (which act as inhibitors) from whole blood samples has been carried out with DEP to leave only cells behind for replication helping in increasing PCR efficiency. Different methods for controlling thermal cycling in PCR are also proposed. One of them is by using Joule heating effect (Hu et al. 2006). Joule heating effect can be generated in the electro-kinetically driven microfluidic channels by making current to flow through

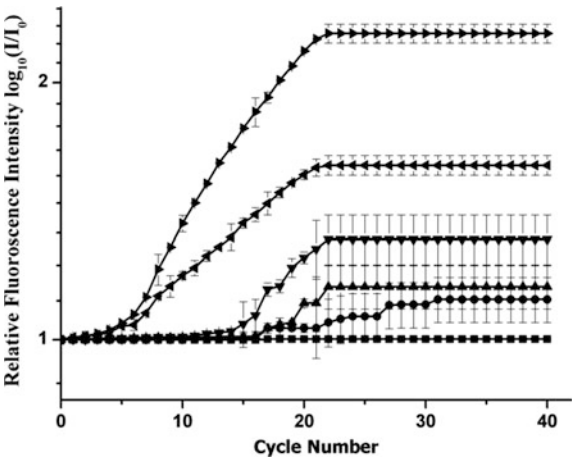

Fig. 5 Plots depicting the trend in fluorescence increase during RT-PCR with time for normal cells black circle—10^2cfu/mL; black diamond suit—10^3 cfu/mL; black up-pointing triangle—10^4 cfu/mL; black down-pointing triangle—10^7 cfu/mL; black right-pointing pointer—10^9 cfu/mL; black square—control. Reprinted with permission from Nayak et al. (2013)

the buffer solution which contains the PCR mix. It removes the need of using external heating component in the system and gets rid of the huge thermal inertia that is being offered by the thermal mass of the chip. This type of chip has successfully shown the amplification of DNA fragment from *E. coli* strains. Similar to RT-PCR or q-PCR, there are also some modifications/advancements offered by changing the environment in which the PCR is being carried out or in the terms of how various constituents of PCR are handled, either as that of the normal liquid PCR or in some alternate way. These modifications are the following:

(i) Solid-phase PCR.
(ii) Droplet-based PCR.

(i) Solid-Phase PCR

Solid-phase (SP) PCR is carried out with either primer or DNA immobilized on a substrate surface instead of being in solution state along with the other reactants. Various methods to attach primers through the 5' end on glass substrates are proposed for the SP-PCR technique (Adessi et al. 2000). It has been observed that for glass substrates, the best immobilization method is obtained through 5′-thiol-modified primers attached to amino-silanised glass surfaces. The reaction can be detected through fluorescent tags or radiolabeling. In this case, as primers are attached to the substrate, two types of amplification occur: one which takes place on the interface between the solution and the glass and the other one on the surface. Initially, the primer hybridization takes place at the surface-attached primers and this is followed by the floating DNA getting attached to this surface layer and gets further multiplied and after that requisite phase gets back to the solution once again. Such phenomena are better known as interfacial amplification. Additionally, the strand for which amplification is completed at the immobilized primer end also takes part in the next amplification cycle after being attached to the surface immobilized primers (remember, the PCR process is about hybridization of a

forward and reverse primers). This near surface multiplication is better known as surface amplification. The single point mutation of *Harvey RAS* gene has also been detected through microring-based SP-PCR and isothermal recombinase polymerase multiplication (Shin et al. 2013). In this work, the primers were attached on the surface via amine modification and the detection was carried out through optical wavelength shift in a silicon-fabricated microring resonator. The optical ring resonator is an optical tool where the surface immobilization of molecules can be transduced into a change in wavelength by light–matter interaction while the scanning beam is being confined into a resonator. The sensitivity in this case was recorded to be around 100 times more than conventional PCR. Another variety of SP-PCR, in which the target DNA from the sample is attached to a membrane after lysis of the containing cells, have also shown better results as compared to those of the culture-based method for detection particularly for airborne microorganisms (example in detection of *E. coli* DH1) (Alvarez et al. 1994). In this case, cell lysis and immobilization of lysed DNA on a membrane are done and subsequently the replication is done on the immobilized lysates using PCR.

(ii) **Droplet-based PCR**

Further extending continuous flow PCR concepts, the droplet-based PCR is executed within microchips. In case of on-chip PCR, the cross-contamination problem (through adsorption of sample on the chip surface) can ruin the efficacy of the PCR process. As it may be a good idea to explore multiple sample amplification on the same microchip, the reusability of the same chip can create cross-contamination between alternate runs and may increase error rates. So keeping this fact in mind, a continuous flow system is designed which may take care of cross-contamination. There has also been the design of wash steps as reported by some groups to explore the multiple usability of the same microchip. The continuous droplet-based PCR is designed by keeping the water/oil immiscibility in mind where a water-based PCR solution is inserted in a flowing stream of oil drop by drop and the drops of PCR mix are further circulated across of different zones maintained at different temperatures without touching the microchip surface. This way the amplification takes place in the particular droplet only leaving the microchip surface uncontaminated and clean. Various working conditions for droplet-based PCR are optimized in term of chip design requirement, thermal mass, thermal resistance, and flow rate needed (Mohr et al. 2007). Figure 6 shows basic concept of droplet-based PCR and one proposed design.

It is observed that low thermal mass for carrier fluid is a desirable condition to achieve heating/cooling of the droplet in a minimum time. A proper dilution to have optimum reaction contents is needed and a desirable flow rate is needed to be maintained uniformly to attain good results. Droplet-based PCR with oscillating flows is also studied (Wang et al. 2005). A silicon-based microchip manufactured in a way so that it has three zones kept at different temperatures as needed by the PCR process for amplification. Single droplet is flown over these in an oscillatory manner over all three zones. Slab gel electrophoresis methods are used to identify the results in one of the shortest times achieved for amplification of samples. For a

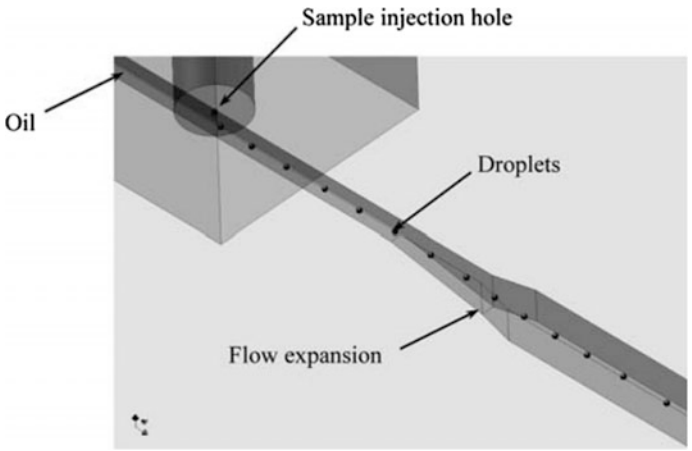

Fig. 6 Concept of droplet-based PCR. Reprinted with permission from Mohr et al. (2007)

similar method, a different technique for transporting the reaction mixture through three different temperature zones is also observed wherein hydrophilic magnetic beads are mixed with the PCR droplet and the movement of droplet is controlled thereby through a magnetic field setup at the bottom of the tray-type reactor (Ohashi et al. 2007).

3 Sensing and Detection Techniques Involved in DNA-Based Biosensors

When analyte becomes detectable in some way, it has to be sensed via multiple strategies. These sensing/detection routes can be varied depending upon the conditions of the system. This section discusses various techniques for carrying out sensing/detection like electrophoresis, EIS, colorimetric sensing, optical sensing, inertial sensing, etc.

3.1 Electrophoresis

Electrophoresis is a detection technique in which particles move under the effect of uniform electric field and the particles are separated on the basis of their respective size. Depending on the field strength, the particles move at some speed towards oppositely charged electrodes, smaller particles travelling at higher speed and vice versa. Electrophoresis can be divided in three categories depending upon the

medium through which particles are passed, and these are gel electrophoresis, surface electrophoresis and capillary electrophoresis.

Gel electrophoresis uses porous gelatinous structure for separating the particles. The gel structure used is agarose (polysaccharide polymer material), with two extreme ends connected to oppositely charged electrodes. The pore size is usually very small that only particles of size of the order of DNA can be passed through this gel; hence, it is mainly used for separating differently sized DNA (Schwartz and Cantor 1984). Electrophoresis is most widely used method for detecting PCR products since long. It works like a confirmation test for PCR. If PCR products contain fluorophore attached to the strands, the total number of molecules present can also be estimated correctly through electrophoresis by just counting. Electrophoresis is also helpful in detecting single base mismatch (Ganguly et al. 1993). Gel electrophoresis is also used for detecting DNA polymorphism (Orita et al. 1989) which is done by digesting DNA through restricting endonucleases and denaturing it in alkaline solution and consecutive electrophoresis.

While separating DNA through gel electrophoresis, size becomes a critical constraint as it is very tough to separate DNA sizes of above than 10 kilo base pair. Hence in such cases, a modified technique for fractionation of DNA is proposed, where DNA travels on the surface rather than through the gel block as in case of gel electrophoresis. This technique works on the similar principle as that of gel electrophoresis. The surface on which DNA travels can be of different types, one being PDMS with channel fabricated on the top through LASER machining (Ghosh et al. 2011). Figure 7 shows the schematic of one such chip fabricated to carry out surface electrophoresis.

In capillary electrophoresis technique, single capillary is used for making DNA to travel from one end to the other. Microchips comprising of capillary have also been fabricated through micromachining process to separate fluorescein and calcein (Harrison et al. 1992) on planar glass substrate.

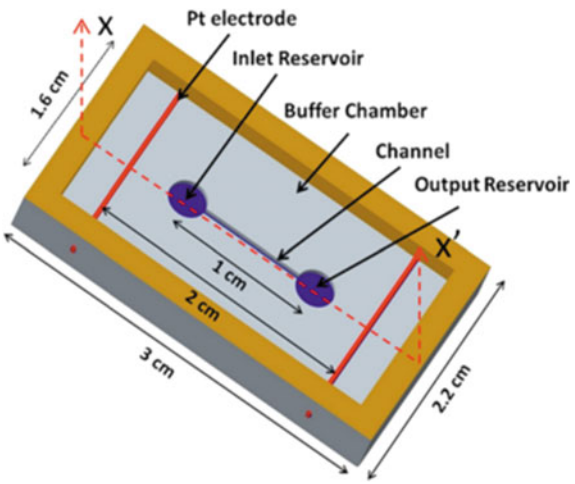

Fig. 7 Schematic of chip to carry out surface electrophoresis (dimensions: reservoirs diameter = 3 mm, width of channel = 400 μm, depth of features = 250 μm). Reprinted with permission from Ghosh et al. (2011)

3.2 Electrochemical Impedance Spectroscopy

EIS is an analysis technique which is used to detect the presence of genes through a change in the overall AC impedance of the medium containing the genes. It has been found that the charge transport phenomena within a solution get significantly altered due to the presence of DNA in this medium. Various DNA lengths further may have different signature impedances. This behaviour can be studied as a electrochemical reaction at the electrode/electrolyte interface, which can be further elaborated using an electrical equivalent circuit (EEC) model, as proposed by Randles (1952). The concept of ion transport in the electrode/electrolyte interface and corresponding equivalent circuit have been illustrated in Fig. 8.

In this figure, the abbreviation IHP expresses inner Helmholtz plane and the abbreviation OHP expresses outer Helmholtz plane. The figure explains the concept of formation of a diffusion layer when the interaction of electrodes occurs while the electrode comes in contact with the electrolyte solution. In this condition, the current level is affected by various factors contributing to the overall ion transport. These components will ultimately lead to the change of impedance. One component is a faradaic component (due to electron transfer across the IHP/OHP interface during the reaction as the electron transfer takes place by crossing over the activation barrier), which determines the polarization resistance, R_p, and solution resistance, R_s. Other component is the non-faradaic part, which comprises of the double-layer capacitor, C_d (due to charging), and gets affected by a change in dielectric constant or thickness of the double layer (Saby et al. 1993). The other non-faradaic part is the Warburg impedance Z_w (due to mass transport of reactant and product across the dual layer).

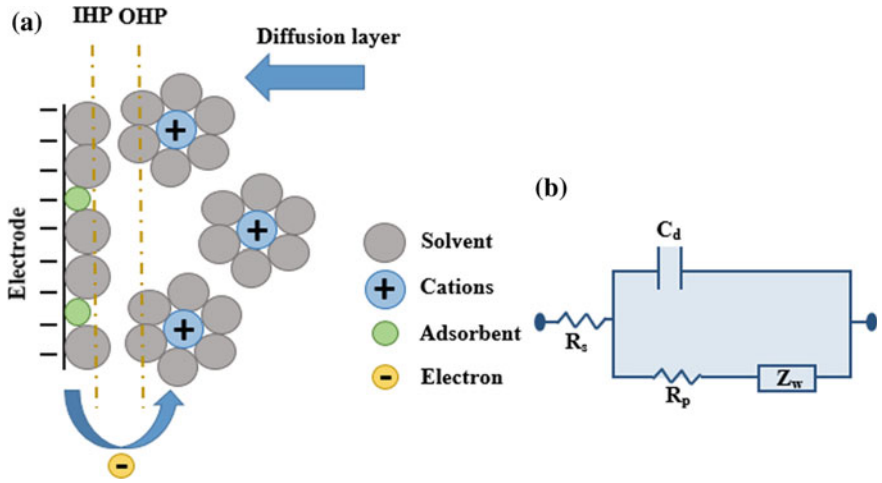

Fig. 8 **a** Interaction of electrode with electrolyte solution. Concept of ion transport. **b** An idealized Randles electrical equivalent circuit for the interface

Identification using EIS technique mainly focuses on identification of molecules with or without the labels attached on the molecule. Measurement techniques have several other aspects, either impedance of molecules can be directly measured while floating in solution or can be done by immobilization on a substrate with appropriate bonding method. In either case, the mechanism is same, but the number of prior steps is different. Various researchers have worked in this field of impedance measurement of molecules immobilised on the substrate surface. Various antibodies, antigens, DNA/proteins, and heavy metal ions are detected by immobilising them on electrode surfaces modified with self-assembled monolayers (SAM) (Berggren et al. 2001). In this assembly, the capacitive sensing has been used for detecting molecules up to a detection limit of 10^{-15} M. In the same context, various bacteria like *E. coli* and *Salmonella typhimurium* are detected on gold IDEs functionalised by biotinylated polyclonal anti-*E. coli* antibodies to neutrAvidin-coated surfaces of the electrode (Laczka et al. 2008). Among the most significant results in this case, a decrease in capacitance was observed due to a decrease in effective dielectric constant of the interface. Application of impedance spectroscopy for detection of *Listeria innocua/monocytogenes* and *E. coli* cells has been elaborated by some groups (Gómez et al. 2002). Impedance measurements are made with the help of micropatterned platinum IDEs on the crystalline silicon substrates. Significant differences in the signal for live and dead cells for even the small order of range 0–20 (limit of detection limit: 1–10 cells/nL) have been observed. Two kinds of suspension media have been used, one where there is lesser ionic content (Tris-Gly-Dext) and the other with high ionic content (LB broth). Significant differences in the impedance have been observed in case of *L. innocua* and *E. coli* suspended in Tris-Gly-Dext for number of cells of the order of 100. No difference in sensitivity has been observed in the two suspension mediums, but LB broth was found to be the preferred medium in two as it did not stress the cells. Impedance curves obtained were examined to show different behaviour for the live and dead cells in this work.

Interfacial interactions between immobilized DNA probes and DNA-specific sequence binding drugs using impedance measurement techniques based on the charge transfer kinetics of the label $[Fe(CN)_6]^{3-/4-}$ were studied (Li et al. 2005). In this both the immobilization of DNA and the DNA drug interaction on the surface of an electrode altered the overall capacitance and interfacial electron resistance and thus removed charge transfer kinetics by preventing redox species from approaching the electrode. A gold nanoparticle-deposited surface has shown higher sensitivity, higher detection limit and thus higher impedance increase after hybridization as compared to pure gold substrates. Sensing and analysis of Tay–Sachs mutants is studied using impedance spectroscopy in sensing and analysis (Bardea et al. 1999) using gold electrodes used for immobilization. The assembly comprised of three components. Initially, a strand of oligonucleotide (with thiophosphate tags) with some part complementary to Tay-Sachs mutant is immobilized in which Tay–Sachs mutant is hybridized, which is further immobilized with another biotinylated oligonucleotide. Finally, avidin is immobilized on this assembly. Nyquist plot showed that the impedance value increases with each

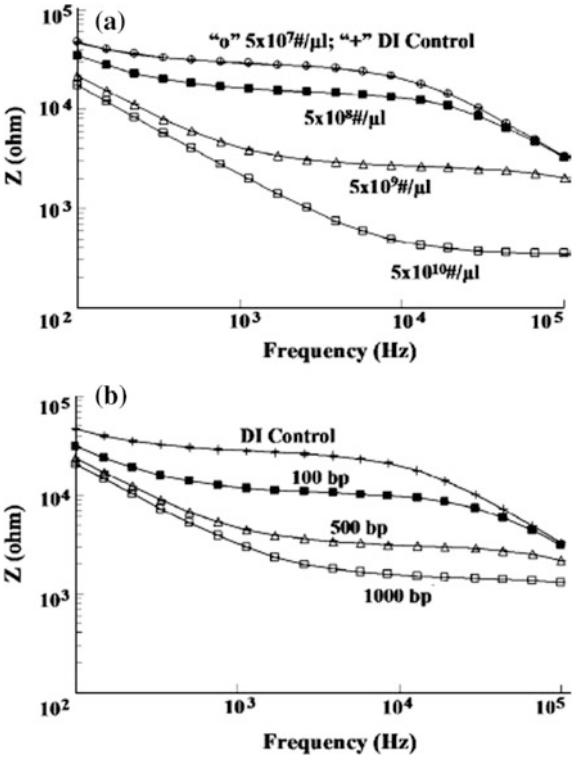

Fig. 9 Impedance magnitude as a function of frequency for a variation in concentration of 400 bp dsDNA molecule and b variation in size of dsDNA where concentration of each molecule was 10^9 molecules/µL. Reprinted with permission from Liu et al. (2008)

immobilization step and reaches a maximum with the addition of the avidin. This identification is specific as no impedance increase was seen with any other combination of the attaching molecules. The study dealing with change in impedance in fluids to examine the effect of length and concentration of free floating dsDNA molecules (Liu et al. 2008) has also been done. Figure 9 depicts the effect of variation of concentration and number of base pair on impedance change.

DNA floating in the fluid generates electrical response under the effect of applied electrical fields due to formation and relaxation of the induced dipole moment. The impedance magnitude was found to decrease as the concentration of dsDNA was increased. Additionally, the impedance magnitude was found to decrease as the length of dsDNA molecule was increased. DNA-based detection using PCR/DEP sensed by impedance measurement has also been extensively carried out (Nakano et al. 2014). In this, the PCR product was immobilized on microbeads and these microbeads were captured on the IDEs, which was sensed by a change in the overall impedance of the system. Microbead-tagged DNA strands have shown positive DEP pattern, which was negative DEP in case of only microbeads. Impedance-based system has shown good sensitivity in a manner to differentiate between various concentrations.

3.3 Colorimetric Sensing

Colorimetric sensing is the most common type of sensing technique in which detection is done through a colour change as a result of change of wavelength of the DNA containing solution. Colour change can be either through direct adhesion or some kind of hybridization. The colour-based detection generally deals with interaction of nanoparticles with DNA in some form.

Colorimetric detection is also observed using hybridization chain reaction (HCR). Two hairpin auxiliary ssDNA probes as immobilized on gold nanoparticles let the gold nanoparticle solutions remain stabilized in a manner so that they do not form aggregates as the salt concentration is increased. But as soon as the target DNA hybridizes with the auxiliary hair pin auxiliary probe, the gold nanoparticles start to aggregate under the effect of similar salt concentrations changing the colour from red to purple because of size effect (Liu et al. 2013). Figure 10a shows a change in colour when hybridization was carried out. It shows how colour varies with different concentrations of (0–6.0 nM) target DNA. Figure 10b shows that with an increase in the DNA constituent in the gold solution the absorbance decreases with an increase in the concentration.

Sensitive DNA detection is also carried out using gold nanoparticles along with asymmetric PCR (Deng et al. 2012). In this technique, selective hybridization takes place on the DNA attached to gold nanoparticles, when correct target DNA is put in

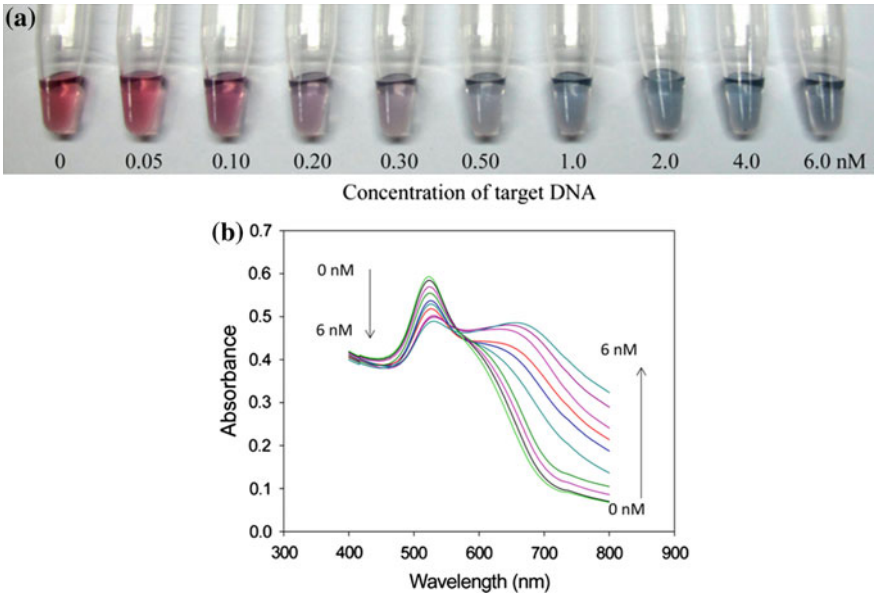

Fig. 10 Photograph showing **a** colorimetric responses of detection system in the presence of various concentrations of target DNA; **b** corresponding absorbance plot. Reprinted with permission from Liu et al. (2013)

the solution, which further aggregates the gold solution and changes its colour from ruby red to purplish blue in the presence of the salt. This aggregation is usually highly specific in nature. Some internally modified DNA sequences incorporated with strands of dATP and dCTP nucleotides using standard NIAK-translation protocol with labelled biotin are also used to test the target DNA (Gebeyehu et al. 1981). It was seen to get detected by streptavidin and calf intestinal alkaline phosphate conjugates in Southern blot of mammalian DNA. Standard colour scheme has been devised to detect the solution concentration in different aspects. DNA-gold nanoparticle conjugated systems can also be used further to detect various ions like mercury (Lee et al. 2007). This is a temperature controlled process and additionally it's an enzyme free process. Depending upon the concentration of the Hg^{2+} ions, the colour change was observed. The Hg^{2+} ions are directly immobilized on the DNA-gold nanoparticle assembly, which is an easy and affordable route of detection. This technique of detecting ions on DNA-gold nanoparticle hybrids can also be used for other ions like silver. These all protocols deal with functionalising gold nanoparticle with some probe DNA in a manner so as to detect the target DNA. Single-strand probe DNA, unmodified gold nanoparticle and water soluble positively charged conjugated polyelectrolyte combinations have been generalized to produce a universal biosensor for detecting various targets like proteins, DNA and small molecules like ions. (Xia et al. 2010). This universal sensor is based on the mechanism of conjugated polyelectrolyte to prevent ability of ssDNA to avoid gold nanoparticle aggregation which is not the case in case of dsDNA. These modification-based techniques have been further simplified by removing the need of immobilizing probe DNA on the nanoparticle surface. Rather direct adhesion of target ssDNA or dsDNA on the gold nanoparticle through electrostatic means has also shown different colour change in the solution showing aggregation (Li and Rothberg 2004). This colour-based detection has shown temperature dependence. Hybridization conditions are different for different type of strands which are to be optimized separately.

3.4 Optical Sensing

Optical sensing needs sophisticated instruments because of the accuracy needs in recording optical data. Therefore the instrumentation deployed should be critically calibrated for better results. In case of optical sensing, the preliminary sensing mechanism is a change in optical properties due to analyte/analyte interaction in the presence of a biocatalyst enabling the sensing to be carried out (Bhattacharya et al. 2007). These optical properties can be changes in fluorescence, reflectance (wavelength/intensity), UV-Vis absorption, chemiluminescence and so on.

Change in fluorescence is a key element for carrying out detection in optical sensing. For sensing of DEP captured molecules, the major sensing technique still remains fluorescence-based sensing. DNA/cells are stained with fluorescent dyes like Acridine Orange which is excited through a specific wavelength (≈502 nm).

And while doing PCR the SYBR green dye is used which shares a similar visibility condition. Various authors have worked with fluorescence-based detection for characterizing the DEP capture process (Saucedo-Espinosa et al. 2016; Bhatt et al. 2017; Tuukkanen et al. 2006; Sonnenberg et al. 2012) and PCR (Kasahara et al. 2015; Bhattacharya et al. 2008; Nayak et al. 2013; Perch-Nielsen et al. 2003). Figure 5 shows the fluorescence intensity increase corresponding to a RT-PCR process with respect to carrying out of the different cycles. It is observed that initially the fluorescence increase is linear and then exponential followed by plateauing for reasons mentioned earlier.

TaqMan probes are also used for rapidly detecting PCR via fluorescence route as TaqMan probe consists of covalently bonded fluorophore at 5′ end and quencher at 3′ end of primer and produces fluorescence at the annealing step in PCR. Quencher restricts fluorophore from producing fluorescence when is present in proximity but during annealing *Taq* polymerase breaks bond between fluorophore and primer and thus gets released to produce fluorescence in a manner to detect the correct bonding. TaqMan assay helps in detection of viruses like West Nile from clinical samples (Lanciotti et al. 2000). Apart from TaqMan probe, molecular beacons also help in detecting PCR product through fluorescence route, like *Adinovirus* (Poddar 1999) amplification has been studied through this route. Apart from fluorescence-based detection, other optical sensing techniques are also devised. SP-PCR techniques based on microring-based silicon devices combining recombinase polymerase amplification has been devised to identify single point mutation of *Harvey RAS*. In this the identification has been done through measurement of optical wavelength shifting in a microring resonator (Shin et al. 2013). Figure 11 shows the shift in resonant wavelength for amplification as compared to the control samples. It can be clearly observed from the figure that the resonant frequency shift is more prominent as time progresses.

Nanopore-optofluidic chip has been characterized for detection of ssDNA using patch clamp technology (Liu et al. 2015). Optical signals generated while passing

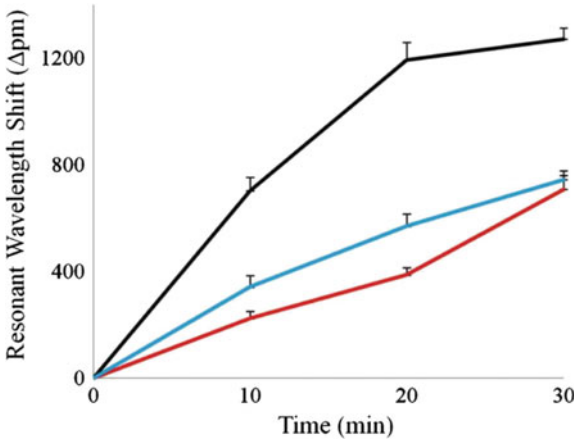

Fig. 11 Resonance wavelength shift shows the results of the amplification of HRAS gene containing 5 ng/μl of human genomic DNA (black) in the ISAD device. Negative controls; (I) distilled water (red), (II) rat genomic DNA from INS-1 cells (light blue) instead of human genomic DNA. Reprinted with permission from Shin et al. (2013)

DNA through nanopores have been further verified by electrical measurement taken consequently. Fluorescent intensity changes for DNA at different positions is plotted and is compared with the corresponding electrical response.

Optical fibres are also heavily used for detecting DNA. These fibres are used as optofluidic devices in which DNA detection is carried out by measuring the shift in reflected Infrared light wavelength (Bertucci et al. 2015). In this the optical fibre containing microchannel and Bragg grating additionally functionalized with peptide nucleic acid (PNA) specific to the gene being detected is used for DNA detection.

3.5 *Inertial Sensing*

Inertial or mass-based sensing is a mechanical sensing platform. As mechanical loads are applied on thin overhanging structures there is a deflection; the magnitude of which can be used to quantitate the force applied. A similar concept is followed while performing inertial sensing. The hanging structures could be vertically growing nano/microstructures or cantilevers. The size scale of micro or nano is particularly explored to perform high sensitivity detection of miniscule targets particularly as the inertial loads of such structures are also miniscule and does not create noise while sensing of deflection is carried out. As some force/load is applied on the cantilever, it deflects and the limit of deflection can be quantitated considering the mathematical formulation as below. The cantilever deflection, □ can approximated using the Eq. 2 (Sader 2001):

$$\delta \cong \frac{3(1-v)L^2}{Et^2}(\Delta\sigma_1 - \Delta\sigma_2) \tag{2}$$

where E is the Young's modulus; v is the Poisson's ratio; t is the thickness; L is the length of beam and $(\Delta\sigma_1 - \Delta\sigma_2)$ is the differential surface stress. Hence while working with the cantilevers, either defection or the resonant frequency with which cantilever vibrates becomes measure for detection. Since Atomic Force Microscopy (AFM) (Binnig and Quate 1986) has been in market, cantilevers have evolved as one of the major biosensors. As in AFM, a tip is used to indent the sample and corresponding force between tip and sample is measured by measuring deflection in tip or change in frequency at which tip vibrates. Hence the frequency at which cantilever vibrates completely depends on the mass/load/force which is exerted on the cantilever. Shift of cantilever resonant frequency (measurements are carried out in a dynamic mode (vibrating cantilevers) is considered in many ways. A specific DNA sequence can be detected by hybridizing it on the capture DNA which is further immobilized by the probe DNA immobilized on the gold nanoparticle (Su et al. 2003). Binding event tends to change the resonating frequency of cantilever and this detection strategy can detect very low concentrations as weight of cantilevers goes down substantially. This platform also provides facility of detecting various DNA by immobilizing various capture DNA on the cantilevers and

comparing the resonating frequency of individual cantilevers. Microcantilever-based optical detection has been devised for detecting DNA mismatch (Hansen et al. 2001). For this purpose, a gold coated Silicon-based cantilever immobilised with thiolated probes are used with no other external labelling. This assembly further exposed to the target DNA resulted in the deflection. Hybridization of one or two mismatch nucleotides produced net negative deflection while correct matching of approximately 10 nucleotide produced net positive deflection providing a base pair wise comparison of individual mismatches while conducting hybridization. Figure 12 shows the design of triangular micromechanical cantilever and corresponding deflection of cantilever when one or two mismatch or 9 or 10 correct matches occurred while carrying out hybridization. The net negative and positive deflection caused is depicted in the figure.

Polymer-based cantilevers have been also heavily utilized for the detection of DNA. Standard spin coating and UV exposure technique is used for fabricating SU8 (photosensitive polymer)-based cantilevers (Calleja et al. 2005). As Young's modulus of SU8 is 40 times lower than that of Silicon, hence sensitivity enhancement by a factor of six is achieved in case of SU8-based cantilevers. These cantilevers have shown a noticeable response for adsorption of ssDNA and subsequent interstitial adsorption of spacer particle.

As a concluding remark, a comparison table (Table 1) is devised to compare various sensing/detection techniques, their advantages/disadvantages and limit of detection in the available literature (limit of detection related to *E. coli* detection).

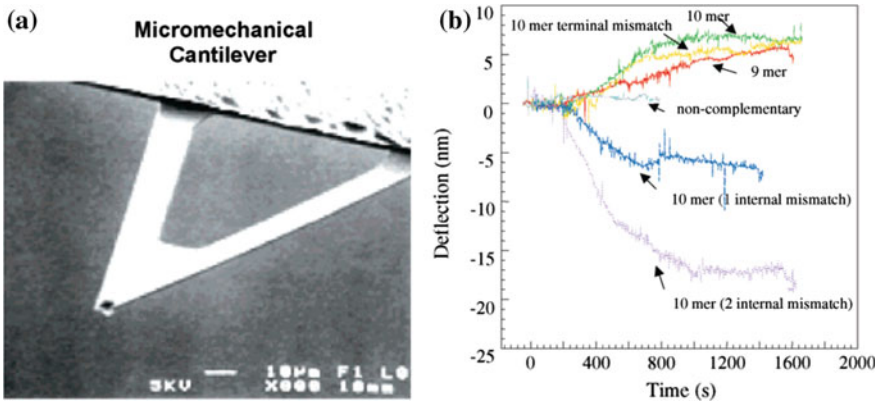

Fig. 12 **a** Structure of micromechanical cantilever; **b** deflection produced through hybridization. Reprinted with permission from Hansen et al. (2001)

Table 1 Comparison table showing limit of detection, advantages, and disadvantages of various sensing/detection techniques

S. No.	Sensing/detection technique	Limit of detection (cfu/ml)	Advantages	Disadvantages
1	Electrophoresis	1000	• Lesser cost involved	• Time-consuming process
2	Electrochemical impedance Spectroscopy	39 Dastider et al. (2016)	• Easy detection • Real-time sensing • Single- or multiple-analyte detection possible • Portable instrumentation • Lesser cost involved	• Low sensitivity
3	Colorimetric sensing	5 Hossain et al. (2012)	• Simple/rapid detection • Portable chips • Cost effective	• Limited quantification detection (only yes/no type, not real time)
4	Optical sensing	5 Fu et al. (2005)	• Real-time sensing • Higher sensitivity	• Most expensive technology • Sophisticated instrumentation required • Instrument is not portable
5	Inertial sensing	100 Leahy and Lai (2017)		• Complicated fabrication procedure • Instrument is not portable

4 Conclusions

The chapter therefore summarizes all DNA detection techniques as utilized for a sensitive DNA diagnostics. A comparison table has been provided to briefly identify the limit of detection, advantages and disadvantages of various sensing methods. The chapter discusses various aspects related to DNA-based diagnostics and applicable area in detail. It also details various principles in DNA detection like DEP, PCR and immobilization and also discusses in detail the various sensing and detection techniques like electrophoresis, impedance-based sensing, colorimetric sensing, optical sensing and inertial sensing. By and large among all diagnostics the DNA-based diagnostics still formulates a vast share of the market and one of the reasons in the sensitivity aspect related to the molecular detection strategies as exist in the DNA-based diagnostic technologies. In fact the DNA-based detection solutions mostly carried out at the molecular levels is still an industry gold standard for diagnostics.

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