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Gold Nanoparticles for DNA/RNA-Based Diagnostics

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Abstract

The remarkable physicochemical properties of gold nanoparticles (AuNPs) have prompted development in exploring biomolecular interactions with AuNPs-containing systems, pursuing biomedical applications in diagnostics. Among these applications, AuNPs have been remarkably useful for the development of DNA/RNA detection and characterization systems for diagnostics, including systems suitable for point of need. Here, emphasis will be on available molecular detection schemes of relevant pathogens and their molecular characterization, genomic sequences associated with medical conditions (including cancer), mutation and polymorphism identification, and the quantification of gene expression.

Keywords

Au-nanoprobes; Au-nanobeacons; LSPR; Nucleic acid; Point of care; SNP; Pathogen

List of Abbreviations

AuNP	Gold nanoparticle
ARMS	Amplification-refractory mutation system
AsPCR	Asymmetric PCR
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
iTPa	Isothermal target and probe amplification
LFA	Lateral flow assay
LOD	Limit of detection
LSPR	Localized surface plasmon resonance
MRSA	Methicillin-resistant staphylococcus aureus
MTBC	*Mycobacterium tuberculosis* complex
NASBA	Nucleic acid sequence based amplification
NP	Nanoparticle
PEXT	Primer extension

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POC	Point of care
QCM	Quartz crystal microbalances
RCA	Rolling circle amplification
RT-PCR	Reverse transcription PCR
SERS	Surface–enhancement Raman spectroscopy
SNP	Single nucleotide polymorphism
SPCE	Screen-printed carbon electrode
SARS	Severe acute respiratory syndrome
SBE	Single base extension
TB	Tuberculosis

General Aspects of Gold Nanoparticles Application for DNA/RNA-Based Diagnostics

Gold nanoparticles (AuNPs) show unique properties for biodetection, namely, optical, electrochemical, and spectral properties. AuNPs can be readily synthesized and promptly bioconjugated with similarly sized oligonucleotides, antibodies, aptamers, and other biomolecules appropriate for biodetection. Due to their nanosized scale, AuNPs have a high surface-to-volume ratio, making them ideal platforms for multivalent interactions based on a high concentration of exposed bioactive molecules.

DNA and RNA characterization for diagnostics purposes relies on the hybridization of a probe to a given target exploring the strand complementarity resulting from specific and stable Watson–Crick pairing. Therefore, a key step toward the establishment of an AuNP-based diagnostics methodology is to create an *AuNP nanoprobe* which is generated by functionalizing AuNPs with bifunctional ligands in which a moiety binds to the particles' surface, while the other allows for specific interaction with biomolecules. For example, AuNPs are easy functionalized with thiolated oligonucleotides [1] (Fig. 1).

The interesting optical proprieties of AuNPs, i.e., their intense vibrant colors in solution, derive from the interaction with light. Upon influence of the oscillating electromagnetic field of light, the AuNPs' free electrons collectively oscillate with respect to the positive metallic lattice [2]. This process is resonant at a

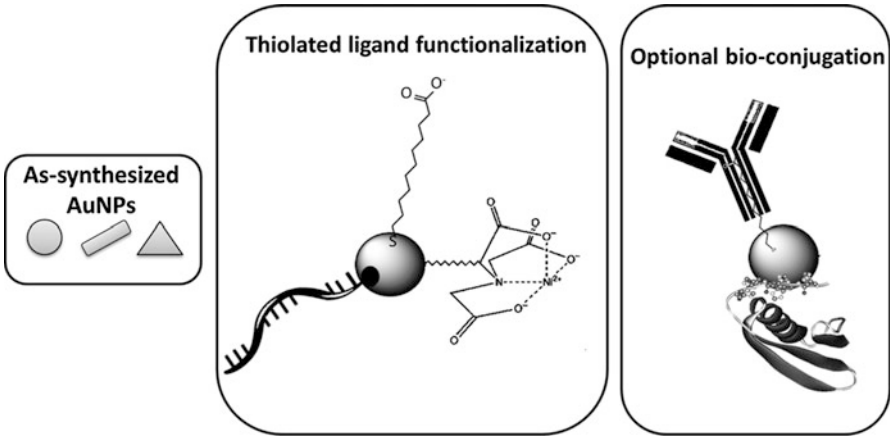

Fig. 1 Au-nanoprobe synthesis. Examples of consecutive steps of nanoparticle synthesis, ligand functionalization, and optional bio-conjugation. AuNPs of different morphologies (rod-shaped, triangular plates, but mostly spherical) can be linked to thiolated bifunctional ligands (11-mecarcaptoundecanoic acid (*MUA*); thiolated nickel(II) nitrilotriacetate (*Ni-NTA*); or single-stranded thiolated nucleic acid oligomers). Optional additional bio-conjugation can include antibodies (e.g., chemically cross-linked to MUA) or a His-tagged protein (binds to Ni-NTA)

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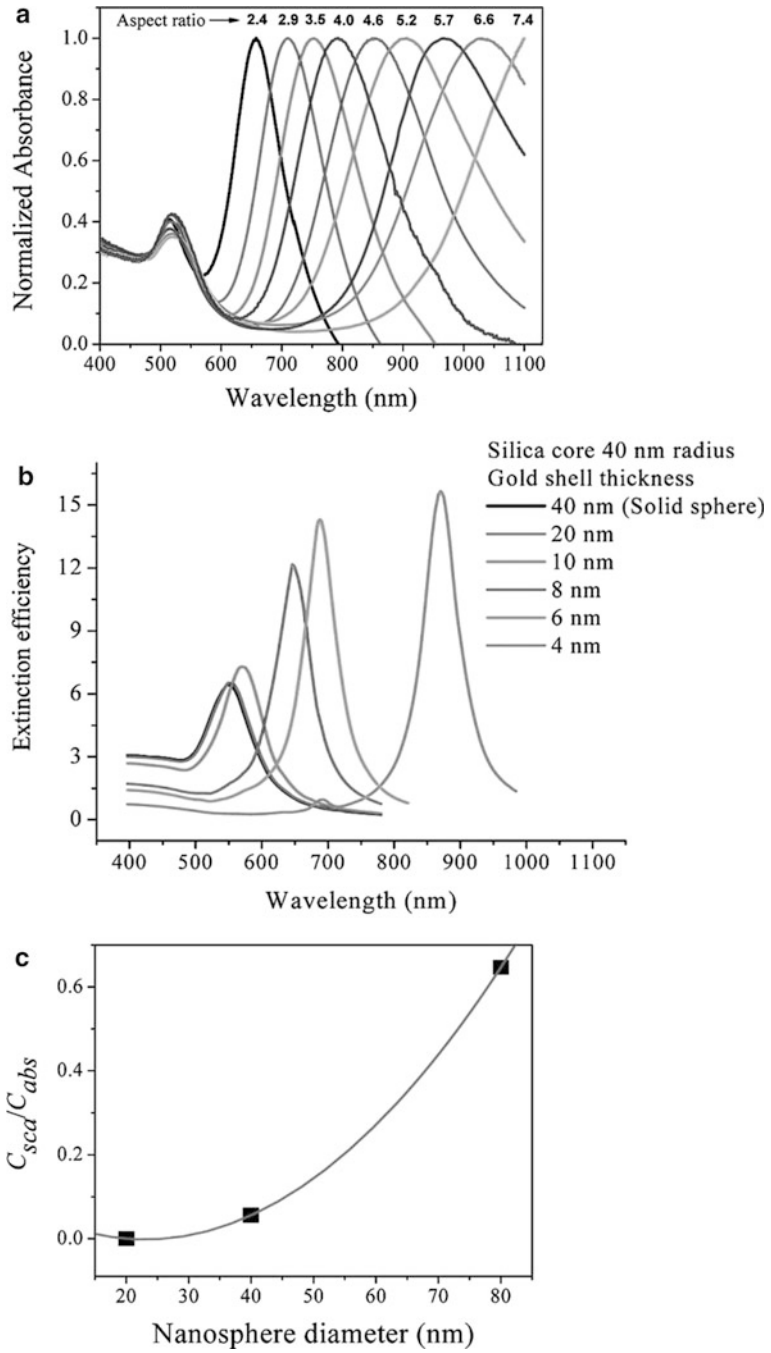

Fig. 2 Size, shape, and composition tunability of the plasmon resonance of gold nanostructures, (**a**) tuning the LSPR frequency of the gold nanorod long-axis mode by synthetically controlling aspect ratio, (**b**) silica core-gold nanoshells show plasmon resonance frequency tunable from the visible to the NIR by changing the shell thickness relative to the core size, (**c**) increase in the plasmon scattering to absorption ratio by increase in particle volume in spherical AuNPs (Reprinted with permission from Jain et al. [3] Copyright 2008 American Chemical Society)

particular frequency and is termed localized surface plasmon resonance (LSPR). Considering AuNPs, both the electric field intensity and the scattering and absorption cross sections are strongly enhanced at the LSPR frequency, which therefore occur in the visible (Fig. 2). Because of this, optical cross sections of metal nanoparticles (10–100 nm) are roughly five orders of magnitude larger than those of traditional

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organic dyes and fluorophores [2]. LSPR may be easily tuned by changing the nanostructure size, shape, composition, or environment [2, 3] (Fig. 2).

Nanodiagnostic methodologies have the ability to make molecular biological tests become faster, more sensitive, and flexible at reduced costs [4]. These new approaches can be integrated with conventional standard analytical methods, improving current techniques, and have been shown to be more sensitive and specific than conventional commercial molecular diagnostics. However, the great majority of these new systems still need further evaluation and validation with clinical samples and targets to transpose these tools from laboratory to clinic.

Despite the variety of nanoscale systems for biomolecular assays, AuNP-based systems have been mostly explored due to their unique physicochemical properties and are becoming a key element of nanotechnology-based detection of pathogens [5]. The development of schemes that can improve the sensitivity and specificity of molecular diagnosis methods and consequently to decrease the impact of a certain phenotype/disease is an easier task since the completion in 2003 of the Human Genome Project. In fact, targets have been revealed and their genetic sequence is well known [6].

This chapter presents several examples of applications involving AuNPs that can be translated into clinical applications. DNA/RNA detection schemes are based on the remarkable optical properties of AuNPs supplementary to the easiness of chemical functionalization through thiol ligands (e.g., thiol-modified oligonucleotides, antibodies, and other biomolecules). Recognition events occur at the nanoscale, i.e., in a one-to-one interaction between analytes and the nanoscale structures that act as signal transducers, allowing for increased sensitivity at lower costs. Each section encompasses a set of approaches based on the nanoscale properties involved in detection, namely: (i) colorimetric sensing depending on interparticle distance, which represents the most developed approach, especially for nucleic acid detection; (ii) fluorescence quenching/enhancement properties of AuNPs; (iii) plasmonics and light-scattering properties, including detection based on surface-enhanced Raman (SERS) and LSPR spectroscopies; (iv) piezoelectric sensors using AuNPs to increase sensitivity of detection based on mass increase; and (v) electrochemical detection methods based on electrical signal enhancement or generation provided by AuNPs (summarized in Table 1). The final section will present the latest advances in the utilization of AuNPs for diagnostics at point of care, using lateral flow devices.

AuNPs for Colorimetric Sensing

Among detection methods based on AuNPs, colorimetric approaches are the most frequent, allowing for simplicity and portability, making them ideal for diagnostics for point of care (POC). These methods rely on the colorimetric changes of an AuNP colloidal solution upon aggregation. The aggregation process can be either mediated by changes to the dielectric medium or upon recognition and binding to a specific target. Colloidal solutions of spherical AuNPs present an LSPR band that strongly depends on interparticle distance. These systems rely on the ability of complementary targets to modulate and control interparticle interactions (e.g., attractive and repulsive forces), which define whether AuNPs are dispersed or aggregated.

Functionalized AuNPs: Cross-Linking Approach

AuNPs modified with thiolated oligonucleotides led to the first application of AuNPs in nucleic acid detection. In their approach, Mirkin and coworkers [7] functionalized AuNPs with oligonucleotides modified with a thiol group at their 3′ and 5′ ends, whose sequences were contiguous and complementary to a target in a tail-to-tail (or head-to-tail) conformation. The hybridization of the two Au-nanoprobes with the target resulted in the formation of a polymeric network (cross-linking mechanism), which brought AuNPs in close vicinity to cause a red to blue color change, which can readily be detected visually, or by UV–vis spectroscopy (Fig. 3b).

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Table 1 Gold nanoparticle based molecular diagnostics

Detection technique	Nucleic acid target	Biological target	Sample amplification vs. direct detection	Detection limit	References
Colorimetric (non-cross-linking aggregation)					
Naked eye, UV–vis spectroscopy or optical monitoring system	DNA	Human	PCR	600 pM	Sato et al. [93]
	DNA	Human	PCR	0.1 pmol	Qin and Yung [94]
	DNA	Human	PCR	18 ng/μl	Doria et al. [35]
	DNA	Human	AsPCR	10 pg	Deng et al. [95]
	RNA	Human	Direct detection	10 ng/μl	Conde et al. [33]
	DNA	*M. tuberculosis* spp.	Direct detection	2 ng/μl	Liandris et al. [31]
	DNA	*Chlamydia* sp.	PCR	–	Jung et al. [96]
	DNA	MTBC	PCR	0.75 ug	Baptista et al. [21] Costa et al. [24]; Veigas et al. [22, 23]; Silva et al. [27]; Bernacka-Wojcik et al. [25]
	DNA	*E. coli*	Direct detection	54 ng	Padmavathy et al. [32]
	RNA	*S. typhimurium*	NASBA	–	Mollasalehi and Yazdanparast [29]
	RNA	*S. Enteritidis*; S. *Typhimurium*	NASBA	5 CFUs	Mollasalehi and Yazdanparast [30]
Colorimetric (cross-linking aggregation)					
Light scattering imaging	DNA	Human	SBE	–	Storhoff et al. [97]
	DNA	MRSA	Direct detection	33 fM	Storhoff et al. [8]
	DNA	HIV; *M. Tuberculosis*; *B. glucanase*	Direct detection	80 pM	Wei He et al. [9]
	DNA; RNA	*C. parvum*	PCR; RT-PCR	10 amol	Javier et al. [10]
Naked eye, UV–vis spectroscopy or optical monitoring system	DNA	Human	PCR	–	Qin et al. [98]
	DNA	Human	RCA	70 fM	Li et al. [99]
	DNA	*MTBC*	PCR	0.5 pmol	Soo et al. [13]
	DNA	*C. trachomatis*	PCR	250 pM	Parab et al. [18]
	DNA	*C. trachomatis*	iTPA	102 copies of Plasmid DNA	Jung et al. [15]
	DNA	*P. aeruginosa*; *S. aureus*; *S. epidermidis*; *K. pneumonia*; *S. marcescens*; *B. cereus*	PCR		Wang et al. [19]
	DNA	*N. gonorrhoeae*; *T. pallidum*; *P. falciparum*; HBV vírus	Non-enzimatic (MNAzyme)	1 nM	Zagorovsky and Chan [20]
	DNA	*Salmonella sp*	Direct detection	37 fM	Kalidasan et al. [16]
	RNA	*M. Tuberculosis*	NASBA	10 CPU/mL	Gill et al. [12]

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Table 1 (continued)

Detection technique	Nucleic acid target	Biological target	Sample amplification vs. direct detection	Detection limit	References
	RNA	*C. parvum*	Direct detection	1.2×10^7 copies/μL	Weigum et al. [11]
	DNA	HPV	AsPCR	14 pM	Chen et al. [14]
	DNA	Kaposi's sarcoma-associated herpes virus	Direct detection	1 nM	Mancuso et al. [17]
Colorimetric (sandwich assay)					
Colorimetric detection using gold label silver stain	DNA	*U. urealyticum*; *C. trachomatis*	Multiplex asPCR	5 pM	Cao et al. [38]
Naked eye or CCD camera (sandwich hybridization)	DNA	HBV	PCR	10 pM	Xi et al. [100]
Colorimetric detection using gold label silver stain – micro array	DNA	Human	Direct detection	0.5 ug	Bao et al. [101]
	DNA	Human	Direct detection	1 ug	Lefferts et al. [90]
	RNA	Avian Influenza Virus (H5N1)	Direct detection	0.1 pM	Zhao et al. [41]
Lateral flow test strips	DNA	Human	PCR	50 pM	Mao et al. [102]
	DNA	Human	PCR or PEXT	–	Litos et al. [103]
	miRNA	Human	Direct detection	1 fmol, 5 amol	Hou et al. [89]
	DNA	*V. cholerae*	PCR	5 ng	Chua et al. [87]
	DNA	E. coli	Direct detection	~0.4 nM	Rastogi et al. [40]
	RNA	HIV-1	NASBA	9.5 $\log_{10}$ RNA copies in 20 mL	Rohrman et al. [88]
Colorimetric (unmodified AuNPs)					
Hyper-Rayleigh scattering (HRS)	DNA	Human	PCR	10 nM	Li et al. [49]
Naked eye or optical monitoring system	DNA	Human	Direct detection	–	Tan et al. [104]
	DNA	Human	Direct detection	6 nM	Chang et al. [105]
	DNA	*MTBC*	PCR	1 ng	Hussain et al. [106]
	DNA	HBV	Direct detection	5 nM	Liu et al. [52]
	DNA	*B. anthracis*	AsPCR	0.1 pmol	Deng et al. [54]
	RNA-DNA	HIV-1	Enzymatic activity (HIV-1 RT)	–	Xie et al. [53]
	RNA	HCV	Direct detection	<100 fM	Shawky et al. [51]
Fluorescence spectroscopy					
Fluorescence spectroscopy Surface-energy transfer (NSET)	DNA	Human	Direct detection	1 nM	Beni et al. [64]
	DNA	Human	ARMS	0.5 nM	Beni et al. [65]
	DNA	Human	Direct detection	5–10 nM	Wang et al. [67]
	RNA	Human	RT-PCR	10.3 fmol	Rosa et al. [107]

(*continued*)

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Table 1 (continued)

Detection technique	Nucleic acid target	Biological target	Sample amplification vs. direct detection	Detection limit	References
	cDNA	HAV; HCV; West Nile; HIV; SARS	RT-PCR	–	Sha et al. [108]
	DNA	*S. enterica* serovar Enteritidis	PCR	1 ng/mL	Zhang et al. [61]
	RNA	HCV	Direct detection	0.3 fM	Griffin et al. [50]
Raman spectroscopy					
Surface-enhanced Raman scattering (SERS)	DNA	Human	SBE	3 pM	Hu and Zhang [109]
	RNA	Human	RT-PCR	2.3 pM	Sun and Irudayaraj [110]
	DNA	HIV-1	Direct detection	0.1 aM	Hu et al. [70]
	DNA/ RNA	HAV; HBV; HIV; Ebola virus; Variola virus; *B. anthracis*	Direct detection	20 fM	Cao et al. [69]
Piezoelectric					
DNA probe functionalized quartz crystal microbalance	DNA	*E. coli*	PCR	1.2×10^2 CFU/mL	Chen et al. [72]
	DNA	*E. coli*	AsPCR	2.0×10^3 CFU/mL	Wang et al. [73]
	DNA	*B. anthracis*	AsPCR	3.5×10^2 CFU/mL	Hao et al. [74]
Electrochemical					
Square wave anodic stripping voltammetry on screen-printed carbon electrode (SPCE) chips)	DNA	*B. anthraci*; *S. enteritidis*	PCR	0.5 ng/mL for *S. enteritidis*; 50 pg/mL for *B. anthracis*	Zhang et al. [78]
Automated System (Light scattering imaging)	DNA	*Mycobacterium* spp.	Direct detection	1.25 ng/mL	Thiruppathiraja et al. [79]
Differential pulse voltammetry	DNA	*S. enterica* serovar Enteritidis	Direct detection	7 ng/mL	Vetrone et al. [80]
Electrocatalytic signal amplification of AuNPs	cDNA	HCV	RT-PCR	–	Li et al. [111]
Differential pulse anodic stripping voltammetry	DNA	Human	PCR	–	Ozsoz et al. [112]
	DNA	*V. cholerae*	AsPCR	3.9 nM	Low et al. [113]
Other					
Automated System (Light scattering imaging)	DNA	Influenza A virus; Influenza B virus; respiratory syncytial virus A and B	PCR	–	Jannetto et al. [114]
Bio-MassCode mass spectrometry	DNA	HIV; HBV; HCV; *T. pallidum*	Direct detection	10^{-18} M	Yang et al. [115]

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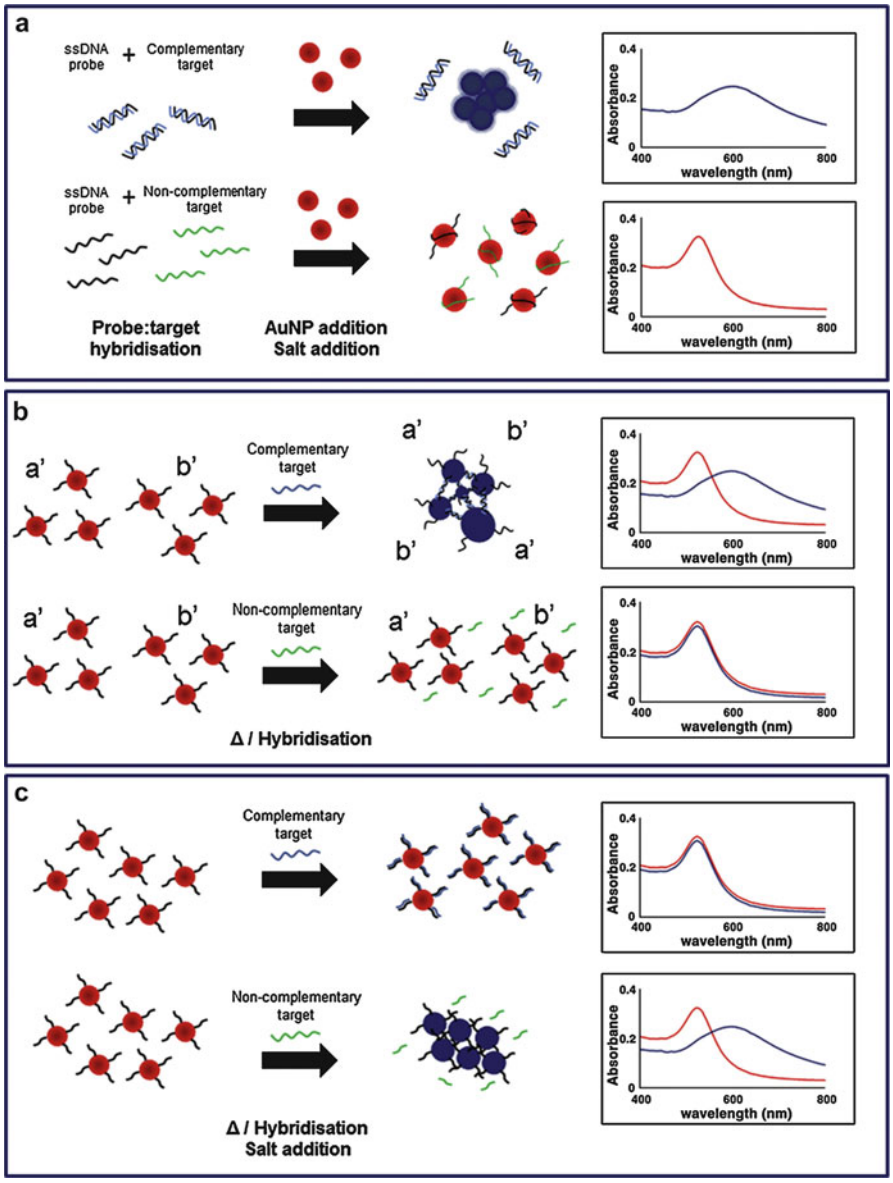

Fig. 3 Gold nanoparticle-based colorimetric assays, (**a**) colorimetric assay based on naked AuNPs, (**b**) cross-linking hybridization assay, (**c**) non-cross-linking hybridization assay (Reproduced from Larguinho and Baptista [92] with permission from Elsevier)

The first application of this approach, for pathogen detection, was described by Storhoff and coworkers [8]. They developed a "spot-and-read" colorimetric detection method for the identification of DNA sequences. In this assay, the color change of DNA-modified gold probes was detected after spotting onto a glass surface illuminated by a light beam. This light-scattering method allowed detection of DNA in zeptomole quantities without signal or target DNA amplification. In comparison to the absorbance-based methods, this approach allowed a 4 order of magnitude increase in sensitivity. This way, it was possible to detect the *mecA* sequence gene in methicillin-resistant *Staphylococcus aureus* directly from genomic DNA samples. The system showed high sensitivity with a limit of detection (LOD) of 66 ng/μL of DNA. Following the same approach, several groups were able to develop methods for human immunodeficiency virus type 1 [9] and *Cryptosporidium parvum* [10] with direct detection capabilities, allowing to circumvent expensive enzymatic DNA amplification reactions. Recently, Weigum

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et al. developed an amplification-free molecular assay for the detection of *Cryptosporidium parvum* oocysts [11]. The assay targeted the *C. parvum* 18 s rRNA, with an LOD of 4×10^5 copies of RNA per μL per reaction mix. The ability to detect the *C. parvum* oocysts without the need for complex amplification is of utmost relevance in resource-limited settings where protozoan detection is needed the most.

Toward the development of naked eye scheme for detection and characterization of pathogen's RNA, Gill et al. integrated a nucleic acid sequence-based amplification (NASBA) and AuNP probes for the specific detection of *Mycobacterium tuberculosis* [12]. The 16S rRNA of mycobacterial RNA was amplified via isothermal NASBA and then hybridized with specific probes. This method showed an LOD of 10 CFU ml^{-1} with a sensitivity and specificity of 94.7 % and 96 %, respectively. Following the same detection approach, Soo et al. designed a set of Au-nanoprobes for *M. tuberculosis* complex strains DNA identification [13], showing a detection limit of 0.5 pmol of DNA. The 2-h assay comprises two main steps: target DNA amplification via single or nested PCR, followed by detection using the specific nanoprobes.

During the last few years, several advances have been aimed at reducing the gap between light-scattering imaging and naked eye sensitivity. Earlier methods used enzymatic amplification techniques, such as PCR, asymmetric PCR, and NASBA [14–19]. Recently, Zagorovsky and Chan reported on the integration of a multicomponent DNA-responsive DNAzyme with colorimetric detection using AuNPs, allowing for nonenzymatic signal amplification [20]. This constitutes a simple and fast colorimetric approach for the detection of DNA targets of bacteria, viruses, and parasites (LOD of 50 pM). This method is also able to detect multiple sequences in parallel. The color readout discards the use of complex equipment that makes it suitable for POC assays. More recently, this same approach was used for the naked eye and direct (amplification-free) detection of Kaposi's sarcoma-associated herpes virus [17] and *Salmonella* sp. [16]. Kalidasan et al. reported the detection of unamplified DNA from *Salmonella* sp. with an LOD of 37 f. for visual detection.

Functionalized AuNPs: Non-Cross-Linking Approach

Baptista and coworkers developed an inexpensive approach for the colorimetric detection of DNA sequences [21]. This method uses just one Au-nanoprobe, instead of the two required in the cross-linking method (see previous section). Detection is achieved after comparison of the solutions' color following salt addition. The presence of a complementary target does not allow nanoprobe aggregation, and the solution retains the original red color; non-complementary/mismatched targets allow gold nanoprobe aggregation, and the solution turns blue. The first clinical application of this strategy was in the rapid and sensitive detection of *Mycobacterium tuberculosis* [22] (Fig. 3c). The gold nanoprobes were functionalized with thiol-modified oligonucleotides harboring a sequence derived from the pathogen's RNA polymerase β-subunit gene sequence. This methodology had been previously tested in clinical samples and, if associated to PCR, shows great sensitivity [21]. This protocol was further used for the rapid detection of *M. tuberculosis* complex (MTBC) members and simultaneous identification of mutations linked to antibiotic resistance [23]. LOD was determined at 75 nM. Using a set of three Au-nanoprobes based on the *gyrB* locus, specific identification of MTBC, *M. bovis*, and *M. tuberculosis* was easily achieved [24]. Considering application at POC, Baptista and colleagues integrated this non-cross-linking method in an optoelectronic platform (an amorphous/nanocrystalline biosensor and a light emission source) that assesses the colorimetric changes. This low-cost simple platform may be of use once integrated in a microfluidic test device for improved TB diagnostics with a tenfold reduction of reagents [25–27]. Recently, in an effort to increase sensitivity and ease of use, this detection strategy was integrated onto a paper-based platform [28]. Differential color scrutiny is captured and analyzed with a generic "smartphone" device to evaluate assay results and perform RGB analysis. The possibility to send the acquired information to a central lab is also considered via 3G technology. GPS

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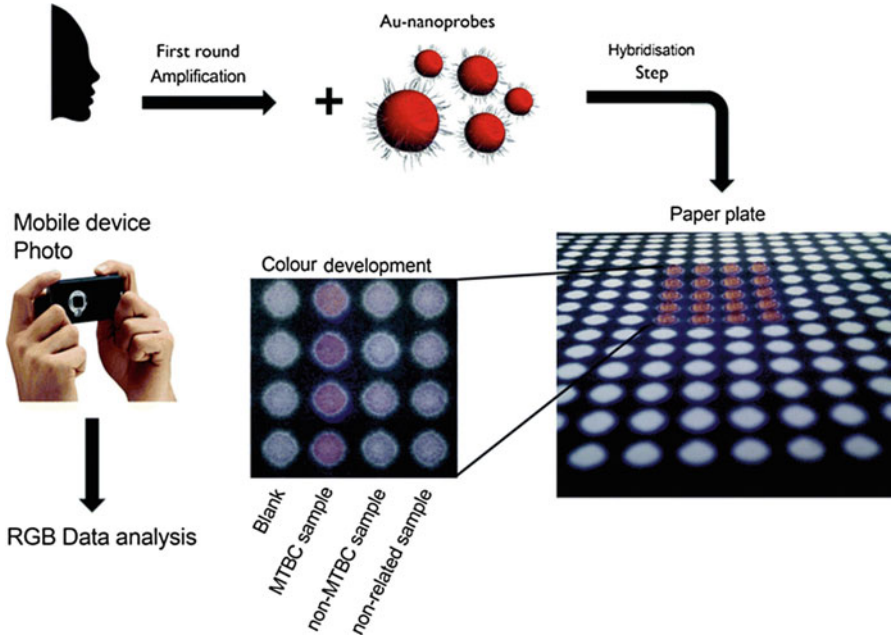

Fig. 4 Au-nanoprobe strategy for the detection of MTBC members. Schematic representation of detection of *M. tuberculosis* using Au-nanoprobes and a paper platform. The colorimetric assay consists of visual comparisons of test solutions after salt-induced Au-nanoprobe aggregation on a [$MgCl_2$]-impregnated paper plate: MTBC Au-nanoprobe alone, blank; MTBC Au-nanoprobe in the presence of MTBC sample, *M. tuberculosis*; MTBC Au-nanoprobe in the presence of a non-MTBC sample; and MTBC Au-nanoprobe in the presence of a noncomplementary sample, nonrelated. After color development, a photo of the paper plate is captured and RGB image analysis is performed (Reproduced by permission of The Royal Society of Chemistry [28])

location may be added to each test image, which would provide actual epidemiologic data on MTBC (Fig. 4).

Following the same trend as the cross-linking method, several optimizations have been made toward higher sensitivity with the integration of NASBA or other RNA amplification methods [29, 30]. Liandris et al. optimized the non-cross-linking approach described above to the detection of TB without the need for target amplification [31]. The detection is based on the fact that double- and single-stranded oligonucleotides show distinct electrostatic behaviors. Hybridization leads to double-stranded DNA formation, which cannot uncoil sufficiently to expose its bases toward the Au-nanoprobe. Therefore, the Au-nanoprobe aggregates under the acidic conditions of the test. More recently, Padmavathy et al. reported on the visual direct detection of *Escherichia coli* without the need for nucleic acid amplification. This approach is able to detect ~54 ng for unamplified genomic DNA, while reducing the overall time for detection to less than 30 min [32].

The non-cross-linking method was further applied to the identification and quantitation of RNA associated with human disease [33]. This system was successfully applied in the detection of BCR-ABL fusion gene mRNA, a hallmark for chronic myeloid leukemia [34]. This approach was capable to detect less than 100 fmol/μl of the specific RNA target (less than 10 ng/μl of total RNA). Also, the non-cross-linking approach could distinguish common-base mismatch (SNPs) within the β-globin gene [35]. The authors could detect three different individual mutations using only one Au-nanoprobe.

Functionalized AuNPs: Sandwich Assays

DNA sandwich methods are very common in various POC systems. Microarrays, streptavidin–biotin stripes, and lateral flow cytometer systems are usually based on this method [36, 37]. Although each

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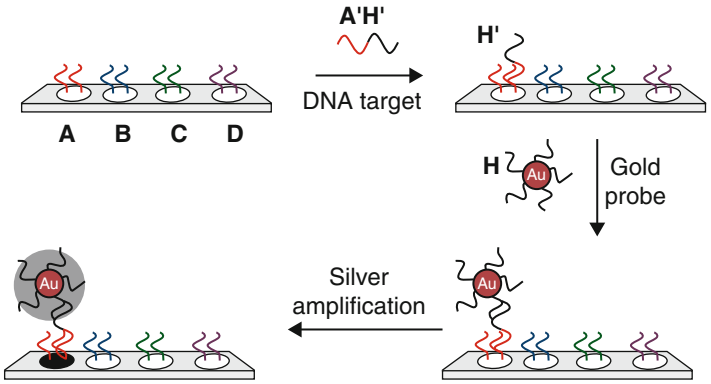

Fig. 5 Microarray DNA detection via AuNPs. DNA hybridization to microarrays and detection using silver-amplified gold nanoparticle probes. Following target hybridization to a capture probe immobilized on the array surface, a secondary Au-nanoprobe is used for detection. DNA target and Au-nanoprobe hybridization can be performed in a single step. An additional signal amplification step may be introduced via silver deposition (Reproduced from Storhoff et al. [48] with permission from Elsevier)

method varies in the building block, all share the same principle: a DNA probe is fixed on a support in the form of stripes or spots, followed by sample DNA hybridization to the fixed probe [38–41]. The second probe, consisting of AuNPs functionalized with 5′-thiol-modified oligonucleotides (the Au-nanoprobe), is then hybridized to a second region of the target sample DNA. Total complementarity with both probes prevents the Au-nanoprobes from being removed by washing and yields the final assay result after signal enhancement by silver deposition (Fig. 5). Sandwich-based assays are being developed in a lateral flow strip scheme with increased sensitivity and are today a suitable alternative for the detection of PCR amplicons [37, 42–47].

Storhoff and coworkers introduced the first application of the DNA sandwich approach for pathogen and human detection, applying it to methicillin resistance *S. aureus*, and human MTHFR gene [48]. Later, Cao and coworkers developed a similar method using a visual DNA microarray using Au and Ag stain together with multiplex asymmetrical PCR. This approach allowed for the simultaneous detection of *Ureaplasma urealyticum* and *Chlamydia trachomatis*. The surface immobilized 5′-end-amino-modified oligonucleotides, acted as capturing probes to bind the complementary biotinylated targets. Au-streptavidin conjugates specifically bind to biotin, and agglomeration was enhanced with silver for enhanced colorimetric signal. The sensitivity of this assay has been much improved by combination with silver/gold deposition to enhance light-scattering properties and further decrease detection limits, allowing visual or optical detection (e.g., image scanner). Using a similar approach, Zhao and coworkers developed a microarray platform using AuNP-based genomic microarray assay for specific identification of avian influenza virus H5N1 RNA [41]. Viral RNA was detected within 2.5 h using capture-target-intermediate hybridization, and AuNP triggered silver staining, without target amplification or any enzymatic reactions (LOD of 100 f. for purified PCR amplicons and $TCID_{50}$ units for H5N1 RNA).

Non-functionalized AuNPs

Unmodified AuNPs have also been used for detection of DNA/RNA biomarkers [49]. These systems rely on the unspecific adsorption of biomolecules to non-functionalized AuNPs, usually profiting from the differential affinity of ssDNA and dsDNA to the AuNPs' surface. ssDNA nucleobases electrostatically interact with the citrate capped AuNPs' surface, thus stabilizing the AuNPs against higher ionic strengths. dsDNA molecules do not adsorb the same way to the AuNPs and, as such, aggregation occurs (Fig. 3a).

Griffin et al. used unmodified AuNPs for the detection and quantification of hepatitis C virus (HCV) RNA. Their approach consists on the adsorption of a synthetic ssDNA probe complementary to the target

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[50]. Whenever the target is present, the ssDNA probes and target hybridize and stop being available to stabilize the AuNPs. Despite a clear colorimetric visual detection, the use of hyper-Rayleigh scattering measurement allowed for an increase in sensitivity of two orders of magnitude. Shawky et al. used the same method using clinical samples to detect the presence of HCV RNA [51]. The isolated RNA was added to a solution containing the complementary oligonucleotide probe, and after a denaturing and annealing cycle, unmodified AuNPs were added. This extremely simple and inexpensive assay, which does not include an RT-PCR step, presented a detection limit of 50 copies/reactions and exhibited a sensitivity of 92 % and a specificity of 89 %. A similar approach using an S1 nuclease to discriminate single mismatches in DNA with unmodified AuNPs was proposed, in which dNMPs adsorb to AuNPs stabilizing them further in relation to ssDNA [52]. In the presence of a mismatch between the synthetic oligonucleotide and the DNA sample, the S1 nuclease degrades ssDNA–ssRNA hybrid into its monomers (dNMPs), which increase the stability of AuNPs in solution. Also using an enzyme-mediated process, [53] tested the activity of ribonuclease H (RNase H) activity of HIV-1 reverse transcriptase. In the presence of RNase H activity RNA–DNA hybrids are formed, otherwise ssRNA remains in solution adsorbing to the AuNPs. [54] took advantage of asymmetric PCR to generate long ssDNA amplicons that stabilize AuNPs in solution after salt addition. However, if no amplification occurs, no long ssDNA amplicons are present, leading to AuNPs aggregation due to the increasing ionic strength (LOD set at 10 ng of target DNA).

Gold Nanoparticles Modulation of Fluorescence

Fluorescence is frequently used for biosensing due to sensitivity and easiness of implementation. The extraordinary quenching of fluorophores by noble metal NPs prompted the development of several new approaches with remarkable sensitivity and specificity. Gold nanoprobes can take advantage of the interesting luminescent properties of AuNPs for molecular diagnostics. In fact, AuNPs can cause fluorescence enhancement or quenching of chromophores in their vicinity. Chromophores within ca. 5 nm of the surface of the AuNP have their fluorescence quenched by non-radiative pathways, while chromophores at larger distances (>10 nm) show a fluorescence enhancement of up to 100-fold [55–58]. The quenching properties of AuNPs have been applied in two approaches: molecular beacons, which rely on NPs functionalized with fluorescent-labeled hairpin structures, and AuNPs nanoprobes with ssDNA that hybridize to another fluorescent-labeled ssDNA probe [59]. Molecular beacons, i.e., AuNPs functionalized with fluorescent-labeled ssDNA where the NP act as a fluorescence quencher via the NP surface energy transfer occurring between the dye (donor molecule) and the NP's surface (acceptor). On the other hand, noble metal nanoprobes rely on NPs functionalized with ssDNA hybridized to a complementary fluorescent-labeled ssDNA probe. Since ssDNA naturally adsorbs to the AuNPs, the proximity of the fluorophore with the nanoparticle is close enough for a 98 % of quenching efficiency. In the presence of a complementary target, dsDNA is formed resulting in a spatial separation of the fluorophore from the AuNPs, thus restoring the fluorescence signal (Fig. 6).

The combination of AuNPs and semiconductor quantum dots (QDs) as a Förster resonance energy transfer (FRET) system has been used to develop fluorescence competition assays for nucleic acid, protein, and antibody/antigen detection where the dye is replaced by QDs [1].

Fluorescence modulation by AuNPs has been used to monitor specific nucleic acid hybridizations. Two different research groups have used this approach to detect synthetic DNA of *Campylobacter* and HCV synthetic RNA, respectively, with single-base mismatches detection capability [50, 60]. Deng Zhang et al. used a bio-barcode-type assay to obtain a fluorescence signal of PCR-amplified *Salmonella enteritidis* DNA [61]. In this type of assay, two types of nanoparticles are functionalized. Firstly, magnetic sulfo-SMCC-modified nanoparticles are functionalized with oligonucleotide sequence complementary to the sample DNA. Secondly, AuNPs are bi-functionalized with an oligonucleotide sequence specific for

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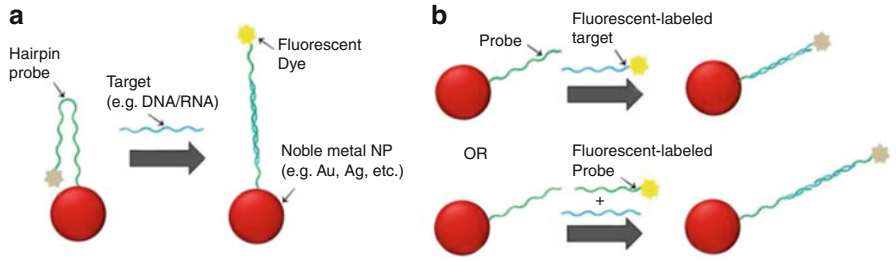

Fig. 6 Fluorescent-based noble metal NPs biosensing, (**a**) molecular nanobeacons, (**b**) direct hybridization of a fluorophore-labeled target or sandwich assay using fluorophore-labeled probe. Distances are not represented to the scale (Reproduced from Doria et al. [59])

the target (in lower percentage), and another sequence that contains a nucleotide sequence 3′ modified with a fluorophore (in higher percentage). Both nanoparticles are mixed with the sample DNA and, in case of total complementarity, the sample DNA serves has linker of both nanoparticles. After the hybridization step, a magnetic field is generated to isolate hybridized NPs from non-hybridized ones, and the thiol bonds are broken releasing the fluorophore-labeled oligonucleotides (LOD of 21.5 fM). Ganbold et al. describe a similar method, but the synthetic probe is functionalized with a fluorophore whose fluorescence is quenched if the ssDNA is adsorbed to the particle but not if there is complementarity to the target [62]. Standard fluorescence signal is measured but there is also the possibility of using sub-aggregation conditions for Raman spectroscopic analysis. The method was tested with influenza A (H1N1) synthetic DNA and was able to discriminate single-base mismatches.

In another application, Dubertret and coworkers used Au-nanobeacons to detect single-base mismatches with 100× more sensitivity than that of conventional molecular beacons [63]. Similarly, Beni et al. used Au-nanobeacons to successfully detect a mutation that occurs in 70 % of cystic fibrosis patients using nM concentrations of DNA target [64, 65]. In the second case, AuNPs can be combined with dye-labeled ssDNA probe and is used to detect specific DNA targets mediated by energy transfer mechanisms (FRET). The method consists in designing probes that identify complementary and contiguous sequences on the target, where hybridization forces the dye into close vicinity of the AuNPs' surface and the fluorescence signal decreases [66]. More recently, Wang et al. proved that the fluorescence quenching/enhancement conferred by AuNP could be used as an SNP genotyping system suitable for point of care [67].

Gold Nanoparticles for Plasmonic-Based Sensing

Plasmonic sensing here refers in the broader sense to detection based on light-scattering techniques, namely, SERS and LSPR spectroscopies. These techniques take advantage of the different size and shapes of AuNPs to detect the biding of molecules and alterations to conformation. Plasmonic nanoparticles act as signal transducers capable of converting minute changes to the local refractive index into sharp spectral shifts [68] (Fig. 2).

Raman Scattering

Raman scattering originates from the inelastic scattering of photons that interact with the analyte molecule, changing its vibrational states [59]. This interaction generates unique narrow spectrum bands that may be enhanced by metal nanostructures, allowing multiplex detection assays. Metallic nanosurfaces associated to SERS allow detection of specific biomolecules and is usually performed by means of a molecule with an intense and characteristic Raman signature (e.g., dye). SERS detection methods show great similarities to those previously described methods in colorimetric and fluorescent assays.

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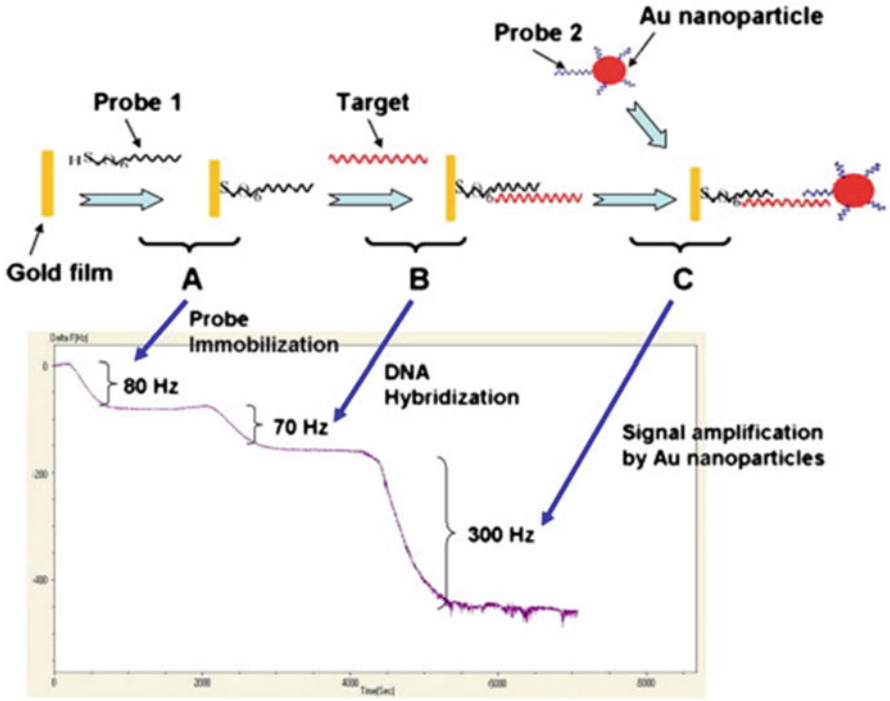

Fig. 7 Time-dependent frequency changes of the circulating-flow QCM sensor, (**a**) addition of Probe 1 (1 μM; P1-30/12 T) for immobilization on the surface of the QCM sensor via self-assembly, (**b**) complementary target oligonucleotides [0.5 μM; T-104(AS)] are subsequently introduced for DNA hybridization, (**c**) additional treatment of the DNA hybridized QCM with Probe 2 (P2-30/12 T)-capped Au nanoparticles. The sequences of Probe 1 and Probe 2 are complementary to the two ends of the analyte DNA (i.e., target sequences) (Reproduced from Chen et al. [72] with permission from Elsevier)

The first proof of concept of this approach for pathogen detection was introduced by Cao et al., with a multiplexed detection of oligonucleotide targets with AuNPs labeled with oligonucleotides and Raman-active dyes [69]. AuNPs help the formation of the silver coating, i.e., SERS promoter for the dye-labeled particles captured by the target molecules. This may be employed in a microarray format. Six DNA targets with six Raman-labeled NP probes were easily distinguished together with two RNA targets with single nucleotide polymorphism (LOD of 20 fM). This allowed for the multiplexed direct detection of hepatitis A virus, hepatitis B virus, HIV, Ebola virus, Variola virus, and *Bacillus anthracis*.

Hu et al. developed a sensitive DNA–SERS biosensor based on multilayer metal–molecule–metal nanojunctions [70]. The sensor could detect as little as 0.1 atomolar of a HIV-1 DNA sequence. High sensitivity may be attained via a bio-barcode approach combined with a silver-enhanced spot test. Isola and coworkers explored a similar approach for HIV detection using Raman-active dye-labeled DNA on Ag or AuNPs [71]. Upon target recognition, the cross-linking cages the Raman reporter in "hot spots" between NPs and, thus, enhances the SERS signal.

Piezoelectric Sensors Using AuNPs for DNA/RNA Recognition

Quartz crystal microbalances (QCM) have been extensively investigated as transducers for hybridization-based DNA sensors, such as for the detection of gene mutations associated with disease and foodborne pathogens. Improving the sensitivity of QCM sensors has been based on probe immobilization and signal amplification strategies that include nanoparticles [72]. Nanoparticles are effective amplifiers for QCM DNA detection because they have a relatively large mass compared to that of DNA targets [73]. The use of AuNPs coupled to the DNA targets acts as "mass enhancers," i.e., signal amplification, thus extending the limits of QCM DNA detection (Fig. 7).

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Chen and coworkers introduced the first nanoparticle-amplified QCM DNA sensor for foodborne pathogens [72], using the sandwich hybridization of two specific probes: one specific to *E. coli* O157:H7 immobilized onto the piezoelectric surface and a second conjugated to the AuNPs as "mass enhancer" and "sequence verifier" by amplifying the frequency change of the piezoelectric part. The oscillation frequency of the piezoelectric sensor decreased with increasing weight at the sensor's surface (i.e., sandwich hybridization involving the target oligo, the sensor's probe, and the circulating DNA-functionalized AuNP). Thus, PCR products amplified from concentrations of 1.2×10^2 CFU/mL of *E. coli* O157:H7 were easily detectable. Wang et al. also used AuNPs to functionalize QCM for *E. coli* DNA detection, employing two sizes of AuNPs to increase sensitivity [73]. First, 18-nm AuNPs were immobilized onto the QCM surface to support the ssDNA probes that will bind specifically to the biotinylated DNA of target bacteria. The gold layer binds a higher number of ssDNA molecules to the QCM, thus increasing sensitivity. The biotinylated DNA from the target organism binds to the sensor, which is recognized by avidin-functionalized 70-nm AuNPs to further amplify the signal. This scheme was capable of detecting bacteria without sample enrichment showing an LOD of 2.0×10^3 CFU/mL. Recently, Hao et al. developed this method to detect *Bacillus anthracis* based on the recognition of a 168-bp fragment of the *Ba813* gene and the 340-bp fragment of the *pag* gene in plasmid pXO1 [74]. A thiol DNA probe was immobilized onto the QCM gold surface to hybridize to the target ssDNA obtained by asymmetric PCR. The DNA-functionalized QCM biosensor could specifically recognize *B. anthracis* (and distinguish from its closest species, *Bacillus thuringiensis*) – LOD of 3.5×10^2 CFU/mL for *B. anthracis* vegetative cells without culture enrichment.

Electrochemical Detection of DNA/RNA Targets Using AuNPs

Other physicochemical properties of AuNPs have also been used in detection protocols, such as electrochemical activity. AuNPs are also extremely useful in electrochemical bioassays, to bind enzymes to electrodes, mediate electrochemical reactions as redox catalysts, and amplify recognition signals of biological processes [1, 75, 76]. Examples of applications in DNA detection include direct detection of AuNPs anchored onto the surface of the genosensor, conductimetric detection, and AuNPs as carriers of other AuNPs or of other electroactive labels [77]. In general, electrochemical biosensors employ potentiometric, amperometric, or impedimetric transducers.

Zhang et al. described an approach that makes use of the bio-barcode method to detect DNA from *Salmonella enteritidis* and *Bacillus anthracis* in a multiplex assay where the characteristic molecular signature sequences are labeled with cadmium and lead ions, respectively [78]. Following magnetic separation, the ions are cleaved from the oligonucleotides and the square–wave anodic stripping voltammetry analyzed on screen-printed carbon electrode (SPCE) chips. The differential signal of both ions allows the parallel read of either DNA signature. Further signal augmentation was attained via introduction of PCR amplification and the detection limit set at 0.5 ng/mL for Cd^{2+} and 50 pg/mL for Pb^{2+}.

Also using an electrochemical approach, it was possible to directly detect *M. tuberculosis* DNA with a detection limit of 1.25 ng/mL [79]. Firstly, AuNP are dual-labeled with a complementary sequence of target DNA and an enzyme alkaline phosphatase. Secondly, Au-nanoprobes are fixed onto indium tin oxide-coated glass plates that work as electrodes, and the extracted DNA followed by a second mix of dual-labeled AuNPs are then added and let to hybridize. Then, in the presence of paranitrophenyl phosphate, if both probes hybridize, the substrate is converted in paranitrophenol generating a signal that can be measured by differential pulse voltammetry.

Vetrone et al. describe a bio-barcode-based assay in which, instead of using a signature DNA sequence, the amount of gold separated via the magnetic nanoparticles is measured [80]. Hydrochloric acid

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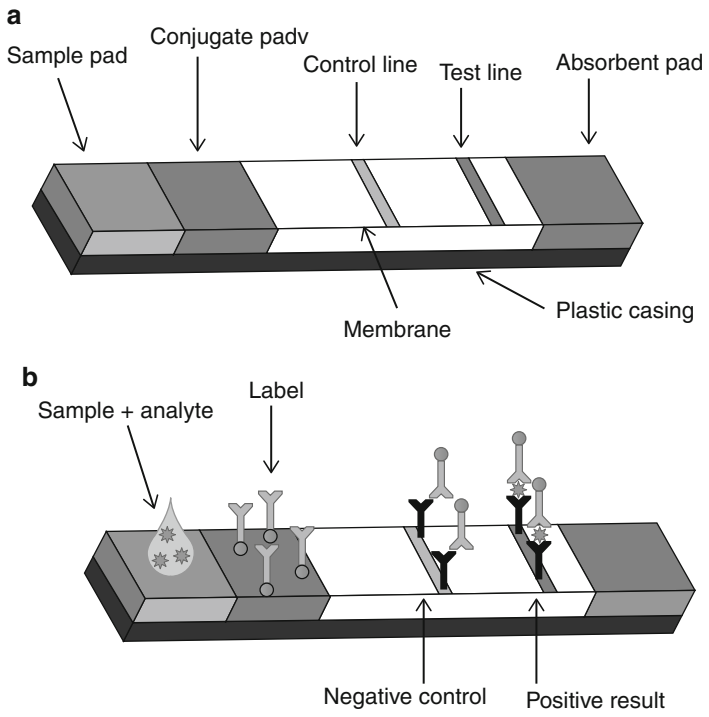

Fig. 8 Schematic representation of a LFA strip, (**a**) sample pad (sample inlet and filtering), conjugate pad (AuNP-antibody conjugates), incubation, and detection zone with test and control lines (antigen detection and functionality test) and final absorbent pad (liquid actuation); (**b**) sample is applied to the sample pad and the analyte binds to the AuNP-antibody conjugates and elutes with the buffer flow, and this analyte-AuNP-antibody conjugates bind to the test line (positive result). If the analyte is absent, the AuNP-antibody conjugates bind only to the control line (negative and control result) (Reprinted with permission from Gubala et al. [36]. Copyright 2011 American Chemical Society)

promotes dissolution on an SPCE's plate that measures Au^{3+} ions. The method was capable to detect unamplified DNA specific of *Salmonella enterica* serovar Enteritidis (*S. enteritidis*) at an LOD of 7 ng/uL.

Multiplex approaches have also been described. Li and colleagues used DNA arrays on gold surface combined with reporting silver nanoprobes to detect herpes simplex virus, Epstein–Barr virus, and cytomegalovirus by differential pulse voltammetry. The silver tag is allowed for the detection of as little as 5 aM of target DNA. Zhang et al. explored anodic stripping voltammetry using a screen-printed carbon electrode chip together with bio-barcoded AuNPs and magnetic NPs, to detect *B. anthracis* and *S. enteritidis* [78].

AuNPs for Diagnostics at Point of Care: Lateral Flow Devices

The lateral flow assay (LFA) or lateral flow immunochromatographic assay was introduced in 1988 by *Unipath* and is the most common commercially available POC diagnostic platform [81]. Most of the success of LFAs is due to the low cost and simplicity of operation. In fact, these devices are currently used in resource-poor or non-laboratory environments [37]. Generally, LFAs consist of a porous white membrane striped with a line of antibodies or antigens that interact with AuNP-antibody nanoprobes visible by the naked eye (Fig. 8). These types of platforms can support competitive or noncompetitive immunoassays. Competitive immunoassays are used for detection of low molecular weight target molecules like pesticides, hormones, and drugs, whereas competitive formats are used to detect high molecular weight targets with at least two binding sites [82]. With a suitably configured system, LODs in the picomolar range may be easily obtained.

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Many LFAs have been developed on the capillary test strip platform during the past 30 years. LFAs are easy to use, disposable, fast to perform, and relatively cheap [83]. Integration of such approaches with gold labels introduce several advantages in lateral flow designs, since they have been shown to be stable in liquid and dry without loss of signal [84]. Today, these platforms are "everywhere," from pregnancy, heart attack, blood glucose, and metabolic disorders to small-molecule detection (e.g., narcotics, drugs, toxins, antibiotics, etc.) and even pathogens, such as anthrax, salmonella, and some viruses. LFAs have been applied to immunodiagnostics, RNA detection, and even identification of whole bacteria. Some of the more recent designs and publications show the detection of DNA without the need of amplification by PCR opening yet another vast field of new applications. In fact, Wilson and coworkers demonstrated how unlabeled PCR products can be detected with an antibody-free lateral flow device at room temperature [85]. Trials are being conducted for massive multi-parallel screening together with LFAs microarrays [86].

Recently several lateral flow strip-based devices have been developed. Chua et al. developed a typical design where the detector reagent recognizes the fluorescein haptens of PCR-amplified DNA target and produces visual red lines with a recorded LOD of 5 ng of DNA [87]. Rastogi et al. optimized this approach via the integration of locked nucleic acid conjugated Au-nanoprobes together with signal amplification protocols for as little as 0.4-nM DNA [40]. More recently, Rohrman et al. presented a lateral flow assay coupling NASBA RNA amplification with Au-nanoprobes and gold enhancement solution to quantitate amplified HIV RNA [88]. These results suggest that the lateral flow assay can be integrated with amplification and sample preparation technologies, allowing viral load testing to monitor therapy response in limited resource setting.

Also, quantification of microRNA was demonstrated by Shao-Yi Hou et al. using of LFA nucleic acid test strips and AuNPs to detect miRNAs as low as 1 fmol [89].

LFAs evolved from a simple device into increasingly sophisticated platforms with internal calibrations and quantitative readouts. Nevertheless, single-use POC test devices are often affected by critical issues associated to dispersion of dried reagents into the sample, sample mixing with reagents, and effective control of incubation time and conditions. Several enabling technologies such as printing and laminating of components and microfluidic technologies are contributing to advances in LFAs. For a deeper perception on this technological perspective, including recent innovations in LFA technology, see the excellent review by Gubala et al. [36].

Some detection kits using AuNPs and their colorimetric sensing capability for human genetic tests can already be found in the market. For example, Nanosphere™ offers FDA-approved assays aimed at identifying typical mutations in coagulation factors F5 (1691G > A), F2 (20210G > A), and MTHFR (677C > T) without the need for nucleic acid amplification or to genotype polymorphisms associated with the drug-metabolizing enzyme CYP450 [90, 91]. Samples are processed through a cartridge where the sample is analyzed via an automated processor and reader.

Conclusion

AuNPs have become one of the most effective transducers and tags used in molecular diagnostics. In fact, the minimum number of AuNPs that can be detected by the naked eye against a white background is around roughly 10^{10}. Assuming one molecule of target molecule per AuNP, ten femtomoles may be detected, which compares negatively to the amount of target analyte in a sample that is usually one million less [82]. An alternative to diminish this difference can combine detection with amplification techniques. These can either focus on the target, such as PCR or antigen concentration, or on the assay development, such as by silver enhancement. The conjugation of silver enhancement and microfluidics allows the

exploitation of multiple protein and nucleic acid targets in a bio-barcode format. Nevertheless, the cost of manufacture of these microfluidic devices remains a problem, and the technology for POC use in detection protocols is still under development; thus, translation to the clinical setting remains a challenge.

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