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Advances in nanodiagnostic techniques for microbial agents

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ABSTRACT

Infectious diseases account for millions of sufferings and deaths in both developing as well as developed countries with a substantial economic loss. Massive increase in world population and international travel has facilitated their spread from one part of the world to other areas, making them one of the most significant global health risks. Furthermore, detection of bioterrorism agents in water, food and environmental samples as well traveler's baggage is a great challenge of the time for security purpose. Prevention strategies against infectious agents demand rapid and accurate detection and identification of the causative agents with highest sensitivity which should be equally available in different parts of the globe. Similarly, rapid and early diagnosis of infectious diseases has always been indispensable for their prompt cure and management, which has stimulated scientists to develop highly sophisticated techniques over centuries and the efforts continue unabated. Conventional diagnostic techniques are time consuming, tedious, expensive, less sensitive, and unsuitable for field situations. Nanodiagnostic assays have been promising for early, sensitive, point-of-care and cost-effective detection of microbial agents. There has been an explosive research in this area of science in last two decades yielding highly fascinating results. This review highlights some of the advancements made in the field of nanotechnology based assays for microbial detection since 2005 along with providing the basic understanding.

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1. Introduction

Infectious diseases have been one of the major threats to mankind in the entire history (Brachman, 2003; Fauci, 2001; Syed and Bokhari, 2011). Devastations caused by microbial pathogens are evident from the Egyptian mummies, Hippocrates writings and history book chapters on great epidemics in middle age Europe and other areas of the world (Brachman, 2003; Wolfe et al., 2007). In the era of globalization, where international travel and trade have been highly facilitated, one of the major challenges medical science facing today is the spread of infectious diseases from one part of the world to the others (Gooding, 2006). Although recent advancements in medical sciences have made remarkable breakthroughs in devising strategies for prevention and control of most of the diseases that cost millions of lives in the history, human battle towards control of many infectious diseases such as (AIDS, Tuberculosis, Malaria, Diarrhea etc.) continues unabated (Hauck, et al., 2010).

One of the many challenges modern medicine facing today is the accurate and early diagnosis of infectious diseases. Although explosive research in this arena has produced many breakthroughs, such as development of highly sophisticated molecular techniques such as Polymerase Chain Reaction (PCR), Ligase Chain Reaction (LCR), DNA sequencing, DNA hybridization assays, DNA Microarrays (Houpikian and Raoult, 2002; Muldrew, 2009) or commercially available dip stick tests, much more needs to be done for rapid, sensitive, cost-effective and point of care diagnosis of infectious diseases (Syed and Bokhari, 2011). Rapid microbial detection from water, food, environmental and clinical samples is indispensable for both public health and security perspectives (Mairhofer et al., 2009; Dutse and Yusof, 2011). Furthermore, developing and resource poor countries still remain largely deprived of expensive molecular techniques in the post genomics era and need low cost devices and system to be used in remote areas (Coloma and Harris, 2009).

Both classical culture media based and modern diagnostic techniques have been valuable tools for the diagnosis of infectious agents. Bacterial agents of many infectious diseases may be grown in the laboratory using culture media and identified by cultural characteristics, biochemical testing, serology and molecular assays. Nonetheless, each of the method has its advantages and limitations. Biochemical and serological testing for the identification of bacterial agents have been in use for almost a century. However, recent advents of modern molecular techniques have revolutionized the infectious diseases diagnosis by increasing the sensitivity and reducing the time taken by the laboratory tests (Bissonnette and Bergeron, 2012). PCR has become one of the most commonly used techniques practiced in diagnostic labs nowadays. Diagnostic kits manufactured by a number of companies offer rapid, sensitive and cost-effective diagnosis of many infectious diseases (Heo and Hua, 2009; Shinde et al., 2011).

Although both classical and modern molecular diagnostic techniques have been found to be highly valuable in the diagnosis of infectious diseases, they are tedious, expensive, less sensitive in some cases and require skilled personnel, which is unsuitable or unaffordable for field situations (Gehring and Tu, 2011; Pfaller, 2001; Sanvicens et al., 2009; Syed and Bokhari, 2011). Furthermore, cultivation of slow growing and fastidious bacteria has always been a problem in the microbiology labs where laboratory test results are awaited to start the treatment. In addition, many bacterial species such as *Treponema palladium* are non-culturable and cannot be grown in the lab (Centurion-Lara et al., 1997). Serology has been hampered by the unavailability of antisera to a wide number of microbial species, poor sensitivity and higher cost. Furthermore, many bacterial and viral agents undergo dormant phases and hence undetectable by the routinely used diagnostic techniques (Speers, 2006).

Molecular diagnostic techniques have not only revolutionized the diagnosis of infectious diseases of both culturable and non-culturable organisms, but they also offer a reliable means of antibiotic sensitivity testing, genotyping as well as classification systems for microorganisms (Pfaller, 2001; Speers, 2006; Mothershed and Whitney, 2006). RT-PCR may be used to determine the drug resistance in viruses, total viral load, genotype or strain characterization (Pfaller, 2001). These robust techniques, although highly sensitive in many cases, are either too expensive, time consuming or do not offer an option of point of care diagnosis. PCR requires DNA extraction from microbial cells and it may also produce false positive and negative results. Moreover, many of the molecular techniques require trained personnel to operate the sophisticated equipments, hence unaffordable by many of the labs in the developing countries. Therefore, there is a need for alternative strategies for microbial detection and identification (Singh et al., 2006).

Recent research in nanotechnology based strategies for microbial detection has produced highly fascinating and promising results. High surface to volume ratio of the nanomaterials greatly enhance the sensing bimolecular interactions by optical, electrical and electrochemical biosensors. Systems that can be automated and miniaturized offer enormous advantage over others, as they may be used in field situations requiring less complicated protocols (Gabig-Ciminska, 2006). Furthermore, disposable dipstick tests seems to be most promising advancement for point of care, rapid, sensitive and cost-effective microbial detection (Syed and Bokhari, 2011).

Nanotechnology has been merged with biosensing to improve sensitivity and detection limit of the biological events due to greater surface area of the sensing surfaces of the nanostructures such as carbon nanotubes, nanowires, graphene, gold film and conductive polymers incorporating into the conducting transducers (Liu et al., 2011). These nanostructures have been used in a number of biosesning applications for the detection of proteins, nucleic acids, microbial toxins, bacterial and viral agents etc. (Doria et al., 2012).

Nanodiagnostics, although in its infancy and little far from commercialization at the desired level, seems to greatly facilitate microbiologists in future developing sensitive and user friendly devices for microbial detection in their labs and field situations. Scientists around the world have been engaged developing cutting edge technologies for this purpose based on novel size dependent properties of the matter of different kinds. The number of research articles published on latest advancements and breakthroughs in this arena of science is ever increasing. This review attempts to highlight some of the advances in nanodiagnostic techniques for microbial detection in recent years.

2. Nanodiagnostics for microbial agents

Nanotechnology deals with the study of creation, manipulation and use of materials, systems, and devices of the size ranging from 1 to 100 nm in at least one dimension (Jianrong et al., 2004). Materials of this size exhibit unique physical and chemical properties due to their higher surface to volume ratio (Kaittanis et al., 2010). The spectrum of study of this science is wide covering both fundamental sciences (such Physics, Chemistry, Biology etc.) and applied sciences (such as Electronics and Material Science) (Kim et al., 2010; Liu, 2006). Potential applications of nanotechnology in medicine are broad, using the unique features of materials at nanoscale size for the treatment as well as diagnosis of diseases at molecular level (Kim et al., 2010). An enormous interdisciplinary research has been witnessed in nanomedicine in the last two decades, a part of which is dedicated to designing nanotechnology

based assay formats for sensitive and early detection of microbial agents or their products (Liu et al., 2006; Kim et al., 2010); Kumar et al., 2011a; Vashist, 2013).

Living systems consist of a number of nanometer sized materials such as proteins, DNA, RNA lipids, ligands, receptors, cells surface molecules etc. Furthermore, nature offers a unique organization of biomolecules in the form of viruses, that are capable of carrying genetic information as well infecting host cells including bacteria. Therefore, biologists have started understanding nanometer sized cellular structures and have been dealing with such small systems for a long time using sophisticated tools like electron microscopes prior to emergence of nanotechnology as a separate science. Since natural processes have been selected over millions of years of evolution and take place spontaneously, nanotechnology devises strategies to study these processes by designing and manufacturing materials and devices having at least one dimension of nanometer. The robust sensitivity offered by these nano-scaled materials offers unique opportunity to understand tiny details of living processes with higher sensitivity. The higher surface to volume ratio of the nanoparticles offers a great opportunity to sense the biological processes with higher sensitivity than the material of larger size. Nanomaterials conjugated with biomolecules (such as antibodies, nucleic acid probes, aptamers etc.) sense and transmit the biological information in minimum period of time, thereby making them promising material for biosensing applications (Kim et al., 2010). Nandiagnostic techniques have been found to be promising by offering means of sensing at single cell or even single molecule level depth. Nanotechnology based assays for microbial detection and identification offer rapid, label free, highly sensitive testing with the option of their onsite utility. For, examples, lab on the chip is one of many nanodiagnostic techniques, for highly sensitive detection of microbes, their cellular processes, and biomolecules such as nucleic acids, toxins, enzymes etc. (Chen et al., 2007; Jain, 2003; Jain, 2007, Huang, 2011).

Biosensors are analytical devices used to detect unknown biological agents or study biological events. A high number of efforts have been made to develop various types of biosensors for the early detection of microbial agents (Setterington and Alocilja, 2012; Syed and Bokhari, 2011). Use of nanomaterials in biosensors introduces many new signal transduction mechanisms in their manufacture which enhance their sensitivity to a greater extent. Due to their submicron size, nanosensors, nanopores, nanoparticles and other nanometer sized sensing elements are revolutionizing the field of biosensing, including the one used for microbial detection and identification (Jianrong et al., 2004).

3. Microfluidic assays or lab-on-a-chip

The field of miniaturized microfluidic system or Lab on a chip (LOC) has gained increasing popularity and has been found to be promising for accurate and point of care microbial detection, which is needed presently more than at any other time (Chen et al., 2007; Mairhofer et al., 2009). LOC based assays have a wide range of applications in rapid, real time and simultaneous detection of microbial agents by combining miniaturized components such as probes, transducer, chambers of a microfluidic lab etc. (Chen et al., 2007; Gabig-Ciminska, 2006). Such systems offer a tremendous advantage of analyzing nano to microliter sized volume of the analyte for analysis by automated systems capable of signal enhancement (Duste and Yusof, 2011; Jin et al., 2009). These devices are not only suitable for use in the diagnostic labs but also for remote areas of the developing countries where diagnostic facilities are very limited or areas like airports for rapid screening of patients for highly infectious diseases or bio-warfare agents (Duste and Yusof, 2011; Mairhofer et al., 2009).

LOC technology attracts a great number of scientists from many different fields such as microbiology, electronics, chemistry, physics and chemical engineering etc. The number of research articles published in this area is ever increasing and a number of interesting results have been published in the last decade. LOC are catching great attention due to both public health as well as security point of view. A high number of research groups as well as commercial organizations are carrying out dedicated research for the developing such miniaturized devices for the rapid and reliable detection of bio-warfare agents (Liu et al., 2006; Mairhofer et al., 2009). For example, Liu et al. (2006) have developed an integrated miniaturized portable device for simultaneous detection and genotyping of a number of pathogenic bacterial species based on nuclieic acid hybridization process. Several other interesting attempts have also been made in this arena. Huang et al. (2011a, 2011b, 2011c) have tried to use such microfluidic channels for simultaneous amplification and quantitative detection of bacterial nucleic in real time. In another interesting study, Ho et al. (2012) developed a microfluidic portable device for simultaneous detection of a number of bacterial species responsible for nosocomial infections as well as study of their antibiotic susceptibility patterns in a single assay format. Lee and Yager (2007) developed a LOC DNA microarray for microbial detection from water samples. Many other groups are working on using microeletrocmechanical systems (MEMS) for simultaneous detection of a number of microbial agents as well as their products, most importantly bio warfare agents, from food and environmental samples (Dutse and Yusof, 2011; Mairhofer et al., 2009). Further advancements in such strategies will definitely lead to our capability to detect a single cell or even molecules in a matter of minutes (Huang et al., 2011a).

4. Nanoparticle based assays

Nanoparticles are one of the major applications of nanotechnology in medicine. Nanoparticle based assays usually consist of a recognition element such as nucleic acid probe, antibody, enzyme, aptamer, or some other biomolecule that binds the specific ligand (such as bacteria, virus, toxin etc.) and nanoparticles for the transduction of this biological event into a measurable signal or as an optical reporter. Nanoparticles have been widely investigated class of nanomaterials for potential applications in microbial diagnostics (Agasti et al., 2010; Liu, 2006; Syed and Bokhari, 2011). Nanomaterials such as nanoparticles, nanotubles, nanorods etc. provide novel platform for pathogen detection by offering higher surface to volume ratio, unique tunable optical and transduction properties and ease of conjugation with the recognition probes (Agasti et al., 2010; Agarwal et al., 2005; Syed and Bokhari, 2011). Indeed, nanoparticles have increasingly been used in the biossensing applications due to their unique size dependent electronic, optical, physical and chemical properties (Agasti et al., 2010). Nanoparticles may be classified on the basis of type of

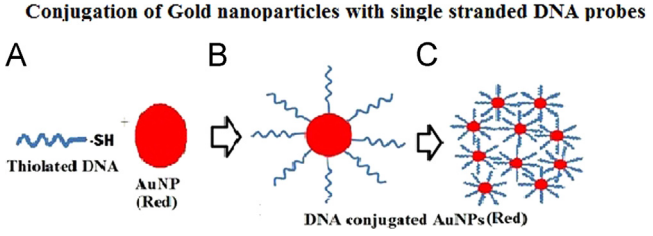

Fig. 1. Conjugation of AuNPs with single stranded thiolated DNA strand.

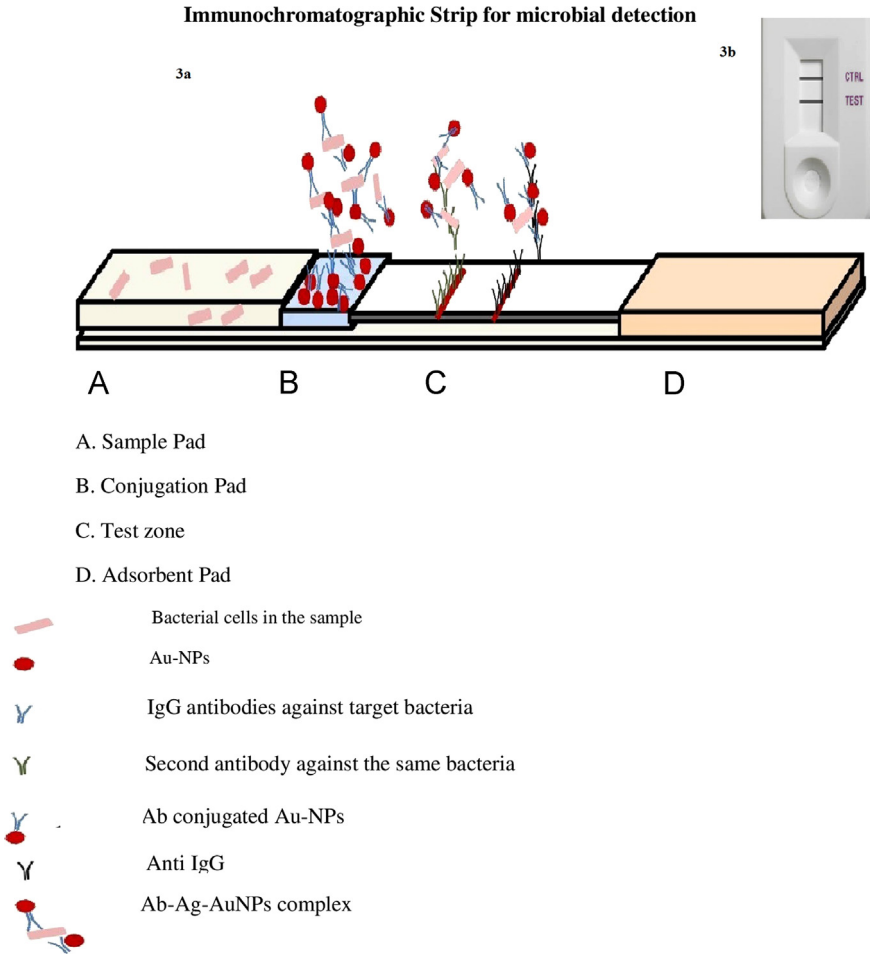

Fig. 2. Diagram of a typical Immunochromatographic strip for microbial detection. The sample pad made of cellulose is used to apply the diluted sample in a buffer. Pores of the nitrocellulose membrane contain antibody conjugated nanoparticles. Once antibody conjugated nanopartilces encounter antigen, Ag–Ab–AuNP complex is formed, which moves towards test zone. A second antibody to the same bacteria fixed at the test line captures the Ag–Ab–AuNP complex and a red line appears due to accumulation of the Au-NPs. Control line of the test zone retains anti IgG to capture all IgGs, acts as appositive control. Excess of the fluid is retained by the absorbent pad.

material they are made of into metallic, semiconductor and polymeric nanoparticles. Gold, magnetic, and fluorescent nanoparticles are widely used in diagnostic applications and will be described in detail in this review. Other classes of nanoparticles such as polymeric nanoparticles are also widely used in diagnostic assays (Oh et al., 2011).

4.1. Gold nanoparticles

Gold has been an exciting material in nanotechnology and has been found to be an ultimate diagnostic material (Kumar et al., 2011a; Syed and Bokhari, 2011). Gold nanoparticles (AuNPs) exhibit a broad spectrum of applications among NP based assays for microbial detection and identification. Unique size dependent optical properties of AuNPs, their inertness in biological fluids and stability make them one of the most robust materials used in nanodiagnostics (Bakthawathsalam et al., 2012; Ray et al., 2007; Syed and Bokhari, 2011). Modulation of their size and shape dependent physiochemical properties may easily be achieved by choosing appropriate method of synthesis. Furthermore, AuNPs may easily be functionalized with thiolated DNA probes (Fig. 1) and protein molecules, which make them superior over other types of NPs (Syed and Bokhari, 2011). Nonetheless, AuNPs have conventionally been used in immunochromatographic strip (ICS) biosensor as well for sensitivity enhancement of many nanodiagnostic assays (Halfpenny and Wright, 2010; Syed and Bokhari, 2011).

ICS seem to fulfill many of the expectations for rapid, point of care, sensitive detection of infectious agents, their toxins as well antibodies raised against them. One of the many unique features of AuNPs is their red color. The colloidal AuNPs appear red and their aggregation in diagnostic tests may easily be detected visually without the aid of any instrumentation. This aspect has been proven very helpful in their utilization in ICS and tube based assays for microbial detection. ICS are the most exciting application of AuNPs that have already reached commercialization (Syed and Bokhari, 2011). Disposable dip stick tests are portable, cost-effective, reliable and rapid method of detecting biomolecules and microbial entities from clinical, environmental and food samples (Syed and Bokhari, 2011; Zarakolu et al., 2002).

ICS have greatly fascinated the microbiologists as their use does not require much expertise, instrumentation and the results may easily be visualized as a red line in the test zone. Furthermore, the ICS is also easy to develop according to the requirements of the study using specific antibodies and commercially available membranes as well as other reagents of analytical grade. The monoclonal IgG antibodies are usually used for the detection of the target antigens due to their specific binding capability (Ho et al., 2004).

A typical ICS consists of 1 Sample pad, 2 conjugate pad, 3 nitrocellulose membrane and 4 adsorbent pad (Fig. 2). The sample pad, made of cellulose, is used to apply the sample, whereas conjugate pad possesses antibody conjugated AuNPs (Ab–AuNPs). The Ab–AuNP conjugate binds the target antigen present in the sample resulting

formation of antibody–antigen-gold nano particle complex (Ab–Au-NP–Ag). This complex moves to the nitrocellulose membrane where it is captured by second antibodies to the same antigen, giving rise to red color of the test line. The second line in the test zone possesses anti-IgG antibodies, which appear red in both positive as well as negative test cases (Peng et al., 2008; Matsui et al., 2011; Zarakolu et al., 2002).

A high number of research articles in the last decade described successful use of ICS for bacterial (Blazkova and Fukal, 2011; Huang, 2007; Yan et al., 2011), viral (Peng et al., 2008), fungal (Thornton, 2008), parasite antigen (Wang et al., 2011a), and toxin (Ching et al., 2012; Engler et al., 2002) detection. Furthermore, some tests have already reached commercialization. A recent publication by (Ching et al., 2012) has reported a detection limit of 5 ng/ml of botulism toxin, one of the most potent toxin and bio-warfare agents known to date. Furthermore, many groups have used aptamers as capture probes instead of antibodies due to their enhanced binding affinity for their ligand (Xu et al., 2009).

Colorimetric assays are also performed for the detection of microbial DNA using AuNPs conjugated with specific DNA/RNA probes complementary to the microbial nucleic acid sequences. Hybridization of the probe conjugated DNA with the target DNA sequence of microbes results in aggregation of the AuNPs visualized with the naked eye (Gill et al., 2008; Bakthawathsalam et al., 2012). DNA may also be covalently linked to the AuNPs by introducing a thiol group (–SH) to the 3′ or 5′ end of the nucleic acid probe, resulting in stable covalent bond between the AuNP and the probe (Syed and Bokhari, 2011).

An enormous research data is available on the use of gold nanoparticle for microbial cells, their product or DNA detection. Hybridization probe strategies remain among the most popular techniques for microbial DNA detection. DNA may be detected by colorimetric aggregation of AuNPs conjugated with complementary sequences specific gene of the target organism (Gill et al., 2008; Kumar et al., 2011a; Ray et al., 2007).

AuNPs have also been used as fluorescent labels in optical imaging and sensing of biomolecules such as proteins. AuNPs may overcome many of the limitations of the classical fluorophores due to higher extinction coefficient, broader absorption spectrum in the visible region, which is usually overlapped with the emission wave length of the FRET donor (Coto-Garcia et al., 2011; Yang et al., 2011).

4.2. Magnetic nanoparticles

Mangnetic nanoparticles (MNPs) have been becoming increasingly popular in nanomedicine due to their higher physical and chemical stability, low cost of production, ease of bio-conjugation and separation and enrichment using a magnet (Goeransson et al., 2010; Koh and Josephson, 2009; Huang et al., 2010). Use of MNPs is of importance because they may be exploited to capture and separate microbial agents from complex matrices (Ho et al., 2004; Huang et al., 2010), resulting in pure cultures for analysis (Padmavathy et al., 2012). Furthermore, use of superparamagnetic nanoparticles (SPMNPs) is of advantage, because their magnetic properties increase dramatically at nanometer size (Maalouf et al., 2008). Antibody or aptamer conjugated MNPs may easily be used for the immunoseparation of the microbial species from the sample (Cheng et al., 2009; Padmavathy et al., 2012). γ-aminopropylethoxysilane (APTES) is a commonly used reagent for the surface functionalization of the nanoparticles for bioconjugation (Padmavathy et al., 2012). Furthermore, magnetic nanoparticles may also be covered by silica or gold shell, whereby magnetic part acts like a core and silica or gold are the shell. These nanoparticles utilize the properties of both magnetic and fluorescent materials (Liu, 2006).

Magnetic relaxation nanosensors (MRNS) are among the most promising nanomaterials for microbial detection with higher sensitivity and specificity. MRNS employ polymer coated nanoparticles which may be conjugated with some biomolecules such as antibody specific for a microbial agent or its product (Kaitttanis et al., 2007). The binding of bioconjugated MRNS with their target ligand brings about changes in samples Magnetic Resonance Signal, which is directly correlated to the analyte concentration in the sample (Kaittanis et al., 2012). In a recent study conducted by Kaittanis et al. (2012) a MRNS was used for the detection of an intracellular parasite *Mycobacterium avium* ssp. *paratuberculosis* (MAP). They used hybridizing magnetic resonance nanosensors (hMRNS) for the conserved DNA sequences of MAP. Binding of hMRNS with the complementary sequences (i.e. IS900) in bacterial DNA resulted in change in magnetic resonance signal indicating presence of the microbe in the sample. The technique does not require highly purified DNA like in the case of PCR and the test may be performed with minimally processed samples. Presence of this slow growing intracellular bacterial species may be confirmed in one hour as compared to culture which takes an average of 12 weeks (Kaittanis et al., 2012). The same group also used MRNS for the study of bacterial antibiotic susceptibility in culture media. The dextran coated iron oxide nanoparticles along with a protein with high affinity to carbohydrates (Concanavalin A) was used in a competition assay. The ConA coated nanosensors responded differently in response to varying bacterial metabolism of the carbohydrates. The group showed that Con-A conjugated nanosensors may be used for the quantification of carbohydrates, bacterial metabolism, antibiotic susceptibility testing in complex matrices such as culture media, blood, tissue samples etc. (Kaittanis et al., 2008).

A study conducted by Huang et al. (2010) reported use of amine functionalized magnetic nanoparticles for bacterial removal from water samples. The amine group confers positive charge to the magnetic nanoparticles that can easily attach the negatively charged bacterial cells. This nonspecific binding may be useful for enrichment of bacterial cells from water samples. In a similar study, Chen and Zhang (2012) used Gentamicin conjugated magnetic silica nanoparticles for the detection of gram positive bacterial species *Staphylococcus aureus*. The amino group of gentamicin makes it positively charged in physiological conditions by having affinity for negatively charged bacteria. A study conducted by Lee et al. (2009) used MNPs to detect bacillus Calmette–Guerin as a surrogate for *Mycobacterium tuberculosis* from sputum samples. MNPs bind bacterial cell wall rendering it superparamagnetic. In the following step bacteria are concentrated in the microfluidic chamber and the spin spin time of the whole sample (*T2*) was measured with Nuclear Magnetic Resonance (NMR). This system could detect as low as 20 cfu/ml of bacterial cells in less than 30 min.

4.3. Fluorescent silica nanoparticles

Florescent nanoparticles have been used by a high number of research groups in their attempts to detect microbial agents with higher sensitivity due to their robust chemical and optical properties. Fluorometric assays are not new to microbiologists, since fluorescence microscopes and spectrofluorometers are widely used in diagnostic as well as research labs. Fluorescent nanoparticles (FNPs) such as silica or organic may easily be bioconjugated and may be used in monitoring microbial presence by fluorescence microscope or using spectrofluorometer (Wang et al., 2007). Furthermore, dye doped silica nanoparticles can detect biomolecules in the samples with higher sensitivity as compared to other fluorescent nanoparticles (Wang, et al., 2007; Tallury et al., 2010).

A single fluorescent silica nanoparticle retains thousands of dye molecules in its matrix, whereas direct fluorescent labeling of antibodies in immunofluorescence assays attaches only a very few dye molecules. Therefore, using these nanoparticles enhances the sensitivity of the assays many hundred folds. Furthermore, compared to other fluorescent nanoparticles, dye doped silica nanoparticles offer advantages of photostability and enhanced luminescence. They have been widely used for the detection of biomolecules such as DNA, antibodies, cell receptors, bacterial cells etc. (Qin et al., 2007).

The matrix of silica nanoparticles may retain both organic as well as metallic dyes (Tallury et al., 2010). The dye may be either attached to the surface of the nanoparticles or contained inside the particles. However, for imaging purpose, nanoparticles with embedded dye molecules exhibit stronger photostability by being protected from the light (Murcia and Naumann, 2005). Furthermore, silica NPs may easily be chemically functionalized and bioconjugated with antibodies and nucleic acids. Chemical modification of the nanoparticle surface to generate amino or carboxyl group enables one to covalently attach antibodies to the fluorescent nanoparticles (Zhao et al., 2004).

Antibody coated dye doped silica nanoparticles have shown enhanced sensitivity for the assays and reduced the detection limit to a single cell level (Li and Xu, 2009; Zhao et al., 2004). This strategy is particularly useful for the detection of fastidious and slow growing bacteria such as *Mycobacterium tuberculosis,* that unusually require very long incubation time and special cultural conditions. Rapid identification of microbes and their antibiotic resistance patterns are prerequisite for a timely and effective treatment as well the management of the disease. Qin et al. (2007) used fluorescent silica nanoparticles for the sensitive detection of *M. tuberculosis* from bacterial mixtures as well as spiked sputum samples. They first reacted the bacterial cells with the monoclonal antibodies to *M. tuberculosis,* that were detected by protein A conjugated RuBpy doped silica nanoparticles. Furthermore, Tan's group at University of Florida used fluorescent silica nanoparticles for multiplexed bacterial monitoring in a single sample. The NPs contained varying concentrations of dye molecules appearing different in color by excitation with same wave length. By conjugating each type of these nanoparticles with different antibodies for separate bacterial species they were able to detect three different bacterial species in the sample, each appearing different in color (Wang et al., 2007). A recent study conducted by Ekrami et al. (2011) using monoclonal antibody against *M. tuberculosis* and proteins A conjugated fluorescent NPs found that *M. tuberculosis* can easily be detected from sputum samples with 97.1% sensitivity and 91.35 specificity, taking culture as a gold standard.

4.4. Quantum dots

During the last two decades, a great deal of attention has been focused on the size dependent optoelectronic properties of semiconductor nanoparticles or quantum dots (QDs) and their potential applications in the life science research, largely in the field of bioimaging (Bera et al., 2010; Kim et al., 2004). QDs are semiconductor crystals of nanometer size, bearing unique optical properties, have emerged as a promising class of nanomaterial for microbial detection (Liandris et al., 2011). QDs, composed of 100–100,000 atoms (Mazumder et al., 2009), possess broad absorption spectra, narrower emission bandwidth with size dependent local maxima (Edgar et al., 2006; Kim and Kim, 2012) and higher biocompatibility (Ma et al., 2010). Different emissions may be excited with the same wavelength, because the emission wavelength is tunable with the size, shape and composition of the QDs (Giri et al., 2011). In contrast to QDs, commonly used fluorophores have two disadvantages; they have low signal to noise ratio and they are not photostable (Edgar et al., 2006).

Bioconjugated QDs offer an excellent opportunity to detect microbial pathogens due to their unique qualities such as long term photostability as compared to conventional organic labels (Decho et al., 2008; Edgar et al., 2006). QDs are able to absorb 10–50 times more photons than the conventional organic dyes at the same excitation photon flux providing more brightness to the sensing system for microbial detection (Kim and Kim, 2012). With minimum interference from autofluorescent particles, enhanced photostability and broader absorption spectra and ease of bioconjugation, QDs may easily be used for biomolecular analysis in the complex matrices (Liandris et al., 2011; Edgar et al., 2006). QDs with Cadmium Selenide (CdSe) core and zinc sulfide (ZnS) shell with excellent fluorescent properties are most commonly used and commercially available type of QDs (Valizadeh et al., 2012).

As a fluorescent label QDs have made tremendous progress in bioassays, such as immunoassays, DNA hybridization assays as well as microbial detection (Ma et al., 2010; Wang et al., 2012). Furthermore, multicolor QDs may be used for multiplexed assay for simultaneous detection of a number of pathogens in a given clinical, environmental or food sample (Hahn et al., 2005). A high number of successful attempts have been made to use QDs for microbial detection in recent years (Liandris et al., 2011; Giri et al., 2011; Decho et al., 2008; Stringer et al., 2008). To mention a few, Tripp et al. (2008) used mAb conjugated Cadmium Telluride (CdTe) QDs for the detection of respiratory syncytial virus. Hahn et al. (2005) used CdTe/ZnS core/shell nanoparticles for the detection of highly pathogenic bacterial species *E. coli* O157H7 to a single cell level. In a multiplexed assay Zhao et al. (2009) detected three food borne pathogenic bacterial species i.e. *S. typhimurium, E. coli H157H7* and *S. flexneri* using MNPs for cell enrichment and QDs as fluorescent tags.

5. Förster resonance energy transfer based biosensors and molecular beacons

Förster or fluorescent resonance energy transfer (FRET) has been one of the promising techniques for the study of cellular processes, viral detection and their susceptibility to drugs. FRET is a non-radioactive technique in which one fluorophore acts as an energy donor while the other is energy acceptor (Kim et al., 2008a, 2008b; Mathur et al., 2008; Tsai et al., 2009). FRET based biosensors have been a widely used strategy for microbial detection in a number of studies, as it provides a fast, sensitive and convenient way of probing distance between the molecules (Kattke et al., 2011). Molecular Beacons (discussed later) are an excellent example of FRET based biosensors. AuNPs are highly effective fluorescent quencher molecules leading to sub-picomolar detection of bioanalytes. Both theoretical calculations and experimental work prove AuNPs as a super quencher, being able to quench fluorescence of a range of dyes with high efficiency. (Halfpenny and Wright, 2010; Yang et al., 2011). AuNPs are considered as super quenchers due to their ability to quench a number of fluorophores (Yang et al., 2011) over a long distance (Mayilo et al., 2009). In a recent study, Yu et al. (2013) utilized gold nanorods (AuNRs) as quenchers and FAM as a FRET donor for the detection of Hepatitis B virus DNA.

Quantum dots may also be used as fluorescent donors in FRET based assays. Wang et al. (2011) used CdSe/ZnS quantum dots as FRET donor and black hole quenchers as FRET acceptors for the detection of *tst* gene encoding for toxic shock syndrome toxin in *Staphylococcus aureus.* In this approach, Wang et al. used ssDNA complementary to the *tst* gene conjugated to CdSe/ZnS quantum dots. A complementary stand with black hole quencher was used

to quench the fluorescence. Detection of the target DNA sequences of *tst* was detected by addition of 10 equivalents of bacterial DNA to the hybrid resulting in the recovery of emission of QDs (Wang et al., 2011b).

Molecular Beacons (MB) are 15–40 nucleotide long single stranded DNA or RNA sequences that fluoresce on binding with the target complementary sequence (Fig. 3) (Larios-Sanz et al., 2007; McKillen et al., 2007). MB have been successfully used to detect target DNA/RNA sequences exploiting FRET phenomenon. The single stranded nucleic acid probe constituting a central sequence complementary to the target DNA sequence forming the loop whereas flanking regions complementary to each other act as a stem (Tan et al., 2000). One end of the nucleic acid probe is attached with fluorophore while the other end with a quencher (Poddar, 1999; McKillen et al., 2007). The quencher is chosen so that its absorption spectrum is overlapped with the emission spectrum of the fluorophore. Once reacted with the target sequence, the central nucleic acid sequence will hybridize with it causing the FRET donor/ acceptor pair to dissociate from each other (Kim et al., 2007; Poddar, 1999). MBs have been successfully used for the study of gene expression in both prokaryotic and eukaryotic cells. However, the most common application of MBs is in real time PCR. Molecular beacons based assays, as with the conventional PCR utilize extension of forward and reverse primers as well as MB for the complementary to the to target sequence to be amplified. As stated previously, MBs fluoresce on binding to the complementary sequence. During the PCR number of amplified DNA segments increases and hence the increase number of MBs hybridizes to the complementary sequences and increased fluorescence (Chen et al., 2000; McKillen et al., 2007).

In the last decade, MBs have been used for nucleic acids of a number of microbial agents using RT-PCR and now it is becoming a routine practice in diagnostic and research labs (George et al., 2012; Kim et al., 2007; Orru et al., 2006).

6. One dimensional and carbon derived nanomaterials

One dimensional (1-D) nanostructures such as carbon nanotubes, nanorods and nanowires have been proven to be most promising materials in biosensing applications. Materials such as zinc oxide, carbon and polymers have been widely been used for the fabrication of nanostructures owing to their enhanced sensitivity, biocompatibility and ease of functionalization and preparation. Noteworthy among all other materials are carbon derived nanomaterials such as carbon nanotubes and graphene (Kumar et al., 2011b). Carbon based nanomaterials, such as one dimensional carbon nanotubes (CNTs) and two dimensional graphene are widely being used as sensing elements in biosensors for microbial detection owing to their unique properties in biosensing applications (Yang et al., 2010). CNTs or graphene modified electrodes have been useful in immobilizing biomolecules and detection of the target microbial species (Qureshi et al., 2009).

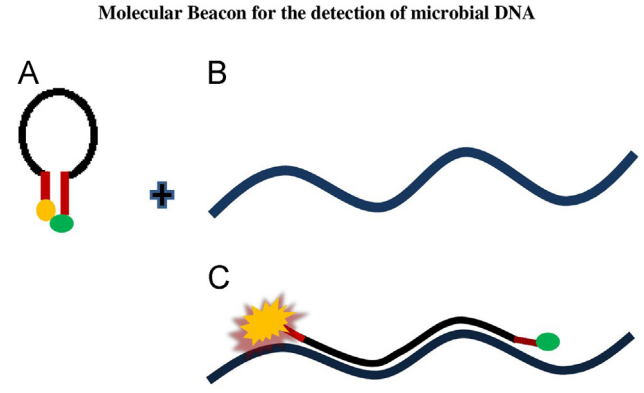

Fig. 3. (A) Molecular Beacon for the detection of the target microbial DNA. A short stretch of single stranded DNA containing both loops (black) and stem (red) structures (A). The central loop of the Molecular Beacon is complementary to the target DNA, whereas the flanking sequences, complementary to each other, are used do give a Molecular Beacon its secondary structure. A Molecular Beacon bears fluorophore at its one end and quencher at the other. Binding of the Molecular Beacon to its complementary sequence (B) of the target DNA brings about dissociation of the fluorophore from quencher (C), resulting in the fluorescence of the fluorescent dye. (B). A commercially available immunochromatographic test trip. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

6.1. Carbon nanotubes

CNTs are considered as the most commonly used building block of nanotechnology (Merkoci, 2006). CNTs are the allotropes of carbon that can have a surface to volume ratio up to 100,000 (Fig. 4). CNTs are significant among all nanomaterials due to their unique chemical, thermal, optical, magnetic, surface and electronic properties and unusual strength (Hirlekar et al., 2009; Moon and Kim, 2010; Vardharajula et al., 2012). CNTs are widely being investigated due to their excellent features which may be exploited to develop nanosesnsors with higher sensitivity. Based on their structure, CNTs are divided into two main groups, namely single walled carbon nanotubes (SWCNTs) and multiple walled carbon nanotubes (MWCNTs) (Ji et al., 2010).

The bio-nano integrated systems combining carbon nanotubes with the recognition or catalytic biomolecules have been remarkable tools for developing biosensors (He and Dai, 2006; Zhang et al., 2007). The high surface area of the carbon nanotubes greatly facilitate immobilization of biomolecules such as antibodies, aptamers, oligonucleotides and proteins without diminishing there structure and bioactivity, and therefore enhance their sensing capability (He and Dai, 2006; Huang et al., 2004; Jain et al., 2012). Furthermore, multiple functionalizations of CNTs are also possible for conjugation with different types of biomolecules or recognition elements. Functionalization of CNTs may be carried out for many purposes, for example, increased solubility, lower toxicity and bio-conjugation and specific binding to the analytes (Vardharajula et al., 2012). Antibody immobilized carbon nanotubes have been successfully used for electrochemical detection of bacterial agents with higher sensitivity and specificity (Jain et al.,

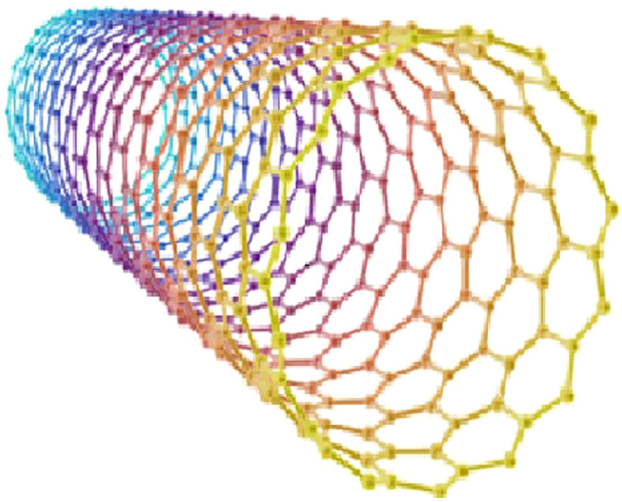

Fig. 4. Three dimensional structure of single walled carbon nanotube.

2012; Zhang et al., 2007). Moreover, protocols have been developed to synthesize single walled carbon nanotubes with functionalities such as carboxyl group for covalently binding with proteins (Zhang et al., 2007).

One dimensional single walled carbon nanotubes (SWCNTs), also called field effect transistors (FETs), have been used to electrically detect biomolecules with higher sensitivity. As the flow of current is solely on the surface, conductance of SWCNTs is highly sensitive to the electrochemical disturbance imposed by the biomolecular interaction (Huang et al., 2011c). FETs have been successfully used to detect bacterial cells up to a detection limit of 100 cfu/ ml of the samples (Huang et al., 2011c; Villamizar et al., 2008). In a similar study, Bhattacharya et al., 2011 used antibody functionalized carbon nanotubes for the detection of avian metapneumovirus (aMPV). Change in conductance was observed upon binding of the specific antibody on the CNT surface with the viral antigens, which was related to the antigen concentration.

Bacterial cells demonstrate affinity to carbon nanotube clusters (CNTCs) and this ability has been utilized for using them in bacterial filters (Kim et al., 2007b). The high binding affinity of CNTC for bacteria and paramagnetic susceptibility was exploited by Moon and Kim (2010) as universal adsorbents for bacterial cells and magnetic separation agents. They successful used CNTCs for binding and capturing bacterial cells of different types, whereas the separation of the cells was carried out by using an external magnetic.

6.2. Graphene

Graphene has become most widely used and investigated nanomaterial since its discovery in 2004. Graphene, a single atom planer sheet of carbon atoms perfectly arranged in a honey comb manner, finds great application in biosensing owing to its extra ordinary physical, electrochemical and optical properties (Fig. 5) (Vashist and Venkatesh, 2013). This recently discovered cousin material of the CNTs is anticipated to be a better alternative of the SWCNTs owing to its better electrochemical, electrical, optical and biocompatibility properties (Monhanty and Berry, 2008). The perfect two dimensional area of the graphene offers uniform functionalization and immobilization of the biomolecules. Furthermore, exceptional electric properties of graphene (such as high charge mobility and tunable conductance) make it an ideal sensing material in electronic biosensors (Huang et al., 2011b).

In recent years, a number of remarkable efforts have been made to develop graphene based electrochemical, electrical and optical biosensors for microbial detection with enhanced sensitivity (Huang et al., 2011b). The graphene based immunosensor developed by Huang et al. (2011b) showed significant conductance increase after exposure to *E. coli* and they were able to detect as low as 10 cfu bacterial cells in 1 ml of sample. Liu et al. (2011) developed graphene based electrochemical biosensor for the detection of rotavirus. The graphene film on the working electrode shows excellent electron transfer property. Immunoglobulins to rotavirus were covalently immobilized on the graphene films and the process of antigen antibody interaction was monitored with cyclic voltammetry.

In a recent study conducted by Mannoor et al. (2013), an interesting application of graphene has been described for microbial detection from tooth enamel and saliva. They printed graphene on water soluble silk, which permits transfer of graphene onto the tooth enamel. By self-assembly of antimicrobial peptides on the graphene surface, they were able to detect bacteria at single cell level.

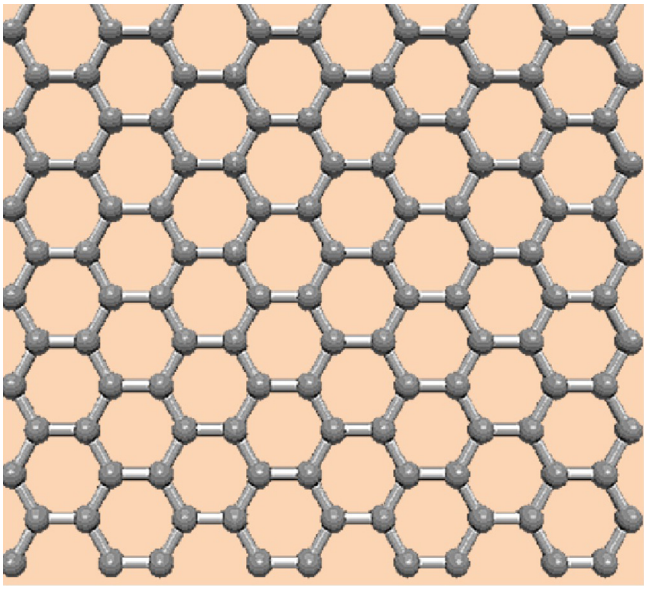

Fig. 5. Graphene structure. One atom think flat layer of hexagonal rings arranged in a honeycomb like style.

6.3. Nanowires

Nanowire FETs have been used for viral, toxin, microbial DNA and bacterial detection (Basu et al., 2004; Mishra et al., 2008; Patolsky et al., 2004,). One direct approach to directly detect the microbial agents is using semiconducting nanowire field effect transistors (FET). Antibody modified surface of the nanowire bring about change in the conductance upon binding of the target antigen. In such as study conducted by Patolsky et al. (2004) nanowire conjugated with antibodies against influenza A virus were used for viral detection. Binding and unbinding of the influenza virus with the antibody modified nanowires brought about discrete conductance changes but not with adenoviruses and paramyxovirus. In a similar study, Zhang et al. (2010) used silicon nanowire based sensor for rapid detection of RT-PCR product of Dengue fever serotype 2. A peptide nucleic acid probe was attached to the silicon nanowire and the complementary nucleic acid sequence of Dengue fever virus was amplified using RT PCR. A change in resistance was observed upon binding of the attached DNA probe with the RT PCR amplified product.

7. Conclusion

The significant advancements in the fields of molecular biology and nanotechnology have made remarkable breakthroughs in the area of microbial diagnosis. Micro and nanoscale transducers, optical imaging systems, integrated electronic devices as well as microbial probes are about to bring about historical breakthroughs. Scientific communities will soon see miniaturized, automated, portable, cost-effective, and point of care devices with higher sensitivity and specificity. Although a number of nanotechnology based devices have already reached commercialization and many of the biologists are familiar with techniques like microarrays, ICS, microfluidic systems, an enormous number of discoveries is being patented or commercialized. Future microbiologists are expected to be familiar with the use of nanotechnology based assays in their labs and it is hoped that the average time taken by each of these diagnostic tests will be reduced significantly. Further, diagnosis of slow growing and fastidious bacteria is also likely to be made quicker and more sensitive in both developing as well as developed countries.

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