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# Nanodiagnosics for Tuberculosis

Bruno Veigas, Gonalo Doria and Pedro V. Baptista\*  
CIGMH, Departamento de Ci4ncias da Vida, Faculdade de Ci4ncias e Tecnologia,  
Universidade Nova de Lisboa  
Portugal

## 1. Introduction

Tuberculosis (TB) remains one of the most serious infectious diseases in the world requiring new and more effective diagnostics and treatments (World Health Organization [WHO], 2010). Several approaches have been developed to improve TB diagnostics, reducing the time from weeks to a few days that still require demanding expertise technical personal for labor intensive and expensive methods, which hamper application in resource-poor countries where the main TB epidemic is observed. Nanotechnology has triggered the development of new and cheaper approaches for biomolecular recognition that may circumvent the current limitations of conventional molecular diagnostic methods used in the global fight against TB. This new era of molecular nanodiagnosics may provide a rapid and sensitive detection of the main TB etiologic agent in humans, i.e. *Mycobacterium tuberculosis*.

Nanodiagnosics can be defined as the use of nano-sized materials, devices or systems for diagnostics purposes. Biological tests measuring the presence or activity of selected analytes become quicker, more sensitive and more flexible when nanoscale particles are put to work as tags or labels, with numerous advantages over more traditional procedures, for example fluorescence and chemiluminescence technology. Here we will provide a closer look into nanodiagnosics systems developed for TB diagnostics and/or *M. tuberculosis* detection and characterization, such as nanoparticle-based systems (e.g. gold, silver, silica and quantum dots) and nanocantilevers. These techniques are already showing to be more sensitive and specific than conventional commercial molecular diagnostics methodologies although many aspects of nanodiagnosics for TB still need further evaluation and validation. Current advances in nanofabrication may enable the construction of cheap and full-automated devices, extending the limits of current molecular diagnostics and enable point- of-care diagnostics.

## 2. Tuberculosis

Tuberculosis is caused by *M. tuberculosis*, a member of the *Mycobacterium tuberculosis* complex (MTBC) and, according to the most current statistics of the World Health

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\* Corresponding Author

Organization, remains one of the most serious infectious diseases in the world, being responsible for 1.7 million deaths and 9.4 million new cases in 2009 alone (WHO, 2010). The emergence of multidrug-resistant TB also represents a serious threat to the TB control and an increasing public health problem (Deun et al., 2010), leading to a global need for rapid drug susceptibility testing. Single nucleotide sequence variations (mutations and/or polymorphisms) within *M. tuberculosis* genome have been associated with antibiotic resistance for all first-line drugs (isoniazid, rifampin, pyrazinamide, ethambutol, and streptomycin), and for several second-line and newer drugs (ethionamide, fluoroquinolones, macrolides, and nitroimidazopyrans), making these sequences ideal targets for the development of molecular drug susceptibility testing (Abebe et al., 2011; Miller et al., 1994; Musser, 1995; Soini & Musser, 2001; Telenti et al., 1993).

The mainstay for TB diagnostics in endemic developing countries is sputum smear microscopy (Perkins, 2009). However, the sensitivity of this technology is low as it can only detect roughly half of all active cases of tuberculosis when properly used – in people with co-infections and in children the sensitivity is even lower. Moreover, though routinely described as a simple technology, microscopy is actually complex, and highly dependent on the training and diligence of the technician, requiring multiple examinations which may take weeks to complete, with the consequence that many patients drop out during the diagnostic process. Several diagnostic approaches have brought incremental improvements for the direct detection, species identification and drug susceptibility testing of mycobacteria that are capable of reducing the laboratorial time from weeks to a few days (Barnard et al., 2008; D'Amato et al., 1995; De Beenhouwer et al., 1995; Griffith et al., 2007; Hillemann et al., 2005; Hirano et al., 1999; Moore et al., 2006; Park et al., 2002; Rossau et al., 1997; Sharma et al., 2003; Traore et al., 2000). Most of these approaches for direct detection of TB and drug susceptibility from clinical specimens, including several commercial tests, rely on complex and expensive DNA amplification based procedures (e.g. PCR), whereas the need is for affordable, simple and high-throughput systems with the possibility to use small amounts of sample (Cheng et al., 2005; Das et al., 2010; Shamputa et al., 2004; Watterson et al., 1998). These molecular recognition assays still need to make the way to widespread utilization in some technological advanced countries, which will definitely delay the required validation and setup for simplified platforms for general use at more remote and less equipped areas.

Some of the diagnostic tools expected to be introduced into control programs will be incremental improvements on existing technologies while others will be radically new. The speed and extent of adoption of new technologies will depend on the balance between the benefits they bring and the degree of disruption their implementation causes. For instance, a simplified microscopy method may see greater adoption than a novel alternative that necessitates changes in the way testing or case notification are carried out. On the other hand, a new method that rapidly identifies all smear-positive and many smear-negative cases might, if suitably robust and specific, see widespread use and could substantially replace microscopy. Point-of-care diagnosis is instrumental to TB control because, despite having the necessary treatment, strategies in some regions are rather ineffective (see South Africa as an example). Identifying new cases very quickly and getting patients immediately on to treatment are crucial in addressing this pandemic. New diagnostic tools for drug resistant TB (TB that is resistant to drugs – multi-drug resistant TB, MDR-TB) are urgently needed for reducing diagnostic time from months to days.

Several new technologies are under development, which will enable the presumptive diagnosis of MDR-TB in just one to two days, compared with two or more months when using conventional culture and drug susceptibility tests. Rapid diagnosis of MDR-TB will have several benefits: earlier treatment of patients, reduction of time spent on inappropriate and ineffective treatment (thereby promoting the development of further drug resistance), and reduction of MDR-TB spreading in congregate settings.

### 3. Nanodiagnosics for TB

Nanotechnology introduced new paradigms for molecular diagnostics – nanodiagnosics, where the increased sensitivity, specificity, speed and reduced cost constitutes an appealing alternative to conventional techniques (Azzazy et al., 2006). Because we are dealing with nanometer sized objects and/or nanometer scale, bulk matter physics does not apply and amazing and extraordinary new properties arise. This interesting field relies on the knowledge and technological developments emerging from transdisciplinary research efforts that bring together a plethora of expertise from areas as diverse as Materials Science, Physics, Biology and Biotechnology, Chemistry and Medicine. From the intersection of these complementarities new and radical approaches can be explored towards application in platforms for biomolecular recognition (e.g. nucleic acids, antibodies, proteins, etc.) that can be miniaturized for point of care utilization and/or for enhanced portability for small laboratory settings in remote areas without the standard access to conventional laboratory equipment and apparatus. Nanodiagnosics have redefined the standards for molecular diagnostics, triggering the development of new approaches in biomolecular recognition and analytical systems, where the most promising approaches include nanoparticles (NPs), nanotubes, nanopores and nanocantilever technologies (Baptista et al., 2008; Branton et al., 2008; Jain, 2007; Rosi & Mirkin, 2005; Wang et al., 2009). Their potential arises from recognition events occurring at one-to-one interactions between analytes and signal-generating nanostructures, allowing for an increased sensitivity and specificity at lower costs.

Different nanodiagnosics systems have been developed for the molecular diagnostics of TB. Despite the wide range of nanoscale systems being used for biomolecular assays in general (e.g. electromechanical, electrochemical) (Azzazy et al., 2006; Das et al., 2010; Jain, 2007), nanoparticle based systems, such as gold, silver, silica and quantum dots (QDs), have been the most widely used for TB diagnostics due to their unique physicochemical properties, that offer greater sensitivity than conventional reporter molecules and can be easily tuned and functionalized by simple chemistry modulation and derivatization (Azzazy, 2009). Table 1 summarizes existing nanotechnology based systems applied to TB diagnostics.

#### 3.1 Nanoparticles

Nanoparticles are typically in the size range of 1–100 nm and can have different shapes and compositions (Liu, 2006). They are structurally robust and have very specific size dependent properties that differ considerably from those observed on microparticles or bulk materials. Depending on their size and composition they exhibit peculiar properties, such as quantum confinement in semiconductor nanocrystals, surface plasmon resonance (SPR) in some metal NPs and superparamagnetism in magnetic materials (Vollath, 2008). They also provide large

Technology	Description	Application(s)	Reference(s)
<b>Noble metal NPs</b>	Detection relies on the evaluation of SPR change upon aggregation and the concomitant colorimetric changes that can be assessed by the naked eye.	> Specific detection of <i>M tuberculosis</i> complex, <i>M. avium</i> complex, <i>M. avium</i> subsp. <i>paratuberculosis</i> , <i>M. bovis</i> and <i>M. tuberculosis</i> . > Detection of <i>rpoB</i> mutations associated with drug resistance.	Baptista et al., 2006; Costa et al., 2010; Liandris et al., 2009; Silva et al., 2008, 2010; Soo et al., 2009; Veigas et al., 2010
<b>Magnetic NPs</b>	Detection by measurement of the spin-spin relaxation time. Minimal sample preparation needed, without the need for sample amplification.	> High sensitive detection of <i>bacillus Calmette-Guérin</i> .	Kaittanis et al., 2007; Lee et al., 2009
<b>Quantum Dots</b>	Fluorescence detection of inorganic fluorophores with size-dependent optical properties, bright and narrow fluorescence. Highly sensitive. Optimal for multiplex assays.	> Conjugation of streptavidin-coated QDs with specific bacteriophage. > Integration with magnetic NPs for the detection of <i>M. tuberculosis</i> and <i>M. avium</i> subsp. <i>paratuberculosis</i> .	Rotem et al., 2006; Gazouli et al., 2010
<b>Silica NPs</b>	Fluorescence detection of NPs with large quantities of fluorophore molecules inside a polymer or silica matrix. Easy conjugation with several biomolecules and fluorophore making. Ideal for multiplex assays.	> Detection of <i>M. tuberculosis</i> by combining luminescent NPs and indirect immunofluorescence microscopy. > Improved two-color flow-cytometry assay by a combination of the bioconjugated fluorescent silica NPs and SYBR Green I to avoid false positives.	Qin et al., 2007, 2008

Technology	Description	Application(s)	Reference(s)
Electro-chemical devices	Electrochemical nanofabricated sensors. Portable microfluidic nuclear magnetic resonance biosensor for rapid, quantitative, and multiplexed detection of biological targets. Reduced cost of the automated sensitive detection. Ideal for point-of-care applications.	> Specific detection of <i>M. tuberculosis</i> by nanostructured zinc oxide (nsZnO) films. > Amorphous/nanocrystalline biosensor integrated into an optoelectronic platform for the specific identification of <i>M. tuberculosis</i> complex members.	Das et al., 2010; Lee et al., 2010; Wang et al., 1997; Prabhakar et al., 2008;

Table 1. Nanotechnology systems for TB diagnostics

surface to volume ratio with the same size range of biomolecules and cellular organelles, allowing a nearly one-on-one interaction between the NP and the biomolecule of interest (Azzazy et al., 2006, 2007; Jain, 2005) and making them of high potential for use in *in vitro* diagnostics. The most promising NPs already applied to TB diagnostics are gold, magnetic and silica NPs, and QDs. Size-dependent properties of the NPs also enable modification of the surface for conjugation with various biomolecules allowing for a wide range of bioassay applications (Salata, 2004).

### 3.1.1 Noble metal nanoparticles

Noble metal NPs have attracted considerable attention in molecular diagnostic applications due to their simplicity and versatility, becoming a critical component in the development of nanotechnology-based detection of pathogens (Liu, 2006). Gold NPs (AuNPs), in particular, have been extensively used due to their unique optical properties with their typical bright red color in colloidal solutions associated with a well-defined SPR band in the visible region of the spectrum (Halfpenny & Wright, 2010). This SPR is originated from the collective oscillation of conduction band electrons at the NPs' surface induced by the interacting electromagnetic radiation of light. The SPR band is weakly dependent on size of the NP and refractive index of the surrounding media, but changes considerably with the composition, shape and inter-particle distance (Johnson et al., 2007). In the latter case, the aggregation of AuNPs leads to a pronounced color transition from red to blue due to plasmon coupling between NPs (Jain, 2007). Another remarkable property of AuNPs is the easiness of chemical functionalization via the use of thiol-ligands (e.g. thiol-modified oligonucleotides, antibodies or other biomolecules) that form quasi-covalent bonds with the NP's gold surface, rendering gold nanoprobe for specific target recognition (Daniel & Astruc, 2004).

Most AuNPs based methods rely on the colorimetric changes of the colloidal solution upon aggregation either mediated by a change to the dielectric medium or by recognition of a specific target. The design of these systems is centered in the ability of complementary targets to balance and control inter-particle attractive and repulsive forces, which determine

whether AuNPs are stabilized or aggregated and, consequently, the SPR band and color of the solution remains unaltered or changes, respectively. For example, a specific complementary target can hybridize to the gold nanoprobe and promote an inter-particle cross-linking aggregation (e.g. when using two nanoprobe with contiguous target recognition) or stabilize the nanoprobe against the changes of the dielectric medium, which otherwise would induce a non-cross-linking aggregation of the nanoprobe in the absence of a complementary target (e.g. exploring the differential salt induced non-cross-linking aggregation of the nanoprobe) (Baptista et al., 2005).

The first application of AuNPs for the molecular diagnostics of *M. tuberculosis* was introduced by Baptista et al. (Baptista et al., 2006). The method consists in differential stabilization of gold nanoprobe in the presence of different DNA targets. The presence of a complementary target prevents nanoprobe aggregation and the solution remains red, while non-complementary/mismatched targets or their absence do not prevent gold nanoprobe aggregation, resulting in a visible change of color from red to blue. The gold nanoprobe were functionalized with thiol-modified oligonucleotides harboring a sequence derived from the *M. tuberculosis* RNA polymerase  $\beta$ -subunit gene sequence suitable for mycobacteria identification. The methodology was tested in clinical samples demonstrating high efficiency when combined with an initial round of PCR for target amplification (Baptista et al., 2006) – see Figure 1.

The attained results have shown a 100% concordance with the available commercial molecular TB diagnostics test INNO-LiPA Rif.TB. Following optimization towards detection of single base mismatches (Doria et al., 2010), this strategy was applied to the rapid detection of MTBC strains and simultaneous characterization of the presence of mutations associated with rifampicin resistance (Veigas et al., 2010). This low-complexity assay enabled the detection of mutations D516V and S531L from MTBC clinical specimens with remarkable sensitivity in just a few hours. Based on the molecular signatures of MTBC members and the most common mutations associated with RIF resistance in *M. tuberculosis*, a two-step approach based on the PCR amplification of a fragment of *rpoB* gene and subsequent hybridization with specific nanoprobe, namely a probe for the *rpoB* locus shared by all the members of the MTBC and a probe specific to MTBC members, was developed. Three additional sets of probes specific for the most common point mutations associated with RIF resistance (D516V; H526D; S531L) were also designed and synthesized. Each set composed of two probes: one complementary to the wild-type sequence and the other complementary to the mutation. A limit of detection could be set at 75 nM, however, for robust single base mismatch determination, 117 nM of DNA target were used per assay. This non-cross-linking approach correctly detected the presence of DNA from members of the MTBC in 83.3% of all samples, when compared to the INNO-LiPA Rif.TB assay. By means of a set of two probes for each mutation associated to RIF resistance to be screened (mutations in codons 516, 526 and 531 of the *rpoB* gene), it was possible to correctly score the presence of at least one of the mutations in 81% of all samples also screened via the INNO-LiPA Rif.TB assay. Following PCR amplification, the method takes only 90 min to yield a colorimetric result which, through the use of a suitable photodetector (e.g. UV/visible spectrophotometer, microplate reader, etc.), may be used in medium throughput analysis at a peripheral laboratory or point-of-care. Fast and reliable identification of MTBC members and mutations within the *rpoB* gene is of great advantage as it is a secondary

marker for isoniazid resistance allowing to predict, with a high degree of confidence, whether the strain is indeed a multidrug-resistant TB (Hillemann et al., 2005).

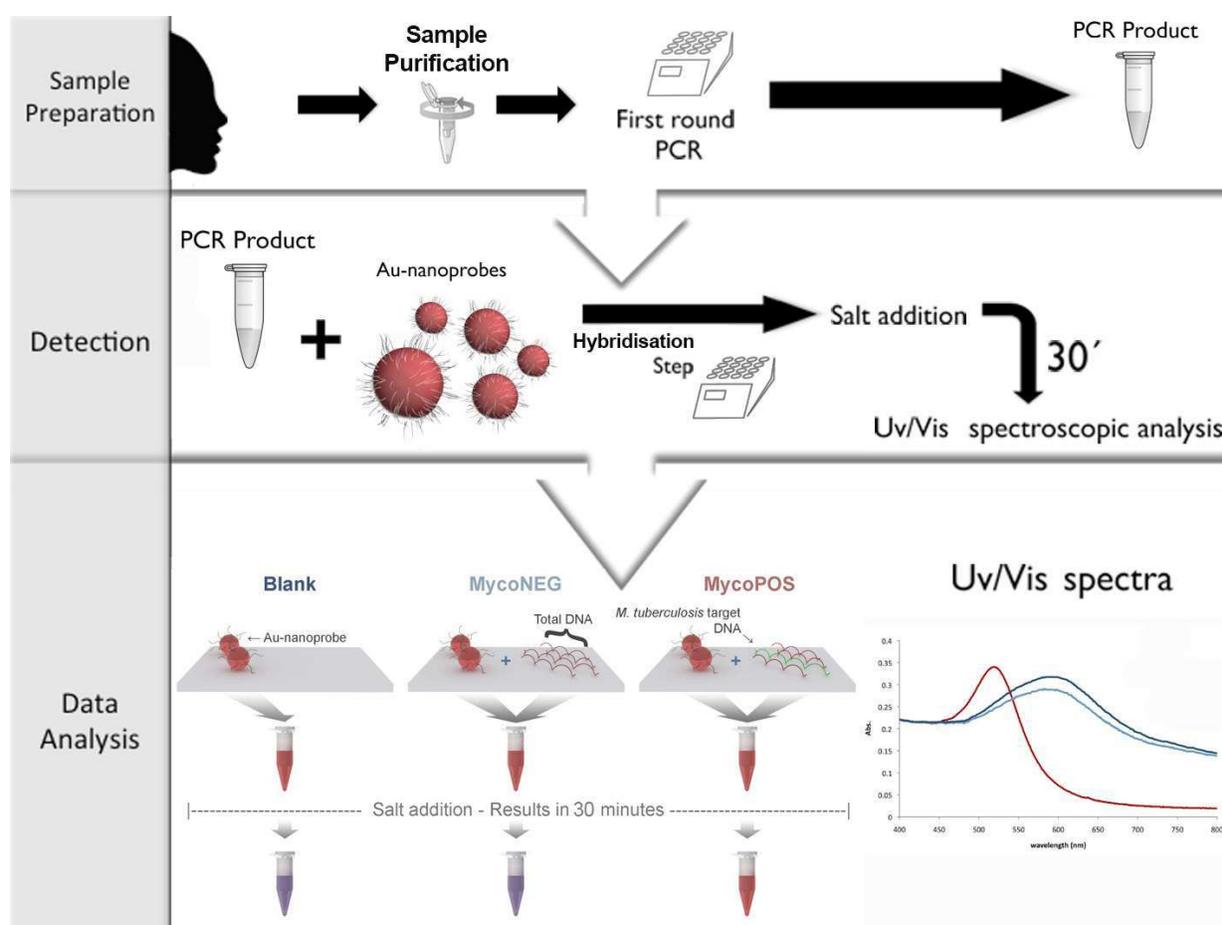


Fig. 1. Non-cross-linking detection of MTBC members. A DNA sample is extracted from a patient and amplified by a first round PCR. The resulting PCR product is characterized using gold nanoprobes and following a non-cross-linking approach that consists of a visual comparison between solutions before and after salt induced nanoprobe aggregation: 'Blank', nanoprobe alone; 'MycoNEG', nanoprobe in the presence of a non-complementary DNA sequence; and 'MycoPOS', nanoprobe in the presence of a complementary DNA sequence.

Optimization of the above strategy allowed detection and identification of members of the MTBC at the species level. Three different nanoprobes based on the *gyrB* locus, allowed the specific identification of MTBC, *M. bovis* and *M. tuberculosis* (Costa et al., 2010). Based on the conserved *gyrB* gene sequence between species from the MTBC, a set of primers was used to PCR amplify a specific 1020 bp fragment of the gene from MTBC species only. *In silico* alignment of the *gyrB* gene sequences showed three regions that allowed discrimination between MTBC members. As proof-of-concept, one probe was designed to identify this genomic region shared by all the members of the MTBC, and two probes were designed to specifically identify *M. tuberculosis* and *M. bovis*, respectively. The MTBC probe positively identified the members of the MTBC used in the assay, while clearly discriminating the non-members. The *M. tuberculosis* and *M. bovis* probes unequivocally identified the respective species. Also, a blind assay using mycobacteria strains isolated from fifteen different clinical

samples showed 100% concordance with the results attained by the *gyrB*-PCR-RFLP method.

Towards a point-of-care application, Baptista and co-workers further integrated the non-cross-linking nanoprobe-based method in an innovative optoelectronic platform that allows an analytical measurement of the colorimetric changes, hence to detect a target without the need of experienced personnel. The device integrates an amorphous/nanocrystalline biosensor and a light emission source with the non-cross-linking method for specific DNA detection. This low cost, fast and simple optoelectronic platform was optimized for the specific identification of MTBC members and the consequent improvement of the laboratorial diagnostics algorithms of TB (Bernacka-Wojcik, et al., 2010; Silva et al., 2008, 2010). The integration of these technologies together with the possibility of miniaturization are of utmost importance for the development of an integrated biosensor suitable for peripheral laboratories and/or point-of-care diagnostics, providing a new tool in the fight against TB.

Recently, Liandris et al. have developed a non-cross-linking approach to the detection of TB without the need of target amplification (Liandris et al., 2009). The method relied on the same non-cross-linking hybridization approach of Baptista and co-workers, whereas the aggregation of the gold nanoprobe was induced by an increasing acid concentration instead of salt. The detection is based on the fact that double and single-stranded oligonucleotides have different electrostatic properties. After hybridization, single-stranded DNA becomes double-stranded DNA. As a result, the double-stranded DNA cannot uncoil sufficiently like the single-stranded DNA to expose its bases toward the gold nanoprobe. Therefore, the nanoprobe undergoes aggregation in an acidic environment. Liandris and coworkers designed an array of gold nanoprobe to collectively detect the main mycobacterial pathogens in clinical samples, namely MTBC, *M. avium* complex and *M. avium* subsp. *paratuberculosis*. A nanoprobe harboring 20 nucleotides was designed to harbor a conserved genus region sequence of 16s–23s ITS DNA of the most common mycobacterial pathogens. In order to obtain an indication of the method's performance on clinical samples, the assay was tested for the detection of *M. avium* subsp. *paratuberculosis* DNA in feces. For this purpose, 12 fecal samples were collected from an equal number of goats from a herd with a well-established record of *M. avium* subsp. *paratuberculosis* and the results were compared to those obtained by a real-time PCR assay. The quantification was performed using *M. avium* subsp. *paratuberculosis* DNA of known concentration, and the standard curve as obtained by real-time PCR. The evaluation of the specificity and repeatability of this non-cross-linking approach indicated a reliable and highly specific detection of a broad spectrum of mycobacteria without cross reactions with related bacteria (the concordance of the two methods with connection to real-time PCR positive and negative sample was defined respectively as 87.5% and 100%). Moreover, the methodology demonstrated to be highly sensitive, where even the lowest concentration of the targeted sequence was easily detected by simple visual observation of the test and the control tubes (Liandris et al., 2009).

Following a cross-linking approach, Soo et al. designed a set of gold nanoprobe to specifically hybridize with target DNAs of MTBC and *M. tuberculosis* strains (Soo et al., 2009). The nanoprobe were oriented in a tail-to-tail arrangement, one probe functionalized via a thiol moiety located at the 5' end of the sequence and other at 3' end, with both sequences being contiguous to each other. This way the hybridization of the nanoprobe

with the complementary target resulted in the formation of a polymeric cross-linked network, bringing the AuNPs close enough to induce a color change from red to blue (Beermann et al., 2007; Li et al., 2006; Liandris et al., 2009; Storhoff et al., 2005). The efficacy of such cross-linking assay was evaluated by analyzing sputum specimens. Results were compared with traditional culture and biochemical identification methods together with patients clinical assessments. The detection limit of this assay was measured using IS6110 DNA amplified from *M. tuberculosis* H37Rv chromosome. This methodology was able to detect as low as 0.5 pmol of DNA target within two hours. The assay comprises two main steps, namely, the target DNA amplification by single or nested PCR, followed by nanoprobe detection. The gold nanoprobe is added to the heat denatured PCR products, and incubated at 55°C for DNA hybridization with increased stringency. In the presence of complementary DNA the nanoprobe aggregated upon hybridization to the target, resulting in decrease in absorbance of the solution at 525 nm. On the other hand, the color and absorbance pattern did not change when specific complementary target DNAs were absent in the solution. The methodology was evaluated by directly and simultaneously detecting MTBC and *M. tuberculosis* from 600 clinical strains and comparing the results with those from conventional culture methods and biochemical identification in combination with clinical assessment. The assay presented 96.6% sensitivity and 98.9% specificity towards detection of MTBC, and 94.7% sensitivity and 99.6% specificity for detection of *M. tuberculosis*.

### 3.1.2 Magnetic nanoparticles

Magnetic properties are largely dependent on the composition and molecular structure of the NPs (Lu et al., 2007). Different materials can exhibit diamagnetic, paramagnetic or ferromagnetic behavior (Sato et al., 2003). In most cases, the particles range from 1 to 100 nm in size and may display supermagnetism when the thermal energy is enough to change the direction of magnetization of the NPs (Neuberger et al., 2005). Superparamagnetic NPs made of magnetic materials (e.g. iron, nickel, cobalt, or alloys of magnetic metals) are preferred for biomedical applications, due to the fact that they behave non-magnetically when they are not under the influence of an external magnetic field, thus preventing undesired self-magnetic agglomeration. In the presence of an external magnetic field gradient, the large magnetic moments of all the atoms align with the field and the superparamagnetic NPs can be manipulated to interact with different biomolecules (Jain, 2007). Removing the external magnetic field causes the NPs to lose their alignment with the field and relax into random directions of magnetization. To make the superparamagnetic NPs biocompatible, they are coated with a material such as silica or polyethylene glycol, and then functionalized with the relevant targeting biomolecule for the desired application, such as antibodies, proteins or oligonucleotides (Jain, 2005). As an example, superparamagnetic NPs have been used in the development of a magnetic immunoassay. The presence of a target analyte allows the superparamagnetic NPs to bind to a magnetic sensor in a sandwich conformation, which creates a local magnetic field that is detected by the sensor once an applied external field is used to induce a magnetic moment in the superparamagnetic NPs, (Sato et al., 2003).

Kaitanis and coworkers designed superparamagnetic iron oxide nanoprobe coupled with a magnetic relaxation methodology to detect *Mycobacterium avium* spp. *paratuberculosis*, in

milk and blood (Kaittanis et al., 2007). The methodology could quickly quantify the bacterial target with high sensitivity, and was not susceptible to interferences caused by other bacteria. The principle underlying the detection by these nanosensors is their ability to change between disperse and clustered (or assembled) state upon target interaction, with a concomitant change in the spin-spin relaxation time of the solution's water protons. This approach, apart from sensitive and fast, is independent of the sample's optical properties and requires minimum sample preparation. More recently, Lee et al. developed a very similar methodology where bacteria were targeted by highly magnetic NPs with a large Fe core and a thin ferrite shell NPs, concentrated into a microfluidic chamber, and detected by nuclear magnetic resonance (Lee et al., 2009). The clinical utility of this diagnostic platform was evaluated by detecting TB using the *bacillus* Calmette-Guérin (BCG) as a surrogate for *M. tuberculosis*. Following liquefaction, the samples were subjected to standard TB diagnostic tests, namely culture and acid-fast bacilli smear microscopy, to be compared with the magnetic NPs-based nuclear magnetic resonance measurements. This methodology shown similar results to those attained with the standard culture-based methods (detection limit of ~20 colony forming units) with the advantage to be less prone to human error and less labor-intensive. The nuclear magnetic resonance-based detection was much faster (< 30 min) and performed on a single microfluidic chip, markedly contrasting with the culture-based test that was time-consuming (> 2 weeks) and facility-dependent (e.g. incubators).

Both these methodologies are in their first stages of development and present great advantages over current techniques such as speed, easiness of procedure and minimum sample preparation.

### 3.1.3 Quantum dots

Quantum dots are inorganic fluorophores with size-dependent optical properties, exhibiting strong light absorbance, bright and narrow symmetric emission bands and high photo stability, due to three-dimensional quantum confinement effects (Chan et al., 2002; Yezhelyev et al., 2006). The size and composition of QDs determine their emission wavelength and color (Coto-García et al., 2011; Michalet et al., 2005; Sukhanova & Nabiev, 2008). Moreover, the QDs can maintain these properties upon conjugation to biomolecules (Alivisatos et al., 2005; Fortina et al., 2005; Salata, 2004) making them preferred fluorescent probes for imaging applications (Halfpenny & Wright, 2010). The fact that QDs do not depend on the presence of a variety of different fluorescence dyes also allows their application for multiplexing analysis (Azzazy & Mansour, 2009). Although QDs are typically insoluble in water, they can be made biocompatible by several strategies including silanization and coating with a polymer shell, thus enabling their utilization in biological systems. Target specificity is achieved by conjugating them to a variety of biomolecules, such as antibodies, streptavidin and oligonucleotides, enabling their application in conventional molecular biological methodologies, such as fluorescent *in situ* hybridization (FISH), immunological assays or northern/southern/western blots (Michalet et al., 2005). In fact, QDs have already been used in a number of biological applications including studies of protein trafficking, DNA detection and dynamic studies of cell mobility (Zrazhevskiy et al., 2010). Rotem and coworkers reported the use of QDs for the detection of pathogenic bacteria combining *in vivo* biotinylation of engineered host-specific bacteriophage with the conjugation of the phage to streptavidin-coated fluorescent QDs (Rotem et al., 2006).

Although the proof of concept only used a single phage–host system, the method may be expanded for the detection of multiple bacterial strains by their specific phages. This concept could be applied to any slow growing pathogen, such as *M. tuberculosis* for TB diagnostics.

Only recently QDs have been used for the detection and imaging of respiratory pathogens and, in particular, for TB diagnostics. Gazouli and coworkers developed and evaluated a detection assay for specific DNA sequences combining fluorescent semiconductor QDs with magnetic beads allowing for a fast identification of two members of the *Mycobacterium* genus (*M. tuberculosis* and *M. avium* subsp. *paratuberculosis*) without the need for DNA amplification (Gazouli et al., 2010). The assay involves two biotinylated oligonucleotide probes to recognize and detect specific complementary mycobacterial target DNA through a sandwich like hybridization. Five 30-bp-long genus-specific probes were designed for the detection of *Mycobacterium* based on the 23S rRNA gene, which is highly conserved among the mycobacterial species. For the detection of *M. tuberculosis* and *M. avium* subsp. *paratuberculosis*, 2 sets of five 30-bp-long probes were designed based on IS6110 and IS900, respectively. Cadmium selenite QDs conjugated with streptavidin and species-specific probes were used to produce a fluorescent signal, while the magnetic beads conjugated with streptavidin and genus-specific probes were used to isolate and concentrate the DNA targets. The minimum detection limit of the assay was defined to be 12.5 ng of DNA diluted in a sample volume of 20  $\mu$ l. In order to obtain an indication of the method's performance with clinical samples, the system was compared with conventional diagnostics methodologies, namely Ziehl-Neelsen staining and real-time PCR. Additionally, to assess the performance of the assay with clinical material, DNA isolated from bronchoalveolar lavage samples, formalin-fixed paraffin-embedded tissues or feces was used for the detection of *M. tuberculosis* and *M. avium* subsp. *paratuberculosis*. With regard to *M. tuberculosis*, the assessment relied on DNA isolated from bronchoalveolar lavage samples from 48 patients with clinical tuberculosis and 12 bronchoalveolar lavage samples from healthy individuals, both confirmed by culture and real-time PCR, with the exception of a bronchoalveolar lavage sample that reacted negatively by the latter method. The overall concordance of this assay was 84.61% and 100% with regard to positive and negative results, respectively. This approach of capturing and detection in two steps by different building blocks minimizes false-positives associated with low specificity. Given that the capture and detection probes of the QD assay are complementary to different genes of the mycobacterial genome, the chances of false-negative results due to DNA fragmentation are inevitably increased. Nonetheless, this weakness can be circumvented by the use of a different set of DNA probes that anneal closer to each other, allowing an assessment that minimizes false-positive results associated with low specificity (Gazouli et al., 2010). Additionally, the method avoids the drawback of PCR-based diagnostic assays that are prone to false-negative results generated by inhibitors commonly found in clinical samples such as feces.

### 3.1.4 Silica nanoparticles

Fluorescence-based detection techniques have been extensively used in both biological research and clinical diagnostics, due to the extremely high sensitivity. Dye-doped silica NPs contain large quantities of dye (fluorophore) molecules inside a polymer or silica matrix, amplifying the fluorescence of each interaction event. This signal enhancement

facilitates ultrasensitive analyte determination otherwise undetectable with conventional fluorescence labeling techniques. Furthermore, the polymer and silica matrix serves as a protective shell, reducing photo-bleaching (Zhao et al., 2007) and allows a high versatility towards different surface modification protocols (Tan et al., 2004). These silica NPs are also more hydrophilic, biocompatible and not subject to microbial degradation, swelling or porosity changes with varying pH (Jain et al., 1998). Several systems based in silica NPs have been widely applied in biological imaging and ultrasensitive bioassays, including cell staining (Santra et al., 2001a), DNA detection (He et al., 2006; Zhao et al., 2003), cell surface receptor targeting (Her et al., 2006; Santra et al., 2001a, 2001b, 2004), and ultrasensitive detection of pathogens (Zhao et al., 2004). Moreover, these NPs can also be conjugated with QDs in a core shell like structure, taking advantage of the capabilities of QDs and taking advantage of silica chemistries versatility, while reducing toxicity (Qin et al., 2007).

The first application of dye-doped silica NPs for TB molecular diagnostics was published by Qin and co-workers, where a rapid immunological method combining highly luminescent RuBpy-doped silica NPs with indirect immunofluorescence microscopy allowed for detection of *M. tuberculosis* in both mixed bacterial and sputum samples (Qin et al., 2007). Later on, this approach was improved by using two-color flow-cytometry and adding SYBR Green I to avoid false positives (Qin et al., 2008). Briefly, *M. tuberculosis* is first recognized by the antibody-conjugated RuBpy-doped silica NPs, and then stained with a nucleic acid dye SYBR Green I to discriminate bacterial cells from background particles, followed by multiparameter determination with flow-cytometry. This way, the population of *M. tuberculosis* dual stained with antibody-conjugated RuBpy-doped silica NPs and SYBR-I could be discerned as a distinct population and the false positives caused by aggregates of NP-bioconjugates and nonspecific binding of NP-bioconjugates to background debris could be decreased dramatically, when compared with the initial one-color approach. Moreover, the decrease of false positives also allowed achieving a higher sensitivity for detection of *M. tuberculosis*. This later dual-color approach allowed for detection of TB in buffer and spiked urine, with higher sensitivities than the conventional flow cytometry techniques, maintaining the same simplicity, speed and usability.

### 3.2 Nano-fabricated devices

Several biosensors for the determination of short sequences from the *M. tuberculosis* DNA have been described (Buijters et al., 2008; Csako, 2006; McGlennen, 2001). Wang and co-workers developed a sensor that relies on the modification of the carbon-paste transducer oligonucleotide probe and their hybridization to complementary strands from the *M. tuberculosis* DNA (Wang et al., 1997). Chronopotentiometry was employed as transducer and the sensor allowed detection down to nanograms per milliliter of *M. tuberculosis* DNA. Prabhakar et al. used cysteine modified NH<sub>2</sub>-end peptide nucleic acid probes and 5'-thiol end labeled DNA probes immobilized onto BK-7 gold coated glass plates for specific detection of *M. tuberculosis* using SPR (Prabhakar et al., 2008). More recently, Das and co-workers developed nanostructured zinc oxide films on conducting indium-tin-oxide coated glass plate to immobilize a DNA probe and specifically detect *M. tuberculosis* based on the strong electrostatic interactions between ZnO and the complementary target (Das et al., 2010). The presence of nanostructured ZnO films allowed to increase the electro-active

surface area for DNA molecules loading and detect genomic target DNA up to 100 pM, which enables the direct detection of pathogens in clinical samples at point of care.

These nanofabricated sensors have the capability of reducing the costs of the automated sensitive detection, making them ideal for point-of-care applications. Moreover, these platforms demonstrate the most promising trends in bioanalytical and biochemical methods, the fusion of different approaches, methods and technologies into a single platform.

### 3.3 Nano-electromechanical devices – nanocantilevers

Nanocantilevers are one of the most promising nanotechnologies for identification of biomolecules capable of providing label-free detection with high sensitivity and specificity (Craighead, 2007). These systems can operate either statically, by measuring absolute cantilever deflection, or dynamically, by measuring resonance frequency shifts. In the *static mode*, the main parameter measured is the differential surface stress produced when molecular adsorption is produced on one side of the cantilever (Fritz et al., 2000). In *dynamic mode*, sensing relies on the observation of the dynamical properties of a resonant cantilever (e.g. vibration amplitude, resonance frequency), which has been increasingly applied for mass sensing (Craighead, 2007; Waggoner & Craighead, 2007). Sensors based on static operation have demonstrated their potential for selective detection of DNA and proteins in liquid. Mass sensors based on dynamic mode can potentially achieve sub-femtomolar sensitivity (Llic et al., 2005). The ultrahigh mass sensitivity is counterbalanced with a very low selectivity due to the device contamination with non-sought molecules and salt debris (Varshney et al., 2008). Thus, in practice small bio-molecules, such as proteins or oligonucleotides, can be detected at low concentrations. Recent developments merged this nanotechnology with AuNPs as sensitizing agents (Wittenberg & Haynes, 2009). These NPs act as mass enhancers, allowing detection of biomolecules at the femtomolar level and beyond, while maintaining the possibility of performing parallel analyses and working with minute sample volumes. Despite these advantages, to our knowledge, nano-electromechanical systems have not yet been applied to the diagnostics of TB.

## 4. Conclusions

In the last decades we witnessed the development of nanotechnology in isolated fields of research, introducing new and revolutionary approaches for molecular detection. Today the most promising advances are made at the interface, merging two or more technological architectures for new hybrid approaches, circumventing current limitations of each existing techniques for biomolecule analysis protocols. Tremendous advancements in the development and performance of new technological approaches for the rapid diagnostics of TB and prediction of drug resistance have been made in the last few years. The obvious advantages of nanodiagnostics based schemes are their ability to provide results within hours, with increased sensitivities and specificities at a fraction of a cost when compared to conventional microbiological and molecular biology methodologies, such as sputum smear microscopy and nucleic acid amplification based techniques. Nevertheless, thus far, only a very small number of these new nanodiagnostics platforms have been translated to the clinical setting for TB molecular diagnostics. The great efforts put into the development of proof-of-concept approaches most of the time lack the connection and the robustness to

make an impact in the analytical laboratory and very few techniques are available for direct application in respiratory specimens (Lee et al., 2009, 2010). It is expected that in the next few years, some of the strategies depicted throughout this short chapter can take their rightful place at the front line of fighting TB.

Future trends in nanodiagnostics will continue through miniaturization of biochip technology to the nanoscale range for point-of-care diagnostics with a sample-in answer-out approach that hampers user-error, thus enabling their use by non-specialized personnel.

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## 6. References

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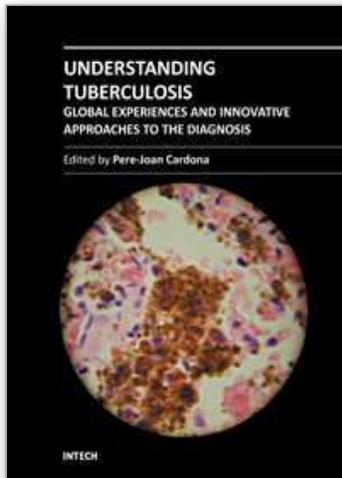
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## **Understanding Tuberculosis - Global Experiences and Innovative Approaches to the Diagnosis**

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Mycobacterium tuberculosis is a disease that is transmitted through aerosol. This is the reason why it is estimated that a third of humankind is already infected by Mycobacterium tuberculosis. The vast majority of the infected do not know about their status. Mycobacterium tuberculosis is a silent pathogen, causing no symptomatology at all during the infection. In addition, infected people cannot cause further infections. Unfortunately, an estimated 10 per cent of the infected population has the probability to develop the disease, making it very difficult to eradicate. Once in this stage, the bacilli can be transmitted to other persons and the development of clinical symptoms is very progressive. Therefore the diagnosis, especially the discrimination between infection and disease, is a real challenge. In this book, we present the experience of worldwide specialists on the diagnosis, along with its lights and shadows.

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Unit 405, Office Block, Hotel Equatorial Shanghai  
No.65, Yan An Road (West), Shanghai, 200040, China  
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元  
Phone: +86-21-62489820  
Fax: +86-21-62489821