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REVIEW

Gold nanoparticles for the development of clinical diagnosis methods

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Abstract The impact of advances in nanotechnology is particularly relevant in biodiagnostics, where nanoparticle-based assays have been developed for specific detection of bioanalytes of clinical interest. Gold nanoparticles show easily tuned physical properties, including unique optical properties, robustness, and high surface areas, making them ideal candidates for developing biomarker platforms. Modulation of these physicochemical properties can be easily achieved by adequate synthetic strategies and give gold nanoparticles advantages over conventional detection methods currently used in clinical diagnostics. The surface of gold nanoparticles can be tailored by ligand functionalization to selectively bind biomarkers. Thiol-linking of DNA and chemical functionalization of gold nanoparticles for specific protein/antibody binding are the most common approaches. Simple and inexpensive methods based on these bio-nanoprobes were initially applied for detection of specific DNA sequences and are presently being expanded to clinical diagnosis.

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Abbreviations

DNA	Deoxyribonucleic acid
ssDNA	Single-stranded deoxyribonucleic acid
RNA	Ribonucleic acid
AuNP	Gold nanoparticle
AFM	Atomic force microscopy
SNP	Single-nucleotide polymorphism
SPR	Surface plasmon resonance

Introduction

Nanodiagnostics can be defined as the use of nano-sized materials, devices or systems for diagnostics purposes. It is a burgeoning field as more and improved techniques are becoming available for clinical diagnostics with increased sensitivity at lower cost [1]. 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. Nanoparticles have high surface areas and unique physicochemical properties that can be easily tuned, making them ideal candidates for developing biomarker platforms.

In this review we will focus on gold nanoparticles (AuNPs) used as basis for the development of methodologies suitable for application in clinical diagnosis. AuNP-based

diagnostics can be broadly divided in three different approaches:

1. utilization of the AuNP color change upon aggregation, the best characterized example being AuNPs functionalized with ssDNA capable of specifically hybridizing to a complementary target for the detection of specific nucleic acid sequences in biological samples [2];
2. use of AuNPs as a core/seed that can be tailored with a wide variety of surface functionalities to provide highly selective nanoprobes for diagnosis [3]; and
3. utilization of AuNPs in electrochemical based methods that can be coupled with metal deposition for signal enhancement [4].

The last approach was recently the theme of excellent reviews [5, 6] and will not be dealt with in this critical review. The first approach based on color changes is by far the most developed in terms of clinical diagnosis and several application examples will be presented. The second approach shows great potential for future applications and future trend lines will be highlighted.

Synthesis and properties of AuNPs

Gold nanoparticles have been widely studied for their unique optical properties arising from their surface plasmon resonance (SPR) [7]. AuNPs have exceptionally high absorption coefficients, allowing higher sensitivity in optical detection methods than conventional dyes. Typically, colloidal solutions of spherical AuNPs are red with the SPR band centered at ca. 520 nm. This band is weakly dependant on the size of the particle and the refractive index of the surrounding media [8–10], but strongly changes with shape [8, 10, 11], and inter-particle distance [12]. The morphology and surface chemistry of nanoparticles is usually controlled by using appropriate synthetic methods; these will be discussed briefly in the end of this section.

The strong absorption of AuNPs has been advantageously used in colorimetric detection of analytes, either by inducing aggregation of AuNPs in the presence of specific analytes [13–18], or by measuring changes in the refractive index of the AuNPs environment, due to adsorption of biological analytes [19]. The great enhancement of electromagnetic field at the surface of AuNPs by interaction with electromagnetic radiation offers other interesting optical properties with great potential for biodiagnostic assays. For instance, AuNPs have been used for single-molecule detection by surface-enhanced Raman spectroscopy (SERS) [20]. In addition to the optical properties of AuNPs, their catalytic activity has also been used as a means of developing highly sensitive detection methods, for example by electrochemical detection that can be coupled with enzyme assays [5, 6, 21] and/or with metal deposition for signal enhancement [4].

The successful utilization of AuNPs in biological assays relies on the availability of synthetic methods generating nanoparticles with the desired characteristics, namely high solubility in water, and adequate morphology, size dispersion, and surface functionalities. Numerous synthetic strategies for the preparation of AuNPs have been reported [22–24]. Most commonly, AuNPs are synthesized by chemical or electrochemical reduction of a gold(III) precursor compound in the presence of a capping agent, i.e. a compound able to bind to the nanoparticle surface blocking its growth beyond the nanometer range and stabilizing the colloid in the particular solvent used. Control over the shape and size of the AuNPs is usually achieved through the careful selection of the experimental conditions, namely reducing agent, reaction time, temperature, and capping agent. A common approach is to use capping agents with strong affinity for gold, e.g. thiol capping agents. This allows the synthesis of AuNPs with good size dispersion but usually only soluble in organic solvents [25] requiring an additional step of extraction of the particles into water. In addition, exchange of strongly binding capping agents is usually cumbersome, which makes this type of AuNP less versatile for biological applications.

Due to its simplicity and high yield, the most commonly used method for preparation of spherical AuNPs for biological assays is the citrate reduction method of Turkevich et al. [26]. The use of citrate as a capping agent is very convenient due to its easy post-synthesis treatment, since it can be easily replaced by other capping agents, e.g. thiol capping agents, bearing an appropriate functionality for binding of the biological analyte of interest. Size control is reasonable, and recent modifications have allowed not only for better size distribution of the AuNPs, but also control of their size within the 9–120 nm range [27]. Several other methods for improvement of size dispersion of spherical AuNPs have been reported [28, 29]. In addition, several methods have been published for preparation of water-soluble AuNPs with SPR bands in the near infrared [30]. This type of AuNP is quite promising for biological applications, allowing the use of AuNPs in biological fluids without interference from absorption of other biological molecules. Furthermore, the simultaneous use of AuNPs absorbing at different wavelengths might allow multiplexed analysis.

Functionalization of AuNPs

Functionalization of Au-NPs involves the use of bifunctional ligands in which a moiety is used for anchorage to the particle while the other is directed to the outer-surface

for specific interaction with biomolecules. For example, thiol-modified oligonucleotides have been used to functionalize AuNPs for specific detection of nucleic acid sequences in biological samples (see below).

Functionalization of Au-NPs with biomolecules other than nucleic acids has also been used in order to develop methodologies suitable for clinical diagnostics. These include:

- antibodies for signal enhancement in immunoassays [21, 31–34];
- carbohydrate functionalization to study specific molecular interactions [35, 36]; and
- surface functionalization with ligands that can be tailored for specific protein binding [3, 37] or direct binding of peptides and proteins to the Au-nanoparticle surface [18, 38, 39].

The specific interaction between biological pairs has also been widely used, e.g. biotin–streptavidin [40, 41] and Ni-NTA–histidine tail [42, 43].

Glyconanoparticles

Carbohydrate functionalized nanoparticles ("glyconanoparticles") have the advantage of increasing the specific interactions between glycans and lectins for biosensing applications [44–46]. Compared with biotin–streptavidin, the carbohydrate–protein interaction is relatively weak but can be successfully enhanced by multivalent interactions—the so-called "cluster glycoside effect" [47]. These multivalent carbohydrate–ligand/protein interactions were successfully used by Tsai et al [46] in a rapid, selective, and quantitative detection method for the carbohydrate-binding protein concavalin A. Penadés et al. have developed a glyconanotechnology strategy to study and evaluate carbohydrate–carbohydrate and carbohydrate–protein interactions, enabling the establishment of models for carbohydrate-mediated biological processes [35]. Multifunctional glyconanoparticles containing lactose were prepared as a possible platform for carbohydrate-based anticancer vaccines, with the potential for tailoring polyvalent anticancer vaccines and drug-delivery carriers [36]. Even though glyconanoparticles are simple and flexible to prepare and have several interesting physical, chemical, and biological properties, their utilization in clinical diagnosis is still under development.

Monolayer-protected clusters for protein binding

Rotello and co-workers have performed seminal work on monolayer-protected clusters (MPCs) bearing organic functions such as multivalent and non-covalent recognition elements [37]. The thiolated ligands used for gold nanoparticle functionalization (cationic, anionic, hydrophobic, etc.) generate a plethora of stable single (MPCs) or mixed (MMPCs) Au-nanoprobes, the latter often including groups like oligo(ethylene glycol) and poly(ethylene glycol) to improve water-solubility and to decrease non-specific protein binding [48, 49]. Recently, utilization of MMPCs in a sensor array based on fluorescence quenching was reported [3]. The system explores a "chemical nose/tongue" approach featuring selective receptors (instead of the more common "lock–key" specific recognition procedures) to detect, identify, and quantify protein targets. The robust characteristics of its components (nanoparticles, polymers), the simplicity of the method, and the tailoring possibilities provided by nanoparticle-surface functionalization, make this array an extremely promising approach to point-of-care diagnosis.

Peptide-capped Au-nanoprobes

The tailoring of the gold nanoparticle's surface by fabricating monolayer-protected nanoparticles was employed in the rational design of peptide-capping ligands [38]. The proposed combinatorial approach enables the synthesis of exceptionally stable AuNPs with properties in aqueous media that are modulated by the amino acid sequence of the appended cysteine-terminated penta-peptide [39]. The design strategy of these peptides combines a strong affinity for gold (cysteine), the ability to self-assemble into a dense layer that excludes water (amino acid residues with hydrophobic side chains), and a hydrophilic terminus to ensure solubility and stability in water [39]. This type of nanoparticle-peptide probe has been successfully utilized in a colorimetric kinase activity method [18] in which the kinase-substrate can be identified by specific binding of gold nanoparticles to the phosphorylated product immobilized on a microarray [50]. This format allows a multiplexed approach and opens the possibility for application in clinical diagnosis and drug discovery.

Specific DNA and RNA detection based on AuNPs

The use of thiol-linked ssDNA-modified gold nanoparticles (herein designated Au-nanoprobes) for the colorimetric detection of DNA targets represents an inexpensive and easy to perform alternative to fluorescence or radioactivity-based assays (Ref. [51] and references therein). In 1996, Mirkin et al. [2] described the use of single-stranded oligonucleotide targets that could be detected using two different Au-nanoprobes such that each was functionalized with a DNA-oligonucleotide complementary to one half of the given target. The hybridization of the two Au-

nanoprobes with the target resulted in the formation of a polymeric network (cross-linking mechanism), which brought the gold nanoparticles close enough to result in a red to blue color change. This cross-linking mechanism, where a nanoparticle is functionalized with several identical ssDNA oligonucleotides, brings each nanoparticle in close vicinity to several others through the specific hybridization of the DNA strands. Therefore, the cross-linking of multiple Au-nanoprobes allows for extensive nanoparticle aggregation, and yields the observed colorimetric change. In this system, the Au-nanoprobes were oriented in a tail-to-tail arrangement—one probe functionalized via a 5′-thiol bond and the other through a 3′-thiol group [51–53]. Recently the same method was elegantly expanded from specific detection of DNA sequences to a real-time screening assay for endonuclease activity [54].

Following a parallel approach, we developed a simple, easy-to-use and inexpensive assay based on a non-cross-linking hybridization method, where aggregation of the Au-nanoprobes is induced by an increasing salt concentration [55, 56]. Our method consists in visual and/or spectrophotometric comparison of solutions before and after salt-induced Au-nanoprobe aggregation—the presence of a complementary target prevents aggregation and the solution remains red; non-complementary/mismatched targets do not prevent Au-nanoprobe aggregation, resulting in a visible change of color from red to blue. The schematic diagram of this "Au-nanoprobe method" shown in Fig. 1 highlights its applications to clinical-derived samples [56]. This Au-nanoprobe method was also used to detect eukaryotic gene expression (RNA) without the need for retro-transcription or PCR amplification steps [55]. The method was able to specifically detect mRNA from as little as 0.3 μg of unamplified total RNA, avoiding the RNA to cDNA conversion step normally utilized by other methods.

We used AFM, a highly suitable technique for producing high-quality images and measurements of individual biomolecules [57], for imaging in parallel to the above described colorimetric detection system, to elucidate the DNA recognition events occurring at the nanoscale, i.e. hybridization of Au-nanoprobes to target double-stranded DNA that had previously been heat denatured to a single-stranded form. Complementary and non-complementary target sequences were used to study the level of specific interaction between the target and the Au-nanoprobes. This furnished direct evidence of the mechanism involved in Au-nanoprobe hybridization and enabled assessment of non-specific interactions between the Au-nanoprobes and the target [58]. An example image of the gold nanoparticles bound to the complementary DNA strands is shown in Fig. 2, in which a PCR-generated fragment from the β-globin gene locus was hybridized with Au-nanoprobes harboring a complementary DNA sequence. Analysis of a large number of hybridization products strongly supported hybridization to the complementary sequence as the primary interaction with the Au-nanoprobes. Bui et al. [59] have also used AFM to image aggregation caused by specific hybridization of ssDNA-modified nanoparticles with target ssDNA. The AFM clearly showed the aggregates of NPs used, and the authors established a linear

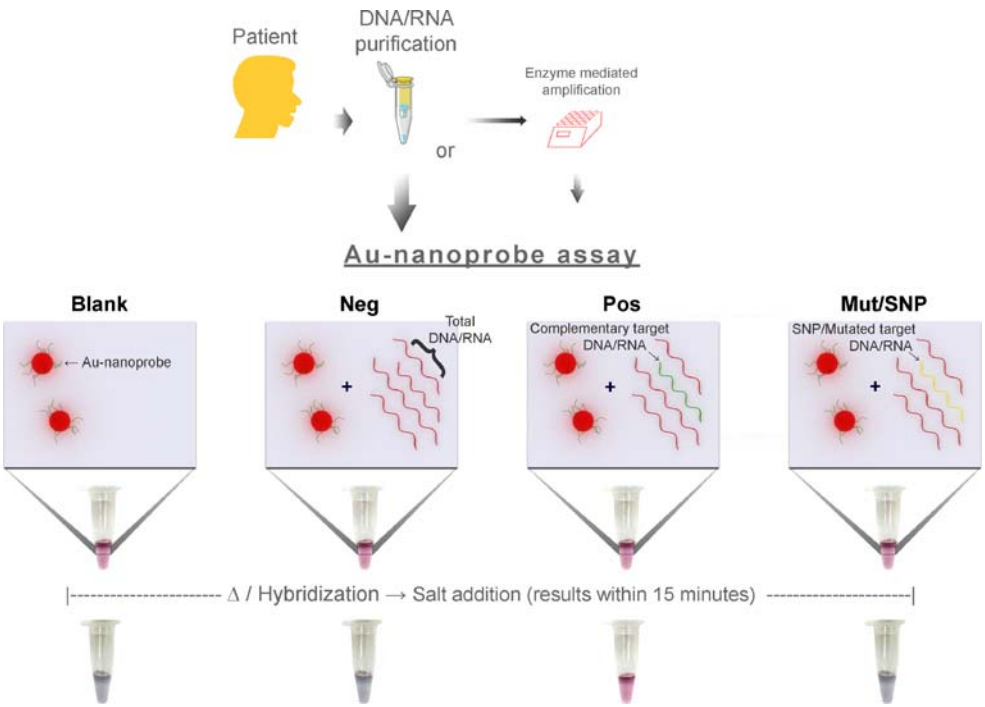

Fig. 1 Au-nanoprobe assay method. After DNA purification from clinical samples, the Au-nanoprobe method can be directly applied to the isolated DNA or to an enzyme mediated amplified product. The method relies on visual comparison of test solutions before and after salt-induced Au-nanoprobe aggregation. *Blank*, Au-nanoprobe alone; *Neg*, negative sample in the presence of non-complementary DNA/RNA; *Pos*, positive sample in the presence of complementary DNA/RNA; *SNP/Mut*, in the presence of DNA harboring SNP or single point mutation

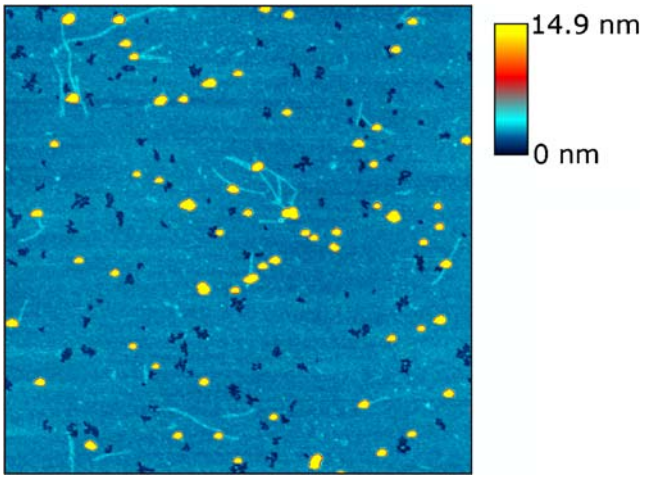

Fig. 2 Tapping mode AFM image of hybridization product of Au-nanoprobes (*yellow*) and target DNA (*blue strings*). DNA is a PCR product of the β-globin gene locus genomic region and the Au-nanoprobes harbor a complementary ssDNA sequence. Image size 3 μm

relationship between the diameter of the aggregates and the concentration of target DNA in solution. However, the actual hybridization was not directly visualized.

AuNPs-based clinical diagnostic methods

AuNPs in clinical immunoassays

Gold nanoparticles have found promising applications in signal enhancement of the standard enzyme-linked immunosorbent assays (ELISAs) where they can be conjugated with the antibodies [60] or coupled with silver-enhancement [61]. Recent examples include the development by Tanaka et al. [60] of a novel enhancement for immunochromatographic test strips where both the primary and the secondary antibodies are conjugated with AuNPs. This experimental set-up increased the limit of detection of the chorionic gonadotropin hormone by an order of magnitude to reach 1 pg mL^{-1}. Sensing platforms have also been developed based on fiber optic evanescent-wave sensors functionalized with self-assembled gold colloids [62] or using a Cy5-antibody-AuNP complex as the fluorescence probe [32]. These sensing platforms may replace the standard ELISA assay since they do not require a secondary antibody and allow sensitivity increases of at least one order of magnitude. The sensitivity of chemiluminescent analysis of antibodies in clinical samples could also be improved by using irregularly shaped AuNPs, which have 100-fold greater catalytic activity in comparison with spherical AuNPs [34]. Anti-IgG derivatized with these irregular AuNPs was used to determine successfully the IgG content of human plasma samples. Electrochemical approaches based on derivatization of electrodes with AuNPs have recently been applied to the label-free detection of the carcinoembryonic antigen (CEA) [33, 63]. The immunosensors obtained showed excellent reproducibility and stability [33], allowing batch fabrication [63]. The use of metal-enhanced fluorescence, a near-field effect that can significantly enhance fluorescence signatures up to fivefold, has found application in immunoassays through silver nanoparticles [64, 65]. Application of AuNPs in this field is a promising area under development [66]. For further applications of signal-amplification technologies in immunoassays and new generations of lateral-flow assays, see Ref. [31] and references therein.

AuNP-based DNA-detection clinical assays

A recent development of the now traditional Au-nanoprobe cross-linking method of Mirkin et al [2], is the bio-barcode system, used for protein detection with attomolar sensitivity. The method involves the capture of the analyte with a magnetic particle featuring recognition elements, followed by binding of a functionalized AuNP with a second recognition agent and "barcode" (marker) DNA strands. After magnetic separation of the sandwich complex, the DNA barcodes are released and the DNA strands detected and quantified using the a Au-nanoprobe sandwich assay followed by silver enhancement [67, 68]. This method was successfully used for measuring the concentration of amyloid-β-derived diffusible ligands, a potential Alzheimer's disease marker present at extremely low concentrations (<1 pmol L^{-1}) in the cerebrospinal fluid of affected individuals [69].

The Au-nanoprobe method we developed has been successfully applied to the detection of single nucleotide polymorphisms (SNPs) and mutations associated with disease or metabolic variation [70]. The technique facilitates characterization of individual genetic variability, which has been associated with individual susceptibility to several multifactorial diseases such as cancer, diabetes, and also with individual response to therapeutics. A rather successful application of the Au-nanoprobe method to clinical diagnosis was in the rapid and sensitive detection of *Mycobacterium tuberculosis* (the etiologic agent of human tuberculosis) in clinical samples [56]. In order to improve sensitivity and easily achieve quantification of target DNA/RNA samples, the detection system was directly integrated in an amorphous/nanocrystalline silicon device, without the need to functionalize the glass surface with the sequence of interest [71].

Other systems based on non-cross-linking DNA hybridization [17, 72, 73] have been described and applied to detection and characterization of human SNP sequences. A considerable increase in output signal and sensitivity can be

achieved by signal amplification by autometallography, leading to an approximately 1000-fold increase of the detection signal [74]; the scattered light is detected with a specifically designed photosensor coupled with an imaging system but with the inconvenience of more complicated experimental set-up and expensive apparatus [17, 75]. Recently, an improvement in non-cross-linking DNA hybridization for SNPs detection was described in which SPR imaging allowed a limit of detection of 32 nmol L^{-1} (19 fmol) without temperature control in 5 min [76] making this a promising method for point-of-care testing for SNPs. A SPR platform was also used for sensitivity improvement of detection of p53 cDNA at sub-attomole concentrations using Au-nanoprobes, allowing detection of a 39-mer target in 15 μL of a 1.38 fmol L^{-1} solution; this has important promise for cancer diagnosis [77].

The double-probe cross-linking method developed by Mirkin and collaborators [78] is considerably more specific than the non-cross-linking mechanisms, as it uses two probes, hence increasing the specificity of hybridization. Furthermore, in this approach the temperature at which probe hybridization occurs is tightly controlled, thus optimizing specificity. However, this strategy makes use of expensive apparatus and technology that might be difficult to implement at point-of-care. The non-cross-linking method, due to use of a single probe, can in principle compete with other currently available methods but at lower cost and with greater simplicity, taking less than 30 min to complete. The color change can be easily visualized with the naked eye, thus rendering it suitable for point-of-care diagnostics.

Only very few Au-NP-based strategies have proven suitable for direct use on biological samples in clinical diagnostics. Most AuNP-based methods are rapid and easy to perform, especially when compared with the most commonly used molecular procedures, such as polymerase chain reaction (PCR) or real-time PCR. Even though these methods are widespread, the high costs involved and the need for highly skilled and trained operation makes them difficult to implement at point-of-care and in remote regions or low-resource countries. The AuNP based methodologies that have already reached the clinical setting may help solve this issue. Even though a large number of methodologies and techniques have been presented for the detection of DNA/RNA through AuNPs, most have done so in controlled experimental settings and/or using synthetic or previously prepared molecules as targets. Few methods have been applied to the detection of DNA/RNA directly in clinical samples; these are summarized in Table 1.

Conclusions and future trends

In the last few years use of AuNPs in clinical diagnosis has developed rapidly and although effective application to clinical samples and targets is still scarce much effort is being devoted to bringing the use of these tools from the laboratory to the clinic. A particularly promising application of the colorimetric detection capabilities of AuNPs is based in specific DNA-functionalized AuNPs ("Au-nanoprobes") for hybridization with a DNA target-analyte, allowing for a

Table 1 Methodologies based on spherical gold nanoparticles for detection of nucleic acid sequences (DNA/RNA) directly in clinical samples. The underlying principle of the methods is listed, with limit of detection, molecular target (gene, *locus*, organism, etc) and type of sample

Detection	Detection limit	Clinical biological targets / sample	Refs.
Naked-eye (dry-reagent dipstick)	2 fmol–25 fmol[a,b]	Hepatitis C virus (HCV) / RNA, human plasma SNPs (mannose-binding lectin gene, MBL2) / genomic DNA, human whole blood	[82, 83]
Naked-eye (electrostatic interactions of unmodified AuNPs)	100 fmol[a]	SNPs associated with long QT syndrome—KCNE1 gene / genomic DNA	[84]
Light-scattering imaging (cross-linking aggregation)	33 zmol	Methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*, (*mec*A gene) / DNA, cultured bacteria	[51]
Naked-eye or UV–visible spectroscopy (non-cross-linking aggregation)	375 zmol–4.2 pmol; 100 nmol L^{-1} [a]	K-ras oncogene / genomic DNA, colorectal adenocarcinoma cell lines; β-thalassemia mutations / genomic DNA, human whole blood *Mycobacterium tuberculosis* / DNA, clinical specimens	[56,70,85]
Light-scattering imaging (sandwich hybridization)	250 zmol–10 amol	Coagulation genes (Factor V, Factor II, and MTHFR—associated with thrombotic disease) / genomic DNA, human placenta; Gene expression in human brain / human brain total RNA; Methicillin-resistant *Staphylococcus aureus* (*mec*A gene) / DNA, cultured bacteria	[73,74,86]

[a] May involve PCR
[b] May involve RT-PCR

rapid, sensitive and inexpensive detection system. These systems have been successfully applied to the detection of specific DNA and RNA sequences, mutations, and SNPs, and to the detection of several pathogens. These strategies could easily become the next generation of diagnostic tools, as they show great sensitivity and specificity that can easily replace conventional molecular methods such as PCR and PCR-based approaches.

Furthermore, a recent nanotechnology development enabling extremely sensitive detection of biomolecules is the use of AuNPs as sensitizing agents for microcantilever-based sensors. Array systems are being developed with more than one hundred cantilevers in parallel [79], enabling low concentrations and small volumes to be probed with microcantilevers [80]. However, the greatest improvement in sensitivity to date has been through the use of nanoparticles as mass enhancers, allowing detection of DNA at a concentration of 23 pmol L^{-1} [81]. It is to be expected that small, high-frequency cantilevers and sensitization with metallic nanoparticles can enable sensitivity of microcantilever-based biosensors to increase to the femtomolar range and beyond, while maintaining the possibility of performing parallel analyses and working with minute volumes.

Future trends of AuNPs in diagnostics encompass their functionalization, allowing tailoring of the AuNP-biomolecule interface and modulation of the properties of the biomolecule either directly functionalized to the AuNP (e.g., thiolated DNA segments) or adsorbed on a suitable surface. Promising examples of the former are mixed monolayer-protected clusters (MMPCs), Au-nanoprobes with specific chemical functionalities that can be used in a "chemical nose/tongue" array to detect, identify, and quantify protein targets [3], and the glyconanotechnology strategy that holds great potential for utilization in clinical diagnosis [36], as it allows for the establishment of carbohydrate-mediated interactions, a highly relevant type of interaction in biological systems.

Although significant advances have been made involving the use of Au-NPs for diagnostics applications, technical and practical problems need to be solved. These include tight control over particle synthesis (size, size dispersion, capping agents) and functionalization, as small variations can drastically change their properties and behavior in diagnostic methods. One should note that most of the proposed systems must still be taken from proof-of-concept to use in generalized laboratorial settings, and from there to common clinical and diagnostic situations. Many questions on sample handling and preparation still need to be addressed, as many of these Au-NP systems are rather unstable in complex media, which may hinder application at point-of-care.

Further developments should include convenient high-throughput analysis, either by multiplexing and use of instrumentation, or by means of different chemistry that may vary the physicochemical properties of the AuNPs. Additional advances will be directed towards creation of integrated platforms to test for a variety of analytes simultaneously without loss of sensitivity, providing fast, specific, and low cost analysis at point-of-care. This could bring from bench to bedside instruments that will enable faster screening of alterations (DNA associated with human pathology, gene expression profiles, biochemical analysis, and pathogen identification), enabling tailored therapeutic intervention, prevention, and reduction of the significant number of hospital admissions that results from therapeutic incompatibility and failure.

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