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Emerging nanotechnology-based strategies for the identification of microbial pathogenesis ☆

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ABSTRACT

Infectious diseases are still a major healthcare problem. From food intoxication and contaminated water, to hospital-acquired diseases and pandemics, infectious agents cause disease throughout the world. Despite advancements in pathogens' identification, some of the gold-standard diagnostic methods have limitations, including laborious sample preparation, bulky instrumentation and slow data readout. In addition, new field-deployable diagnostic modalities are urgently needed in first responder and point-of-care applications. Apart from compact, these sensors must be sensitive, specific, robust and fast, in order to facilitate detection of the pathogen even in remote rural areas. Considering these characteristics, researchers have utilized innovative approaches by employing the unique properties of nanomaterials in order to achieve detection of infectious agents, even in complex media like blood. From gold nanoparticles and their plasmonic shifts to iron oxide nanoparticles and changes in magnetic properties, detection of pathogens, toxins, antigens and nucleic acids has been achieved with impressive detection thresholds. Additionally, as bacteria become resistant to antibiotics, nanotechnology has achieved the rapid determination of bacterial drug susceptibility and resistance using novel methods, such as amperometry and magnetic relaxation. Overall, these promising results hint to the adoption of nanotechnology-based diagnostics for the diagnosis of infectious diseases in diverse settings throughout the globe, preventing epidemics and safeguarding human and economic wellness.

Contents

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Nomenclature

AFM	atomic force microscopy
Ag NPs	silver nanoparticles
AIDS	Acquired Immunodeficiency Syndrome
Au NPs	gold nanoparticles
CTAB	cetyl trimethylammonium bromide
D-Ala	D-alanine-terminated peptidoglycan
D-Lac	D-lactate-terminated peptidoglycan
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
FISH	fluorescence *in situ* hybridization
FRET	fluorescence resonance energy transfer
HAADF-STEM	high angle annular dark field scanning transmission electron microscopy
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
K_d	equilibrium dissociation constants
LC–MS	liquid chromatography mass spectrometry
MALDI-TOF-MS	matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry
MAP	*Mycobacterium avium* spp. *paratuberculosis*
MDR-TB	multidrug-resistant *Mycobacterium tuberculosis*
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MRSA	methicillin-resistant *Staphylococcus aureus*
MudPIT	multidimensional protein identification
NIR	near infrared
OD	optical density
PCR	polymerase chain reaction
PDMS	polydimethylsiloxane
Qdots	quantum dots
R2	spin–spin relaxivity
RAPID-PCR	random-primed polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SQUID	superconducting quantum interference device
STEM	scanning transmission electron microscopy
T2	spin–spin relaxation time
UV	ultraviolet
UV–vis	ultraviolet–visible
XDR-TB	extremely drug-resistant *Mycobacterium tuberculosis*
ΔT2	change in spin–spin relaxation time

1. Introduction

Infectious diseases cause significant human pathogenesis and mortality throughout the world, surpassing cardiovascular diseases and cancer [1]. Although affluent developed countries have made great progress in sanitation and technological advances to identify and control most infectious diseases, problems remain with food contamination, hospital-acquired pathogens, and sexually transmitted diseases [2,3]. In poor developing countries and even in rural areas of developed countries, infectious diseases are a major problem mainly because of not only poor sanitation but also the lack of efficient technologies to identify and treat these conditions in a timely manner [2,3]. Furthermore, additional transmission routes involving mosquitoes, co-habitation in close contact with infected animals and contaminated water, socioeconomic trends and political instability of several developing nations are additional factors that synergistically contribute to the spread of infectious diseases [4]. Thus, improving the living conditions and diagnostic protocols in poor rural areas is critical in controlling the spread of disease before becoming a worldwide pandemic. Also, as modern global traveling facilitates the spread of the disease faster than ever, developing fast, simple and accurate methods to identify infectious diseases is of timely importance.

Infectious diseases are caused by contagious agents (pathogens) that are capable of inducing disease with symptoms that can be manifested within a couple of minutes, or after a couple of hours to days or even years after the initial infection. These pathogenic agents are subject to transmission from either an infected individual or vector (such as ticks, birds or pigs) to a healthy individual [4]. The complexity and broad range of pathogens that cause disease, in addition to the prolonged incubation time of some of these agents before clinical symptoms of the disease are present, make the diagnosis of some of these conditions even more challenging. Pathogens that cause disease can be listed within various groups, such as bacteria, viruses, fungi, protozoa, parasitic worms, and prions. Their unique characteristics, ways of transmission, as well as any associated disease biomarkers, such as toxins, antigens and nucleic acids are listed in Table 1. The diversity of these pathogens resides not only on the nature of the disease they inflict in the host, but also in their size and shape (see also Scheme 1).

Nanotechnology presents a great opportunity to develop fast, accurate and cost effective diagnostics for the detection of pathogenic infectious agents [5,6]. Due to the presence of unique properties in nanoscale materials, devices able to report the presence of a pathogenic agent in clinical or environmental samples can be designed. The properties observed in nanomaterials are different from those observed in the bulk (micron-size) material due to their small size (1–100 nm) and large surface area, resulting in enhanced surface reactivity, quantum confinement effects, enhanced electrical conductivity and enhanced magnetic properties, among others [6]. Most importantly, modifications of the nanostructures' surface can alter dramatically some of their properties [7,8]. Hence, a single binding event can be potentially recorded. Because of these phenomena, multiple nanostructures have been engineered to detect particular molecular targets in biodiagnostic applications, including pathogen detection. This article focuses on reviewing some of the most promising nanotechnologies available or under development for the detection of pathogens that cause diseases.

2. Current technologies for infectious agent diagnosis and their limitations

2.1. Isolation, growth and microscopy

Traditionally, the presence of most pathogens such as bacteria, fungi, protozoa and worms is determined microscopically, usually after growth in pure culture. Typically, a sample from the infected individual is taken and observed in the microscope for the presence of the pathogen. For bacteria or fungi, subsequent confirmation is based on the growth patterns in differential media and via additional biochemical tests. These methods, although highly specific, have several limitations. First, microscopy-based methods work with samples containing a high amount of pathogens. Second, growth pattern methods usually require

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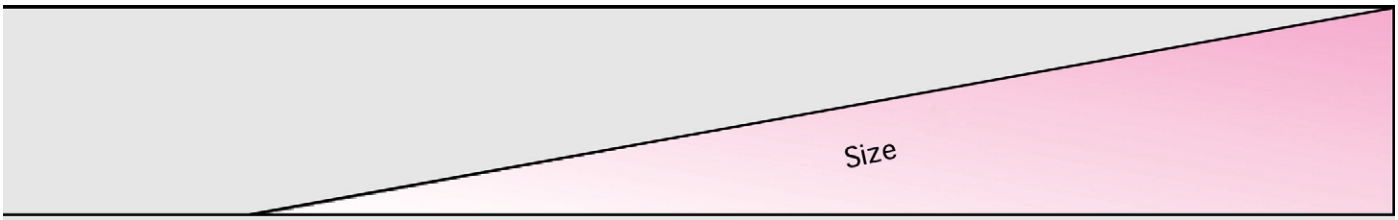
Table 1
Typical infectious disease agents. Differences in size, morphology, infection mode, pathogenesis mechanisms, clinical symptomology and disease highlight the need for development of sensitive and specific pathogen identification modalities in diverse settings.



	Prions	Viruses	Bacteria	Fungi	Protozoa	Parasitic worms
Major characteristics	– Misfolded proteins –No autonomous replication	– Capsid-coated genome – Host-dependent replication	– Unicellular prokaryotes – Autonomous metabolism and replication	– Osmotrophic eukaryotes – Environmental or animal infection	– Single cell –Heterotrophic eukaryotes – Water and soil infection	– Eukaryotes – Residence inside the body
Transmission	– Contaminated food/fluids	– Contaminated blood/body fluids/ aerosols	– Contaminated food/aerosols – Environment – Hospitals	– Environment – Vectors	– Environment – Vectors	– Vectors
Biomarkers	– Misfolded proteins	– Epitopes – Genomic sequences – Circulating antibodies	– Epitopes – Toxins – Genomic sequences – Circulating antibodies	– Toxins – Circulating antibodies	– Epitopes – Proteins	– Epitopes – Proteins
Disease	– Creutzfeldt–Jakob –Scrapie –Bovine spongiform encephalopathy	– Hepatitis – AIDS – Hemorrhagic fever	– Intoxication – Meningitis – Tuberculosis	– Candidiasis – Cryptococcosis – Sporotrichosis	– Malaria – Toxoplasmosis – Dysentery	– Schistosomiasis – Lymphatic filariasis

the growth of the pathogen in a particular medium followed by at least a 24-h incubation period to yield results. Third, as some microorganisms cannot grow easily in culture, their identification is even more challenging. These limitations are even more significant in the identification of viruses that due to their small size (aprox. 100 nm) cannot be studied using conventional optical microscopy and require the use of an electron microscope for their visualization. Finally, the culture and growth of viruses in the laboratory require extensive protocols to grow them before analysis.

2.2. From microscope-based detection to nucleic acids

With the discovery of the DNA and the development of Polymerase Chain Reaction (PCR), microbiologists adopted a molecular-based diagnostic frameset in the last quarter of the 20th century. Specifically, instead of hunting for the microorganism as an entity itself, they started looking for genes and proteins associated with its virulence and disease patterns. Consequently, there was a rise in genome sequencing and the deposition of the annotated genome in databases [4]. Based on this information, DNA microarrays have been developed, identifying DNA segments corresponding to an organism's genome. These methodologies are highly sensitive and selective, achieving detection down to the single pathogen. Despite this, the major limitation of the gene chip is that it cannot provide critical information about a pathogen's specific RNA (transcriptional) and protein (translational) levels. Furthermore, it cannot address how these parameters are modulated by factors and processes that may alter the microorganism's growth, as well as how the host's immune response affect the microorganism's expression patterns at the transcriptional and translational levels. Apart from PCR, several other molecular diagnostic methods have been introduced, such as RAPID-PCR, checkerboard hybridization, ligase chain reaction, ribotyping using restriction length polymorphisms, and pulsed-field gel electrophoresis [4]. Despite their distinct advantages, all of these methods require undamaged microbial DNA and have to be performed in a laboratory setting by experienced personnel and expensive instrumentation and reagents. Therefore, the associated cost of these molecular diagnostic modalities is high enough, prohibiting their wide-scale use at the points-of-care and in developing nations.

2.3. Detection of pathogen markers with antibodies

In addition to microscopy- or nucleic acid-based techniques, the identification of a pathogen can be achieved via immunoassays targeting specific proteins or carbohydrate moieties unique to the pathogen. Such methods include, agglutination tests, fluorescent immunoassays, enzyme-linked immunosorbent assays (ELISA) and Western blot analyses [4]. Although these methods are very sensitive, they detect a pathogen's molecular 'fingerprints', such as proteins and cell wall epitopes. Hence, as these assays do not provide any direct information about the presence and metabolic state of a microorganism in the sample, the administration of suitable antibiotic or other therapeutic agents cannot be achieved. Additionally, as these methods regularly utilize an antibody for the recognition of bacterial targets, proper handling and storage is needed in order to prevent antibody

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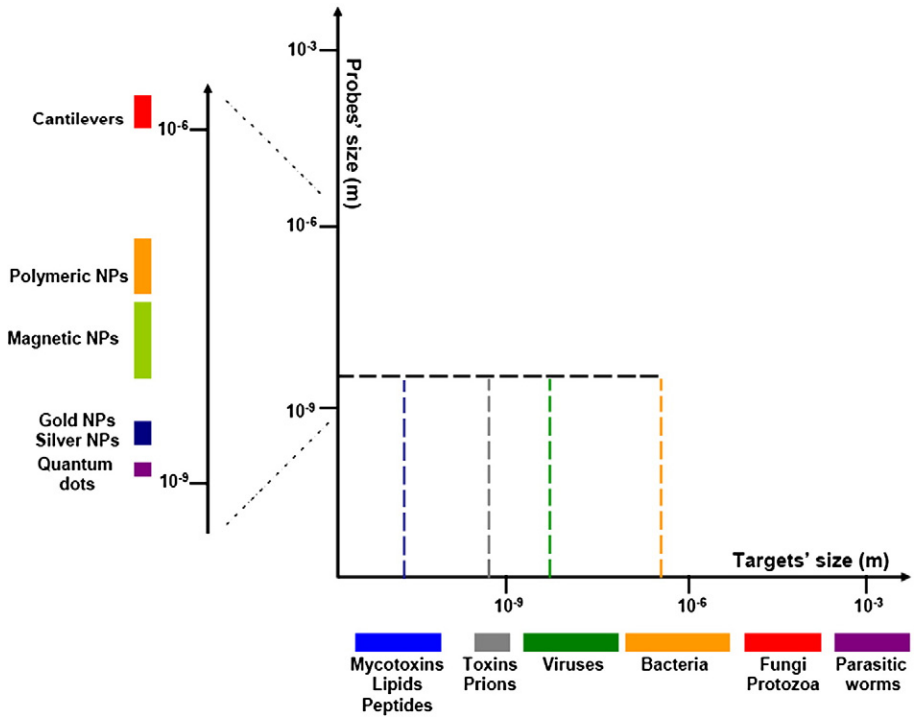

Scheme 1. Size distribution of widely used nanosystems in comparison with the most common types of infectious disease agents. A particular nanoparticle (represented by a black horizontal dashed line) could interact differently with targets of various sizes, such as peptides, toxins, viruses and bacteria (blue, grey, green and orange dashed lines, respectively). In this particular case, the nanoparticle has a size of 100 nm, whereas virulence factors and disease markers are smaller (e.g. lipids, peptides, DNA, toxins), and pathogens can be of roughly equal (e.g. most viruses) or bigger size than the nanoparticle (e.g. bacteria, fungi, etc.). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

denaturation. Furthermore, these assays are affected by the clinical sample's nature, because the specimen, for instance blood, often times needs to undergo processing before being analyzed. Similarly, flow cytometry [9–11] and phage typing [10] face limitations imposed by the sample's characteristics, as optically opaque or viscous samples can interfere with the readout, plus the assay's portability is hindered due to the bulky instrumentation. Likewise, magnetic microbeads have been used for the magnetic separation and identification of microbes through PCR and flow cytometry [11,12], yet these methods require several intermediate steps and have limited capabilities in complex biological samples.

Pathogenesis caused by intracellular pathogens, such as *Mycobacterium tuberculosis* among others, relies on the survival of the microorganism within host cells, such as macrophages, dendritic cells and T helper cells [13]. This mode of infection and the frequent absence of clinical symptoms hamper these pathogens' detection and prevent treatment and containment. Consequently, the physician cannot proceed to the assignment of an appropriate treatment course, delaying the clearance of the pathogen from the body. Current tests cannot detect intracellular pathogens in biological fluids, such as the blood and lymph fluid, as most of these tests are based on fluorescence emission in the absence of optical interference. Hence, the need for developing optical-independent diagnostic modalities is pivotal for the detection of intracellular pathogens in body fluids and biopsies, with no or minimal sample preparation steps. Furthermore, these modalities should be able to facilitate multi-sensing capabilities, detecting not only the microorganism per se, but genomic fragments and protein markers. This can corroborate bacterial identification and prevent misdiagnosis due to cross-reactivity [4], which is common in serological assays.

2.4. Detecting toxins — approaches for a challenging task

Toxins are virulence factors that affect the host cell's physiological processes and plasma membrane integrity, causing cytotoxicity [14]. For instance, toxins, like listeriolysin O from *L. monocytogenes*, create pores in and disrupt the phospholipid bilayer, causing host cell's lysis [15]. Other toxins, such as Shiga toxin, inhibit protein synthesis, which in turn activate apoptotic and necrotic cascades [16,17]. Furthermore, toxins, like anthrax and botulism, alter the host cell's signal transduction cascades. These alterations initiate the disease state and, ultimately, lead to the host cell's death [18]. Hence through exploitation of numerous mechanisms, bacterial toxins affect host cell's homeostasis and induce cell death, aiming to the release of nutrients needed for microbial survival and colonization. Particularly, intracellular pathogens that have a potent toxin in their virulence repertoire utilize the toxin as a key exit mechanism, a tool for further infection and manifestation within the body.

Even though toxins are not usually transmitted among infected individuals, their devastating effects on tissues and whole organs merit the development of sensitive, fast and reliable diagnostics methods and therapeutics to minimize their harmful effects. Most importantly, toxins might remain in environmental, food or clinical samples long after the corresponding pathogens have been dead. Therefore, screening these samples for the presence of active toxins is highly important to minimize intoxication and economic losses, particularly during a pandemic. Current toxin detection methods utilize antibody–antigen interactions in ELISA [19], Western blots [20,21], antibody microarrays [22], surface plasmon resonance biosensors [23], and antibody-coated polystyrene microbeads [24]. These methods are relatively sensitive and have multiplexing capabilities, but require homogeneous or purified samples. Although time-consuming and laborious, these purification procedures are critical for assay sensitivity and specificity, as they minimize background noise. A major limitation of these assays is their spectrophotometric or fluorimetric toxin determination mode. Thus, limited biological and environmental samples can be screened. To circumvent this obstacle, toxin detection techniques that utilize mass spectrometry, such as liquid chromatography mass spectrometry (LC–MS) [25] and multidimensional protein identification (MudPIT) [26], have been

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developed and achieved high sensitivities. However, the lack of portability and user-friendliness as well as the sophisticated instrumentation, prevent the broader use of these diagnostic methods.

2.5. Limitation of current technologies — the potential of nanotechnology

Conventional molecular diagnostic techniques are widely used in laboratories throughout the world to identify pathogenic agents with high degree of sensitivity and reproducibility. However, most of these techniques cannot be utilized in the field (e.g. airports and food distribution centers) or in developing countries where resources are scarce, because they often require sophisticated, expensive instrumentation that needs to be used by trained personnel. Additionally, the high cost and short shelve half-life of some reagents, such as enzymes and DNA primers, limit the application of most conventional pathogen detection techniques in developing nations. Furthermore, despite their sensitivity, current technologies, like ELISA and PCR, require extensive sample preparation and have long readout times, which delay prompt response and disease containment. Hence, taking advantage of the unique electrical, magnetic, luminescent, and catalytic properties of nanomaterials, faster, sensitive and more economical diagnostic assays can be developed that can assist in the battle against microbial pathogenesis. Apart from striving for sensitivity and speed, nanotechnologists have geared their efforts towards the development of nanotechnology-based systems that are affordable, robust and reproducible, making them suitable for applications even in rural areas of developing nations. For instance, researchers try to formulate cheap and stable nanoparticles via novel facile synthetic routes, making nanoparticle-based diagnostic assays globally accessible and deliverable. Moreover, using innovative approaches, nanotechnology has the potential to build assays that can be performed in opaque media, like blood and milk, without any sample preparation, providing fast and reliable results in simple and user-friendly formats as we describe in the following sections.

3. Available nanotechnologies

Nanotechnology offers many technological advances for pathogen detection. The use of nanoparticles as labels in conjunction with novel detection technologies has led to improvements in sensitivity and multiplexing capabilities [5,6]. Metallic nanoparticles composed of gold or silver have many optical and electronic properties, derived from their size and composition [27]. When coupled to affinity ligands, these nanomaterials have found important applications as chemical sensors. For example, gold nanoparticles conjugated with specific oligonucleotides can sense complementary DNA strands, detectable by color changes [7,8]. Other nanoparticles including fluorescent quantum dots and carbon nanotubes have been used in various applications including DNA detection, and the development of immunoassays for the detection of bacteria and toxins [28–32].

The properties of the nanomaterials used for pathogen detection can be tailored by changing the size, shape, composition and surface modification of the nanomaterial. Particularly, their electronic, spectroscopic (emissive, absorptive), light scattering and conductive properties can be modified by engineering the nanoparticles' structural parameters, including their size, composition, self assembly and binding properties [6]. In addition, recent years have seen an explosion in the development of surface patterning techniques that promise on generating nanoscale arrays of pathogen targeting ligands. These developments could revolutionize the way how one can detect pathogens and infectious diseases. Below, some of the most commonly used nanosystems for the detection of pathogens are described. It should be noted that a particular nanosystem, for example a nanoparticle of 100 nm in size including its coating and targeting ligands, can be designed to sense targets that might be smaller (i.e. lipids), bigger (i.e. bacteria) or of equal size (viruses) to the nanoparticle (Scheme 1). Therefore, it should be expected that this particular nanoparticle would interact differently with targets of different sizes, resulting in different response patterns that are unique to the particular type of interaction that the nanoparticle exhibits with the pathogen.

3.1. Gold nanoparticles

Gold nanoparticles (Au NPs) can be synthesized either in an aqueous or organic environment [33]. Conventional techniques for aqueous synthesis involve the reduction of $AuCl_3$ with trisodium citrate, followed by the addition of a capping agent to stabilize the Au NPs by introducing adequate electrostatic repulsion between individual particles keeping them well dispersed in the medium [33]. Alternatively, organic-based Au NPs synthesis offers excellent control and improved size uniformity [33]. Surface chemistries of Au NPs can be controlled by grafting functionalized organic thiol molecules or thiol-containing polymers, as the gold surface exerts strong affinity towards sulfhydryl groups leading to the formation of relatively strong covalent bonds. Hence, further surface modification can be done simply by using thiolated functional molecules, facilitating conjugation of various probes, including antibodies and nucleic acids. Successful formation of Au NPs is associated with the presence of a surface plasmon band in the preparation's UV–vis absorbance profile. The surface plasmon band arises from the coherent existence of free electrons in the conduction band, due to the small particle size. The band shift depends on the particle size, chemical surrounding, adsorbed species on the surface, and dielectric constant [34]. Changes in the local dielectric constant of the nanoparticles by absorbed biomolecules or the biomarker-induced agglomeration of the nanoparticles cause plasmonic band shifts [34]. Hence, this unique characteristic of gold, as well as silver, nanoparticles allows the use of the surface plasmon band shifts for several diagnostic applications by recording the alterations in the UV–vis absorbance spectrum. Apart from absorption, gold nanoparticles were used as tags for the detection and quantification of numerous targets using fluorescence, Raman scattering, electrical conductivity, atomic and magnetic force techniques [5,6].

3.2. Silver nanoparticles

Silver nanoparticles (Ag NPs) were first used as a new generation of antimicrobial agents to prevent infections [35]. Generally, these nanoparticles can be synthesized using methods yielding spherical, elongated (rod-shaped) or truncated (triangular) Ag NPs [35]. Initially, silver seeds can be prepared by rapidly injecting 10 mM $NaBH_4$ into an aqueous solution of 10 mM $AgNO_3$ and 1 mM sodium citrate and aged for 1.5 h [35]. Spherical silver nanoparticles can be prepared by reducing an aqueous solution of $AgNO_3$ (1 mM) by adding silver seed solution and aqueous solution of sodium citrate, under boiling conditions until the color of the solution becomes greenish-yellow [35]. Similarly, the elongated and triangular shaped silver nanoparticles can be prepared by adding silver seed, 0.1 M ascorbic acid, 0.1 M CTAB and 1 M NaOH solution to the aqueous solution of $AgNO_3$ (10 mM) to accelerate particle growth [34,36]. The *in situ* reduction of Ag^+ ions and introduction of surface functional groups can be achieved in one-pot reactions, using various biopolymers. Other than small reducing agents, such as sodium citrate and hydrazine hydrate, various functional polymers, including poly(acrylonitrile), poly(N-vinyl pyrrolidone), poly(vinyl alcohol), polyacrylic acid and polyacrylamide, were used for the reduction and surface coatings of the silver ions [37]. Hence, these functional Ag NPs can perform conjugation chemistries to connect various targeting ligands, such as proteins, antibodies, peptides, oligonucleotides and small molecules. Ag NPs exhibit a surface plasmon band, usually at 422 nm for spherical nanoparticles, which can be used for colorimetric detection and quantification of targets. Also, these nanoparticles can be used for the microscopic identification of targets using either high angle annular dark field

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(HAADF) or scanning transmission electron microscopy (STEM), and electrochemical approaches.

3.3. Quantum dots

Fluorescent quantum dots (Qdots) are semiconductors with unique optical properties when compared to conventional semiconductors, organic fluorescent dyes and proteins [6]. Qdots can be prepared using a variety of methods ranging from molecular beam epitaxy to electron beam lithography, yet colloidal synthesis is the most common technique for Qdots preparation. Specifically, in the case of hot solution-phase synthesis, periodic group II and VI (CdSe, CdTe, ZnSe, etc) or III and V elements (InP, InAs, etc), are dissolved in a solution with a stabilizing agent or polymer. Thus during the synthesis procedure, these salts enter into a phase where they associate forming core-shell Qdots capped with either a polymer or other stabilizer [38]. Furthermore, a simple 'cap exchange' approach can be used to anchor for instance bifunctional mono- or di-thiols to the Qdot surface, whereas the other functional end-group remains available for further functionalization and conjugation chemistries. Mechanistically, it is the band-gap that determines in which frequencies the Qdot will respond to. Hence, by engineering their band-gap (such as by changing their size), Qdots can emit light in different wavelengths upon excitation, having broad and diverse applications. For instance, simply by modulating their size, Qdots can be excited at a given wavelength and have tunable emission from the ultraviolet (UV) to the near infrared (NIR) region. Apart from being tunable, Qdots are highly bright and extremely photostable, making them suitable for various biomedical applications such as sensing and detection of biomarkers including antigens and pathogens, immunolabeling of cells and tissues, detection of cancer *in vitro* and *in vivo* [28,39–42]. Qdot-based FISH (fluorescence *in situ* hybridization) probes have been widely used for the detection of the Y-chromosome in fixed human sperm cells [43]. Furthermore, Qdot-FRET-based nanosensors mediated the ultrasensitive detection of low concentrations of target DNA in the diagnosis of genetic diseases [44,45].

3.4. Fluorescent polymeric nanoparticles

Polymeric nanoparticles are easily prepared using a linear or branched polymer that promotes the encapsulation of a fluorophore within the nanoparticle's cavity or hydrophobic microdomains [46–48]. Several synthetic routes can be utilized yielding stable monodispersed nanoparticles in aqueous media. The resulting nanoparticles, such as fluorescent silica ones, can be filtered and concentrated. As the presence of the polymeric coating protects the fluorophore, these nanoparticles are stable under diverse conditions [46–48], surpassing organic dyes in photostability and versatility. Additionally the presence of functional groups on the polymer coating, including amines, carboxylic acids and esters, confers facile attachment of probes. Surface functionalization and targeting can be achieved via numerous conjugation chemistries, such as "click" and carbodiimide [49]. Detection is mainly achieved with the use of a fluorescence spectrometer, flow cytometer, and fluorescence-recording microtiter plate reader [46–48].

3.5. Magnetic nanoparticles

Magnetic nanoparticles have been used for a long time in the clinic and the molecular biology laboratories [5,6]. For instance, superparamagnetic iron oxide nanoparticles, composed of a magnetite/maghemite core, have been utilized as contrast agents for magnetic resonance imaging (MRI). Also, magnetic nanoparticles conjugated to antibodies have been used for the immunomagnetic separation of nucleic acids, proteins, viruses, bacteria and cells [5,6]. Iron oxide nanoparticles are primarily synthesized via water-based protocols, involving the alkaline precipitation of iron salts. To enhance water stability and add surface functionality, often times the iron oxide core is coated with polymers, such as dextran, polyacrylic acid and silica. Modulation of the nanoparticles' shape and magnetic properties can be engineered via different strategies, including the time of addition of the polymer, higher temperature and the use of particular capping agents [50]. Further surface modification can facilitate the addition of functional groups, such as amino and carboxylic acids, making subsequent conjugations easy. Hence, iron oxide nanoparticles can carry diverse ligands, such as peptides, small molecules, proteins, antibodies and nucleic acids. Therefore, iron oxide nanoparticles have been used for the identification and quantification of several targets, including mRNA, DNA, viruses, bacteria and cells [50–54]. Furthermore, enzymatic and metabolic activities were monitored with these nanoparticles [55–57]. Detection with magnetic nanoparticles can be achieved with the use of magnetometers or superconducting quantum interference device (SQUID), which record alterations in the magnetic properties of the particles upon molecular interactions with a target [58–60]. Also, detection and quantification can be accomplished with magnetic relaxometers and magnetic resonance imaging (MRI), by monitoring the changes in the spin–spin relaxation time (T2) of the solution's water protons due to nanoparticle association with a target [50–54].

3.6. Nanochips and nanoarrays

Nanochips can be prepared for the fast identification of biomolecules, including toxins and antibodies, using gold or silica nanoparticles supported on thin silicon layers [5,6]. Following sample incubation, the nanochips are subjected to different procedures in order to achieve signal amplification. Due to their small size and use of currently available instrumentation, nanochips can screen samples in a high-throughput format requiring minute sample volumes [5,61]. Furthermore, apart from quantifying a target, nanochips can identify if proteins undergo post-translational modifications, such as proteolytic cleavages and phosphorylation, using matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF-MS) [5].

In addition to nanochips, nanoarrays have been used to further expand the emerging field of DNA arrays [5,6,62]. Through different manufacturing approaches, nanoarrays have been fabricated using inkjet printing, electrochemistry, microfabricated surface patterning, or photolithography with either pre-assembled mask or micromirror-based apparatus [5]. Specifically, the nanoarray can be constructed on diverse surfaces, such as silicon, silanes, hydrogels, metallic and polydimethylsiloxane (PDMS), among others. Hence, detection of a target can be achieved with AFM, surface plasmon resonance, optical waveguides, nanowires, microfluidics and electrical current detectors. The nanoarray can utilize various probes, including antibody, nucleic acid and small molecule, which can be either deposited in 1–30 μm droplets or via fluidics-enhanced molecular transfer operation [62]. In addition to these entities, colloids and Qdots can be further applied leading to the construction of multiplexed nanoarrays capable of screening minute samples in large libraries of potential pathogenic agents or therapeutics.

3.7. Fiber-optic-based biosensors

For the rapid detection of analytes, researchers have modified traditional ELISA plate assays, developing sandwich-format fiber-optic-based platforms [63–65]. Immobilization of a capturing moiety (antibody, aptamer, and ligand) to a fiber optic or capillary tube mediates the capturing of the molecule of interest, whereas a secondary probe (i.e. antibody) conjugated to a fluorophore facilitates target detection. Following fluorophore excitation, the emitted light is recorded by portable instrumentation, which can quantify the target based on the fluorescence intensity [65].

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3.8. Cantilever-based arrays

Due to their high sensitivity, cantilevers can be used for the detection of molecular targets. These cantilevers resemble atomic force microscopy (AFM) tips and can be decorated with nucleic acids, small molecules and antibodies [6]. Upon binding of a target, the cantilever deflects a couple of nanometers, facilitating detection. Since the deflection is proportional to the amount of target binding, cantilever-based arrays are quantitative [6]. Advantages of these arrays include the absence of any enzymatic amplification steps such as PCR, the detection of nucleic acids and proteins without the need of labels (i.e. fluorophores), and the ability to be used in microfluidic setups.

4. Approaches for detection of infectious diseases

4.1. Pathogen identification through surface marker recognition

Pathogens express different markers on their surface to serve a variety of purposes. Receptors, glycoproteins, glycopeptides, lipoproteins, carbohydrates, and lipids participate in host infection, adhesion, immune system evasion, nutrient uptake and transport, among other activities. Despite their wide repertoire of functions, these entities share a common feature; that is, they are exposed to the extracellular milieu. Hence, these biomolecules are attractive pathogen association markers in nanotechnology, as nanoprobes can easily access them without having to cross any biological membranes or barriers, such as the thick peptidoglycan layer. Utilizing this fundamental property, nature and the immune system have evolved clearance mechanisms to recognize these surface markers through highly specific antibody–antigen associations. Acknowledging the high specificity of antibodies towards microorganisms' surface epitopes, nanotechnology has utilized antibodies as targeting ligands in a wide array of diagnostic modalities.

For the fluorescent-based quantification of bacteria, Tan et al. used antibody-conjugated dye-doped silica nanoparticles [46,47]. After a brief incubation with the specimen, any unbound nanoparticles were removed through short centrifugation rounds, and single bacterium detection was achieved in less than 20 min [46]. Apart from detecting a single *E. coli* O157:H7 in processed ground beef samples, other bacterial species were quantified, such as *Salmonella* and *Bacillus*, indicating that this method can be used for both Gram-negative and Gram-positive microorganisms [46]. Although, both the fluorescent nanoparticle quantification method and the gold-standard method of plate-counting yielded similar results, the nanoparticle assay's readout time was faster than that of the plate-counting (16–18 h). Also, an advantage of the nanoparticle method is its' high-throughput capability using a microtiter plate reader, which can achieve bacterial quantification from 1 to 400 bacterial cells. Thus, its clinical utility is significant, especially for the detection of highly infectious agents where exposure to low concentrations may be lethal.

Viruses have also been identified using fluorescent nanoparticles. Specifically, europium(III)-chelate-doped nanoparticles were able to quantify as little as 5000 virions per mL via a sandwich-based immunoassay, utilizing microtiter plate-conjugated capturing antibodies and nanoparticle-bound detecting antibodies [66]. Furthermore, using this method the presence of adenovirus was quantified in nasopharyngeal patient samples, demonstrating that fluorescence is not compromised in slightly heterogeneous matrices [66]. In an interesting approach, Nie et al. have achieved the detection of the respiratory syncytial virus via dual-color emitting quantum dots and fluorescence energy transfer nanobeads [67]. Contrary to other approaches, this design relies on the principle of discrete flight times of photons after excitation of the nanoparticles (Qdots and nanobeads) by a single source [67]. Specifically, nanoparticles that do not associate with a target emit photons that reach the detector at different times [67]. However, nanoparticles that bind to a target emit photons that have identical travel times, as the photons' point of origin is common [67]. Hence, this approach does not require the removal of unbound nanoparticles as these nanoparticles have a discrete state, which can be easily differentiated from that of the nanoparticles interacting with a target. Also, there is no need to conjugate an antibody on a solid surface, providing faster detection kinetics and good assay sensitivity. Despite its novelty and sensitivity, its' clinical and field applicability may be limited as this method utilizes a microcapillary flow system and a fixed-point confocal microscope. In other reports, researchers using a single quantum dot construct have achieved the high-throughput quantification of human T cell leukemia virions, demonstrating the ability of quantum dots to detect targets via readily available fluorescence microtiter readers [68]. Thus, one would anticipate the use of such diagnostic modalities in the field detection of viruses and in the determination of novel antiviral inhibitors. Recently, quantum dot barcodes have been used for the detection of viruses, such as HIV and Hepatitis, via a sensitive handheld diagnostic system [69].

A major challenge for field diagnostics that employ antibodies as targeting ligand is the need to maintain the antibody's structure and prevent thermal denaturation. Considering this challenge, gold nanoparticles and fluorescent π-conjugated polymer constructs have been used for the fluorescent-based identification of microorganisms without the need of antibodies [70]. In this technique, the electrostatic interaction between cationic gold nanoparticles and anionic polymers led to fluorescence quenching [70] (Scheme 2). However, in the presence of bacteria, the negatively charged bacterial cell wall caused displacement of the nanoparticles' polymer [70] (Scheme 2). Hence, the interaction between the nanoparticles and bacteria and the concomitant dissociation of the polymer from the nanoparticles induced the release of the polymer's quenched fluorescence, leading to enhanced fluorescence emission (Scheme 2). A library of three nanoparticle preparations was prepared and distinct fluorescence emission patterns were observed for each organism, including *E. coli*, *B. subtilis*, *L. lactis* and *S. coelicolor* [70]. Subsequent quantitative analysis for pattern recognition through linear discriminant analysis led to the construction of a signature plot, having each microorganism's characteristic fluorescence emission [70]. Consequently, this approach can be utilized for the affordable and robust identification of microorganisms without the need for heat labile antibody-conjugated probes. It should be noted that as this method is at its infancy, the detection threshold was high ($OD_{600} = 1$; 1×10^9 colony forming units). However, with further optimization and the use of more responsive polymeric conjugates that employ a diversified association with the nanoparticles, a higher sensitivity should be achieved.

Although pathogen detection has been predominantly achieved with optical methods, alternative strategies have been sought to detect targets in opaque media and complex matrices. For example, as few as 25 *Salmonella enterica* bacteria were detected using silicon nitride cantilevers [71]. In this case, bacterial detection was achieved by monitoring the cantilever's surface bending, which was directly associated to the amount of bacteria associating on the cantilever [71]. Other researchers have achieved quick and specific bacterial identification using an assay that relies on the specific interaction between bacteriophage and bacteria. Particularly, it utilizes the bacteriophage's ability to infect certain bacteria and not others. Thus bacterial lysis leads to changes in the sample solution's composition, which can be assessed electrochemically through variations in the electric field of nanowell apparatuses [72].

Lastly, among the most innovative pathogen detection methods are those that employ magnetic nanoparticles. In early studies, antibody-carrying magnetic nanoparticles have been used for the detection of a target being immobilized on a mylar film, via superconducting quantum interference device (SQUID) [58]. While any free nanoparticles quickly relaxed by Brownian motion thus not affecting the signal, the nanoparticles that bind to the target undergoes Néel relaxation

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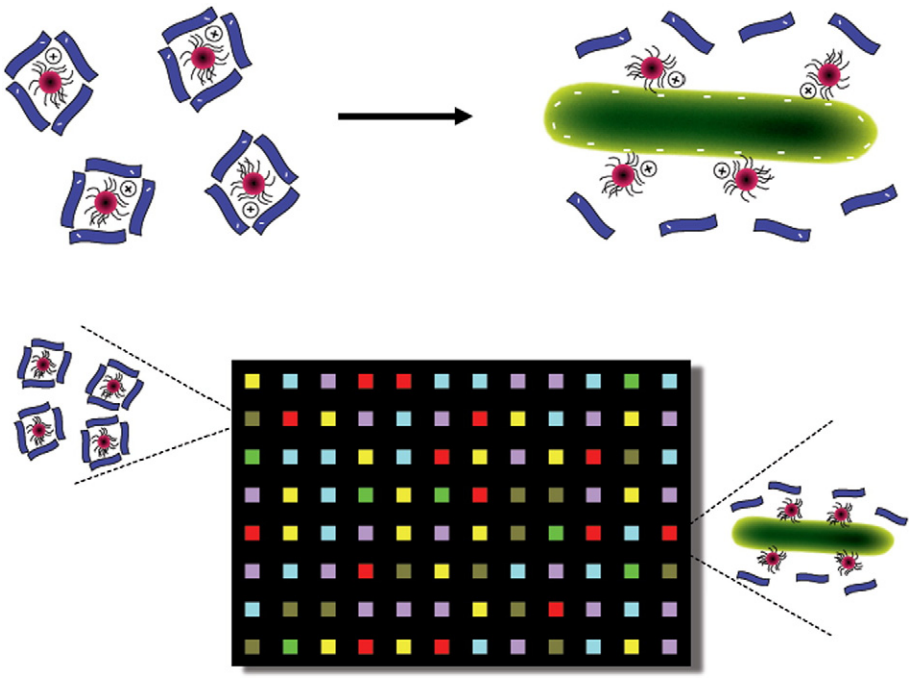
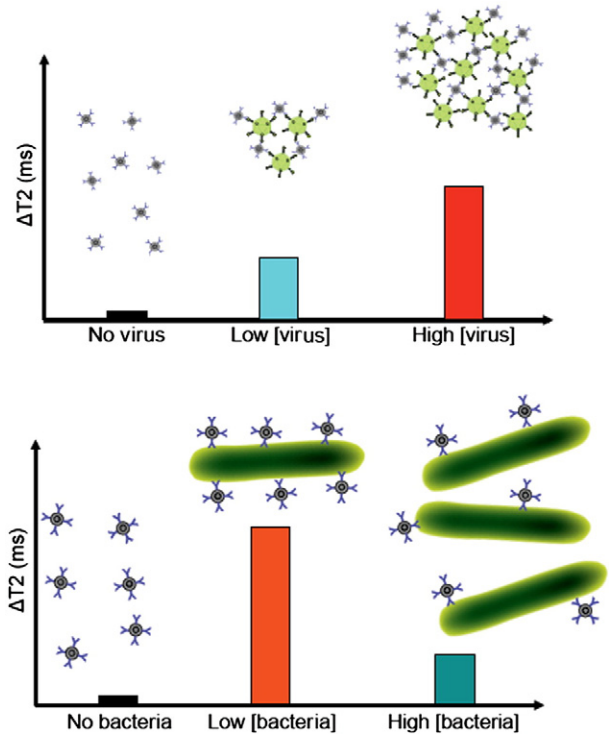


Scheme 2. Fluorescence-based detection of bacteria using cationic gold nanoparticles and anionic fluorescent π-conjugated polymers. In the presence of the bacterial anionic cell wall, there is displacement of the polymer leading to fluorescence emission. Discrete fluorescence emission patterns corresponding to different microorganisms can be obtained in a high-throughput format. Adapted from reference [70].

leading to a gradually dissipating magnetic flux that the SQUID could detect [58]. Thus based on this principle, the food-borne pathogen *L. monocytogenes* has been quantified with a detection limit of 6×10^6 bacteria [59]. Further improvements in SQUID-based detection have been accomplished with the development of immunomagnetic reduction assays [60]. Briefly, these assays' underlying principle relies on the magnetic nanoparticles' differential oscillations in the presence or absence of a target during exposure to ac magnetic fields [60]. Interestingly, in the presence of the avian flu virus the anti-H5N1 magnetic nanoparticles clustered. This specific interaction affected the nanoparticles oscillatory mode, as compared to those of the corresponding negative controls [60]. Additionally, the oscillatory pattern obeyed a target-concentration-dependent pattern, facilitating sensitive H5N1 quantification [60].

Perez et al. have utilized iron oxide nanoparticles that behave as magnetic relaxation switches altering the spin–spin relaxation times (ΔT2) of adjacent water molecules, upon target recognition [53,54]. With the aid of either a benchtop magnetic relaxometer operating at 0.47 T (20 MHz) or an MRI at 1.5 T (60 MHz), antibody-carrying dextran-coated iron oxide nanoparticles were able to quickly detect as little as 5 viral particles of Herpes Simplex or Adenovirus in 10 μL 25% serum samples [54]. However, the sensitivity was slightly affected when detection was performed in 100% serum samples, where the detection threshold was 10 virions per 10 μL [54]. Other molecular targets such as DNA/RNA, proteins, and enzymatic activities (proteases, telomerase and myeloperoxidase) have been detected using this technology. However, all these targets are smaller or of equal size (in the case of a virus) to the nanoparticles used (Scheme 3). Surprisingly, in the presence of targets larger than the nanoparticles, such as bacteria, an interesting phenomenon occurs [51]. Initially, in the absence of any bacteria the nanoparticles are in a dispersed state (Scheme 3). However, addition of even a few bacteria induces the assembly of the nanoparticles on the bacterial surface [51]. This interaction induces prominent changes in the T2, as compared to the corresponding T2 values of the sterile control, allowing bacterial quantification at very low concentrations (low colony forming units) (Scheme 3) [51]. On the other hand, at higher bacterial concentrations, the nanoparticles switch to a quasi-dispersed-like state, which resembles the nanoparticles in sterile control [51]. Hence, at high bacterial concentrations the T2 is proximal to that of the negative



Scheme 3. Detection of viruses and bacteria using magnetic relaxation. An increase in the concentration of the virus facilitates clustering of the nanoparticles and high changes in the spin–spin relaxation times (ΔT2). On the other hand, at low concentrations of bacteria high ΔT2 are obtained due to nanoparticle assembly on the bacteria surface. ΔT2 decreases as the bacterial concentration increases because the nanosensors switch to a dispersed-like state, reminiscent of the one observed in the sterile medium. Adapted from references [54] and [51].

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control (sterile medium) (Scheme 3) [51]. Accordingly, utilizing spherical iron oxide nanoparticles, *Mycobacterium avium* spp. *paratuberculosis* (MAP) was quantified in whole milk and blood within less than 30 min [51]. In the absence of bacterial interference caused by the presence of several other bacterial species (10^6 colony forming units), roughly 16 MAP colony forming units were quantified, whereas under interference conditions the detection threshold was compromised (~39 MAP colony forming units) [51]. Notably, detection and quantification of this bacterium particularly at low concentrations was achieved in blood [51]. Furthermore, this single-step nanoparticle assay was able to determine if blood samples from an individual were MAP positive or negative [51]. This demonstrates the direct clinical utility of this assay which can expedite diagnosis due to its specificity and elimination of laborious sample preparation procedures. Considering these findings, it has been hypothesized that nanoparticles with higher R2 relaxivity may be more sensitive probes for pathogen identification in complex media, achieving bacterial detection and lower detection thresholds [50,51]. Most recently, dextran-coated iron oxide nanorods with high relaxivity ($300\ mM^{-1}\ s^{-1}$) were synthesized and used for the detection of MAP, achieving a detection limit of 6 MAP colony forming units in whole milk within 5 min [50].

In similar studies, vancomycin-conjugated iron oxide nanoparticles were able to quantify *S. aureus* with comparable to the aforementioned anti-MAP nanosensors' sensitivities, using a handheld diagnostic magnetic resonance system [52]. Interestingly, instead of an antibody, the nanoprobe targetability was mediated via a vancomycin–peptidoglycan bacterial cell wall interaction [52]. Overall, these data demonstrate that the magnetic relaxation switches can achieve enhanced sensitivity by engineering their relaxivity and geometry, using currently available instrumentation such as compact relaxometers [51,52], without the need for MR imagers as the measurement is one-dimensional and tomographic analysis is not required. Lastly, magnetic relaxation switches can be adopted in chip biosensor formats to mediate the multiplexed identification of infectious agents in complex media with portable magnetic relaxivity devices, suitable for routine and first response screening under diverse settings and conditions.

4.2. Pathogen detection using nucleic acids

In nature, differences among organisms arise from their variations at the genomic level, or differential alterations in the expression of their genes and protein modifications. Scientists have sequenced the genome of numerous pathogens, identifying unique nucleic acid signatures that are not present in the human genome. Acknowledging the numerous advantages of nanotechnology, nanoparticle conjugates of nucleic acids have been designed as probes for the fast identification of several pathogens.

Early reports by Mirkin et al. have demonstrated the unique interaction between gold nanoparticles and DNA, leading to distinct shifts in the gold nanoparticles' surface plasmon resonance peak [7,8]. Apart from fast and highly specific, the single-strand-DNA-carrying nanoparticles facilitated the real-time DNA detection with micropatterned gold diffraction gratings and gold nanoparticles [73]. Both the diffraction grating and the nanoparticles had thiolated oligonucleotides as probes, which were complementary to non-overlapping sequences of the anthrax lethal factor DNA sequence [73]. Therefore, hybridization between the complementary target sequence and the probes facilitated the association of the target sequence and the gold nanoparticles with the diffraction grating, causing changes in the local dielectric environment that subsequently affected the incident light's diffracted pattern [73]. As this pattern is directly associated with alterations in the local microenvironment, various target DNA concentrations were quantified, with an estimated detection limit of 40 fM [73]. Corroboration of the assay's specificity was achieved by examining the diffraction pattern upon incubation of the nanoparticles and the diffraction grating with an HIV sequence [73]. In this case, nominal diffraction response was observed, which was 25 times lower than that of the target sequence [73]. Considering its sensitivity, specificity, portability, and simplicity, this method may be ideal for point-of-care diagnostics. Furthermore, other researchers utilized gold nanoparticles for the colorimetric detection of DNA-binding molecules, allowing the optical identification of molecules binding to DNA via the solution's color changes. Collectively, naked eye profiling of pathogens and identification of effective antimicrobial DNA/RNA-intercalating agents should be feasible with these nanosystems. In line with this, researchers developed a colorimetric "spot-and-read" assay for the detection of the *mecA* gene, which is found in methicillin-resistant strains of *S. aureus* [74]. Upon hybridization of the gold nanoparticles with the target DNA sequence and spotting on an illuminated glass waveguide, visual changes were observed, leading to bacterial DNA quantification in the zeptomole range ($zM = 10^{-21}\ M$) [74]. However, although a highly innovative technique, as with all nucleic acid-based methods, this assay requires bacterial isolation, lysis and isolation of bacterial DNA which can limit its application in field diagnostic and in developing countries.

Another pathogen-associated nucleic acid detection method utilizes the gold nanorods' second-order nonlinear properties [75,76]. Specifically, researchers were able to quantify HIV-1 DNA with high specificity using hyper-Rayleigh scattering spectroscopy that monitors changes in the light's parameters upon target recognition [75]. Simply, using gold functionalized nanorods and without any modification or enzymatic amplification, researchers have detected 100 pM of the HIV-1 *gag* gene, which encodes one of the viral structural proteins (p55) [75]. Interestingly, this approach was very selective, as single base-pair mismatches in the target sequence affected the hyper-Rayleigh scattering [75]. Additionally, the signal intensity from the complementary target sequence can be increased up to 45 times with a slight modification of the system, drastically improving the assay's detection threshold [75]. This may be particularly important for the identification of highly contagious pathogens or scarce microorganisms isolated from various samples. Furthermore, gold nanorods and hyper-Rayleigh scattering facilitated the detection of Hepatitis C virus, a single-stranded RNA virus, without performing any amplification [76]. Using this technique, 80 pM of Hepatitis C viral RNA was detected with single nucleotide selectivity [76]. Hence, apart from viral RNA, potentially pathogen-related messenger and ribosomal RNAs can be detected, with applications in the clinic and the pharmaceutical industry, as single point mutations may completely abrogate a drug's antimicrobial efficacy.

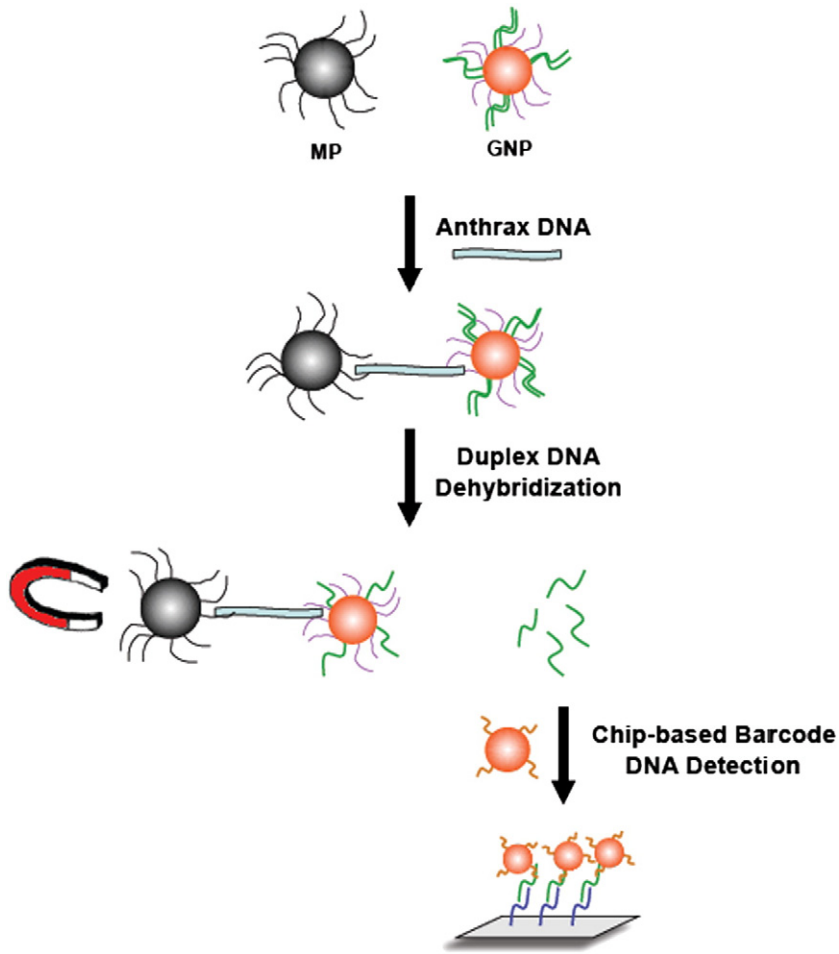
As most conventional nucleic acid detection methods achieve their sensitivity through a polymerase-mediated amplification, research efforts have been geared towards the achievement of higher sensitivities in nanotechnology-based assays through either short PCR rounds or assay-intrinsic signal enhancement. For instance, after performing PCR amplification on clinical samples for various rRNA genes, Sanguinetti et al. have identified the presence of various Mycobacteria, using a Nanochip microelectronic array and fluorophore-conjugated species-specific DNA oligonucleotide probes [77]. In a high-throughput format, the probes were able to identify the presence of bacteria such as *M. avium*, *M. xenopi*, *M. intracellulare*, and *M. chelonae*, whereas nominal signal, comparable to the negative control, was observed in samples containing other pathogens [77]. Hence, this method may be beneficial for the identification of complex diseases, involving various microorganisms or microbiota shifts. Instead of using PCR, Wang et al. have developed a visual gene-detecting technique for Hepatitis B and C viruses through a sandwich hybridization assay that uses gold nanoparticles and silver staining [78]. Serum samples from patients and control individuals were screened on a Hepatitis B, Hepatitis C, or a dual (Hepatitis B and C) gene chip that had directly immobilized oligonucleotides and supplemented oligonucleotide-conjugated gold nanoparticles, serving as capturing and detection probes respectively [78]. As this method is fast and cheap, and requires no sophisticated instrumentation for readout, rural clinics and response teams may

benefit from this technology. Likewise, the attractive strategy by Nam et al. was devised for the detection of anthrax lethal factor DNA with remarkable sensitivity (500 zM) and single nucleotide specificity [79,80]. The assay's sensitivity relied on an elegant intrinsic signal enhancement mechanism, utilizing iron oxide microparticles and gold nanoparticles [79,80]. Specifically, the magnetic iron oxide microparticles carried a short stretch DNA probe, whereas the gold nanoparticles carried a different short DNA probe and a DNA duplex, which served as the amplifier (Scheme 4) [79,80]. The two particle preparations were incubated with the isolated target DNA, followed by magnetic separation and DNA duplex dehybridization via heating at 60 °C [79,80]. The latter step resulted in the release of the non-conjugated strand (barcode) of the DNA duplex, facilitating signal enhancement, as multiple bar-code strands were released per target recognition event (Scheme 4) [79,80]. Despite its' impressive sensitivity, as the nanoparticle-barcode detection requires a couple of hours for data readout and multiple isolation steps, its' field utility may be limited, yet its' laboratory applicability should be foreseen as this method is robust, cheap and does not use protein-based enzymes.

4.3. Detecting toxins and infectious diseases secreted markers

As toxins are potent biomolecules causing pathogenesis in a wide-range of populations, quick identification of these agents is critical. Within the last years, frequent recalls of produce at a global level occurred, resulting in severe economic losses and damaging international trade relations. Additionally, the delayed identification of intoxicated products, such as milk, spinach, nuts, and meats, has caused significant cases of intoxication, even in countries with stringent product handling regulations and vigilant public health monitoring agencies. Hence, this indicates that current toxin identification methods have limitations, including their complexity, high cost, and limited point-of-care utility. Considering these, and the possible use of toxins as bioterrorism agents, nanotechnology has provided us with a plethora of toxin detection approaches, achieving low detection and portability, while maintaining low cost and user-friendly setups.

Among the most prominent and affordable nanotechnology-based toxin detection venues are colorimetric assays that utilize nanoparticle probes. For instance, using thiolated lactose derivatives and gold nanoparticles, researchers were able to detect cholera toxin through molecular mimicry, as the nanoparticles' coating resembled the extracellular matrix terminal portion of GM1 ganglioside which is found in the apical membrane of intestinal epithelial cells [81]. The detection and quantification were achieved visually and spectrophotometrically, through color changes in the nanoparticle suspension (red to deep purple) and shifts in the nanoparticles' plasmonic band, as increases in the toxin concentration facilitated concomitant red shifts in the UV–visible absorption spectra [81]. The assay provided results within 10 min, having a detection limit of 54 nM (3 μg/mL) and not being susceptible to interference caused by ions and proteins [81]. Similarly, by modifying the nanoparticle probing moiety and molecular mimicry, gold nanoparticles conjugated to globotriose were able to detect the Shiga-like toxin, as the toxins B subunit



Scheme 4. Barcode-based detection of *B. anthracis* DNA using magnetic microparticles (MP) and gold nanoparticles (GNP). After magnetic isolation and DNA dehybridization, release of the barcode DNA occurs. The barcode DNA can be separated using magnetic isolation and can be detected using a chip-based setup that utilizes gold nanoparticles and silver staining enhancement. Adapted from references [79] and [80].

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specifically interacted with the nanoparticles' carbohydrate moieties that mimicked Gb3 (globotriaosylceramide) found on intestinal microvilli and renal epithelia [82]. Because Shiga-like toxin intoxications by bacterial strains, such as *E. coli* O157:H7, cause bloody diarrhea and in severe cases hemolytic uremic syndrome that may lead to death, researchers have engineered carbohydrate-carrying glycopolydiacetylene nanoparticles for the rapid and selective quantification of this toxin [83]. Similar to the gold nanoparticles, recognition of the toxin by the nanoparticles resulted in changes in the nanoparticles' absorption profile, thus making these nanoparticles attractive agents in biorecognition assays [83].

Since portability is a key element for the detection of toxins, antibody-carrying gold nanoparticles have been immobilized to immunochromatography strips and allowed the visual detection of aflatoxins in grain samples [84]. This assay's detection limit was 0.5 ng/mL, and results were obtained in just 15 min, matching HPLC analysis data [84]. Therefore, as no sophisticated equipment is needed and results are obtained visually, farmers and health officials may use the gold-nanoparticle-based test strip method for the rapid identification of toxins. In addition to their use as direct colorimetric probes, nanoparticles have been used as capturing entities to mediate the isolation and sensitive detection of toxins. Iron oxide nanoparticles conjugated to aflatoxin M1 antibodies were able to magnetically isolate the toxin from milk, allowing the toxin's quantification in standard ELISA formats [85].

Striving for multiplexity and higher assay sensitivity, nanoparticle-based fluorescent probes have been devised for the detection of toxins. Due to their high fluorescence, quantum dots were conjugated to antibodies for the identification and quantification of several toxins in sandwich immunoassay formats [31]. Specifically, using a microtiter plate reader, cholera toxin, Shiga-like toxin 1, staphylococcal enterotoxin B and ricin were quantified in a high-throughput format [31]. Notably, the tunable toxin-detecting quantum dots were able to determine the exact toxin concentrations in samples that had combinations of these toxins, facilitating simultaneous quantification without the need for multiple isolation and incubation rounds that typical immunoassays require [31]. Interestingly, when europium nanoparticles were used in fluorescent immunoassays, detection sensitivities of 10 pg/mL were achieved [86]. This assay not only surpasses 100 times in sensitivity the corresponding ELISA ones, but it had 100% reproducibility versus ELISA's 36% [86]. Furthermore, with these nanoparticles the anthrax toxin protective antigen was detected in serum and blood samples, demonstrating the clinical utility of these assays [86]. In a different approach, after separation with antibody-carrying magnetic nanoparticles, staphylococcal enterotoxin B was quantified using fluorescence-based enzymatic nanotransduction [87]. Through amplification of DNA templates conjugated to antibodies that recognized the captured toxin, the signal intensity increased, leading to a detection threshold of 0.11 ng/mL [87].

As high sensitivity and selectivity are desired for several clinically important toxins, biosensors are attractive alternatives for lab-based diagnostics. For instance, gold nanoparticles tethered on a supported GM1-containing lipid bilayer were able to detect the presence of cholera toxin, via alterations in the diffusion coefficients of the gold nanoparticles [88]. Comparing this ligand-based method with fluorescent immunoassays, the biosensor assay was sensitive more than 100 times [88]. Likewise, by employing molecular topology mimicry, immobilization of lipoic acid-anchored lactosyl and galactosyl ceramide on a sensor chip via self-assembled monolayer deposition led to the detection of ricin within 5 min [89]. Due to the surface plasmon resonance analysis's sensitivity, low ricin concentrations were quantified (10 pg/mL) [89]. Hence in an attempt to merge the sensitivity of surface plasmon resonance and the facile construction of microfluidic devices from polydimethylsiloxane (PDMS), researchers have tethered GM1-containing lipid vesicles on gold nanoglassified surface [90]. This was achieved using the vesicles' biotin moieties that anchored to the surface-interacting streptavidin molecules. Although not as sensitive as other methods, ricin concentrations above 10 ng/mL (0.2 pM) were detected, suggesting that further improvements in the device architecture and signal amplification may significantly improve this multiplexing-capable system [90].

Apart from detecting toxins, it is often desirable to identify secreted markers, such as antigens and antibodies produced by the host. Especially, the latter is of major clinical significance, mainly in the cases of intracellular pathogens and asymptomatic carriers who may participate in the transmission of a disease without knowing that they have been infected with an infectious agent. Nanotechnology has addressed antigen and antibody detection by employing a diverse repertoire of methodologies, ranging from barcode amplification to immunochromatography, and from colorimetry to magnetic relaxation. For instance, for the battle against HIV/AIDS, researchers have managed to detect 0.1 pg/mL of the p24 capsid antigen via nanoparticle-based barcode amplification [91]. This method in addition to being highly specific and yielding no false-positive results, it was more sensitive than ELISA and was able to detect HIV infection 3 days before ELISA could do so [91]. Considering that the nanoparticle-barcode assays was used for the detection of amyloid β, an Alzheimer's disease hallmark, in cerebral fluid [92], it is envisioned that prions could be detected with this method, potentially preventing the spread of related spongiform encephalopathies and the annihilation of presumably infected cattle herds. Furthermore, elegant studies by Rotello et al. have demonstrated that through fluorophore displacement specific proteins can be identified, without the need for antibodies [93]. The underlying principle for this diagnostic method relies on the gold nanoparticle quenching of charge complementary fluorophores, and fluorophore activation upon binding of a target protein to the nanoparticles' terminal groups [93]. Hence, this methodology may be attractive for rural sensing and developing world applications. Considering these necessities, researchers have utilized immunochromatography and colloidal gold nanoparticles for the fast point-of-care detection of Herpes Simplex Virus and anthrax protective antigen antibodies in serum and whole blood [94,95]. In addition to their robustness and specificity, the immunochromatographic test strips were able to detect 3 μg/mL in serum and 14 μg/mL in whole blood, indicating their potential field application [94]. Finally, through magnetic relaxation, *Brucella* and influenza antibodies were detected in biological samples, with sensitivities of 0.3 nM and 0.1 pM respectively [96,97].

4.4. Determining drug resistance/susceptibility through the monitoring of a pathogen's metabolic activity

An important clinical and public healthcare parameter is the rapid determination of a pathogen's drug resistance. The gold-standard protocol for the determination of antimicrobial susceptibility relies on the growth of the microorganism in the presence of various drug concentrations. In this assay, the appearance of turbidity in bacterial cultures in the presence of a drug typically suggests growth and active metabolism; hence the drug is either ineffective or is present at a concentration that cannot suppress growth. However, lack of turbidity indicates that the drug effectively kills the bacteria and concomitantly, the bacteria are not resistant to that particular drug. Despite its high sensitivity and easiness, the turbidity method, similar to other metabolism-based susceptibility assays, provides results at least within 24–48 h [98,99].

Alternatively, most traditional molecular biology-based methods, as well as contemporary nanotechnological strategies, although fast and sensitive cannot assess if a pathogen is viable or not, as these methods depend on either immunologic or nucleic acid-mediated detection, which do not necessarily reflect a pathogen's metabolic state. Therefore, treatment of bacterial infections frequently relies on empirical associations, sometimes leading to prescription of

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ineffective drugs and application of selective pressure mediating drug resistance. Consequently, within the last 20 years there has been an increase in the emergence of antibiotic-resistant microorganisms with concomitant elevated pathogenesis at the global level [100,101]. Concurrently, there has been a decrease in the development of antimicrobial agents, due to shifts in the pharmaceutical industry's research and development priorities [1]. Thus, the prevalence of drug-resistant strains of *Mycobacterium tuberculosis* (MDR and XDR-TB) and methicillin-resistant *Staphylococcus aureus* (MRSA) in the developing and developed nations respectively, indicate that drug resistance has become a major public health problem [100,101]. An indication of the problem's magnitude can be obtained through several reports, stating that MRSA infections cause more deaths than HIV/AIDS in the United States and bacterial infections are among the leading causes of mortality in the industrialized world [100–102]. Therefore, novel antimicrobial susceptibility systems have to be fast, sensitive and cost-efficient in order to assess if a pathogen is resistant to first-line antimicrobial agents, and the agents' effective dosage.

Several molecular approaches can be employed for the assessment of bacterial drug resistance, in addition to the gold-standard metabolism-based turbidity method. For instance, the expression of protein, carbohydrate and glycolipid markers on the bacterial wall, as well as mutations in the genomic level, can facilitate assessment of antimicrobial resistance. Limitations of contemporary microbial susceptibility assays include extensive sample preparation protocols, long incubation times, the use of expensive reagents and limited multiplexed capabilities. Especially in the developing world, where drug resistance of *M. tuberculosis* is prevalent, performing these laborious molecular assays is prohibitory. Thus, clinicians and health care providers rely on culturing methods that are simpler and cheaper. However, results are obtained after a significant amount of time and in some cases may be inconclusive due to contamination or inadequate sample size. In addition to assay logistics, another critical facet in combating infection diseases in the developing world is the identification of the most effective, yet most affordable, drug. Therefore, screening a sample for bacterial susceptible to multiple antimicrobial agents is too expensive and cumbersome in these countries using currently available microbiological methodologies. This leads to unsuccessful treatment of the infection and potential development of drug resistance strains of the disease. Hence, developing robust, affordable and faster antimicrobial susceptibility methods can assist in the administration of effective antibiotics and alleviate the socioeconomic burden associated with microbial pathogenesis in these countries.

Recently, several steps have been made to this direction, including nanotechnological studies that focused on the simulation of the interaction between multidrug resistant bacteria and antibiotics using cantilever assays and nanomechanical deflections. Specifically, as vancomycin, a last resort antibiotic for MRSA, interacts with D-alanine-terminated peptidoglycan moieties (D-Ala mucopeptides) in the surface of the bacterium, researchers have developed arrays of silicon cantilevers to assess the formation or absence of interactions between D-alanine peptidoglycan and vancomycin at clinically relevant concentrations [103]. The aim of this particular work was to study the interaction between vancomycin and D-Ala-mucopeptides, ultimately leading to the development of sensitive assays for MRSA detection and screening arrays for potential drugs that inhibit vancomycin binding to these mucopeptides. Several factors can lead to antibiotic resistance. For instance, antibiotic resistance can arise upon subtle changes in the bacterial cell wall, such as when an amide (D-Ala) group of the bacterial cell wall's peptidoglycan moieties is converted into an ester (D-Lac). In these studies, the cantilevers were initially coated with either D-alanine (D-Ala) peptidoglycan from vancomycin-susceptible bacteria or D-lactate (D-Lac) peptidoglycan from vancomycin-resistant bacteria. After incubation with vancomycin, significant cantilever defection was observed in the D-Ala cantilevers. However, minimal defection was obtained with the D-Lac cantilevers, as vancomycin cannot form hydrogen bonding with the D-Lac peptidoglycans. As little as 10 nM of vancomycin caused significant D-Ala cantilever deflection, making this assay clinically relevant, as the typical blood serum vancomycin concentration is within the range of 3 to 27 μM [103]. Most importantly, no D-Lac cantilever deflection was observed in the presence of 7 μM vancomycin in blood, suggesting that this assay can evaluate the interaction between a resistant bacterium and an antibiotic at the fundamental single hydrogen bond level, thus expediting the screening of current drugs and the development of novel antimicrobial agents. Interestingly, the vancomycin–mucopeptide interaction equilibrium dissociation constants (K_d) obtained with the D-Lac and D-Ala cantilevers correlated well with the values obtained through surface plasmon resonance and solution-phase UV spectroscopy measurements, indicating that this nanotechnology-based approach is equally reliable to standard methodologies yet more sensitive (10 nM vs 30 nM detection threshold) [103].

A limitation of the cantilever method is that it does not provide any information about the metabolic state of the microorganism infecting a patient [3,104,105]. Therefore, a physician cannot assess if an antibiotic agents will suppress bacterial growth and viability. Following a different approach, recent studies reported the assessment of bacterial drug resistance through the monitoring of bacterial metabolic activity, thus having wider clinical and industrial implications. Among the most interesting nanotechnology-based approaches for assessing the efficacy of antibiotics is the use of screen-printed carbon electrode arrays and cyclic voltametry [106]. In these studies, concentrated bacterial cultures were initially incubated with different antibiotics and then supplemented with an oxidative solution of ferricyanide and dichlorophenolindophenol, followed by amperometric measurements using carbon electrodes [106]. Within less than one hour, the electrodes recorded the amperometric response currents from the reduction of ferricyanide to ferrocyanide, which are markers of electron transfer due to bacterial metabolic activity and respiration [106]. Thus, it was observed that under inhibitory antibiotic concentrations the amperometric currents decreased [106]. This allowed determination of chloramphenicol's *E. coli* inhibitory concentration which suppressed 50% of the bacterial metabolic activity, potentially facilitating the rapid identification of other effective antimicrobial agents [106]. The use of high concentrations of bacteria (OD $\geq$ 0.1; 1×10^8 colony forming units), the need for specialized solutions (buffer with growth media and oxidizing cocktail), the absorption of antibiotic by the electrode and the non-user-friendly data readout may hinder this method's use in the field and poor rural areas. However, the pharmaceutical industry and government agencies might adopt it due to its sensitivity and promptness.

To circumvent these problems, researchers focused on the monitoring of nutrient utilization, targeting clinical sample conditions, speed and reliability. As previous studies have reported the use of carbohydrate-coated gold and iron oxide nanoparticles for the quantification of glucose via surface plasmon resonance shifts and magnetic relaxation respectively [107,108], researchers hypothesized that these nanoparticles can be used for the quantification of complex carbohydrates and the subsequent determination of bacterial metabolic activity in complex media. Recently, Nath and colleagues have synthesized dextran-coated gold nanoparticles, which in the presence of a carbohydrate-specific clustering-inducing agent (Concanavalin A) exhibited starch-concentration-dependent differential clustering [27]. Notably, at high carbohydrate concentrations extensive clustering was observed with concomitant large plasmonic shifts, whereas at moderate to low concentrations smaller nanoclusters and less prominent plasmonic shifts were observed (Scheme 5) [27]. Based on these findings, *E. coli* bacteria (1×10^6 colony forming units) were initially incubated for two hours in the presence of various ampicillin concentrations [27]. Subsequently, aliquots of these bacterial cultures were incubated with the dextran-coated gold nanoparticles and Concanavalin A, and within an hour they spectrophotometrically

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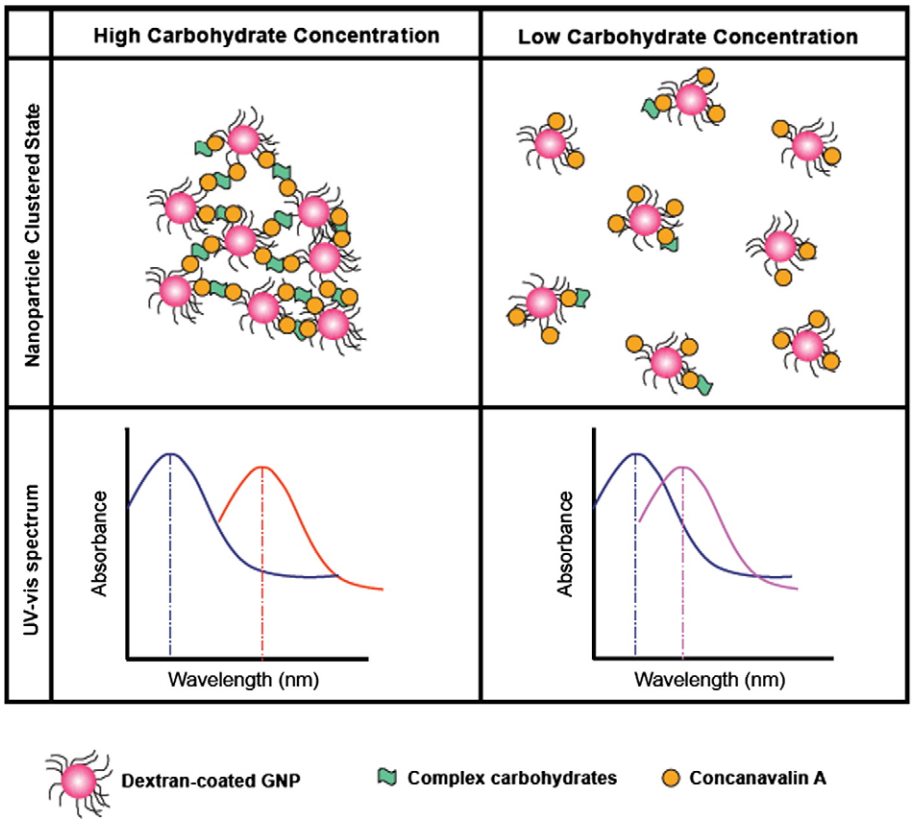

Scheme 5. Determination of antimicrobial susceptibility using gold nanoparticles (GNP). When the levels of complex carbohydrates are high, addition of Concanavalin A facilitates the extensive clustering of the GNP, with large shifts in the plasmonic band. Alternatively, at low carbohydrate concentrations, the nanoparticles' clustering is less pronounced and the resulting plasmonic shifts are smaller. Adapted from reference [27].

determined that ampicillin concentrations of 2 μg did not inhibit growth, whereas at 4 μg bacterial metabolic inhibition was achieved [27]. Furthermore, utilizing these gold nanosensors, Nath et al determined that the minimum inhibitory concentration for this bacterial strain was 8 μg of ampicillin, and corroborated these findings with the gold-standard turbidity method [27]. Although the nanoparticle-based and the turbidity method provided identical results, the nanoparticle-based achieved antimicrobial susceptibility determination within an overall readout time of 3 hours versus the turbidity method that required 24 h [27]. Additionally, using a microtiter plate reader, a 96-well plate was screened within a couple of minutes, facilitating assessment of bacterial drug resistance in a high-throughput format [27].

Acknowledging the need that bacterial drug resistance has to be achieved in clinical or opaque samples, researchers developed an iron-oxide-nanoparticle-based assay to facilitate assessment of drug susceptibility in blood with magnetic relaxation [56]. Two different assays have been devised relying on either a competition or direct binding format. In the competition format, dextran-coated iron oxide nanoparticles competed with the solution's carbohydrates for binding to Concanavalin A [56]. On the other hand, in the direct binding format, Concanavalin A was conjugated to silica-coated iron oxide nanoparticles, mediating the nanoparticles' binding to a sample's non-utilized carbohydrates [56]. In initial studies, the iron oxide nanosensors facilitated starch quantification and monitoring of bacterial growth through the changes in the spin–spin relaxation times (ΔT2) [56]. Similar to the dextran-coated gold nanoparticles, it was reasoned that samples with inhibitory concentrations would exhibit shifts in the relaxation times proximal to the sterile medium due to the comparable levels of free complex carbohydrates in solution (Scheme 6) [56]. Alternatively, it was hypothesized that samples with non-inhibitory antibiotic concentrations, thus having lower levels of carbohydrates as a result of active bacterial metabolism, should have demonstrated larger ΔT2, facilitating the determination of antimicrobial susceptibility even in opaque media (Scheme 6) [56]. Hence, researchers determined if different microorganisms were resistant or susceptible to ampicillin. Within 2.5 hours, the dextran-coated iron oxide nanosensors determined that *E. coli* and *S. sonnie* were susceptible to 8 μg of ampicillin, whereas *S. marcescens* was resistant to this antibiotic (Scheme 7) [56]. Confirmation of these results was achieved through the turbidity method after a 24-h long incubation, demonstrating that the nanoparticle method is equally sensitive and reliable, yet faster, than this reference procedure (Scheme 7) [56]. Interestingly, Kaittanis et al. determined these microorganisms' drug susceptibility in blood, obtaining identical values with the turbidity method and the magnetic relaxation method performed in non-blood-containing growth media [56]. Considering these findings, it was reasoned that the direct bonding format would provide faster results. In studies with silica-coated Concanavalin A-carrying iron oxide nanoparticles, it was found that these nanoparticles could evaluate bacterial susceptibility with high sensitivity in just five minutes after addition of the bacterial aliquots [56]. Taken together, these findings, as well as those of the carbon electrode array and dextran-coated gold nanoparticles, indicate that nanotechnology-based antimicrobial susceptibility assays can be as sensitive and robust as traditional methods, however providing faster results which can be useful for the prevention of epidemics and expedite drug development.

5. Concluding remarks

Nanotechnology is poised to revolutionize the way how pathogen and infectious diseases diagnostics are performed in the 21st century. By enabling the fast, cost effective and sensitive detection of infectious agents, engineered nanoprobes and nanodevices could facilitate the

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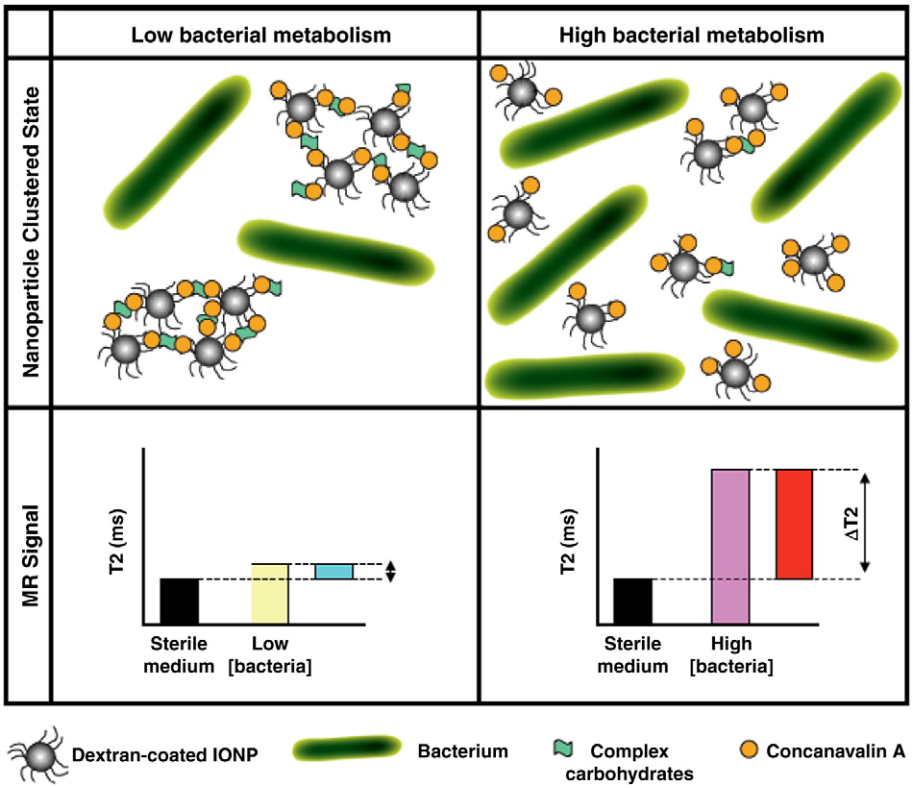
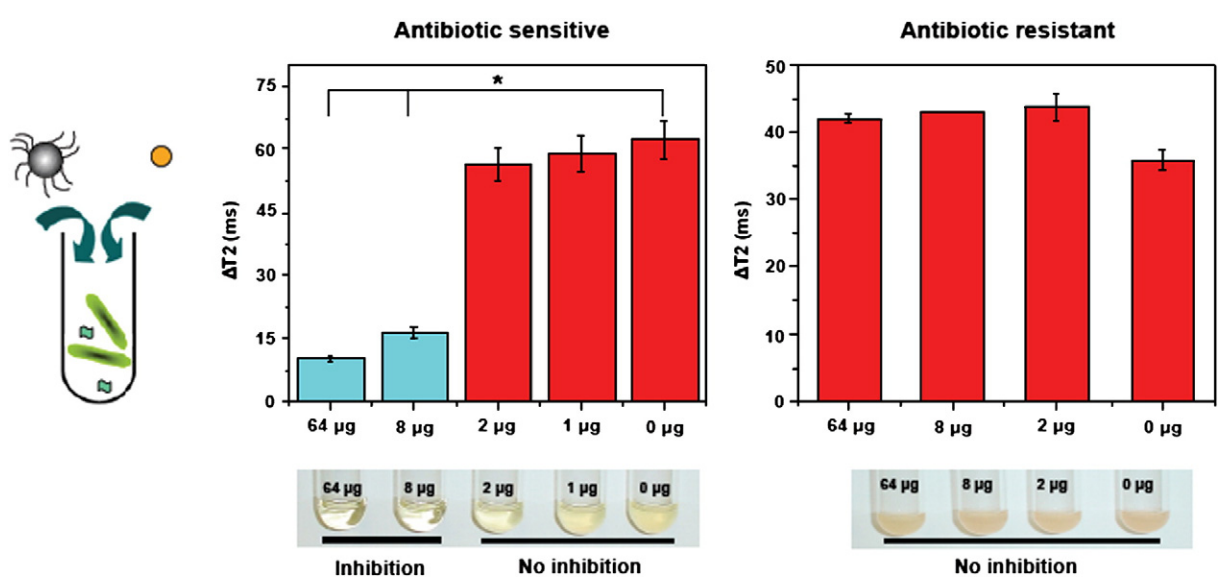


Scheme 6. Assessment of bacterial metabolic activity with magnetic relaxation. At small bacterial populations, addition of Concanavalin A induces the formation of large nanoassemblies of dextran-coated iron oxide nanoparticles (IONP) that have a T2 similar to that of the sterile medium, as the carbohydrate levels are comparable. As the bacterial population expands and carbohydrates are consumed, smaller nanoassemblies are formed in the presence of Concanavalin A, leading to higher T2 than that of the sterile medium. Adapted from reference [56].

robust and high-throughput screening of pathogens in biological and environmental samples. This can be achieved via the unique interactions these nanoprobes and nanodevices have upon recognition of the molecular target or pathogen. Inorganic nanoparticles, semiconductor quantum dots, carbon nanotubes, polymeric nanoparticles, as well as cantilevers and nanochips, all have the potential to be useful in the design of sensitive pathogen diagnostics. Particularly, nanoparticles are very versatile, because in principle could substitute the use of fluorescence molecules as tags in most diagnostics applications. Future trends in nanotechnology will continue in the design of nanostructures to build miniaturized devices, requiring less sample volumes. In addition, as the field of nanotechnology progresses into the design of smart 2-D and 3-D nanoassemblies, new devices that facilitates fast and sensitive pathogen detection in minimal sample volumes are



Scheme 7. Determination of drug resistance using dextran-coated iron oxide nanoparticles and Concanavalin A. The antibiotic sensitive microorganism (*E. coli*) had a minimum inhibitory concentration of 8 μg of ampicillin, which was assessed within 2.5 h with the nanoparticle method. The gold-standard method (turbidity assay) provided results after 24 h. The nanoparticle method was also able to determine the resistance of a microorganism (*S. marcescens*) to ampicillin. Adapted from reference [56].

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expected. Finally, as drug resistance strains continue to emerge, in the forthcoming years we would expect the design of nanosystems that not only are able to sense the presence of a pathogen, but also assess if this pathogen is resistant to a particular drug.

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