9 Microfluidic Diagnostic Systems for the Rapid Detection and Quantification of Pathogens

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Abstract

This article reviews past and current research directed towards developing microfluidic systems that are able to rapidly detect the presence of pathogens and provide additional clinically relevant information (i.e. antibiotic susceptibility, etc.) about them. It is estimated that pathogens are directly responsible for 15 Million deaths worldwide annually. Many of these deaths could be prevented as a result of rapid and/or point-of-care diagno-

sis. Microfluidic systems in use and under development seek to fulfill this significant need.

The technical objective of these systems is to detect and estimate the concentration of pathogens of interest within clinical samples. This can be achieved by the detection and quantification of any of the following: (a) whole pathogen cells; (b) metabolites released or consumed by the pathogen; and (c) proteins / nucleic acid sequences that are specific to the pathogen of interest. The type of pathogen assayed for consequently forms the basis behind the classification of the wide varieties of microfluidic systems encountered within this article. However, irrespective of the actual target assayed for, the microfluidic systems have to overcome twin problems: that of low pathogen concentration and/or the presence of interferents. Hence various strategies, such as culturing, filtration, electro-kinetic separation, magnetic separation, enzyme linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), etc., are used to amplify and/or isolate the target pathogen prior to detection. This challenge is tackled using optical, electrical and mechanical techniques (or a combination of them). In this review, attempts have been made to highlight how the physics at the micro-scale has influenced the design of the separation and detection schemes employed.

9.1 Introduction

9.1.1 Infectious pathogens and their prevalence

Infectious pathogens have posed a considerable threat to global health throughout history. Such pathogens are responsible for the black death (caused by *Yersinia pestis*) of the fourteenth century and the influenza pandemic that occurred between 1918 and 1920, each of which claimed more than 50 million lives [1-3]. More recently, the acquired immunodeficiency syndrome, AIDS (caused by the human immunodeficiency virus, HIV), was first recognized in 1981, and severe acute respiratory syndrome (SARS) emerged in 2003. Additional examples of infectious diseases and their causative agents include hemolytic uremic syndrome and bloody diarrhea (*Escherichia coli* O157:H7), tuberculosis (*Mycobacterium tuberculosis*), anthrax (*Bacillus anthracis*), pneumonia (*Streptococcus pneumoniae* and Avian influenza virus), malaria (Plasmodium), hepatitis (Hepatitis A, B, C, D, and E virus), and hemorrhagic fever (Ebola virus) amongst many others (see Figure 9.1).

More than 50 new, or newly discovered, pathogens have been identified over the past 30 years [1]. In addition to these new discoveries, there exists the constant threat of re-emerging pathogens, which often occur throughout new or extended geographic regions. West Nile fever is one infectious disease that has recently (1999) made a reappearance within a new geographical location, the United States [4]. This disease is the result of a flavivirus transmitted via mosquitoes. Bioterrorism and the growing phenomenon of antimicrobial resistance further compound the difficulties posed by infectious pathogens. One familiar example of a bioterrorist attack took place in 2001 with the mailing of letters filled with anthrax spores that ultimately claimed the lives of 5 people and caused illness in 13 others [6]. The issue of antibiotic resistance is not necessarily new, but, is growing for a variety of reasons, including the inappropriate utilization of antimicrobials [7, 8]. Infectious pathogens that have developed antimicrobial resistant strains over the last several years include pneumococci, enterococci, staphylococci, *Plasmodium falciparum*, and *Mycobacterium tuberculosis* [5].
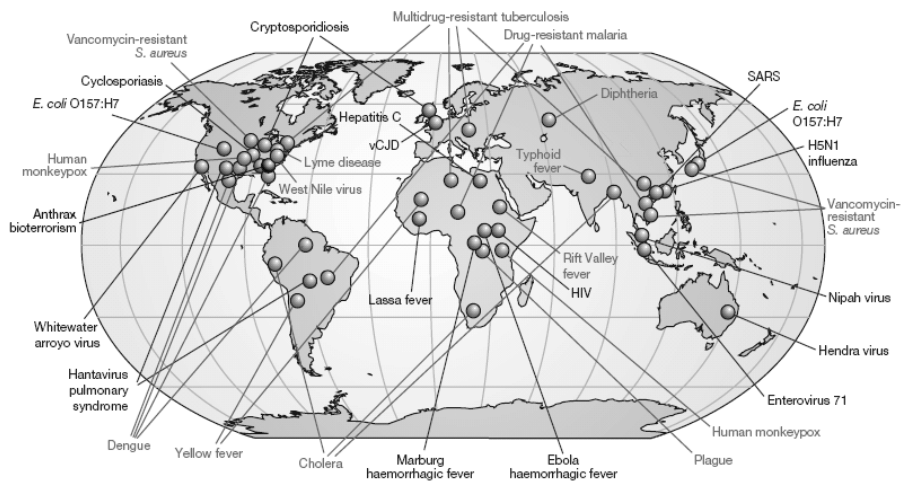

Fig. 9.1 The global prevalence of infectious diseases. Reprinted with permission from Macmillan Publishers Ltd: Nature [2], copyright 2004; and from the University of Chicago Press: Clinical Infectious Diseases [5], copyright 2001.

As can be seen from Figure 9.1, the prevalence of infectious diseases is a global phenomenon. It is estimated that approximately 25% (15 million of 57 million) of annual worldwide deaths are directly attributable to infectious diseases [2]. The toll of these diseases is much greater in the developing world than it is within the developed world [9], with at least half of all deaths being caused by infectious pathogens [10]. However, even within developed countries, microbial pathogens are not a trivial public health issue. An appreciation of their significance can be gained by examining relevant sepsis statistics. Sepsis is a condition that occurs when toxin-producing bacteria, and other pathogens, infect the bloodstream. These pathogenic organisms are often introduced into the blood stream via infected organs, such as the kidneys (upper urinary tract infection) and the lungs (bacterial pneumonia), or during a hospital stay via various routes (e.g. intravenous lines, surgical wounds, surgical drains, and open-skin wounds like bedsores). While not attracting much publicity, sepsis is one of the leading causes of death in the US, with an estimated 750,000 people annually developing severe sepsis and over 215,000 (close to 30%) of those people succumbing to the infection [11]. Within the US, sepsis claims more lives annually than myocardial infraction [12], lung cancer, or breast cancer [13]. Perhaps a greater cause of concern is that, unlike most other leading causes of death, the fatality rate associated with sepsis has not decreased significantly over the last few decades [14]. Prevention and early detection leading to prompt treatment are essential since minor infections can rapidly turn life threatening [15]. Multiple studies have shown that the earlier bacteria can be detected within the bloodstream and the appropriate antibiotic can be delivered, the more successful the treatment is in fighting off the infection [16-18]. Diagnostic systems therefore play an integral role in facilitating an effective response to be provided against these infections.

9.1.2 Traditional pathogen detection methods

The ability to detect infectious pathogens is vital to mounting a quick and effective response to outbreaks of pathogen-caused disease. The clinical microbiologist, nurse, or technician is thus often tasked with answering the question, "Are there pathogens present in my sample (be it a clinical sample, food sample, environmental sample, etc), and if so, what is/are their identity/identities, and in what number are they present?" In general, the answer to the first part (what identities) may vary from just a few (< 5) to hundreds, and the number of each type may vary over several orders of

magnitude (from a few to millions of colony forming units (CFU) per ml of sample).

The traditional, and most general, technique designed to answer this catch-all question, is the plate-culture method [19]. This method was first popularized by Robert Koch in the last quarter of the 19th Century, and is still extensively used today with only minor modifications. The standard procedure is to dilute the available sample serially over a few orders of magnitude and to spread approximately 0.1 ml of each of these serially diluted sub-samples uniformly over the surface of a petridish (usually 3-4 inches in diameter) containing a bed of agar gel. This gel contains nutrients that support the growth of microorganisms. Once the liquid suspension is spread out, bacteria in the (sub)sample adhere to the agar gel. The plates are then kept at a temperature favoring growth (typically 37ºC), and after an incubation period during which bacteria on the agar proliferate, bacterial colonies that can be visually observed are formed (assuming the initial presence of target bacteria). The number of colonies established is then counted. For a limited range of dilutions of the original sample, it is possible to distinguish individual colonies, and these counts are used to establish the concentration of bacteria in the original sample.

The plate-culture technique is relatively simple and provides a snapshot of the number of bacteria in the original sample at the time that it was collected. However, it does have a number of drawbacks. To begin with, the plating technique utilizes a significant amount of material, is tedious, labor intensive, and takes a long time to provide results (depending on the species and the medium, the time required may range from overnight, to days, or to weeks). The process becomes even more labor intensive if one seeks to screen a sample for multiple bacteria. In this case, multiple petridishes are required and selective media (a medium that supports the growth of only a limited class of bacteria, sometimes even just a single species) have to be designed for each of the target pathogens. Given that in many cases it is not possible to design growth media that are selective for all the individual targets, differences in colony morphology have to be relied upon in order to distinguish different targets and obtain an estimate of their concentrations in the sample. Unfortunately, identifying bacteria based on colony morphology requires very specialized training, and the readings obtained are subject to errors in observer judgment. A more subtle drawback is that the blend of nutrients present in the agar gel may not support the growth and colony formation of all the different types of bacteria present in the (sub)sample spread on the plate. It is also possible that different types of bacteria present may grow/proliferate at very different rates on the agar. It

is likely that non-growing or slow-growing bacteria may not be seen, in which case they will not be considered. For many applications, this is a significant limitation. For instance, it is believed that a large fraction of microbes residing in the intestines of humans and other mammals, whose absolute and relative numbers have clinical significance, have never been cultured on plates [20].

An analogous technique used to determine the viral count of samples is to first grow a "lawn" of bacteria/eukaryotic cells on a petridish, and then spread onto it a suitably serially diluted sample suspected to contain virus particles. As the virus infects and subsequently lyses the cells, clear zones or "plaques" are created in the lawn. Each plaque is analogous to individual colonies of bacteria. However, as is often witnessed with bacterial culture, not only do different viruses differ in their ability to form plaques in the cells used to make lawns, but even different strains of the same virus may also exhibit such differences [21].

The inherent limitations of culture-based detection methodologies have resulted in interest directed at, and effort devoted to, developing alternate pathogen detection and quantification methods. The main goals of these developed platforms are to speed-up the detection process and to improve accuracy. These aims have resulted in differing technologies that employ tools such as flow cytometry, standard micro-well plate enzyme-linked immunosorbent assay (ELISA), and nucleic acid based techniques. Most diagnostic devices to date, with a few exceptions, have been designed for use within the developed world with well-equipped laboratories and trained technicians [10]. However, there is now a consumer-driven demand for rapid and point-of-care (POC) diagnostic devices, which culture-based and other commonly employed detection techniques will not be able to meet due to long detection times and a lack of portability. Considering the scarce resources that characterize the developing world, such technologies also will not suffice in these regions. In these regions, a detection device must be portable, robust, rapid, and simple to operate.

9.1.3 Microfluidic techniques

Microfluidic technologies are ideally suited to meet the challenges described for the rapid and POC detection of pathogens. While the goal of portability is perhaps the leading cause behind the development of microfluidic devices, such systems offer many other advantages. These include reduced detection times, reduced biological sample (and reagent) require-

ments, integrated devices that combine sample processing with detection on a single chip, and channel geometries and dimensions that often aid in the isolation and/or detection of the target pathogens.

There exists a large number of microfluidic diagnostic systems that employ different approaches to rapidly establish the presence of the pathogen of interest in a sample of constrained size. In many cases, these techniques also estimate target pathogen concentrations to varying degrees of certainty. In general, these techniques achieve their objective by detecting/measuring any of the following:
(a) whole pathogen(s)
(b) metabolites released or consumed
(c) genetic material or proteins specific to the target pathogen

These differing types of targets provide one way of classifying the large number of microfluidic systems either in the market or under development. The next section of this review, which reports on developments in the recent past, has been divided accordingly.

9.2 Review of Research

9.2.1. Pathogen detection/quantification techniques based on detecting whole cells

In "real world" samples, the concentration of whole bacterial cells is often low, and/or there are a large number of other interfering species present, complicating pathogen detection and quantification. For instance, at the time when patients begin to manifest clear clinical symptoms of sepsis, they often have bacterial loads of only 1-30 CFU (Colony forming Units)/ml, and even for patients who are near death, the load may still be as low as ~1000 CFU/ml [22]. In addition, these low numbers of bacteria need to be detected in the presence of blood cells, of which there are millions per milliliter. Microfluidic diagnostic systems thus have to deal with two distinct, but inter-related problems, increasing the concentration of the target pathogen cells (in absolute terms and/or with respect to that of the other cells) and detecting/quantifying their presence. The easiest way to approach this overall problem is to tackle these two challenges individually. First, isolate the target cells and second, devise a platform for detection and quantification. It is expected that during this isolation process, not

only will the absolute concentration of targets increase, but that interfering species will be discarded as well.

In many cases, especially those in which isolation of the target species from the sample medium is desired, the specificity of antibody-antigen interactions can be leveraged to enhance the separability of the target species from the sample medium. Antibodies are proteins synthesized by the immune system of animals (including humans) as a targeted response to an infection by a foreign body or pathogen (typically referred to as an antigen). The conformation of these proteins is such that they bind to specific proteins on the surfaces of the invading pathogens. Since different strains of pathogens often exhibit different proteins on their surfaces, it is possible to generate antibodies that are strain specific -- such as those developed for *Escherichia coli (E. coli)* O157:H7, a particularly virulent strain of *E. coli* [23]. It is also of importance that only a small part of the antibody molecule actually binds to the target antigen. The other end of the molecule, the terminal end, can be chemically modified and immobilized on to a wide variety of surfaces like glass or silica (silicon wafers with a thin oxide layer or glass) [24], polymers such as acrylates [25], phthalates [26], polyethylene [27], and metals such as gold [28]. As will be discussed later, once target pathogens bind to their corresponding immobilized antibodies, various fluidic, electrokinetic, and other schemes may be used to both quickly isolate them and to rapidly estimate their number.

9.2.1.1. Isolating pathogens

The following methods (culturing, filtration, magnetic, and electrokinetic) are some of the more common techniques utilized to isolate samples prior to attempts at detecting pathogens present within a given sample.

Culturing techniques
If the target pathogen is a viable organism, an increase in its number can be facilitated via reproduction by supplying a balanced set of nutrients. Traditional microbiology research has provided standard growth media formulations for wide-ranging bacteria cultures, making it relatively simple to reproduce a bacterial pathogen. In fact, there are special media formulations that selectively support the growth of particular classes of microbes [29]. One such example is Eosin-Methylene Blue (EMB) agar, which is toxic to gram-positive bacteria allowing the growth of only gram-negative bacteria.

Most bacterial cells are slightly heavier than water and other commonly used aqueous growth media, and so the cells will sediment. During aqueous culture, standard practice is then to keep the bacterial cells suspended in the growth medium using shakers, stirrers or other forms of agitation [30]. This also serves to facilitate the transport of oxygen and nutrients to the surface of the cell, and prevents toxic wastes excreted by the cells from building up near their immediate vicinity. Additionally, the cultures are usually maintained at a constant temperature (typically 37ºC) by housing the growth vessels within incubators. Achieving similar local flow regimes and temperature profiles around proliferating cells maintained within reactors at volumes on the order of micro-liters is a non-trivial task. To resolve these challenges, microfluidic valves, mixers, and heaters have to be incorporated to culture the cells [31-34]. Additionally, sensors to measure and regulate temperature, dissolved oxygen concentration, and pH within the micro-reactor have to be developed and integrated. One such micro-reactor is shown in Figure 9.2.

Additional challenges in micro-reactor development include considerations relating to the volume, scales, and fabrication materials typically encountered in microfluidic systems. For instance, polydimethylsiloxane (PDMS), a material commonly employed in biomicrofluidic systems, is permeable to air. This material property may lead to sample losses due to evaporation [35]. While the rate may be low (micro-liters per hour or lower), the sample losses cannot be neglected when the operation volume is on the order of micro-liters and the reactor is to be maintained for hours.
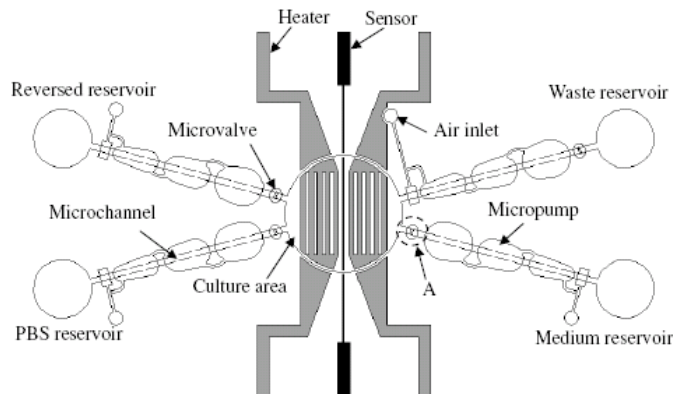
Fig. 9.2 An example of a microfluidic system used for culturing cells. Besides the micro-reactor used to maintain the cells, the system also contains micro pumps, micro check-valves, reservoirs (for growth medium and buffers), feed channels, a micro temperature sensor, and micro heaters. Reprinted with permission from the Institute of Physics Publishing Ltd.: Journal of Micromechanics and Microengineering, [32]. Copyright 2007.

Potential corrective measures available include housing the microfluidic device in a humidity controlled box [36], or specially reformulating the material used to reduce losses due to evaporation [37]. Another issue of concern involves the use of a single micro-reactor for culturing multiple samples, which may be required depending on the cost of the micro-reactor. Under these circumstances, the formation of biofilms can be problematic. Biofilms are formed when bacteria and other microorganisms adhere to solid surfaces in contact with water and secrete a complex polymeric substance that both anchors them to the solid surface and protects them from bacteriocidal substances in the water [38]. Such biofilms will intermittently shed cells housed within them, which can lead to contamination of the samples being cultured. Biofilms are also difficult to get rid of due to the nature of the polymer matrix in which the microbial cells are housed. The challenges associated with biofilm formation are much more acute in microfluidic systems due to the high surface area to volume ratios encountered [39], and consequently using disposable systems is preferred. If for some reason a disposable system cannot be employed, complex bioreactor designs may be required to avoid biofilm formation. One such design involves the continuous transport of small, isolated volumes within a micro-channel loop [40].

As described above, the replication of macro-scale bacterial growth conditions within microfluidic devices is a non-trivial task. Therefore, in designing a diagnostic system that requires pathogen growth through culture, the potential benefits of micro-reactor culture should be weighed against the inherent challenges.

Filtration techniques
Filtration is conceptually a simple method for increasing the concentration of target species. The sample suspension being investigated is allowed to pass through a filter whose pore size is smaller than that of the target, resulting in the target pathogen being retained in the excluded filter cake (retentate). The retentate may then be resuspended in a volume of liquid much smaller ($1/10^{th}$ or even less) than the original sample. At the macroscale, filtration is more commonly employed for applications like envi-

ronmental monitoring and food safety, where large sample volumes (1L or larger) and low bacterial loads (~1 CFU / 100 ml or lower) are commonly encountered. On the other hand, clinical samples are typically much smaller. Given the limited aliquot size that can be introduced into a microfluidic device, the use of filtration at the micro-scale may also be necessitated. For example, consider the case of a liquid clinical sample with a bacterial concentration of 100 CFU/ml. If the aliquot size to be employed within the microfluidic device is 100 μl, then it can be expected that the aliquot will contain 10 CFU on average. However, if the aliquot size is further reduced to 10 μl, then the average colony count within an aliquot will only be 1 CFU, meaning there is a significant chance that the aliquot drawn will contain no bacteria (even though it was present within the original sample suspension). The ability to concentrate the bacteria from the original sample suspension through filtration would thus be beneficial in preventing false negative test results.

Fabricating filters within microfluidic systems poses several challenges, which have been discussed elsewhere [41]. In brief, three types of filters are typically found; arrays of pillars within a micro-channel that are usually arranged in a regular geometric pattern [42, 43], micron-sized "holes" along one of the walls (usually the "floor", which is the wall opposite the fluid inlet within the chamber/channel) of the channel [44, 45], and a micro-channel embedded porous matrix that typically provides a distribution of pore sizes [46-49].

A single filter with a modal pore size smaller than that of the target pathogen will retain the target, which may then be subsequently assayed [50]. However, if there are too many other non-target species larger than the pore size, this may lead to the system quickly fouling, which complicates subsequent target detection within the filter cake. This issue can normally be resolved through the on-chip use of two filters, one with a pore size slightly larger than the target pathogen and the other with a pore size slightly smaller than the target pathogen [51]. In this case, the material trapped between the two filters is likely to contain a higher concentration of target particles. It is still possible that a rapid buildup of filter cake on the upstream filter could occur, however, the use of cross-flow and electrokinetic stirring [52] can be employed to remove the cake.

Magnetic techniques
The use of magnetic techniques to isolate biological samples is a familiar technique on the macro scale [53, 54], and is potentially attractive for use within microfluidic systems for the isolation and manipulation of target

pathogens. Of particular interest is the magnetic susceptibility of a particular cell [55], or of a magnetic particle that can be attached to a particular cell [56-58]. An example of a cell that exhibits magnetic susceptibility is the red blood cell. Deoxygenated red blood cells are the most paramagnetic cell within the body as a result of the free electrons associated with the iron atom contained within the heme ring of hemoglobin. A continuous separation of red blood cells from whole blood has been achieved in a microfluidic system by placing a small ferromagnetic wire along the length of a microchannel and applying a uniform external magnetic field to create a high gradient magnetic field locally [55].

In the majority of cases of interest, the target pathogen will not be naturally magnetic. Consequently magnetic particles with the capability of selectively binding to the target pathogen must be employed, and there exists many examples of their use within the literature [56-59]. Such particles are advantageous for a variety of reasons, including that they offer a considerable surface area for pathogen capture, and that magnetic handling is generally not impacted by changes in pH, temperature, etc. [60]. Magnetic particles are typically composed of an interior magnetic core and an outside shell that is made of substances ranging from polystyrene to silica to polysaccharide. The idea fundamental to the use of magnetic particles is that some form of interaction between the particle and the pathogen can be created. One prominent example relies upon the conjugation of antibodies specific to a given pathogen onto the surface of the magnetic particle. After the introduction of a sample containing the target pathogen, this pathogen will bind to the conjugated capture antibodies while other substances within the sample should remain unattached. This specific technique was employed by Lien et al in an effort to isolate Dengue virus serotype 2 and enterovirus 7 [58]. This isolation employed Dynabeads[®] (Dynal Biotech, Norway), which are hydrophobic superparamagnetic polystyrene beads of approximately 4.5 μm in diameter. After allowing for the target virus pathogen to bind to its respective antibody on the surface of the beads, the beads were then isolated via a magnetic field created using an integrated magnet of microcoils. While holding the beads in place, the remaining suspension could be flushed from the region, allowing for the isolation and concentration of target pathogen.

As described above, the magnetic isolation of a cell or magnetic particle is reliant upon the induction of a magnetic field within the vicinity of the cell or magnetic particle to be trapped. The selection of an appropriate magnet to fulfill this challenge is thus of importance, and in general, either permanent magnets or electromagnets are employed. To date, most work has re-

lied upon supplying a magnetic field from outside the microchannel or microchamber [60]. The development of integrated miniature permanent magnets and electromagnets has progressed and many examples of their use can be found in the literature [59, 61-63]. However, improving the performance of these internal magnets remains an ongoing endeavor. For a detailed discussion of magnetic theory and magnet integration with microfluidic chips, the reader is directed towards several comprehensive reviews that also provide excellent discussions on the implementation of magnetic nano- and microparticles within lab-on-a-chip designs [60, 64, 65].

Electrokinetic techniques

Electrophoresis and dielectrophoresis are the two main available electrokinetic techniques for pathogen isolation. These technologies rely on both the surface charge and the dielectric properties of bacterial cell membranes to isolate the pathogen target from the matrix or suspension in which they are dispersed. Electrophoresis refers to the migration of charged particles suspended in a fluid down the potential gradient of an externally applied electric field. Typically, particles, which are broadly defined here as including both biological cells and macromolecules such as proteins and nucleic acids, migrate with a constant velocity that arises when the viscous drag forces on them equal that of the coulombic forces propelling them down their potential gradients. The velocity of the particle per unit field gradient is referred to as its "electrophoretic mobility". The net charge that the particle carries can be a function of the pH of the solution (most proteins and many cells have a net positive charge at low pH and a net negative charge at high pH), and the pH value at which the particle carries no net charge is referred to as its isoelectric point (pI). Particles with different electrophoretic properties can be separated from each other using various schemes, yielding different techniques such as "field flow fractionation", "zone electrophoresis", "isoelectric focusing", etc. that have been described elsewhere [66, 67]. It has also been known for some time that when suspended in common buffers, bacteria of different species have different electrophoretic mobilities, and that their isoelectric points are also different [68]. These differences have been attributed to the differences in the surface composition (surface charge density) between the different species/strains of bacteria [69]. Such differences in the electrophoretic behavior of cells have been extensively used at the macro-scale to separate various types of biological cells from each other and from non-biological material [70].

Capillary electrophoresis (CE) is a form of electrophoresis where the separands migrate, along with the buffer in which they are suspended,

from one end of a capillary to the other due to electro-osmotic flow produced by an applied direct current (DC) voltage. Due to the electrophoretic velocity of the seperands relative to the buffer, the seperands elute at a different time than when they would have had they flowed along with the buffer. By definition, CE is a "microfluidic" technique. Though traditionally performed within specially constructed capillaries, it can also be performed within channels housed in microfluidic chips [71]. The narrow dimension of the capillary (or micro-channel) plays a key role in the success of the technique by preventing the development of recirculation vortices that tend to arise due to temperature differences generated by the joule heating of the ionic current through the system. The Grashof Number, which gives the ratio of buoyancy forces to viscous forces on a fluid element, and thus predicts the formation of natural convection vortices, scales as the cube of the characteristic length. Consequently, reducing the characteristic length of the system suppresses natural convection that is driven by temperature induced density differences. The high surface area to volume ratio within a microfluidic device also enables relatively rapid heat transfer between the capillary interior and the ambient, thereby further preventing the onset of natural convection.

CE has been used extensively to sort "bioparticles", such as viruses, bacteria, eukaryotic cells, and even sub-cellular organelles. Detailed reviews are available that summarize the results obtained by various researchers in this area [72, 73]. Due to their varied electrophoretic mobilities, different types of bacterial cells elute at different times from the capillary. The species being eluted can be detected using methods such as UV absorbance or laser induced fluorescence [74]. Capillary zone electrophoresis (CZE), which is the most commonly employed form of CE, and is characterized by the constant value maintenance of the pH along the whole separation pathway, has been used to separate significantly differing bioparticle types, such as gram positive and gram negative bacteria [75, 76]. A more sophisticated form of CE, known as capillary isoelectric focusing (CIEF), establishes a pH gradient along the separation path and has been employed in the more difficult separation of bacteria of similar sizes [77].

There are drawbacks, such as lowered throughput, to the use of CE. Another significant disadvantage with CE stems from the fact that extremely high voltages (kilo-Volts) must be used to drive the electro-osmotic flow (EOF) through the capillaries. This high voltage requirement not only necessitates the use of bulky (and dangerous) high voltage supply units, but also produces extensive electrolysis (gas bubbles). Special provisions must then be included within the device to allow for the escape of air [78], while

also compensating for the fact that if air bubbles do enter a micro-channel or capillary, then EOF alone is not sufficient for displacing the bubbles within the conduit [79].

To avoid the drawbacks inherent in using DC methods such as electrophoresis, a method like dielectrophoresis (DEP), that requires the use of alternating current (AC) rather than DC voltages can be employed. In contrast to DC, within AC the polarity of the electrodes changes once every half-cycle of the AC signal. If frequencies higher than ~1 kHz are used, the half-cycle time period is shorter than the charge transfer time at the electrode for most electro-chemical reactions, and consequently most Faradaic reactions, which produce air bubbles, are suppressed [80, 81]. In characterizing DEP, it is convenient to consider it the AC analog of electrophoresis. DEP is a process in which migration, in the presence of an electric field gradient, of suspended particles (not necessarily charged) either towards, or away from regions of high electric field strength occurs. The migration of a particle towards a high-field region is termed positive DEP, while the opposite is known as negative DEP. Whether the particle experiences positive or negative DEP is given by the sign of the Claussius-Mossotti factor, a complex term that depends on the values of the conductivity and dielectric constants (permittivity) of the particle and the suspending medium [82].

At low frequencies (ω<1 KHz), a particle's dielectrophoretic behavior is almost completely dominated by differences in conductivity, while at high frequencies (ω>100 MHz) its DEP behavior is dictated by differences in permittivity. However, for frequencies that can be practically realized, ~10 kHz to 100 MHz (high enough not to generate bubbles, but low enough to not require expensive equipment), DEP behavior depends in a complex manner on both of these quantities. Qualitatively though, the effect of conductivity is more pronounced at lower frequencies and that of permittivity at higher frequencies. In standard buffers, such as phosphate buffered saline (PBS), both the conductivity and the permittivity of bacterial cells are typically lower than that of the medium. Consequently, bacterial cells generally do not display any cross-over from negative to positive DEP (have negative DEP for all frequencies). However, when these cells are placed in low conductivity suspensions (~100 mS/cm), they display positive DEP at lower frequencies and cross-over to negative DEP in the range of 100 KHz to 100 MHz [83]. Since dielectrophoretic phenomena rely on field-induced dielectric polarization at the cell membrane, and the composition and properties of the membrane vary from species to species, the cross-over frequency (the particular frequency at which the DEP behavior changes

from positive to negative) is generally a function of the species and strain of the cell [84]. Thus, there can exist certain frequencies where the DEP behavior of target cells is different from that of other cells (and non cellular impurities in the system), allowing for the isolation of the target cells from complex suspensions containing many other species [85-88]. However, it must be considered that prolonged storage, or other sample handling processes that impact the physical composition of the membrane can change the cross-over frequency of the target cell [89, 90].

Perhaps the main disadvantage associated with DEP is that typical obtainable particle velocities are low. It can be shown that the DEP force on a particle scales quadratically with particle radius and linearly with the applied voltage. This quadratic dependence on particle radius in conjunction with practical limitations on the applied voltage render the particle velocity produced by a DEP force miniscule, typically on the order of 10 μm/sec for bacteria and 1 μm/sec for viruses. Concentration times on the orders of hours are thus required. Also problematic is the fact that the field gradient necessary to drive DEP motion can only be achieved with relatively narrow inter-digitated electrodes, whose field penetration depth is limited by the electrode width. As a result, DEP channels are usually less than 50 microns in transverse dimension. The slow capture and the small transverse dimension produce extremely low throughputs for continuous flow kits. For example, for a 5 cm-long device employing a 50 micron transverse dimension and a 10 micron/s DEP velocity, pathogen capture can only be ensured if the throughput is less than 25 nanoliters per second. At this flowrate, it would require more than 10 hours to process a 1 ml sample. This low throughput and batch volume can be somewhat alleviated with a massively parallel array for laboratory use, but such arrays cannot be easily designed for a miniature and portable diagnostic kit.

Bacteria isolation and concentration by DEP can be sped-up by using fluidic forces (convection) to transport suspended particles (of all types, including the target pathogen) to a specific localized region, and then capturing the targets utilizing DEP forces. This basic idea has been extensively applied in a variety of manners to capture different targets by the corresponding author of this review and collaborators. This can be accomplished by generating converging flows with stagnation points, and imposing the electric field gradient on the particles at the electrode stagnation points (or lines) where the viscous drag is weakest. In such systems, while all suspended particles are swept towards the stagnation point along with the fluid flow, only particles (such as the target pathogen) that are directed by the electric field (due to positive or negative DEP) towards the stagna-

tion points are captured. Converging flows of this nature have been generated by AC EOF at symmetric electrodes [91], by AC EOF generated with serpentine coils [92], by rotational vortices generated using an ionic wind [93], and by a combination of DC bias and circulatory AC EOF [94]. It is possible to generate fluidic velocities of approximately 10 mm/s, which represents a thousand-fold increase in the speed at which target particles move towards the collection zone compared to simple DEP. Once collected at a "point" (a volume of a few pico-liters), the pathogens can then be detected via a variety of detection mechanisms.

The rapid dielectrophoretic isolation and/or concentration of pathogens within continuous flow-through systems has been accomplished utilizing methods such as integrated "3-dimensional" (3D) electrodes. Such designs employ aligned electrode pairs fabricated either along the sidewalls [95] or on the top and bottom [96, 97] of channels through which the particle-laden fluid flows. This 3D configuration produces a high field normal to the flow direction and penetrates across the entire height of the channel. Therefore, bacteria present through the entire depth of the channel (as opposed to just those near the bottom, as is the case for single electrode devices) can be deflected and/or captured while liquid flows unimpeded. Through tailoring the orientation of the 3D electrode, the particles can be directed to different streamlines/exits, or can be prevented from flowing past a point in the channel altogether, creating a DEP force field cage through which particles can not pass [98-101]. DEP fields from 3D electrodes have been exploited to generate a continuous sorting and trapping device, which is shown in Figure 9.3, that is capable of sorting and collecting three different types of pathogens at a rate of ~300 particles/sec [102].
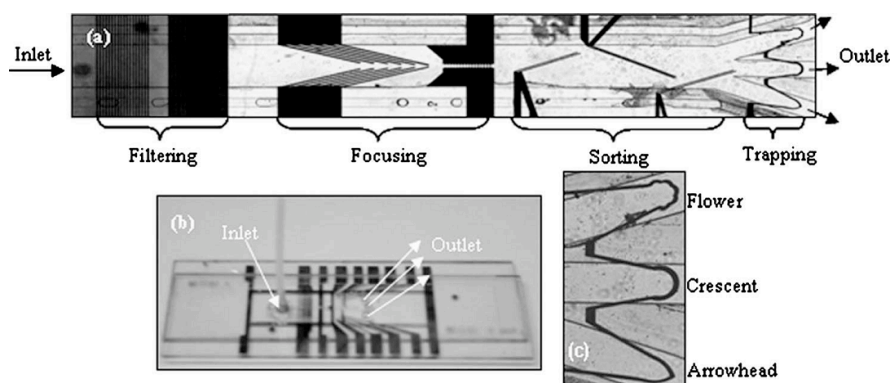
Fig. 9.3 An integrated chip that uses 3-D electrodes to sort pathogens into 3 different classes on the basis of their individual DEP cross-over frequencies. A zoomed in image of the four stages on the integrated chip is provided in (a), while (b) shows that the stages are housed within two glass slides which are detached in this image for clarification. Finally, a schematic for the trapping electrodes that each trap a different respective target particle by their negative DEP mobilities is given in (c). Reprinted with permission from Biomicrofluidics [102], copyright 2007, American Institute of Physics.

The unique physical and electrical properties of nano-particles, such as carbon nano-tubes (CNTs), can also be harnessed to isolate target species dispersed in the sample. Due to their ability to store charge and their long, narrow geometry; very strong dipoles are induced by exposing CNTs to a high frequency AC field. This focused field, especially at the tips of the CNTs, leads to high field gradients, which serve to attract pathogen particles to the CNTs by DEP. CNTs may be embedded onto the "floor" of the micro-channel [103], or may be deliberately dispersed into the pathogen containing suspension [104]. In the latter case, on average the CNTs are closer to the target pathogens. Additionally, at lower AC frequencies, CNTs are themselves attracted to electrodes embedded within the microfluidic device. Their cylindrical geometry confers upon them a much higher DEP mobility than is typically observed for spherical or ellipsoidal pathogens. Consequently, suspension dispersed CNTs will drag along the surface of embedded electrodes as they flow through a micro-channel, thereby bringing trapped pathogens to the electrode surface. This approach, as illustrated in Figure 9.4, has been successfully utilized to enhance the trapping rate of target pathogens by a factor estimated to be over 1000.

9.2.1.2 Estimating pathogen load

Electrical techniques

In an aqueous solution, charge is carried between any two electrodes by ions in the solution. The presence of particulate matter (such as pathogens) physically obstructs the movement of these charge carrying ions, and thereby leads to a higher resistance (or impedance, when using an AC signal) between the electrodes. Typical pathogen loads (~1000 particles/ml) have a volume fraction of ~10^{-12}, and consequently this change in resistance/impedance is not significant. It may, however, be discernable if the suspension is made to pass through a narrow slit, only slightly larger than the pathogens, that possesses electrodes on two opposite ends [105], or if the target pathogens can be made to adhere to or congregate at a surface on

or very near an electrode. Quite often, the surface of interest may be treated to enhance the adhesion of cells [106], or specific antibodies may be immobilized to capture (and hence detect) only the target pathogenic entities [107, 108].
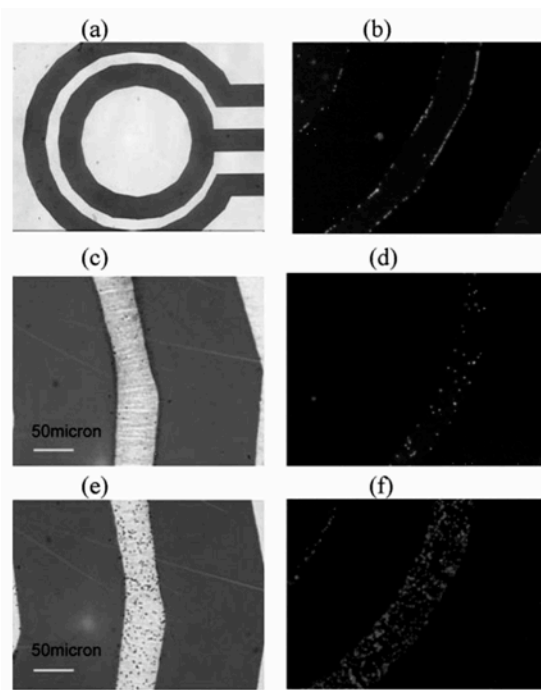

Fig. 9.4 Actual and fluorescent images of fluorescent nano-particles trapped between two electrodes at 1 MHz. (a) and (b) are without CNTs, (c) and (d) are with pre-assembled CNTs, and (e) and (f) are with a dispersed suspension of particles and CNTs [104].

The above mentioned electrical detection techniques suffer from drawbacks that make it difficult for them to handle "real world" samples. The former, passing the suspension through a narrow slit with electrodes on two opposite ends, is plagued by the high likelihood of the slit being clogged by random particulate matters present in field samples. The latter method, forcing the target pathogens to adhere or congregate at a surface on or near an electrode, suffers from two main drawbacks. First, particles other than the target may get deposited or adhere non-specifically to the surfaces being monitored, thereby yielding misleading results. Second, the targets may take a long time to diffuse to the surface, thereby degrading the performance of the system. This latter problem can be overcome by

coupling the detection system to the systems described above for isolating the targets [109]. Another potential approach is to use a specific property of the pathogens that enable them to provide a unique and recognizable signature. One such property that can be exploited is the ability of bacteria to store charge. Direct measurements [110] of the amount of charge in an individual bacterium ($\sim10^{-13}$ Coulombs) and their zeta potential (~20 mV) suggest that an individual bacterium, if considered a solid sphere of radius 1 μm, has an effective dielectric constant of $\sim10,000$. This remarkably high number probably arises due to the presence of proteins (such as those forming the various ion channels and regulators) that are extremely efficient in capturing electrons. In contrast, the dielectric constant of aqueous solutions is typically about 80, and so the presence of even a few bacteria should greatly alter the dielectric constant of the solution. Unfortunately, the "double layer" capacitance at the electrode solution interface effectively screens this bacterial capacitance that is dispersed in the bulk solution, especially when operating at frequencies below 1 MHz [111]. However, by modulating the geometry and positioning of the electrodes in a manner that increases the bulk resistance (R), and hence increases the RC time of the medium, the measured reactance ("imaginary" or "out-of-phase" component of the measured impedance) can be made sensitive to bulk capacitance. Consequently signatures of bacterial presence and proliferation at concentrations low enough (100-1000 CFU/ml) to be applicable to many "real world" problems have been detected [112].

Optical techniques

Optical detection strategies are arguably the most widely employed platforms for microfluidic analyte detection. Such strategies are diverse, and examples of pathogen microfluidic detection based on absorbance [113], chemiluminescence [114], fluorescence [115, 116], light scattering [117], Raman spectroscopy [118], and refractive index techniques [119] can be found in the literature. A comprehensive treatment of these techniques is beyond the scope of this text, and consequently those readers looking for a rigorous discussion are referred to many excellent reviews [120-126].

Each of the detection techniques listed above have specific advantages, however, fluorescence detection schemes are the most widely employed. Such techniques are commonly employed in conjunction with microfluidic immunoassays [127, 128], electrophoresis separation schemes [116, 129], and polymerase chain reaction (PCR) amplification of DNA [130, 131]. Fluorescence is popular due to its high sensitivity, allowing for single molecule detection [132, 133], high selectivity, and easy integration of fluorescent tags within the microfluidic system. The joining of fluores-

cence-based detection platforms with on-chip assay and separation schemes has become prevalent. For instance, Xiang et al. produced an immunoassay device that was capable of detecting *E.coli* O157:H7 at a limit of detection of 0.3 ng/µl [127]. A membrane-based immunoassay system was developed by Floriano et al. that incorporated fluorescent detection of *Bacillus globigii* at a detection limit of as little as 500 spores [128]. Real-time PCR chips that employ fluorescent detection schemes have been developed for the amplification of hepatitis B virus [130] and *E. coli* O157:H7 stx1 [131]. Electrophoretic separation of PCR amplicons and subsequent fluorescent identification has been demonstrated for the detection of *E.coli* O157:H7 and *Salmonella typhimurium* at a starting concentration of as low as 6 copies of target DNA [116].

While fluorescent-based detection schemes are popular within microfluidic diagnostics, the majority of these techniques still rely on relatively bulky external equipment, such as confocal microscopes, preventing them from truly meeting the goals established for an ideal microfluidic diagnostic device. Most commonly, a microscope, connected to either a CCD camera or a photomultiplier tube, is focused onto the microchip at an appropriate location [125]. Efforts to miniaturize optical detection platforms are currently underway, and are discussed in detail elsewhere [120, 126]. Light emitting diodes (LEDs) coupled with optical filters and silicon photodiodes offer the potential for developing a miniaturized optical detection platform [134]. In producing a hand-held device designed for real-time PCR, Higgins et al developed a miniature optical platform employing two LEDs, at wavelengths of 490 nm and 525 nm, along with a miniaturized PMT [135]. This system rapidly and successfully detected *Bacillus anthracis* and *Erwinia herbicola*.

Flow cytometry
Flow cytometry is an integrated technique for counting, examining, and sorting particles suspended in a fluid. There are three key components of a typical flow cytometer, the first being a fluidic mechanism that causes all of the particles in a suspension to line up in a single file as they flow down a channel. The second key component is a set of detectors (such as lasers of different wavelengths) that can probe individual particles, along with the fluid stream, flowing past the detector obtaining information (such as whether a given cell has taken up a particular fluorescent dye) that indicates one or more specific properties of the cell. This information, along with the known velocity of the particle traveling down the channel, can then be taken advantage of by the third key component of the flow cytometer to steer target particles/cells to specific downstream collection cham-

ber(s) while the remaining particles are discarded. Although conventional state-of the-art flow cytometers can measure and subsequently sort particles based on a combination of as many as ten parameters [136] and/or achieve throughputs as high as ~10,000 cells per second [137], they do suffer from a number of drawbacks. Besides requiring expert operators, they also require large volumes of sheath fluid (~1 L of sheath fluid per 1 ml of sample) and high performance pumping systems to operate, thereby making them non-portable and prohibitively expensive for routine diagnostic procedures in the clinical setting.

Extensive research has been conducted to design and fabricate miniature versions of flow cytometers that replace conventional glass capillary based systems with microfluidic chips that employ integrated optics and hydro-dynamic or electrokinetic based flow-switching systems for collecting cells of interest. An excellent review of microfluidic flow cytometry has been provided by Huh et al. [138]. These research efforts, along with others directed towards developing microfluidic pumps, valves and other peripherals [139-141] have already yielded commercial bench-top flow cytometers, such as the Agilent 2100 Bioanalyzer® (Agilent Inc.), the Cyflow® ML (Partec GmbH) and the Mycrocytometer® System (Micronics Inc.). While these systems, like their macroscale counterparts, are primarily designed for mammalian cells, they have also been employed by some researchers to quantify bacteria [142, 143]. This is a difficult task though as bacterial cells are typically 10-100 times smaller than mammalian cells, making them difficult to confine to a single file in a fluid stream, and thus providing unsatisfactory results. Consequently, there have also been efforts to develop cytometers specifically for bacterial cells [143-145].

The main drawback of most of these portable microfluidic cytometers (including those under development) is that they typically handle very small volumes of sample (for instance, 10 μl for the 2100 Bioanalyzer), which may not be suitable for operating a real-world sample. Research to increase the throughput of such devices continues, especially for bacterial diagnostics [146]. As is the case for other microfluidic flow-through systems, the likelihood of particulate contaminants in real word samples clogging the device, or otherwise adversely affecting measurements, remains a challenging problem.

Mechanical techniques (cantilevers)
Cantilevers are beams that are fixed at one end and free at the other. The weight of the beam (when subjected to gravitational forces) and external

forces, if any, flexes the beam. The beam's elasticity in turn attempts to restore it to its original shape, thus leading the system to have a characteristic natural frequency of vibration. This frequency, known as the principle resonant frequency, is a function of the effective mass and the spring constant of the beam. Therefore, when the mass of the beam increases or the spring constant changes due to the adhesion of particles (like bacteria) to it, the vibration of the beam is dampened, and the principle resonant frequency decreases [147]. This effect is utilized to detect the presence of bacteria of interest in many microfluidic systems. Typically, antibodies specific to the target pathogen are immobilized on the cantilever, either by physical adsorption [148] or chemically [149], prior to exposing the system to the sample of interest. Ideally, washing with plain buffer removes all particles adhering non-specifically to the cantilever, and the resonant frequency can subsequently be measured. This is typically accomplished through an external instrument, such as an "optical lever" or interferometer, or through changes in the piezoelectric resistance or capacitance of the beam as measured via internally embedded circuit elements [150]. The change in the resonant frequency from its value prior to the introduction of the sample of interest is related to the amount (mass) of bacteria adhering to the cantilever, thereby also allowing for a quick quantification of the target species as well.

Cantilevers can be fabricated in silicon based systems using either bulk [151] or surface [152] micromachining. These techniques allow the fabrication of multiple cantilevers within a small surface area [153]. Since different cantilevers can be coated with different antibodies [154], the potential for simultaneous detection of multiple pathogens exists. However, coating multiple antibodies on adjacent micro-cantilevers is a complex task, requiring multiple surface treatment steps (one for each type of antibody being coated/immobilized). Furthermore, this process is made more difficult by problems of "stiction" (fluid remaining stuck underneath the cantilever due to surface tension), that require special treatment procedures like freeze-drying [155], "critical point drying" [156], application of laser pulses [157], etc. after each step involving a different solution.

Once these systems are deployed for biosensing, the cantilevers are subjected to multiple stresses that affect their performance. Flow and mixing of the sample solution can give rise to shear forces that bend (and may even break) them. Non-specific adsorption of random species from the sample and electrochemical reactions can change the mass of the cantilever. Moreover, only some of these effects may be corrected for using a reference (control) cantilever in addition to the sensing cantilever. In addition

to sharing the problems associated with non-specific adhesion with most antibody-based systems, having their antibodies anchored to a non-dispersed surface also requires that the targets have to diffuse to the surface (thereby requiring a long time with solutions having low counts of the target species). Thus, while they have been used in the laboratory to detect bacteria suspended at reasonably high concentrations (10^6 / ml and above) in buffers [148, 151] such cantilever based systems may not have the degree of ruggedness that will be required to handle a large number of real world samples.

9.2.2 Pathogen detection/quantification techniques based on detecting metabolites released or consumed

When living pathogens respire, they take in sugars, proteins, and other molecules from the environment and release carbon dioxide, pyruvic acid, and large numbers of other compounds. This changes the properties, like pH, dissolved concentration of gasses (oxygen and carbon-dioxide), and electrical conductivity, of the suspending medium. If an appreciable change is detected in these properties, it is taken to imply that pathogens are present that are causing the observed change. A generic growth medium (such as Tryptic Soy Broth, Luria Broth, or Beef Broth) supports the growth of multiple pathogenic and non-pathogenic microbes. Therefore, the use of such media only allows the determination that there exist some viable cells within the sample of interest. That being said, however; selective media can be designed that supports the growth of a limited number of pathogens, typically being those from the same genus. For instance, the Cornell Modified Growth Medium supports the growth of Mycobacteria (a slow growing class of bacteria whose members include the organism causing tuberculosis in humans) while suppressing the growth of other microorganisms [158]. The elapsed time prior to detection (of a specified change in medium properties) can be used to predict the initial pathogen load. This is fairly intuitive given that the greater the initial number of micro-organisms in the sample, the higher will be the overall rate of metabolism occurring within the sample, and the faster a detectable change in medium properties will occur. The initial load against elapsed time to detection (TTD) calibration for a particular system (device-medium-organism) can be obtained relatively easily. These methods span a variety of techniques, from those based on detecting the activity of a specific enzyme [159], the release of a specific metabolite like carbon dioxide (radio-labeled [160] or otherwise [161]), looking for an increase in the electrical conductivity [162], a change in the pH (typically observable as a color change within

most culturing medium) [163], or finally a change in the oxygen tension of the medium as a whole [164].

A large number of high throughput, but not necessarily "microfluidic" devices rely on this basic principle. These include the Bactec,™ which detects the amount of radio-labeled carbon dioxide released; the ESP® Culture System (TREK Diagnostic Systems), which detects a decrease in oxygen tension; Coli-Check™ swabs, which use Bromocresol Purple as an indicator to measure a decrease in pH due to bacterial metabolism, and the Bactometer™ (Bactomatic Ltd.), Malthus 2000™ (Malthus Instruments Ltd.), and RABIT™ (Don Whitley Scientific Ltd.) systems that each use electrical impedance. These devices are typically designed to handle samples of ~1-10 ml, and are used for a variety of applications, such as environmental water quality testing, food safety, and veterinary and medical diagnostics. As these devices/methods have greatly simplified handling procedures and incorporated many other features (such as automated read-outs) that aid in enabling high-throughout operation, they have acquired broad acceptance in the applied microbiology community. However, the common operating principle, monitoring the sample for effects of microbial metabolism, fundamentally limits how quickly these devices can deliver reliable results. In other words, their "time-to-detection" is still quite high, often being comparable to that of the traditional plating technique. In some cases, such as when the initial bacterial load is very low (~100 CFU/ml or lower), the automated techniques may actually take more time than the traditional plating technique (in which the TTD is independent of bacterial load). This is because the amount of metabolite processed by an individual bacterium is extremely small. Given that the specific oxygen consumption rate for $E.$ $coli$ has been estimated to be ~20 milli-moles of oxygen per hour per gram (dry weight) of bacteria [165], and a typical bacterium has a dry weight of ~10^{-12} grams [166], a fairly active individual $E.$ $coli$ bacterium consumes ~2×10^{-14} moles of oxygen per hour. A typical well-oxygenated sample will have a dissolved oxygen concentration of ~2×10^{-4} M (2×10^{-7} moles in 1 ml of sample). If the sample has a moderately low initial concentration of bacteria to begin with (~1000 CFU/ml), then the hourly consumption of oxygen for 1000 bacteria (~2×10^{-11} moles) represents a 0.01% change in the concentration of dissolved oxygen. Such a change is so small that it is practically undetectable. Given the exponential growth nature of bacteria, a subsequent change in the concentration of the metabolite being monitored of 2-5% in 8-12 hours for $E.$ $coli$ would be expected. At that point, the presence of the original 1000 could be confirmed. However, in this case, the TTD would be comparable to that obtained with the traditional plating technique. Lower initial loads would fur-

ther increase this TTD, and slower metabolizing/growing bacteria would further stretch the TTD. Consider that the detection times for mycobacteria are typically 10-45 days using such automated techniques [164].

Many microfluidic counterparts of these above devices essentially apply the same principles to micro-liter (or smaller) volumes rather than the 1-10ml volumes characteristic of the above devices. Bashir and co-workers have used both impedance [167] and pH [168] as the quantities monitored to register the occurrence of bacterial metabolism. Gas sensors [169] and biosensors for specific chemicals [170] have also been incorporated within microfluidic systems for detecting/monitoring bacterial growth and proliferation. Interestingly, with cells confined to small volumes (on the order of picoliters) and consequently being in close proximity to the electronic sensing elements, it has also been possible to use thermal sensors [171] to record bacterial metabolism. The TTD of the microfluidic versions of the metabolism based detection systems, however, is limited by the same basic principle that stymies their macrofluidic counterparts.

As discussed earlier, if large aliquots need to be sampled due to concerns arising from expectedly low bacterial loads, a pre-concentration step may have to be performed upstream of the reactor. Devices have been developed [86, 172] that employ dielectrophoresis to concentrate bacterial cells from a comparatively larger volume to a smaller one where they are subsequently incubated. In general, as shown in Figure 9.5, there exists an inverse logarithmic relationship between initial bacterial load and time to detection (TTD). This concentration process cuts the TTD by increasing the initial load.
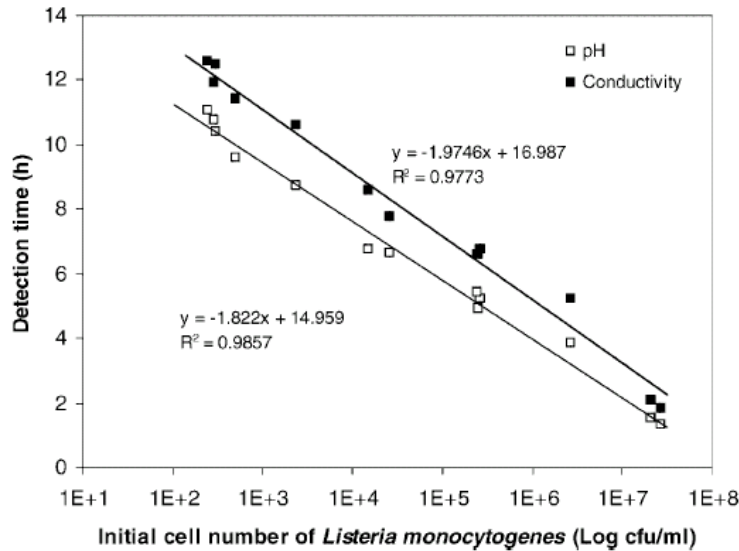
Fig. 9.5 Graph showing the inverse logarithmic relationship between initial pathogen load and time to detection (TTD) for systems that detect the presence of pathogens based on a change in the physical properties of the system (such as conductivity or pH) brought about by the pathogens' metabolism. Reprinted with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.: Biotechnology and Bioengineering, vol. 92, issue 6,copyright 2005, [168].

Despite the benefits provided by pre-concentration, monitoring bacterial metabolism may not be the most sensitive way to detect the presence of pathogens as evidenced by the high TTDs that are typically encountered. However, this detection platform does have a number of other redeeming features, such as the relative ease with which these devices may be fabricated, assembled, and coupled to automated fluid handling systems and electronic readouts. In addition, there exists the advantage of the recognition within the diagnostic community at large of principles involved within these devices that have been previously utilized in pathogen detection strategies. The Association of Official Analytic Chemists International (AOAC) subjects new techniques to two stages of approval: a "first action" that allows its incorporation in commercial devices under supervision, and a "final action" that approves the method for widespread use after the new method has satisfactorily met its benchmarks in the commercial setting. Impedance based methods for detecting bacterial metabolism in microfluidic systems have been accepted as a "first action" method. It would hence not be surprising to see a commercial microfluidic device of this type in the near future.

9.2.3 Pathogen detection/quantification through microfluidic immunoassays and nucleic acid based detection platforms

The following section discusses immunoassays and nucleic acid based assays, which are two vital pathogen detection technologies, along with efforts to create miniaturized counterparts. Immunoassays, protein binding assays utilized for the detection of antigens and antibodies, are most commonly carried out as an ELISA. Nucleic acid based assays utilize genetic material, most commonly DNA (and sometimes RNA), for detection and quantification. The fundamental methods developed for nucleic acid diagnostics are enzymatic DNA restriction, nucleic acid hybridization, PCR, and fluorescence-based techniques [173]. Of these techniques, DNA amplification procedures utilizing PCR and PCR-based methods (real-time PCR, stand displacement amplification, nested PCR, etc) are of particular interest given that DNA is present at a low concentration within a typical biological sample. Consequently genetic identification discussion within this section will focus on microfluidic PCR-based technologies. Several excellent reviews of pathogen identification through diverse nucleic acid based assays can be found [173, 174].

9.2.3.1 Microfluidic immunoassay (ELISA) pathogen identification

Immunoassays are arguably the most heavily utilized quantitative pathogen detection platform [175, 176]. These assays take advantage of the specificity of formation of an antibody-antigen complex, which is a result of the fact that such antibodies are produced based on a specific antigen. Therefore, in use within a diagnostic assay, a given antibody will remain specific to the antigen by which it was developed. As mentioned above, one of the most fundamental immunoassay techniques is the ELISA. There are several different ELISA types, including direct, competitive, and sandwich formats. The sandwich ELISA will likely be one of the primary immunoassay platforms employed on-chip given that it generally exhibits impressive sensitivity and specificity traits, along with favorable kinetics [176]. This assay, as it is traditionally performed, is presented in Figure 9.6 and the associated caption.
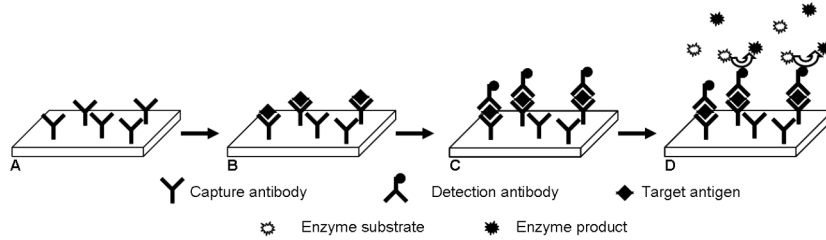
Fig. 9.6 Basic schematic (minus the rinsing and blocking steps) for a traditional sandwich ELISA. In the first step (A), a capture antibody specific to the target antigen is attached to the surface of a microwell plate. This is followed with a rinsing step, a blocking step, and an additional rinsing step. Sample solution, containing target antigen, is added next, allowing for the target antigen to bind to the capture antibody (B). After an additional rinsing step, a detection antibody, which is generally covalently bound to an enzyme, is introduced and allowed to selectively bind to the captured target antigen (C). Finally, after a last rinse, a substrate molecule is added and converted into a product molecule via the bound enzyme (D). This product molecule is utilized as a means of detecting and quantifying the target antigen.

ELISA assays have become commonplace within diagnostics and have been tailored to a wide variety of detection targets. These assays are designed to detect an antigen directly associated with the pathogen or an antigen/antibody produced within a host system as a result of the invasion of the pathogen. Several examples include the detection of *Escherichia coli* (*E. coli*) O157:H7 [113, 177, 178], exotoxins from *Salmonella* and *Clostridium botulinum* [179, 180], and *Helicobacter pylori* [117]. One commercial detection platform based on a sandwich ELISA assay is the VIDAS system marketed by Biomerieux Inc. [178]. This system, which captures bacteria utilizing an antibody specific to an antigen expressed on the surface of the cell, is capable of detecting *campylobacter*, *E. coli* O157:H7, *Listeria spp*, *Listeria monocytogenes*, *Salmonella*, *Shigella*, and *Staphylococcal* enterotoxins [178]. Another commercial use of the ELISA is within HIV detection. Currently, the majority of HIV testing centers within the United States employ an ELISA that targets antibodies produced as a result of the invasion of the virus [181]. The sensitivity and specificity of this test are both greater than 99% [181]. A positive ELISA result for HIV is typically followed up with a repeat ELISA and then a Western Blot test for final validation.

Conventional ELISA assays are typically performed on a multi-well plate, and are static in that transport of the target molecule to the surface-coated molecule is through diffusion. This process is hampered in terms of being

both labor and time intensive, with assay times ranging from at least a few hours to a few days. These drawbacks can often lead to inconsistencies in the obtained assay results and are problematic in producing a rapid diagnostic assay that can be utilized for POC healthcare solutions. The development of microfluidic ELISA platforms will likely relieve these stresses. While microfluidic-based ELISA has not received the same attention that nucleic acid based microfluidic technologies have, the trend is beginning to change [182, 183]. Commercial microfluidic immunoassays are beginning to appear, with one example being the Triage® system developed by Biosite® Inc. (San Diego, CA, USA) to detect several targets, including *Clostridium difficile*. Microfluidic devices are attractive platforms for immunoassays given that they can alleviate the time and labor difficulties of conventional ELISA as transport is through both diffusion and convection, transport distances are greatly reduced, and the assay can largely (if not completely) be automated. Moreover, such devices have the potential to be fabricated and operated inexpensively, and provide the opportunity to integrate the assay with on-chip sample preparation and target detection, providing a micro-Total Analysis System (μ-TAS).

The majority of developed microfluidic-based ELISA platforms have either utilized the surface of a microchannel [113, 176, 184-186] or the surface of numerous micro- or nano-beads that are trapped by various means within a microchannel or a microchamber [187-189]. One of the key issues involved in localizing an immunoassay to the surface of a microchannel wall is the efficient immobilization of antibody (or protein) to the surface while preventing non-specific protein adsorption that would both interfere with the assay and also reduce the available surface area for target protein immobilization. This issue was discussed in a review written by Lee [190] and has been considered in several publications [176, 185, 191]. In developing a microfluidic immunoassay device for the detection of *E. coli* O157:H7, Bai et al. functionalized a poly(methyl metacrylate) (PMMA) surface with poly(ethyleneimine) (PEI) to introduce amine groups to the polymer channel walls [191]. This was followed by glutaraldehyde addition to provide aldehyde groups for protein binding. An affinity purified antibody to *E. coli* O157:H7 cells was then allowed to adsorb to the surface of the channel via the aldehyde groups, followed by the introduction of a sample suspension containing inactivated cells which was allowed to bind to the immobilized antibody. Finally, peroxidase-labeled affinity purified antibody to the cells was added. The peroxidase enzyme catalyzed a substrate to product conversion, producing fluorescence for detection purposes. This surface treatment method improved antibody binding, and compared to untreated PMMA microchannels, an approximately 45 times

greater signal and 3 times greater signal/noise ratio was achieved. Additionally, this microchannel device required only 2 minutes for *E. coli* O157:H7 capture and required a mere 5-8 cells within the initial sample aliquot, both of which are an improvement compared to traditional multi-well plate ELISA.

The use of beads within an immunoassay microfluidic device increases the complexity of the device and requires methods for trapping and handling the beads on-chip [192]. However, the use of beads is becoming more commonplace [187-189, 193] because of inherent advantages, such as an increased available surface area to volume ratio (which in-turn increases the real-estate that can be taken advantage of for protein binding), further reduced antigen-antibody molecular diffusion distance, potential for multiplexing, ease of manipulation, and ease of performing surface chemistry modifications. One specific example of a bead-based microfluidic immunoassay platform was provided by Liu et al. for the detection of a marine iridovirus [187]. In their work, antibody-coated microbeads (~2.89 μm in diameter) were trapped within a microfluidic channel via an included filter assembly. After incubation with the iridovirus, detection was provided through the insertion of quantum dots attached to the detection antibody. In comparison to a traditional ELISA scheme, this approach was able to detect the marine iridovirus at a significantly improved limit of detection (22 ng/ml as opposed to 360 ng/ml), in a shorter time period (<0.5 hours as opposed to >3.25 hours), and with less demand on the availability of antibodies (0.035 μg used as opposed to 0.5 μg).

9.2.3.2 Microfluidic PCR based pathogen identification

The identification of pathogens through genetic analysis techniques is becoming more conventional. Several examples include the detection of *Helicobacter pylori* [194], *Mycobacterium tuberculosis* [195-197], *Neisseria gonnorhoeae* [198], *Streptococci* (Group B) [199], and *E. coli* O157:H7 [200]. The genetic identification of pathogens is dependent upon the presence of sufficient genetic material (i.e. DNA or RNA). However, it is typical that the target pathogen is present at a dilute concentration, complicating detection. To alleviate this difficulty, DNA amplification becomes essential. This is typically achieved through implementation of the PCR, which incorporates three cycled reaction steps; a denaturing step run at 92-96ºC, an annealing step run at about 40-65ºC, and finally an extension step run at about 72ºC.

PCR-based methods within pathogen detection have gained popularity because of several improvements over traditional culturing procedures, namely regarding assay speed and reliability. The same push for improved assay speed and reliability are now driving the development of microfluidic PCR-based pathogen detection technologies, with the ultimate goal being the development of POC diagnostic devices that meet the goals laid out earlier. Several of the advantages offered in miniaturizing the PCR process are similar to those expected with the miniaturization of immunoassays and include a reduction in sample and reagent volumes and a corresponding reduction in reagent costs, increased portability and disposability, and integration with upstream sample preparation and downstream analysis techniques to provide the highly desired μ-TAS. One notable advantage specific to microfluidic-based PCR is a decreased time of amplification as a result of low thermal capacities and large heat transfer rates (because of large surface area to volume ratios) [201, 202]. Though the miniaturization of PCR offers several advantages, the first micro-PCR device was not described until 1993 [203]. Currently, microfluidic chip based PCR can operate with reaction volumes of less than 200 nl [204-208], and can offer reductions in reaction time through improvements in the on-chip heating and cooling rates. A conventional thermal cycler provides heating and cooling rates of approximately 1-2ºC/s, however, a heating rate of 175ºC/s and a cooling rate of 125ºC/s has now been reported within a microfluidic design [209]. Commercial microfluidic based PCR devices have arisen as well, and have been employed in detecting a variety of pathogens. For instance, *Mycobacterium bovis* was detected using the GeneXpert® system produced by Cepheid (Sunnyvale, CA) [210]. Within this system, a filter is employed to capture organisms from a clinical sample, which is then followed by cell lysis. Real-time PCR of the released nucleic acids can then be conducted for pathogen identification. This system is of particular interest as all of these steps, organism isolation, cell lysis, and real-time PCR detection are performed on a single, disposable cartridge. Another example of a commercial microfluidic based PCR device is the BioMark™ 48.48 Dynamic Array developed by the Fluidigm® Corporation (San Francisco, CA). This system is capable of running up to 2304 reactions per chip while only 96 total liquid loading steps are required. The advantageous offered by this microfluidic chip, especially the large number of reactions that can be run in parallel, were beneficial to a study aimed at probing individual bacteria within the lignocellulose-decomposing microbial community that exists within wood-feeding termites [211].

Within the literature, there exist several applications of microfluidic PCR amplification of specific pathogens. Some of these examples include;

Dengue II virus [212], *E. coli* SK [213], *Salmonella typhimurium* [116, 214], severe acute respiratory syndrome (SARS) [215, 216], BK virus [217], *Campylobacter jejuni* [218], Human Papilloma virus (HPV) [219], HIV [220], *Mycobacterium tuberculosis* [221], Influenza viral strain A/LA/1/87 [222], *Neisseria gonnorhoeae* [223], *Bacillus anthracis* [224], Hepatitis B [225], and Hepatitis C [226]. Another example, *E. coli* O157:H7, is a food-born pathogen that has been identified as the culprit in several severe human infection outbreaks [227]. This pathogen is particularly dangerous given that a minimal concentration is required for human infection (as low as 10-100 CFU/g) [228], and that the organism produces many clinical manifestations [229]. It has been estimated that there are 73000 cases and 61 deaths per year within the United States alone that are attributable to this pathogen [230]. Traditional detection protocols, namely identifying the bacteria in stool cultures, can be time-intensive [230, 231], requiring at least a 16 hour culture period [232]. In view of this limitation, Koh et al. have described a microfluidic device created from poly(cyclic olefin) for the amplification of *E. coli* O157:H7 and subsequent on-chip detection through electrophoresis and fluorescence identification of amplicons [116]. The static amplification was carried out within a gel-valve isolated reaction chamber with volumes as small as 29 nL using *E. coli* O157:H7 serotype specific primers. Heating was accomplished using a localized printed ink-based heater that allowed for a heating rate of ~12°C/s. A detection limit of approximately 6 DNA copies present within the initial solution fed to the PCR was determined. Several additional examples of on-chip PCR of *E. coli* O157:H7 can be found within the literature [233, 234].

There are several issues that complicate the rapid, microfluidic genomic detection of pathogenic substances. These concerns include low concentrations of biological targets and a typically complex biological sample matrix. Taken in context, there is thus some balance between minimum sample volume, rapid assay time, and detection sensitivity that must be considered in practical device fabrication. Considering this, Yang et al. amplified a DNA segment on-chip directly from an *E. coli* K12 strain (often referred to as colony PCR) [232]. The polycarbonate device employed a serpentine PCR channel for continuous amplification of volumes as large as 40 μl, and target DNA was provided through thermal lysis of the *E. coli* cells. The amplification was carried out using these cells both within and without the company of 2% sheep blood, and amplification from as few as 10 cells was observed. To block potential PCR inhibitors present within the biological sample matrix, the PCR mixture was supplemented with a specific buffer (Q buffer). Another potential means around this is to isolate

the target genomic DNA after on-chip lysis. This was accomplished after thermal lysis of *E. coli* cells through capture onto probe tagged magnetic particles by Yeung et al. [235]. After DNA isolation, asymmetric PCR was performed to produce amplified single strand DNA that was subsequently detected through electrochemical methods. Other examples of microfluidic amplification of target DNA directly from a biological sample can be found within the literature [211, 217, 218, 236-238].

There remain many key considerations regarding the miniaturization of PCR that must be resolved. Some of these platform design criteria can be seen in comparing the devices described by Koh et al. [116] and Yeung et al. [235]. This includes the PCR format, which can be static with the PCR mixture held within a reaction chamber while the chamber temperature is cycled through the normal temperature ranges, or continuous with the PCR mixture transported through a channel and different regions of the channel held to appropriate temperatures. Each of these designs present different challenges, such as achieving a rapid and precise temperature control and fluid manipulation. Additional considerations include providing for on-chip heating and cooling (typically through either contact or non-contact methods), preventing sample evaporation (which is an increasing problem with a reduction in sample volume), ensuring sufficient sample volume to provide enough starting target DNA, preventing cross-contamination, preventing sample adsorption to surface walls, integrating sample preparation and detection platforms with the PCR on-chip, the material(s) employed for device fabrication, and providing for real-time PCR. As would be expected, the various materials available for construction of microfluidic devices each have different properties that will impact some aspect of the PCR reaction. Thus far, most microfluidic PCR devices have been fabricated utilizing either silicon or glass [116, 202, 239]. Silicon is advantageous for its high thermal conductivity and glass is advantageous because it lends itself well to integration with optical detection schemes. However, the use of these two materials presents specific challenges. For instance, bare silicon is opaque, complicating the integration of optical detection methodologies, and it is known to inhibit the PCR reaction through sample adsorption to the surface wall [239]. As is also the case with silicon chips, glass carries with it a high fabrication cost, preventing the realization of a truly disposable chip. This lack of disposability increases the risk of cross-contamination through multiple uses [226]. A potential means of accounting for cross-contamination involves control of the micro-chamber surfaces. To accomplish this end, Prakash et al. silanized a glass microchamber surface prior to carrying out the PCR reaction [220]. Following the reaction, the silanized surface of the microchamber was removed and a

fresh silanization was carried out. In this manner, cross-contamination was controlled by ensuring sample antigen from a previous amplification did not remain on the wall of the microchamber during future amplification reactions. Another attractive solution to the cross-contamination problem involves the use of certain polymer substrates in the construction of microfluidic chips. Polymers, such as polydimethylsiloxane (PDMS), are relatively inexpensive, potentially allowing for the realization of a truly disposable chip, which would obviously circumvent the problem of cross-contamination. PDMS is also an attractive material as it provides good optical properties and exhibits limited PCR sample adsorption. Consequently there are several examples of the use of PDMS within microfluidic PCR designs [202, 213, 217, 239]. However, the use of PDMS is not without its own difficulties. One such difficulty is that PDMS is permeable, providing the possibility of sample losses. This problem becomes more significant as sample volume is reduced within a microfluidic chip. To avert this obstacle, the use of a vapor barrier has been suggested [240]. In one report, a polyethylene vapor barrier layer was implanted within the PDMS from which the device was fabricated [240]. Amplification was then carried out on 1.75 µl of PCR solution, with yeast genomic DNA as the target. The use of the vapor barrier was shown to reduce fluid loss by at least 3-fold, significantly enhancing the amplification. Finally it should not go without mentioning that sample evaporation is not a problem solely confined to polymer-fabricated devices, as this issue must also be considered when reducing sample volume within silicone and glass devices. Steps taken to minimize this sample loss include the use of mineral oil [209] and microvalves [241]. Recently, valve-less strategies based on diffusion-limited evaporation mechanisms for reducing sample evaporation were shown to be effective by Wang et al. [242]. To accomplish this aim, long and narrow channels were built into the microchip. As convective airflow is not present within these channels, sample evaporation then becomes limited by the lengthy diffusion time of vapor traversing the long, narrow channel. To supplement this strategy, efforts to decrease the liquid evaporation driving force included thermal isolation and vapor replenishment utilizing water.

The above discussion highlights only a few of the design issues facing the development of microfluidic PCR devices, and the reader is thus referred to several informative reviews that discuss this topic and also provide descriptions of the current state of microfluidic PCR technology [183, 201, 202, 239, 243-245]. Ultimately, it is unlikely that one particular design and design material will suffice for all potential uses of microfluidic PCR devices. It is expected that the final decision of which materials and designs

to employ will be dependent on the specific intended use of the chip and the associated integrated components.

9.3 Future Research Directions

The research described within this chapter is ultimately intended to fulfill the need for more effective microfluidic diagnostic devices. These research efforts generally proceed along two inter-related fronts that can be viewed as being analogous to the concepts of "hardware" and "software". The "hardware" aspect of this research is directed at designing better micro-devices (like pumps, sensors, etc.), formulating methods to fabricate these micro-devices in or on materials that are biocompatible (or desirable from some other standpoint), and in devising ways to package these systems and interface them with the outside (macro) world. The "software" aspect of this research is directed towards arriving at newer and more effective strategies for isolating and/or detecting target pathogens.

Thus far, most groups have been focused upon specific areas, such as examining the selective amplification of a target species, developing separation processes for desired isolation goals, and producing biosensors for target species isolation. These efforts have produced very interesting and encouraging results. However, the development of a practical and robust device that fulfils the pathogen detection goals laid out in the introduction of this chapter will likely require considerable focus on combining these varied approaches into an integrated microfluidic system. Such a system will conceivably (at least when describing first generation devices) employ a short pre-culture step in order to increase the concentration of detection targets. This step will be followed by relatively simple separation steps (perhaps utilizing phenomena such as DEP), and then the device will end with a sensitive biosensor of some type. In designing this system, it will be important to consider that the optimization of target pathogen detection (i.e. detecting low number of target pathogens with high specificity) might require the operation of the initial device stages (selective amplification and separation) sub-optimally.

The development of effective microfluidic devices for pathogen detection will have to consider the refined detection parameters that will continually arise from increased understanding of the role microbes play within disease. For instance, recent work [246] has uncovered links between periodontal disease and the infestation of gums by archaea (a microscopic or-

ganism that is believed to have evolved separately from bacteria and other cellular organisms). Periodontal disease, in turn, has been identified as a contributing factor to endocarditis, atherosclerosis, stroke, and preterm delivery of low-birth-weight infants. Unfortunately, little is currently known about the exact mechanisms by which archaea could lead to these complications. Additionally, the protocols for quantifying the presence of such microscale organisms have either not been standardized, or are known only to a few expert researchers. Consequently, diagnostic devices (both micro- and macro-scale) that can successfully detect such microbes have yet to be developed.

Furthermore, diagnostic devices have largely been developed according to the conventional medical paradigm of "one disease, one pathogen." This model states that if a patient's symptoms match those of a particular disease, then the symptoms are expected to be caused by one (or a few related) type of pathogen that has been previously associated with the respective disease in question. However, recent research has suggested that microbial "communities" may in fact either be responsible for and/or contributing towards several aggravating conditions, such as obesity [247] and Inflammatory Bowel Disease (IBD) [248, 249]. In such instances, the relative number, pathogen-host and pathogen-pathogen metabolic interaction, and spatial distribution of the micro-organisms likely determines their resulting impact (regarding both what the impact is and its severity) on the host. All of these factors add up to create an altered and probably increased demand on the capabilities of future diagnostic devices. Consequently, it is highly likely that the manner in which diagnostic devices, particularly microfluidic diagnostic devices, are developed will have to be rethought.

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