EXPERT REVIEWS

Gold nanoparticles for molecular diagnostics

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Gold nanoparticles (AuNPs) exhibit a unique phenomenon, known as surface plasmon resonance, which is responsible for their large absorption and scattering cross-sections, which are four to five orders of magnitude larger than those of conventional dyes. In addition, their optical properties can be controlled by varying their sizes, shapes and compositions. AuNPs can be easily synthesized and functionalized with different biomolecules including oligonucleotides. Numerous methods have been utilized for detecting AuNPs such as colorimetric, scanometric, fluorescence, surface-enhanced Raman scattering and electrochemical techniques. These unique aspects have permitted the development of novel AuNP-based assays for molecular diagnostics which promise increased sensitivity and specificity, multiplexing capability, and short turnaround times. AuNP-based colorimetric assays in particular show great potential in point-of-care testing assays. This review discusses properties of AuNPs and their utilization for the development of novel molecular assays.

Keywords: bio-bar-code assay • colorimetric detection • electronic detection • Förster resonance energy transfer • gold nanoparticles • light scattering • scanometric detection • surface-enhanced Raman scattering • surface plasmon resonance

Fluorescent organic dyes are widely used for the detection of nucleic acids. However, these dyes suffer from photobleaching and can only be detected by a few techniques. On the other hand, gold nanoparticles (AuNPs) are not susceptible to photobleaching and their absorption and scattering cross-sections are superior to those of conventional dyes. The unique optical properties of AuNPs allow the detection of zeptomolar concentrations of nucleic acids, therefore, offering five orders of magnitude higher sensitivity than fluorescence-based techniques [1]. Furthermore, changing the sizes, shapes and compositions of AuNPs allows the tunability of their optical properties, unlike conventional dyes, and permits their utilization for simultaneous detection of multiple targets. AuNPs can be detected using various techniques, such as colorimetric, scanometric and electrical detection, to name a few, which make their use attractive in a wide range of biomedical applications including molecular diagnostics.

Structure & functionalization of AuNPs

AuNP mixtures are associated colloids and can be prepared easily by the reduction of auric acid with sodium citrate. The particles can have sizes ranging from 0.8 to 250 nm and the size can be varied by changing the sodium citrate concentration [2,3]. Another type of AuNPs are gold nanoshells, which have dimensions ranging from 10 to 300 nm and are composed of a dielectric core, usually silica or gold sulfide, surrounded by a thin gold shell [4–7]. The gold shell is formed using the same chemical methods that are used to form AuNPs and, therefore, the surface properties of gold nanoshells are identical to those of AuNPs [4].

AuNPs can be functionalized with different biomolecules, such as antibodies, carbohydrates, peptides, proteins and oligonucleotides [8]. The biomolecules can be conjugated directly to AuNPs through binding of gold to sulfur-, phosphor-, nitrogen- or oxygen-based ligands, or through noncovalent interactions between the biomolecules and capping agents on the AuNPs, such as citrate [3]. Alternatively, the functionalization maybe carried out by using biotinylated biomolecules and strept(avidin)-coated AuNPs [2]. Specifically, for the detection of nucleic acids, the AuNPs are generally functionalized with oligonucleotides by using the biotin-(strept)avidin interaction or by conjugation of free thiol groups or disulfides to oligonucleotides, which are then reacted with AuNPs forming strong gold–sulfur bonds [2].

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Surface plasmon resonance & optical properties of AuNPs

When a AuNP is exposed to light, the oscillating electric field component of light interacts with the free conduction-band electrons at the surface of the AuNP causing their collective dipolar oscillation, which is referred to as surface plasmon. When the surface plasmons have a frequency similar to that of the excitation light, this is called surface plasmon resonance (SPR) (Figure 1). For colloidal AuNPs with a diameter of 20 nm, SPR occurs in the visible region at 520 nm, which is responsible for their intense red color [9–11]. SPR enhances the various optical properties of AuNPs including absorption and scattering [3]. AuNPs have large molar extinction coefficients (~10^9 $cm^{-1}M^{-1}$ for 30-nm AuNPs), which increase with particle size [12]. The absorption cross-section of AuNPs is four to five orders of magnitude larger than that of the strongest absorbing rhodamine-6G dye molecules [3]. Furthermore, the light scattering from 80-nm AuNPs is 10^5-fold higher than the light emission from fluorescein molecules. Additionally, the light-scattering signal is resistant to quenching, unlike molecular fluorophores [13,14]. In surface-enhanced Raman scattering (SERS), SPR plays a role in enhancing any spectroscopic signals from molecules adsorbed on the surface of AuNPs. The reported SERS enhancement factors were on the order of 10^{14}–10^{15} [15]. This enormous enhancement is a result of two main factors. First, there is enhancement of the local electromagnetic field at the particle surface due to SPR in the metal nanoparticles. Second, there is a chemical enhancement, which is a result of charge-transfer electronic transitions between the adsorbed molecules and the metal nanoparticle [3].

The frequency and intensity of SPR absorption and scattering bands depend on the composition, size and shape of AuNPs, the dielectric properties of the surrounding environment, as well as interactions between adjacent AuNPs [9–11]. Changing the size of the AuNPs changes their optical properties in two ways. First, increasing the size of AuNPs, increases the scattering contribution to the total extinction (the sum of absorption and scattering) of AuNPs. Therefore, a 20-nm AuNP exhibits absorption only with almost no scattering whereas the scattering contribution increases in an 80-nm AuNP [11,13]. This is why larger AuNPs are used in light-scattering-based applications. Second, as the size of AuNPs increases, the absorption and scattering wavelengths increase [11,13]. Therefore, the color of the AuNPs can be changed by varying their sizes. As for gold nanoshells, the SPR extinction wavelengths can be varied by changing the ratio between the thickness of the gold nanoshell and that of the inner core (as the core/shell thickness ratio increases, the SPR wavelength increases) [4,6]. This allows the synthesis of gold nanoshells with SPR that ranges from the visible to the near-infrared region. On the other hand, the ratio of scattering to absorption of the incident light can be controlled by varying the absolute size of the gold nanoshells [4,6]. The interaction between adjacent AuNPs is known as plasmon–plasmon interaction and is used in the colorimetric detection of nucleic acids. Colloidal gold, which is red in color, changes to blue upon aggregation (referred to as red shift) due to plasmon–plasmon interactions, which lower the energy of the SPR absorption band [16–18].

Detection of AuNPs

Colorimetric detection

Cross-linking method (two-probe method)

In this method, two sets of AuNPs (13 nm) functionalized with different oligonucleotides are used to detect a ssDNA target [19–21]. Each set of the AuNP-labeled probes is complementary to one end of the target and the two probes align in a tail-to-tail fashion onto the target. Hybridization of the AuNP-labeled probes to the target causes aggregation and a change in the solution color from red to blue. Thermal denaturation reverses this process and restores the color of the solution back to red. The solutions can be spotted onto a white C18 reverse-phase thin-layer chromatography (TLC) plate (spot test) to further enhance color differentiation and to acquire a permanent record of the test [19,20]. In the presence of a perfect match, the spot is blue, otherwise, a red spot will be obtained. UV-vis spectroscopy can also be used to observe the color shift [21,22]. This colorimetric method of detection reduces cost since it eliminates the need for fluorophore-based detection systems. It also provides an easy and quick method for detection of nucleic acid targets. The melting profiles of the AuNP-labeled probes hybridized to complementary target DNA are much sharper, occurring over a range of temperature much narrower than the ones obtained using unlabeled probes or conventional fluorophore-labeled probes. The sharp melting profiles facilitate the identification of single-base mismatches simply by measuring absorbance or observing the color change as a function of temperature [19–22]. The sharp melting profiles are due to the high density of oligonucleotides on the surfaces of the AuNPs and their ability to bind to complementary DNA in a highly cooperative fashion. In addition, the sharpness increases with nanoparticle size, with 50-nm AuNPs capable of displaying a melting transition of only a single degree Celsius [22]. Using 50-nm AuNPs rather than 13-nm AuNPs, a detection limit of 50 pM can be achieved because the molar extinction coefficients increase as particle size increases [21].

Changing the composition can produce NPs with different SPR, which allows multiplexing. For example, NPs consisting of a core of silver and a monolayer gold shell will produce a yellow-to-dark brown color shift in the presence of the target. Cu and Pt could also be used as cores, producing NPs with different optical properties while retaining the surface chemistry and stability of the regular AuNPs [23]. Mirkin and coworkers used AuNP probes and Ag/Au core-shell probes for detection of single-nucleotide polymorphisms (SNPs) [24]. The AuNPs were functionalized with oligonucleotides complementary to the wild-type target and the Ag/Au core-shell NPs were functionalized with oligonucleotides complementary to the SNP-containing target. The wild-type target was added to two solutions, each containing one type of probes, and the solution temperature was increased from 20 to 70°C. At different temperatures, aliquots of the solutions were then spotted on a C18 reverse-phase TLC plate. The gold

solution in the AuNP probe system exhibited a higher melting temperature (T_m), detected by a color change from blue to red, than the Ag/Au core-shell probe system designed to detect the mutant target. In a second experiment, when the mutant target was added to the same two solutions, the opposite results were obtained. The Ag/Au core-shell probe system showed a higher T_m than the AuNP probe system. Therefore, in this method, the Ag/Au core-shell system served as a control to ensure accurate results but it could be used for the simultaneous detection of two different DNA targets.

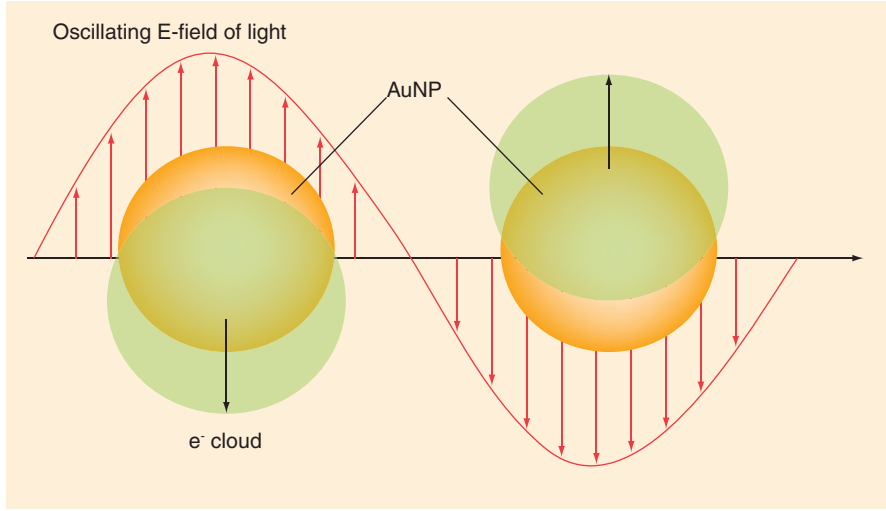

Figure 1. Surface plasmon of a AuNP. The oscillating electric field component of light induces a collective dipolar oscillation of the free conduction-band electrons of the AuNP. AuNP: Gold nanoparticle.
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Non-cross-linking method (single-probe method)

Sato *et al.* developed a non-cross-linking method in which they used AuNPs functionalized with one type of oligonucleotide [25]. Upon the addition of the target DNA, with a NaCl concentration higher than 0.5 M, the AuNPs aggregated and the solution color changed to purple. The advantage of this method is that the aggregation process is much faster than that of the cross-linking method, which requires tens of minutes to hours at room temperature.

AuNPs functionalized with peptide nucleic acids

Another approach for the colorimetric DNA detection involves AuNPs modified with peptide nucleic acids (PNAs). Short PNAs composed of six bases can hybridize efficiently while attached to NPs. In addition, PNA-labeled AuNPs can act as highly selective sensors because of the superior single-base mismatch selectivity of PNAs, which is further enhanced on the surface of AuNPs. The mismatch selectivity of PNA-functionalized AuNPs was around five-times greater than that of DNA-functionalized AuNPs [26]. This approach relies on the ability of PNA-modified AuNPs perfectly hybridized to the target DNA to resist aggregation in high salt concentrations. If the target DNA has a mutation, the AuNP colloid will aggregate at lower salt concentrations within minutes [26].

Non-functionalized AuNPs

AuNPs are typically charged and are highly sensitive to changes in the dielectric constant of the solution. Therefore, the addition of NaCl to a solution of unmodified citrate-stabilized AuNPs shields the surface charge and leads to their aggregation [27]. Li *et al.* exploited the fact that citrate-capped AuNPs are stabilized by the presence of short ssDNA, which adsorbs on the negatively charged AuNPs and prevents their salt-induced aggregation. This is because ssDNA can partially uncoil (due to transient structural fluctuations) exposing its bases and attractive van der Waals forces between the bases and the AuNPs allow the adsorption of ssDNA. In addition, the AuNPs and the ssDNA attract counter-ions from the solution forming dipoles and the attractive electrostatic forces between them cause further irreversible adsorption of the ssDNA. On the other hand, dsDNA does not adsorb on the AuNPs due to repulsion between its negatively charged phosphate backbone and the AuNPs. This observation was used for the detection of specific sequences and SNPs in PCR-amplified genomic DNA [28,29]. Adding unlabeled ssDNA probes to a solution containing denatured genomic DNA results in hybridization of the probes to their complementary sequences. AuNPs are then added to the solution but the probes are unavailable to stabilize them. Therefore, the salt in the hybridization solution causes the AuNPs to aggregate and the color of the solution changes from red to blue. In the absence of the target DNA, the probes stabilize the AuNPs and prevent their aggregation resulting in no color change. The advantage of this assay is that it does not require functionalization of the probes, the DNA target or the AuNPs. This method could also be applied using fluorescently tagged probes that are quenched upon adsorption to the AuNPs, indicating the absence of the target. Fluorescence detection, on the other hand, would be an indication of the presence of the target DNA [30]. Both methods were also used to detect RNA targets [31].

Scanometric detection

Taton *et al.* developed a microarray for DNA detection using AuNPs [32]. In this method, DNA capture strands are immobilized on a glass chip. The target DNA hybridizes to the capture strand. An oligonucleotide functionalized with a AuNP binds to the remaining portion of the target DNA. After hybridization, a thermal stringency wash removes any nonspecifically bound target, allowing 10:1 selectivity for detection of perfect matches versus single-base mismatches (corresponding to a selectivity that is over three-times that observed using fluorophore-labeled probes). This is a result of the sharp melting profiles of the AuNP-labeled probes, which allows the discrimination of a perfect match from

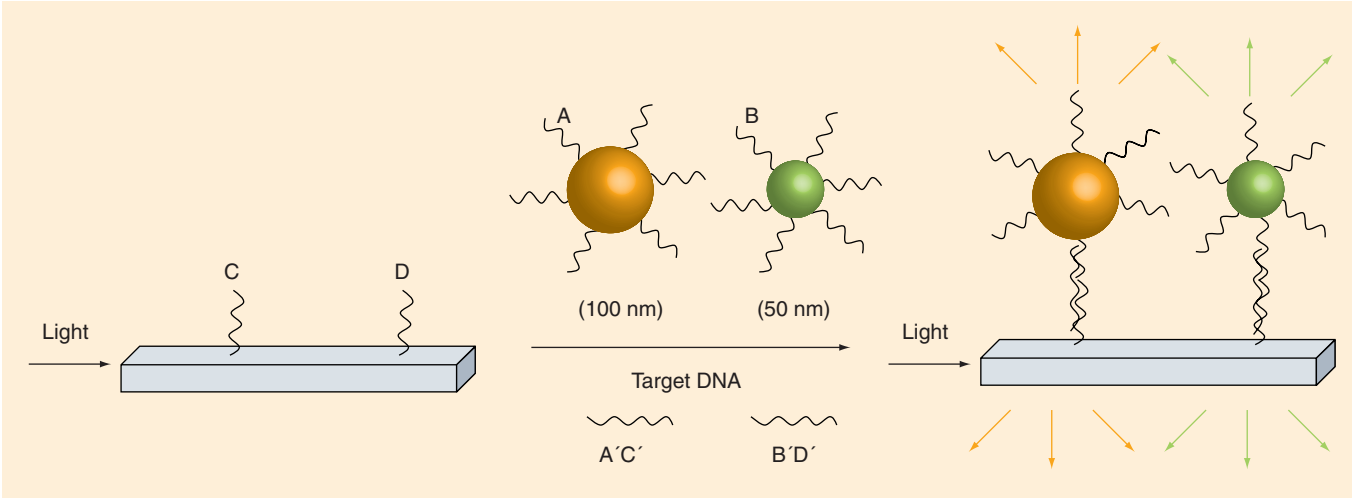

Figure 2. Array for DNA detection based on the light-scattering properties of gold nanoparticles. Capture strands C and D are immobilized on the array slide. DNA targets A'C' and B'D' hybridize to C and D, respectively. The 100- and 50-nm AuNPs functionalized with A and B oligonucleotide strands then hybridize to targets A'C' and B'D', respectively forming a sandwich. After illumination in the plane of the slides, the scattered light is imaged using a microscope. The 50-nm AuNPs scatter green light while the 100-nm AuNPs scatter orange light.
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a single-base mismatch at the optimal stringency temperature. The target signal is amplified by adding a silver enhancer solution that deposits silver metal on the AuNPs and the array is then vizualized using a flatbed scanner (hence the term 'scanometric'). Silver amplification darkens specific spots on the array where the sandwich forms. The spots could be easily imaged with the scanner or even seen with the naked eye. Silver amplification increases the scanned intensity by a factor of 10^5 and allows the visualization of the NPs at low target concentrations. It also allows the quantification of target hybridization based on the imaged grayscale of the darkened area. The detection limit is as low as 50 fM, which represents an increase of two orders of magnitude in sensitivity over that of the analogous fluorophore system [32].

Light-scattering detection

An array for DNA detection based on the scattering properties of AuNPs was developed in which AuNP-labeled probes (50 and 100 nm) were used to simultaneously detect two DNA targets hybridized to capture strands immobilized on a glass slide [33]. After hybridization, the arrays are washed to remove unhybridized target and probes. White light is then illuminated in the plane of the slides and the scattered light is imaged using a microscope. The 50-nm AuNPs scatter green light while the 100-nm AuNPs scatter orange light (Figure 2). This assay can also detect single-base mismatches. Four capture strands, each containing one of the four possible nucleotides (A, T, C and G) at a particular sequence position, are immobilized on the array. A target DNA is added, which will be complementary to one of the capture strands. The arrays are then washed and resubmerged in hybridization buffer. Gradually raising the temperature of the buffer causes the dissociation of the hybridized mismatched DNA targets (no scattered light was observed) leaving the perfect match hybridized as long as this temperature did not exceed the T_m of the perfect match. The dissociation occurs in real time in the order of the thermal stability of the base pairs (T:T ≈ C:T < G:T < A:T). This allows the determination of the optimal stringency temperature, selectivity and target sequence as the experiment proceeds. The detection limit of this method is 1 pM and its single-base mismatch selectivity is 5:1. Using additional AuNPs of different compositions or sizes would allow identification of more than two target sequences.

Storhoff *et al.* used microarrays for the detection of DNA targets where the scattered light is captured using an imaging system developed by Nanosphere Inc. (IL, USA). This detection system uses silver enhancement and permits an approximately 1000-fold increase in detection sensitivity when compared with Cy3-based fluorescence. This system allowed direct detection of *mecA* gene in methicillin-resistant *Staphylococcus aureus* unamplified genomic DNA samples. The detection of SNPs in unamplified human genomic DNA samples was also possible and the reported detection limit of this technique was 200 fM [34]. Detection of a single-base mismatch in a 250-bp PCR amplicon of Factor V Leiden gene was achieved with a detection limit of 100 aM [35].

The ClearRead™ assay, developed by Nanosphere, provides a microarray-based gene-expression system that uses unamplified total human RNA as the target nucleic acid. Total human RNA is hybridized to complementary capture strands immobilized on a microarray slide. Oligo-dT_{20}-modified AuNPs then hybridize to the poly-A tail of the captured mRNA followed by silver amplification. The resulting scattered light of the silver-enhanced AuNPs is imaged by a scanner (Verigene ID™). The detection limit was 0.5 µg of unamplified total human RNA [36].

Electrochemical & electrical detection

An electrochemical method for the detection of DNA hybridization was developed in which streptavidin-coated magnetic beads are bound to biotinylated capture strands. Biotinylated synthetic target DNA hybridizes to the capture strand and streptavidin-coated AuNPs are then added, which bind to the biotinylated targets. The hybridization leads to the bridging of the AuNPs to the magnetic beads. Magnetic separation removes the non-hybridized DNA. Afterwards, the AuNPs are dissolved using a hydrobromic acid/bromine solution, and potentiometric stripping measurements of the dissolved AuNPs is then carried out at carbon electrodes. The detection limit was 15 nM. Further signal amplification and lower detection limits of 1.5 nM and 32 pM, are achieved by depositing gold or silver, respectively, onto the AuNPs before the electrochemical stripping of the dissolved metal [37,38]. In another study, magnetically induced solid-state electrochemical detection of DNA hybridization was carried out. This was achieved through magnetic collection of the magnetic bead/DNA-probe hybrid/AuNP assembly onto the surface of an electrode transducer to allow direct electrical contact of the silver tag. This solid-state electrochemical transduction offers high selectivity and sensitivity, with a detection limit of 150 pg/ml (1.2 fmol). The elimination of the acid dissolution greatly simplifies the assay, shortens the detection time and eliminates the need for toxic bromine solutions required for the dissolution of the silver tags [39].

An electrochemical genosensor was developed to detect the hybridization of AuNP-labeled probes to their complementary target DNA, which is immobilized on a pencil graphite electrode. The hybridization results in a characteristic signal at approximately +1.20 V corresponding to the oxidation of AuNPs, which is measured by using differential pulse voltammetry. The signal is enhanced due to the large surface area of the electrode and the abundance of oxidizable gold atoms in each AuNP. The detection limit of the Factor V Leiden mutation from PCR amplicons was 0.78 pM. In addition, homozygous and heterozygous mutations could be distinguished by comparing the oxidation signals of the gold in each case [40].

Park *et al.* developed a method for the electronic detection of DNA on microarrays using AuNPs as signal transducers and amplifiers (Figure 3) [41]. Capture DNA strands are immobilized on a chip between two electrodes separated by approximately 20 µm. One end of the target DNA then hybridizes to the capture strands, while the other end hybridizes to AuNP-labeled probes. Silver amplification increases the size of AuNPs, thus bridging the gap between the two electrodes. This allows the passage of current from one electrode to the other and, therefore, is indicative of the presence of the target. The detection limit of this method was 500 fM and its single-base mismatch selectivity was 100,000:1 using a salt stringency wash. This selectivity is much higher than that of the analogous experiment using a molecular fluorophore probe (2.6:1) and the scanometric method developed by Taton *et al.* [32] with a thermal stringency wash (10:1). This salt dependency eliminates the need for temperature control on the chip, making it an easy hand-held assay, which could be used in point-of-care testing. Multiplexing can also be achieved using larger arrays of multiple electrode pairs with different oligonucleotide capture strands in each electrode gap [41].

Quartz-crystal-microbalance measurements

Weizmann *et al.* developed a method for the electronic detection of DNA using microgravimetric quartz-crystal-microbalance measurements and AuNPs [42]. The experiments were performed using a frequency analyzer and quartz crystals sandwiched between two electrodes. This detection is based on the fact that hybridization of the target causes a change in the mass associated with the crystal, which results in a detectable frequency change. In this method, a DNA capture strand, which is complementary to one end of the target, is immobilized on the quartz crystal. One end of the target DNA hybridizes to the capture strand and the other end hybridizes to a biotinylated oligonucleotide forming a sandwich. Avidin-coated AuNPs then bind to the biotinylated oligonucleotides leading to signal amplification. The AuNPs catalyze the reduction of $AuCl_4^-$ by NH_2OH resulting in deposition of gold on the AuNP-conjugates, which further enhances the signal achieving a detection limit of approximately 1 fM [42]. This method can also be employed for the detection of single-base mismatches (Figure 4). Here, the DNA capture strand is immobilized on the crystal. The target strand hybridizes to the capture strand and a biotinylated base complementary to the mutation site is added along with a DNA polymerase (Klenow fragment). A AuNP–avidin conjugate then binds to the biotinylated base followed by gold deposition. The single-base mismatch in the analyzed DNA was detected with a reported sensitivity of 3×10^{-16} M [43].

Förster resonance energy transfer & nanometal surface energy transfer

Förster resonance energy transfer (FRET) is the nonradiative transfer of excitation energy from a donor to an acceptor through dipole–dipole interactions only when the distance between them is smaller than the Förster distance (typically less than 10 nm). The Förster distance is the distance between the donor and acceptor at which energy transfer efficiency is 50%. The following equation shows the relationship between energy transfer efficiency (E), the donor–acceptor separation distance (r) and the Förster distance (R_o) [44]:

$$E = \frac{R_0^6}{R_0^6 + r^6}$$

The emission spectrum of the donor must also overlap with the absorption spectrum of the acceptor for FRET to take place [45]. AuNPs are used as energy acceptors because of their superior quenching ability, which is due to their large molar extinction coefficients [12]. However, when metal NPs are used as acceptors, the energy transfer occurs through a phenomenon known as nanometal surface energy transfer (NSET). This phenomenon is similar to FRET; however, more dipolar interactions occur due to the presence of free conduction band electrons in the metal NP that

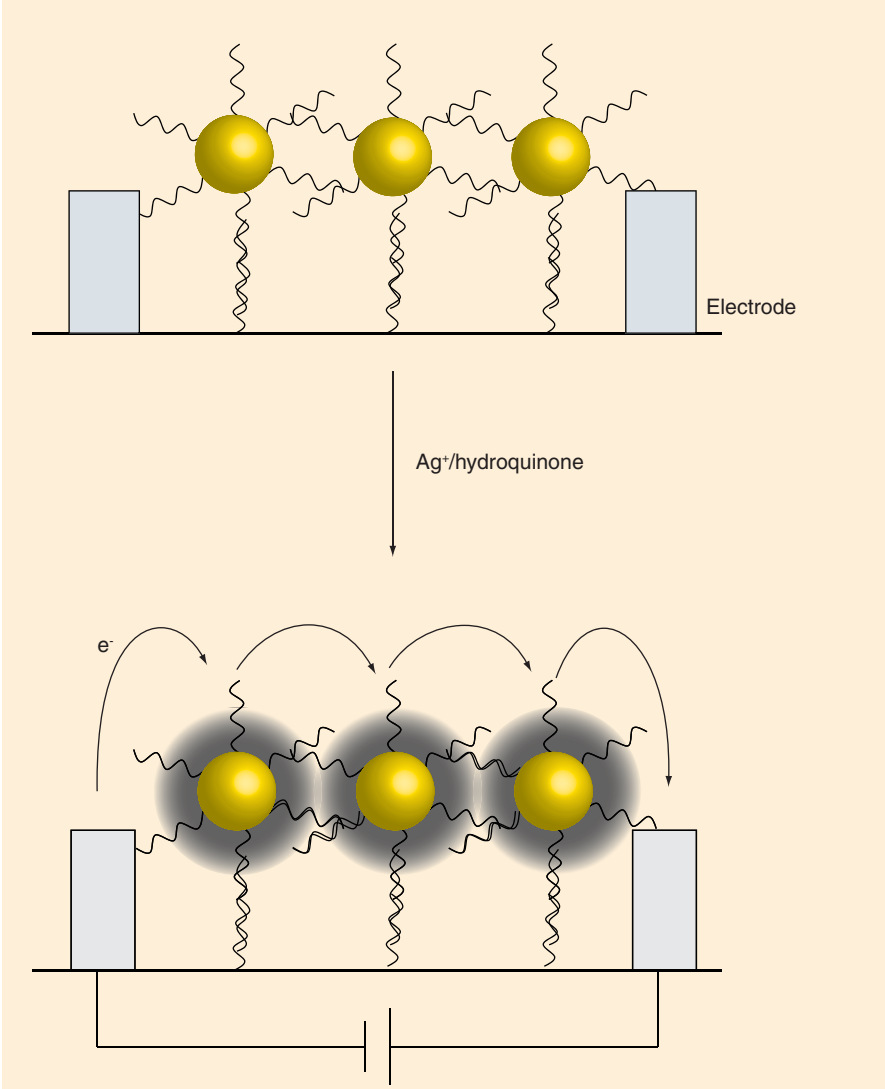

Figure 3. Electrical DNA detection using gold nanoparticles (AuNPs). Capture strands are immobilized on a microarray between two electrodes. One end of the target DNA hybridizes to the capture strands and the other end hybridizes to AuNP-labeled probes forming a sandwich. Silver amplification increases the size of the AuNPs and bridges the gap between the two electrodes allowing current to flow from one electrode to the other, thus indicating the presence of the target DNA.
Modified from [41]. © 2002 AAAS.

provides numerous dipole vectors on the surface of the metal ready to accept energy from the donor. This increases the probability of energy transfer, making it more efficient than FRET. This also results in a $1/R_o^4$ distance dependence versus $1/R_o^6$ for FRET [46–48]. Therefore, in NSET, energy transfer distances are nearly twice as long as the typical Förster distances in FRET. Additionally, the same NP could be used to quench dyes of different emission frequencies, from the visible range to the near-infrared [12]. Yun *et al.* attached a 1.4-nm AuNP to the 5′ end of a DNA strand and a fluorescein molecule to the 5′ end of the complementary strand. On hybridization of the two strands, NSET took place and analysis of the energy transfer on different DNA lengths showed that NSET continued to operate up to a distance of 22 nm [46].

Ray *et al.* developed a AuNP-based NSET assay to monitor the cleavage of dsDNA by nucleases [12]. First, a AuNP was conjugated to fluorophore-labeled ssDNA through sulfur–gold bonds. The fluorophore at the end of the ssDNA loops back and adsorbs to the surface of the AuNP forming an arch-like structure in which both the 3′- and 5′-ends are attached to the AuNP. In this state, a quenching efficiency of nearly 100% was observed (static quenching). After hybridization with the complementary target DNA, the distance between the fluorophore and the AuNP was approximately 6 nm. In this state, fluorescence was quenched by the AuNP through NSET. After cleavage of the dsDNA by S1 nuclease, the fluorescence signal was enhanced by a factor of 120 [12].

Griffin *et al.* designed a NSET probe for the homogenous detection and quantification of a synthetic hepatitis C virus (HCV) RNA target [49]. They used fluorophore-labeled ssRNA probes reversibly adsorbed on a AuNP forming arch-like structures. The target RNA then binds to the complementary RNA probe and the dsRNA is released into the solution, which changes color from red to blue owing to aggregation of the AuNPs. The fluorescence of the dye is restored and the intensity of fluorescence is directly proportional to target concentration. It was found that the quenching efficiency increases by 1000-fold as the size of the AuNPs increases from 5 to 70 nm. On increasing the size of the AuNPs, the sensitivity of the assay also increases reaching a detection limit as low as 300 fM of RNA when the particle size is 110 nm. NSET was observed up to 40-nm distances. The assay also showed high selectivity with the ability of detecting single-base mismatches [49].

Dubertret *et al.* used molecular beacons attached to AuNPs for the detection of DNA targets [50]. These molecular beacons consist of a ssDNA, with a hairpin structure, attached to a AuNP (quencher) at one end and a dye (donor) at the other (Figure 5). In this state, the AuNP and the dye are in close proximity and, therefore, fluorescence of the dye is quenched. In the presence of the complementary target DNA, the hairpin structure opens and, therefore, increases the distance between the dye and the AuNP, thus restoring the fluorescence of the dye. The use of AuNPs increased the sensitivity of molecular beacons up to 100-fold as compared with conventional molecular beacons attached to organic quenchers. In competitive hybridization assays, the ability of molecular beacons to detect single-base mismatches is eightfold greater when attached to AuNPs than organic quenchers.

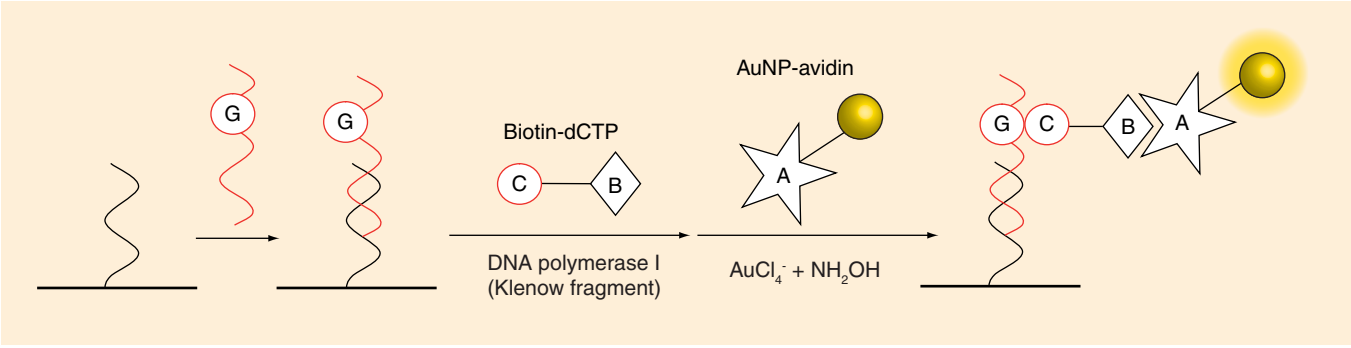

Figure 4. Microgravimetric quartz-crystal-microbalance measurements for the detection of single-base mismatches using AuNPs. A DNA capture strand is immobilized on a quartz crystal. The DNA target containing a mismatch hybridizes to the capture strand. In the presence of polymerase (Klenow fragment), a biotinylated base complementary to the mutation site is added to the dsDNA assembly. A AuNP–avidin conjugate then binds to the biotinylated base followed by gold amplification.
AuNP: Gold nanoparticle.
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In addition, the AuNP-labeled molecular beacon demonstrates a quenching efficiency of nearly 100% and a single-base mismatch selectivity of 25:1 versus 4:1 for conventional molecular beacons [50].

Maxwell *et al.* designed a probe that acts in a similar manner to molecular beacons but has a different structure. A 2.5-nm AuNP is attached to oligonucleotides, each with a thiol group at one end and a molecular fluorophore at the other. The fluorophore adsorbs to the AuNP forming an arch-like structure. In this state, the AuNP quenches the fluorophore with a quenching efficiency of almost 100%. Upon hybridization of the target DNA, the structure opens and, in this state, the fluorophore is separated from the AuNP by approximately 10 nm and fluorescence is restored. This probe has the advantage of being insensitive to temperature since it does not have a stem in contrast to regular molecular beacons with a stem-and-loop structure. Therefore, its background fluorescence increases very little with temperature [51].

FRET has been observed in nanoscale assemblies between oppositely charged quantum dots (QDs) and AuNPs in solution making it possible to develop FRET-based sensors with a donor quenching efficiency close to 100% [52]. When ssDNA attached to a QD was hybridized to the complementary oligonucleotide functionalized with a AuNP, fluorescence quenching was observed [53,54]. In one assay, upon hybridization of the two strands, the QD and the AuNP were at opposite ends and the quenching mechanism was reported to be through FRET [53]. In another assay, the QD was attached to the 5′ end of one strand and the AuNP to the 3′ end of the complementary strand such that upon hybridization the QD and the AuNP become adjacent to one another [54]. The two strands could be separated by the addition of ten equivalents of the unlabeled complementary oligonucleotide to the hybrid and, hence, fluorescence is restored to the QDs. The advantages of using AuNPs and QDs together in FRET-based applications are lower background signal, increased sensitivity and the ability to label both the AuNP and the QD with multiple biomolecules depending on the desired application [55].

It is worth mentioning that contradictory results to energy-transfer quenching have been reported in which 5–8-nm AuNPs enhance the fluorescence of surface-bound fluorophores [56]. In addition, Dulkeith *et al.* reported that fluorescence is quenched by AuNPs, however, not by FRET or NSET, but by phase-induced suppression of the radiative rate [57]. Furthermore, it was proposed that small AuNPs (22 nm) are expected to quench fluorescence because absorption is dominant over scattering. On the other hand, larger AuNPs (100 nm) are expected to enhance fluorescence of fluorophores emitting at wavelengths longer than 600 nm since scattering is dominant above 600 nm [58].

Surface-enhanced Raman scattering

SERS is one of the most sensitive diagnostic methods available, since the spectroscopic bands in Raman spectroscopy are much narrower than the bands obtained in fluorescence spectroscopy and the spectral window is much broader. This makes multiplexing possible and requires only a single laser beam to excite different Raman dyes as opposed to multiple laser beams needed to excite different fluorophores [14]. Multiplexing is accomplished by using Raman dye-labeled oligonucleotides attached to AuNPs (~13 nm) that hybridize to different DNA targets. Capture strands on a chip then bind to this complex and the nonspecifically bound target is then washed away. The presence of the target is detected by silver staining while the identity of the target is established by the Raman scattering pattern of the dye. Using this method, Cao *et al.* were able to detect simultaneously six different DNA targets and in a separate experiment two RNA targets with SNPs. The detection limit of this method is 20 fM [59].

Qian *et al.* developed SERS AuNP beacons for the detection of DNA in a single step without the need for washing or silver deposition [60]. In this method, AuNPs (40 or 60 nm) are labeled with a Raman reporter molecule, then functionalized with ssDNA, and are stabilized with low-molecular-weight thiolated polyethylene glycols. Each target DNA strand binds to two AuNPs forming a sandwich thus bringing two or more particles within a certain range (10–20 nm) resulting in plasmonic coupling (i.e., plasmon–plasmon interactions) and enhancement of Raman scattering. Using the appropriate wavelength of the excitation light (according to the particle size), the SERS signals from single AuNPs without plasmonic coupling (off-state) are very weak, while intense signals

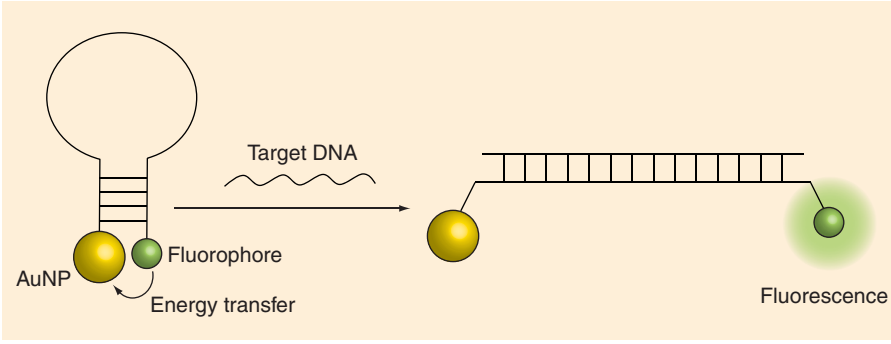

Figure 5. Molecular beacon attached to a AuNP for DNA detection. In the absence of the target DNA, fluorescence is quenched due to the close proximity between the AuNP (acceptor) and the fluorophore (donor). In the presence of the target DNA, the molecular beacon opens to allow hybridization. This increases the distance between the AuNP and the fluorophore causing it to restore its fluorescence.
AuNP: Gold nanoparticle.

are detected from AuNP aggregates with plasmonic coupling (on-state). The signals could be turned on and off by changing the temperature. The SERS beacons display excellent sequence specificity and ability to discriminate single-base mismatches [60].

AuNPs in surface plasmon resonance measurements

Surface plasmon resonance (SPR) is an analytical technique in which a noble metal film, located at the interface between a prism and the sample, has the ability to detect real-time changes in the dielectric constant/refractive index induced by molecular adsorption or DNA hybridization events at the surface of the film [61]. DNA hybridization events lead to a change in SPR, which can be measured by scanning-angle SPR, SPR wavelength shift, or SPR imaging. The most commonly utilized technique is the scanning angle SPR. In this method, a single wavelength is used for excitation and the reflectivity of an approximately 50-nm-thick gold film is measured as a function of the angle of incidence [35]. Conventional SPR is unable to measure extremely small changes in refractive index. Using AuNP-labeled probes hybridized to the surface-bound target DNA in a sandwich format leads to an amplification in the SPR response, leading to a larger than tenfold increase in angle shift. This corresponds to a more than three orders of magnitude improvement in sensitivity because of increased surface mass, the high dielectric constant of AuNPs, and electromagnetic coupling between the AuNPs and the Au film. The detection limit of this technique is approximately 10 pM for 24-mer DNA targets [62]. Yao *et al.* used a carboxylated dextran film immobilized on the surface of the SPR sensor to prevent nonspecific adsorption of AuNP-labeled probes. This led to further SPR signal amplification and a lower detection limit of 1.38 fM for a 39-mer DNA target [63].

Laser diffraction

This method is also based on changes in refractive index and detects real-time DNA target hybridization in a sandwich format using capture strands immobilized on micropatterned chemoresponsive diffraction gratings and AuNP-labeled probes. Three laser beams are passed simultaneously through the gratings. Hybridization of target DNA with the capture strands changes the refractive index contrast of the grating and results in changes in diffraction efficiency. The AuNP-labeled probes lead to signal amplification due to plasmon absorption of the AuNPs, which leads to a modulation of the local refractive index contrast. The reported detection limit of this method was 40 fM, which was extrapolated from signal magnitudes and errors observed for a 1 pM target. This method also allows multiplexing by using AuNPs of different compositions, sizes and shapes [64].

Applications of AuNPs for molecular diagnostics

Detection of bacterial DNA

Storhoff *et al.* developed a homogeneous DNA detection method based on the scattering properties of AuNP-labeled probes (without the use of microarrays). In this method, the oligonucleotides labeled with AuNPs hybridize to the target in solution (see 'Cross-linking method' section) causing the color of the solution to change to blue. The samples are then spotted onto a glass slide that is illuminated with white light in the plane of the slide. AuNPs (40 and 50 nm) attached to unhybridized oligonucleotides scatter green light, while those attached to hybridized oligonucleotides scatter yellow-to-orange light. This method was used to detect *mecA* in methicillin-resistant *S. aureus* genomic DNA samples. The detection limit was 33 fM of total genomic DNA when four 50-nm AuNP-labeled probes were used instead of only two probes. Monitoring scattered light from AuNPs rather than reflected light has many advantages. First, sensitivity is 10^4-fold higher than that of the absorbance-based spot test. Second, this technique enables the detection of AuNP-aggregates in the presence of an excess of non-aggregated particles, which makes it suitable for the detection of low target concentrations [65].

Detection of viral DNA

Glynou *et al.* developed a dry-reagent strip biosensor using AuNPs for the detection of dsDNA. Biotinylated PCR products are hybridized with a poly(dA)-tailed oligonucleotide and applied on a strip. The strip is immersed in a buffer and as the buffer migrates upwards, it rehydrates AuNPs functionalized with oligo(dT), which then bind to the target DNA through poly(dA)/(dT) hybridization. Moving further up the strip, the hybrids are captured by immobilized streptavidin contained in the strip, thus generating a characteristic red band. Excess AuNPs form a second red band, as a control, by hybridization to a specific probe in the strip. The detection limit of this test was 2 fmol of amplified DNA as opposed to 16 fmol detected by agarose gel electrophoresis and ethidium bromide staining. The sensor was used for the detection of HCV in plasma specimens from 20 patients. In this experiment, HCV RNA was extracted and amplified by RT-PCR; the bands were visualized within 10 min. Quantitative data could also be obtained by scanning the strip and performing densitometric analysis of the bands [66].

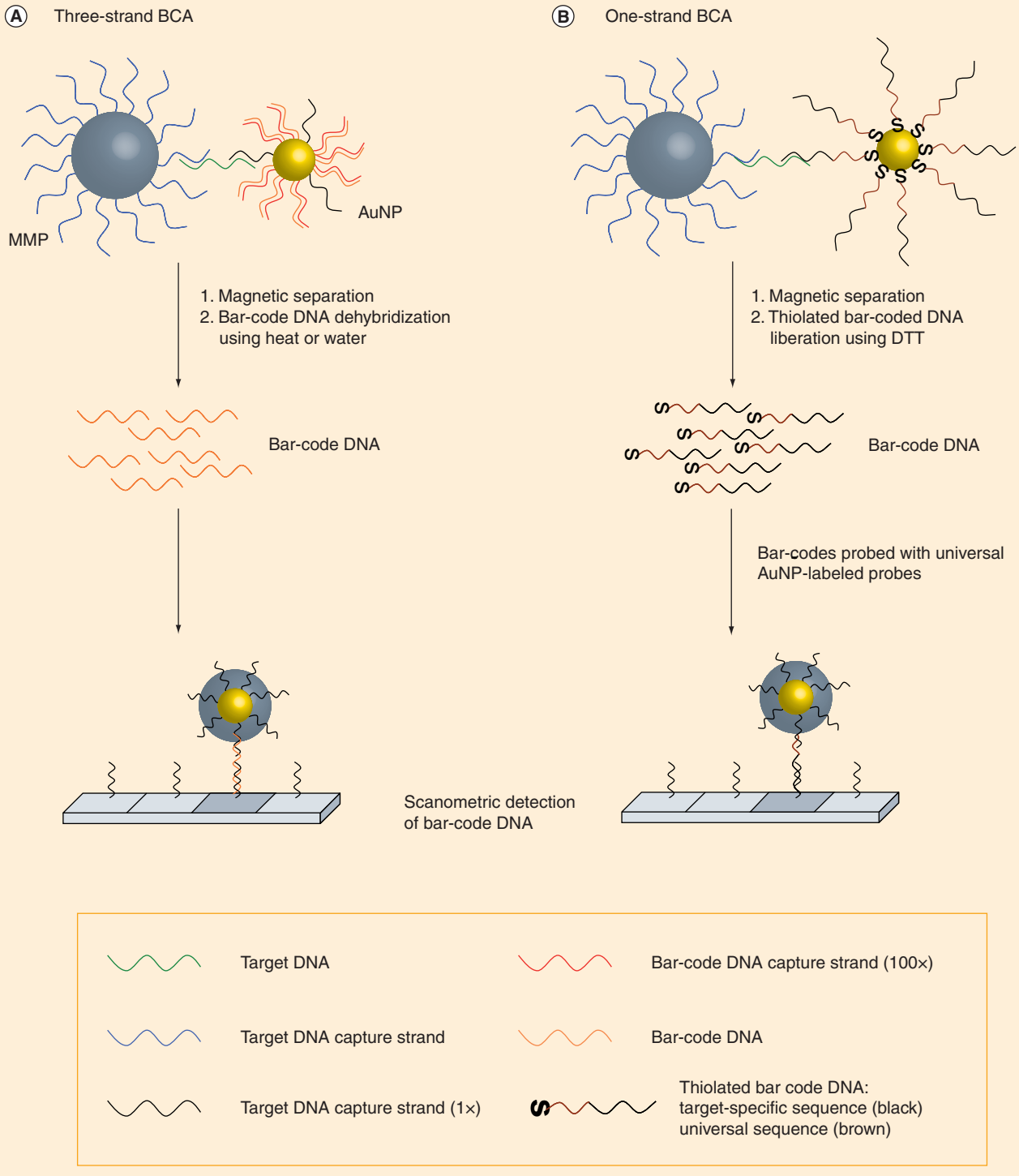

Figure 6. The BCAs for DNA detection. (A) Three-strand BCA in which the AuNP is functionalized with target DNA capture strands, bar-code DNA capture strands and bar-code DNA. The hybridized bar-code DNA is released using heat, water or both. **(B)** One-strand BCA in which the AuNP is functionalized with thiolated bar-code DNA containing a target-specific sequence (black) and a universal sequence (brown) and is liberated using DTT. Bar-codes in both formats are detected using the scanometric method.
AuNP: Gold nanoparticle; BCA: Bio-bar-code assay; DTT: Dithiothreitol; MMP: Magnetic microparticle.
Modified with permission from [1]. © 2005 American Chemical Society.

Detection of SNPs

A microarray-based method for multiplex SNP genotyping for unamplified human genomic DNA was developed using the sandwich assay format [67]. The scatter signal is imaged using Nanosphere's Verigene ID™ imaging system. All possible genotypes for three genes involved in thrombotic disorders were detected with a detection limit of 50 fM. This method is simple, rapid, robust and suitable for multiplex SNP profiling at the point of care. In this technique, each SNP was detected by either of two allele-specific capture strands and a gene-specific AuNP-labeled probe. Placing the SNP site within the capture strands permits the detection of the two possible alleles in the same reaction well [67]. On the other hand, placing the SNP site within the AuNP-labeled probe requires separate wells to detect the two alleles [34].

Doria *et al.* developed an assay using the non-cross-linking method for the detection of specific DNA and RNA sequences [27,68,69]. SNP sites within amplicons of the β-*globin* and *p53* genes were detected. Upon the addition of high salt concentration (2 M), the presence of the complementary target prevented the aggregation of AuNPs and the solution remained red. On the other hand, mismatched targets caused aggregation resulting in a color change to blue. A disadvantage of this assay is that the best reproducibility was achieved with DNA concentrations between 18 and 36 µg/ml and below this concentration, the difference in color between perfect and mismatched targets was not clear [27]. Using this method, the authors were able to detect eukaryotic gene expression (without converting the mRNA into cDNA) with a detection limit of 0.3 µg of unamplified total RNA [68]. They were also able to detect *Mycobacterium tuberculosis* DNA in clinical specimens [69]. It is worth noting that the color change in this study is the opposite of that reported by Sato *et al.* [25] and this is due to two main differences. In the method of Doria *et al.*, a high salt concentration (2 M) was used, which led to the aggregation of AuNPs in the solution containing no target that would have remained red if lower salt concentrations had been used. In addition, large PCR products were used in the method of Doria *et al.* as opposed to targets having the same length as the AuNP-labeled probes which were used in the method of Sato *et al.* The unhybridized parts of the large PCR products act as ssDNA, thus, stabilizing the AuNPs and preventing their aggregation [68].

A three-step colorimetric method for SNP detection using AuNPs and DNA ligase reaction was developed. First, two AuNP-labeled probes hybridize to the target DNA strand. DNA ligase then joins the probes only if they are perfectly matched to the template, while no ligation occurs between mismatched ones. At this stage, both solutions (perfect match and mismatch) are purple in color due to the close proximity of the AuNPs of both probes. Finally, a thermal treatment denatures the formed duplexes. The solution containing the perfect match stays purple as the ligase has joined the two AuNP-labeled probes, whereas, the solution containing the mismatch changes to red since the AuNP-labeled probes separate. This method was used to detect a SNP of a *K-ras* oncogene, which is important for diagnosis of colorectal cancer [70]. This method was also used to identify two SNPs simultaneously of the β-thalassemia gene using a multistep temperature elevation analysis monitored by UV-vis spectroscopy [71].

Sato *et al.* developed a microfluidic chip for SNP detection that could be suitable for point-of-care testing if accompanied with a portable SPR device. The chip consists of a Y-shaped microchannel (100-µm width × 25-µm depth) with two inlets, one for the target DNA hybridized with the AuNP-labeled probes and the other for a NaCl solution. AuNPs with perfectly matched duplexes are selectively deposited onto the bottom of the microchannels in the presence of high salt concentration. Using SPR imaging, discrimination of the target DNA is achieved within 5 min with a detection limit of 32 nM [72].

AuNPs in PCR

The addition of 0.7 nM concentration of 13-nm AuNPs to a PCR mixture was found to increase the amplification yield of a PCR reaction [73]. The detection sensitivity was increased five- to ten-fold in a conventional PCR and at least 10^4-fold in a real-time PCR. In addition, the reaction time could be shortened, and the heating/cooling rates could be increased. This is attributed to the superior heat-transfer property of the AuNPs. When the number of cycles in real-time PCR was shortened by half, the addition of AuNPs allowed the detection of 100 copies/µl of DNA. However, without the AuNPs, at least 10^6 copies/µl of DNA were needed to reach a detectable signal [73].

Nucleic acid lateral flow devices

Nucleic acid lateral flow (NALF) devices are easy and inexpensive to produce in large volumes and do not require high expertise for performing the tests or interpreting the results [61]. Wilson and coworkers developed a NALF device for the detection of specific PCR products [74]. Capture oligonucleotides are covalently attached to a nitrocellulose chromatographic strip. When the device is dipped into a liquid sample containing the PCR products, one end of the target DNA hybridizes to a AuNP-labeled probe, which migrates up the strip. The other end of the target DNA then hybridizes to the immobilized capture strand producing a pink line that indicates the presence of the target [74]. The dry-reagent dipstick developed by Glynou *et al.* for the detection of HCV is another example of a NALF device [66]. Finally, another dipstick using AuNP-labeled probes was developed for the detection of amplified *Trypanosoma brucei* DNA. The detection limit of the test was 5 fg of pure *T. brucei* DNA and the reported sensitivity and specificity were 100% [75].

Bio-bar-code assays

Mirkin and coworkers designed a novel technique for DNA detection with high sensitivity and selectivity that may serve as a substitute for PCR [76]. The assay employs magnetic microparticles (MMPs) and AuNPs. The MMPs are functionalized with DNA-capture strands complementary to one end of the target DNA. The AuNPs are functionalized with DNA-capture strands, complementary to the other end of the target DNA.

Additionally, hundreds of bar-code DNA-capture strands that are hybridized to bar-code DNA are also attached to the AuNPs. In the presence of the target DNA, a sandwich complex is formed consisting of the target DNA hybridized to the MMP and the AuNP. Both particles are then magnetically separated and the unreacted components in the solution are washed away. Water is added and the system is heated to dehybridize the bar-code DNA, which is detected scanometrically, as described previously. The detection limit is 500 zM which is equivalent to ten strands in a 30 µl sample. The advantage of this technique is that signal amplification is carried out instead of target amplification thereby reducing the risk of contamination. This assay can also differentiate between single base mismatches under stringent conditions [76]. In addition, it is also suitable for multiplexed DNA detection by designing bar-codes for any desired target [77].

Mirkin and coworkers developed another AuNP-labeled probe that can be used in the BCA. Instead of having three kinds of strands on each AuNP, this probe requires only thiolated bar-code DNA strands which consist of a target-specific sequence and a universal sequence. The adsorbed thiolated DNA strands can then be liberated from the surface of the AuNP using dithiothreitol (DTT), which simplifies the assay and increases its quantitative capabilities by offering a dynamic range from the low-attomolar to the mid-femtomolar target concentrations. The detection limit is 7 aM using the scanometric detection method [1]. The two kinds of BCA are shown in Figure 6. Based on the DTT system, another BCA for DNA detection of multiple targets was developed and the detection limit was found to be 5 pM in only 40 min using capillary DNA analyzer (ABI, USA) as opposed to approximately 6 h using the scanometric technique [1,78]. Mirkin and coworkers were also able to detect bacterial genomic dsDNA using a modified BCA. In this assay, genomic DNA is isolated from bacterial cells and cut with a restriction endonuclease. dsDNA is then heat denatured and blocking oligonucleotides are added to bind to regions of the target that flank the probe binding sites and to prevent dsDNA rehybridization. This makes these regions accessible for the AuNP- and MMP-labeled probes to bind. The detection limit was found to be 2.5 fM [79].

Conclusion & expert commentary

Recently, dramatic technical developments related to the use of AuNPs in biomedical applications have been achieved. These include the ability to synthesize AuNPs of different sizes, shapes and compositions, the ability to functionalize AuNPs with a variety of biomolecules, and the development of numerous techniques for detection of AuNPs. These technical achievements that are based on clear understanding of the physical properties of AuNPs, have permitted the development of numerous molecular diagnostic assays and prototypes with superior sensitivity, specificity and multiplexing capabilities compared with conventional assays. The contribution of AuNPs to molecular diagnostics is expected at two levels: improving current assays and introduction of new diagnostic strategies. Employing AuNPs in PCR has been shown to result in enhanced assay performance characteristics. In SPR techniques, the use of AuNP-labeled probes has increased sensitivity by more than three orders of magnitude. Additionally, AuNPs have permitted the development of novel assay strategies that promise to take molecular diagnostics to the next level. The BCA can offer PCR-like sensitivity while eliminating the risk of target contamination. Furthermore, unmodified AuNPs can be used for nucleic acid detection in simple and fast colorimetric assays without the need for expensive instrumentation.

Five-year view

More AuNP-based assays for molecular diagnostics are expected to shift from merely proof-of-concept experiments to commercial assays for clinical testing. AuNPs utilized in different assays for detection of SNPs are expected to significantly contribute to the growing market of SNP diagnostics. Also, AuNPs have the potential to develop inexpensive point-of-care tests for simultaneous detection of low concentrations of multiple targets in clinical specimens. For example, a NALF prototype device for the detection of up to seven amplified nucleic acid targets in less than 20 min at room temperature has been developed using AuNPs [101]. This would be of extreme importance for developing countries with scarce laboratory resources and poor infrastructure. Finally, AuNPs are also expected to be utilized for the development of more microfluidic devices. This would allow such sophisticated diagnostic tools to be implemented in several laboratory settings. A disposable microfluidic chip for protein detection based on the BCA has been developed that could detect down to attomolar concentrations of protein. This system could potentially be adapted for detection of nucleic acid targets [80,81]. During the coming 5 years, it is expected that AuNPs will mainly be used for developing multi-analyte NALF devices, microfluidic chips and other point-of-care tests. The majority of such devices will be used for the detection of SNPs and nucleic acids of infectious agents.

Key issues

- Gold nanoparticles (AuNPs) exhibit a unique phenomenon known as surface plasmon resonance, which significantly enhances their optical absorption and scattering.
- By changing sizes, shapes and compositions of AuNPs, their unique optical properties can be manipulated making them amenable to multiplexing.
- AuNPs can be detected by various techniques such as colorimetric, scanometric, light scattering, electrochemical, electrical, quartz-crystal-microbalance measurements, Förster resonance energy transfer, nanometal surface energy transfer, surface-enhanced Raman scattering and laser diffraction methods.
- AuNPs can be used for the detection of bacterial and viral nucleic acids as well as single-nucleotide polymorphisms in clinical specimens.
- AuNPs can also enhance the performance of conventional techniques, such as PCR, quartz-crystal-microbalance measurements and surface plasmon resonance measurements.
- More microfluidic chips and nucleic acid lateral flow devices based on AuNPs are being developed for nucleic acid detection.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

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•• of considerable interest

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