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A Review of Gold and Silver Nanoparticle-Based Colorimetric Sensing Assays

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ABSTRACT

The nanoparticle colorimetric-based methods have been extensively used for rapid detection, however there are few limitations which can be kept under control or avoided by understanding the crucial parameters involved in these reactions. This review addresses the main parameters

32 that influence colorimetric-based methods and provides a rational classification of the current
33 approaches, by focusing particularly on gold nanoparticles (AuNPs) and silver nanoparticles
34 (AgNPs). The AgNP and AuNP-based colorimetric assays can be very efficient and sensitive
35 especially for biomolecule identification and for metal ion detection in environmental screening.
36 Specifically, this review highlights the detection of metal ions through their coordination with
37 nanoparticle stabilizing ligands. The review also addresses various approaches based on label-
38 free aptasensors to better understand their role as smart colorimetric sensing devices.

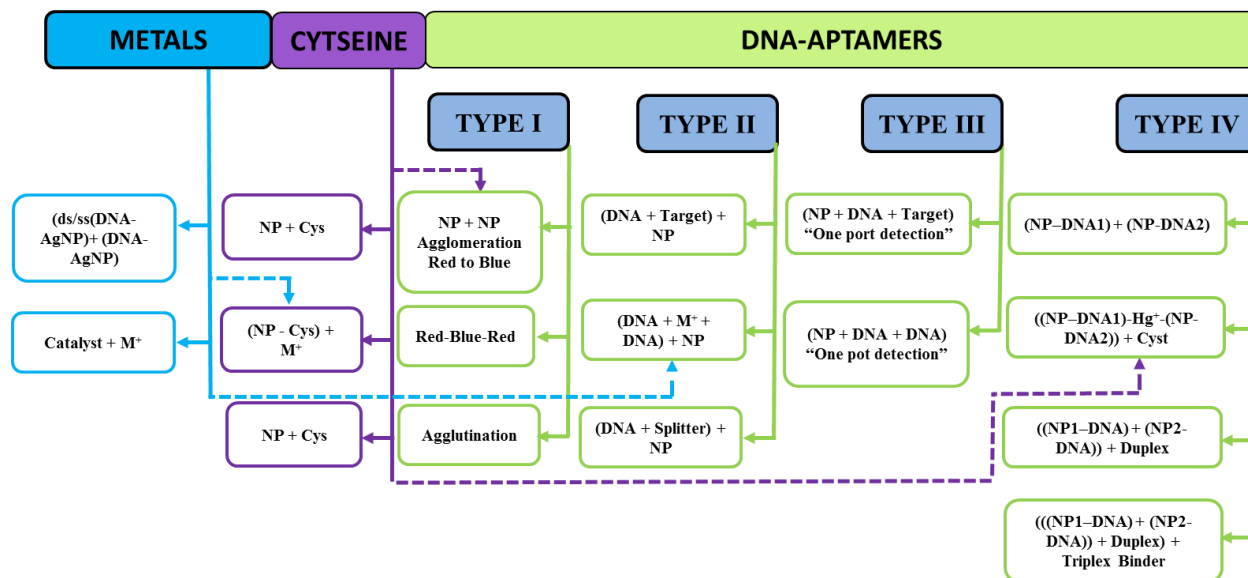
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40 **1. Introduction**

41 The term *nanoparticle* (NP) defines any small object (from 1 to 100 nm) that behaves as a
42 unit concerning its transport and properties. Indeed, as their properties change in function of their
43 size, particles can be classified based on their diameter. Thus, ultrafine particles (or NPs) are
44 particles with a diameter between 1 and 100 nm, whereas fine particles have a diameter between
45 100 and 2,500 nm. For tailored applications, NPs can be capped with a variety of different
46 anionic and cationic ligands, from displaceable small molecules to polymer coatings. The choice
47 of capping ligand depends on the NPs used that can range from conductive inks to biomedical
48 tools.

49 Among the known nanoparticles, gold and silver NPs (AuNPs and AgNPs) have been
50 widely studied because of their unique optical, electrical and photothermal properties. AuNPs
51 and AgNPs show unique optical features in well-dispersed solutions, depending on their level of
52 aggregation which is mostly determined by their specific surface plasmon resonance (SPR)
53 profiles ^[1]. Metal-noble NPs are small enough to confine their electrons and produce quantum
54 effects. This is a key parameter for naked-eye colorimetric sensing applications, because
55 modifications of their surface charge are transformed into a visible color change. Furthermore,
56 NPs also have a very high extinction coefficient that depends mainly on their size, shape and
57 inter-particle distance. Such properties enable them to compete with analytical techniques, like
58 absorbance or fluorescence spectroscopy. Colorimetric-based assays have been developed by
59 exploiting the color changes associated with the aggregation of metal-noble NPs ^[2]. Due to their
60 adaptability, high sensitivity, low cost and versatility (Figure 1), AgNP- and AuNP-based assays
61 have been used for the detection of metal ions ^[3], small molecules ^[3b, 3f, 4], proteins ^[3f, 4z, 5],
62 deoxyribonucleic acidDNA ^[3f, 5i, 6] and enzymes ^[5a, 6g, 7]. AuNPs are often used as sensing

63 elements to develop sensitive, selective, simple and label-free colorimetric assays [7h, 8].
 64 Consequently, NP used as detection agents could be considered as a sort of “litmus test” for
 65 target molecules [5i, 9].



66
 67 **Figure 1: Possible colorimetric detection approaches for metals, ligands and macromolecules.**
 68

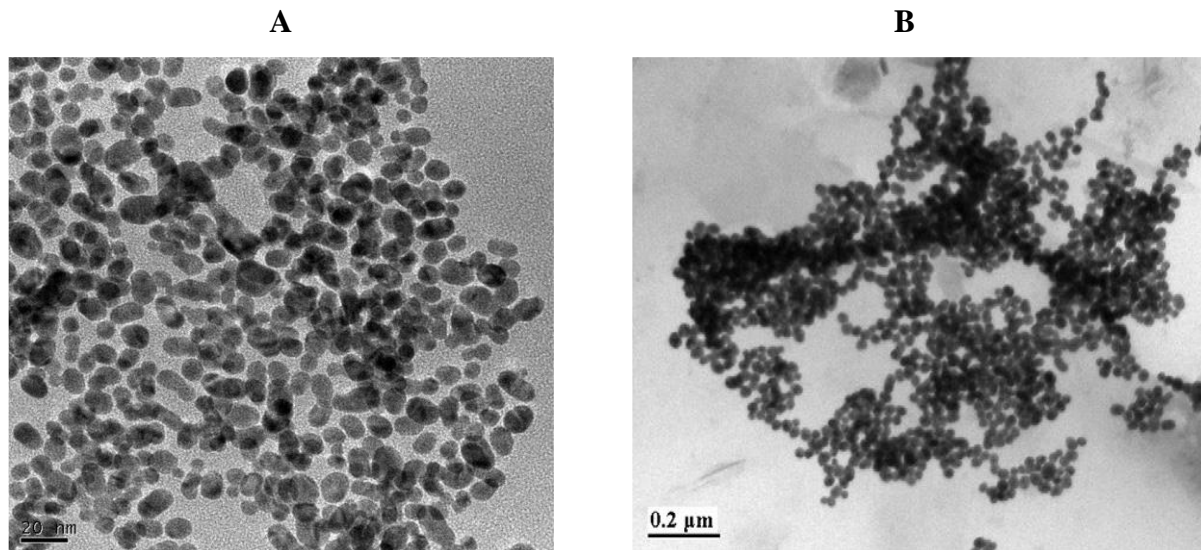
69 Although analytical methods, such as high-performance liquid chromatography,
 70 electrophoresis, voltammetry and fluorescence spectroscopy, are the preferred approaches for the
 71 detection of various macromolecules, sensitive, fast and high-throughput screening methods are
 72 still required [4x]. NP-based colorimetric methods are quick and user-friendly detection
 73 approaches that take advantage of various chemical mechanisms. For instance, such methods can
 74 be used for the rapid detection of influenza viruses through the binding between the influenza
 75 virus envelope protein hemagglutinin and sialic acid-stabilized AuNPs [10], or for high-
 76 throughput screening of endonuclease inhibitors [7e].

77 In this review, the colorimetric approaches that allows for the naked-eye detection of color
 78 changes through ultraviolet-visible (UV-Vis) absorption spectroscopy without fluorescence
 79 detection methods are addressed. Specifically, aptamer-based applications for the detection of
 80 metal ions, small ligands and biological macromolecules will be described. Accordingly, the
 81 methods have been subdivided on the analytes and the NP’s surface modifications and not on the
 82 basis of the used NPs, as the colorimetric performances of AgNPs or AuNPs are very similar.
 83 The applications involving aqueous/water-soluble stabilized NPs with no solid surface arrays,
 84 such as glass will be highlighted. To ensure that all key possibilities are fully explored, the

85 different sections herein have been divided as follows: detection of metals, small molecule
86 (cysteine, dopamine) and oligonucleotides (cross-linking).

87 **2. Gold and Silver NP synthesis, morphology and properties**

88 The most commonly used methods for the synthesis of AgNPs and AuNPs involve reducing
89 AgCl or AgNO₃ and HAuCl₄ with sodium citrate and sodium borohydride. The AuNP mixture is
90 boiled with vigorous stirring in a round bottom flask fitted with a reflux condenser for
91 approximately 10 min. Color change from yellow to wine red is observed within a few seconds
92 [4s, 4z, 11]. The AuNP solution concentration can be calculated following Beer's law, using the
93 extinction coefficient of $2.7 \times 10^8 \text{ M}^{-1} \text{ cm}^{-1}$ at $\lambda=520 \text{ nm}$ [12]. For AgNPs, 1% tri-sodium citrate is
94 added to 0.3 mM silver nitrate solution, and the mixture stirred for 5 min. After a drop-by-drop
95 addition of 1 mM sodium borohydride solution in the dark, the resulting mixture is stirred at
96 room temperature for 2 h. The bright yellow AgNPs are then filtered through a Millipore syringe
97 (0.45 μm) to remove the precipitate [41]. NPs are then characterized by diffusion light scattering
98 (DLS) to calculate their hydrodynamic radius in solution, or by transmission electron microscopy
99 (TEM) to describe their morphology (Fig. 2).



100 **Fig. 2. TEM images of (A) 13 nm citrate-capped AuNPs from wine-red solution [4s] and of (B)**
101 **guanine-induced AgNP aggregates [13].**

102
103 For colorimetric assays, AgNPs have some advantages compared with AuNPs.
104 Specifically, AgNP extinction coefficients are higher than those of AuNPs of the same average
105 size [7h], but with AuNPs being more popular. This could be explained by the fact that AgNPs
106 functionalization usually leads to their chemical degradation and thereafter the AgNP surface
107 can be easily oxidized, thus reducing their stability [4f, 7h, 14]. Indeed, Manuco *et al.* reported that
108 AuNPs are stable for more than 1 month at room temperature, whereas AgNPs only for about
109 two weeks. This difference could be linked to the different reaction constants of thiolated gold
110 and silver [15]. The high extinction coefficients and distance-dependent optical properties of
111 AuNPs accounts for the high sensitivity of AuNP-based colorimetric assays. Moreover, color
112 changes can be easily observed by the naked eye, thus making them attractive for DNA-related
113 colorimetric assays [4c, 4k, 6b, 7h, 8]. The DNA adsorption kinetics by AgNPs are slower than those
114 by AuNPs and they cannot be accelerated by adding salt at neutral pH. This unique property of
115 the specific molecular recognition of DNA-related colorimetric assays accounts for the difficulty
116 of attaching DNA to AgNPs at neutral pH [16].

117 Similarly, AgNPs are good candidates as optical sensors because they display distance-
118 dependent optical properties [4f, 17]. Their stability can be improved by producing Ag/Au core-
119 shell NPs that retains the Ag core optical properties. However, oligonucleotide-modified Ag/Au
120 alloy particles are not as stable as oligonucleotide-modified core-shell particles and irreversibly

121 aggregate in comparable conditions ^[17b]. Furthermore, nanoparticles, such as nanorods, prisms,
122 bipyramid of materials, have different SPR wavelengths ^[15]. For this reason, sensing platforms
123 based on AuNPs optical properties in combination with the molecular recognition of ligands,
124 such as alkyl thiols, antibodies, nucleic acids, and proteins, are active areas of research ^[3d].
125 Mirkin's group developed aptamer-based colorimetric assays for macromolecules using the more
126 stable AuNPs, despite AgNPs having a greater extinction coefficient ^[6c, 18].
127 NP colloidal stability can be adjusted by modifying the surface charges that affects electrostatic
128 stabilization, and NP aggregation can be induced through loss (or screening) of surface charges.
129 Basically, when AgNPs and AuNPs are exposed to light, they oscillate the electromagnetic field
130 of light. This induces a collective coherent oscillation of conduction band electrons, giving rise
131 to SPR. The SPR band intensity and wavelength depend on the factors that affect the electron
132 charge density on the particle surface. According to Mie's theory, these factors include the metal
133 type, particle size, shape, structure, composition and dielectric constant of the surrounding
134 medium ^[19]. Thus, unmodified AuNPs are red while AgNPs are blue or maroon due to their
135 specific and size-dependent SPR absorption. Addition of salt triggers electrostatic repulsion
136 between negatively-charged NPs and antiparticle changes, resulting in NPs aggregation and,
137 consequently, specific color and wavelength changes ^[3e, 4t, 6a, 13]. Hence, by monitoring the
138 changes in absorbance, it is possible to understand the characteristics of the enhanced scattering
139 effect in aggregated NPs compared with non-aggregated NPs ^[10]. Consequently, the band gap
140 energies can also be used to improve the knowledge on NPs sensing and catalytic properties ^[20].
141 The SPR profile is characteristic of the NP surface modification by small molecules, metal ions
142 and bio-macromolecules ^[21].

143 **3. Detection of metals ions (aggregation induced by interparticle cross-** 144 **linking)**

145 AgNPs and AuNPs colorimetric changes are due to the particle surface modification and
146 aggregation. This can be achieved by NPs aggregation induced by interparticle bond formation
147 (cross-linking aggregation) or by modifying colloidal stabilization (non-crosslinking
148 aggregation) ^[7a, 22]. In the non-crosslinking system, aggregation is driven by the London/van der
149 Waals attractive forces between NPs ^[7a]. Therefore, the presence of specific functional groups,
150 such as hydroxyl (-OH), carboxyl (-COOH) and amine (-NH₂), on the NPs surface plays a

151 critical role in aggregation. By modulating the strength of the NPs intermolecular ion and surface
 152 chemistry, this method can be improved [23]. For example, highly charged nucleotides or
 153 uncharged nucleosides can bind to citrate-capped AuNPs with the displacement of weakly bound
 154 citrate ions through metal–ligand interactions. This can increase AuNPs stabilization or trigger
 155 their aggregation, respectively, through gain or loss of surface charges [7a].

156 **Table 1: Detection of metal ions**

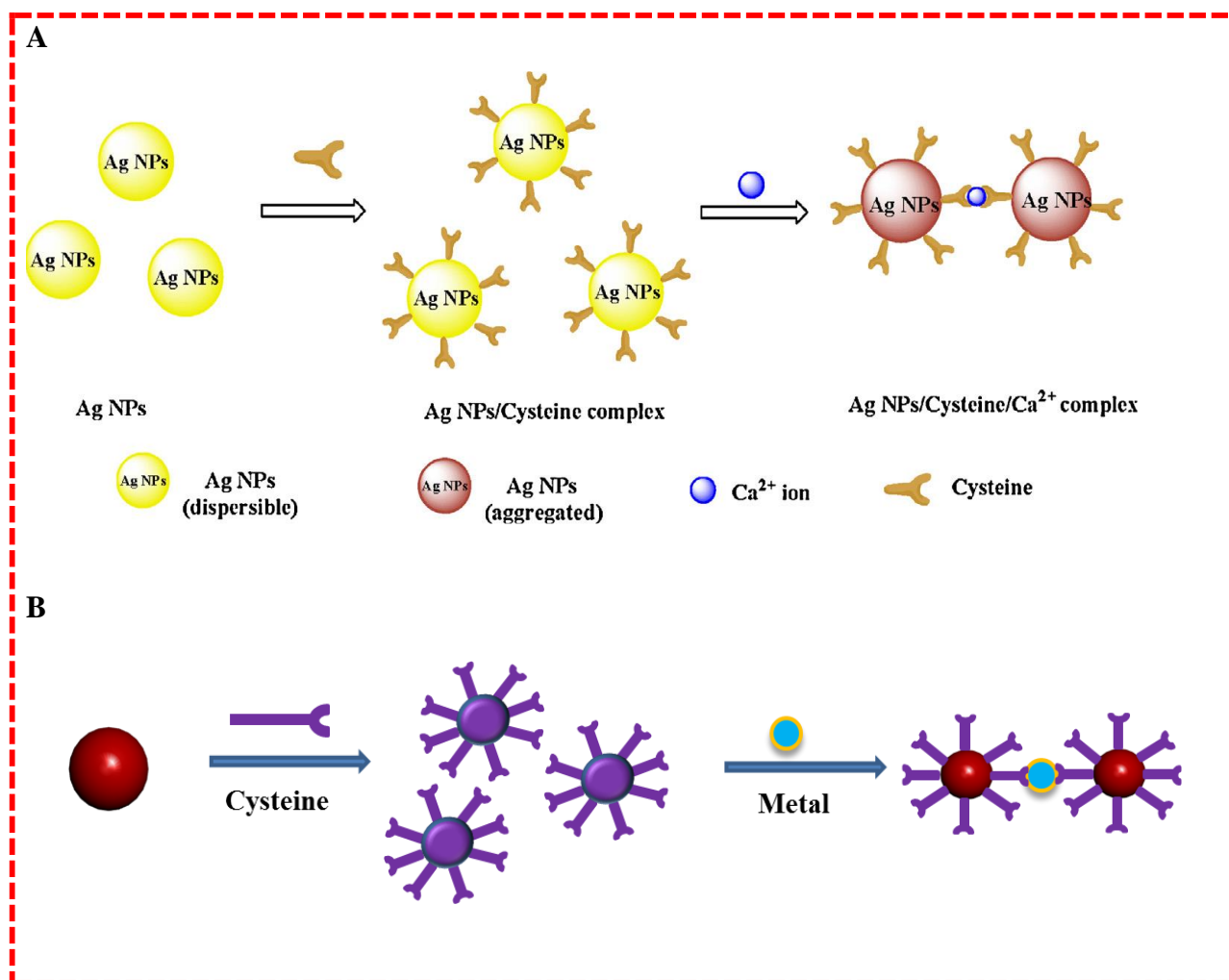
Nanoparticle	Analyte	Sample/interferent	Functionalization	Analytical performance	Ref
AuNPs	Mercury Hg ²⁺	River water and tap water	Citrate-AuNPs + 2, 2'- bipyridyl (Bipy)	LOD: 38 nM LDR: 0.2 to 2.0 μM	[24]
AuNPs (20 nm)	Mercury Hg ²⁺	Target-doped blood serum	AuNPs and conjugated polyelectrolyte	LOD: 50 μM	[3f]
AuNPs (12 nm)	H ₂ O ₂	Interferent solutions at different concentration	Citrate-AuNPs	LOD: 1.3 μM LDR: 1.3 to 41 μM	[4a]
AuNPs (17±2 nm)	Cr ³⁺	Tap water and underground water	Mercaptosuccinic acid- AuNPs	LOD: 0.6 μM LDR: 0.6 to 1.4 μM	[11a]
AuNPs (13 nm)	Ag ⁺	Bimetallic conjugate mixtures	Au–PolyT and Ag–PolyA	LODs: 86.8 nM	[14]
AuNPs (13 nm)	Pb ²⁺	Divalent metal ions	DNAzyme–AuNPs	LOD: 0.1 to 4 μM	[3a]
AuNPs (13 nm)	Pb ²⁺	RNA-cleaving DNA enzyme	Aptamer–AuNPs	LDR: 0.020 to 200 μM LODs: 10 μM	[7i]
AuNPs (10 nm)	Pb ²⁺ , Pd ²⁺ , Hg ²⁺ , Pt ²⁺	Standard solutions	Peptide-AuNPs	LODs: nM/ppb range	[3d]
AuNPs (3.5 nm)	K ⁺	Monovalent cations (Li ⁺ , Na ⁺ , Rb ⁺ , NH ⁴⁺) and bivalent (Ca ²⁺ and Mg ²⁺)	Aptamer–AuNPs	LOD: 1.0 mM	[3e]
AuNPs (13 nm)	SCN ⁻ : thiocyan ate	Tap water and common ions	Citrate-AuNPs	LOD: 140 nM	[4s]
AgNPs (9.5±2.0 nm)	Ca ²⁺	Serum and artificial cerebrospinal fluid	AgNPs and cysteine	LOD: 0.1 mM	[4t]

157 LDR, linear dynamic range; LOD, limit of detection.

158 Recently, colorimetric measurements based on metal-induced aggregation of small
 159 molecules (for instance, cysteine, dopamine), peptides and DNA-functionalized NPs have

160 received considerable attention ^[3b, 3c, 4i, 11c]. Indeed, aggregation results in changes in the inter-
161 particle distances, leading to a shift of the SPR absorption band of AgNPs to a longer wavelength
162 ^[4t]. These cross-linking aggregation-based approaches have been used for the detection of metal
163 ions (Table 1). Farhadi *et al.* described the development of a sensitive and selective colorimetric
164 assay based on the interaction between cysteine, sodium dodecyl sulfate (SDS)-capped AgNPs
165 and calcium ions. In the presence of calcium ions and NaCl, AgNPs aggregation was induced,
166 leading to a yellow-to-red color change ^[3c] (Fig. 3). Mukherjee and co-workers used a similar
167 approach for cysteine detection with AgNPs in the presence of Cr³⁺ ^[3b]. As in the case for
168 colorimetric assays in which a ligand is used for complexation, these approaches ultimately are
169 effective for detection of both ligands and metal ions. For instance, in the presence of Cu²⁺,
170 ascorbic acid rapidly induces AuNPs aggregation as a result of the Cu⁺-catalyzed 1,3-dipolar
171 cyclo-addition of azides and alkynes (click reaction). Thus, the AuNPs solution color changed
172 and this was observed by naked eye for LOD of 3 nM ^[4i].

173



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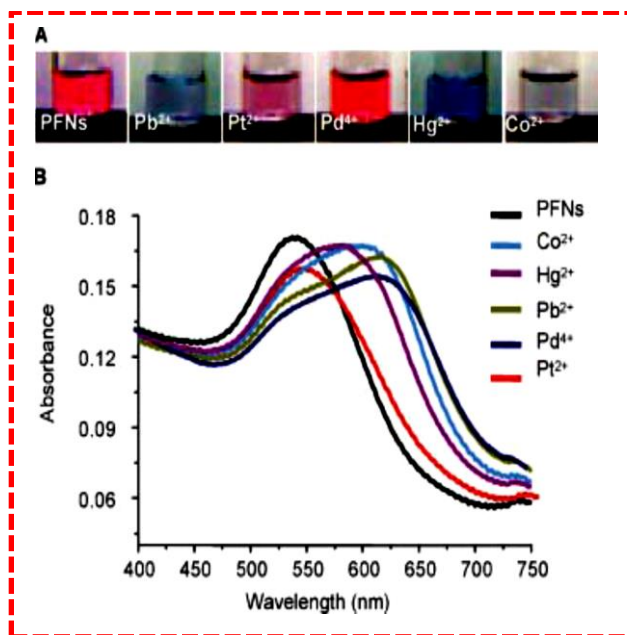
176 **Fig. 3. Schematic illustration of the strategy for cysteine detection using (A) AgNPs and (B) AuNPs.**
 177 **The simultaneous presence of cysteine molecules and Ca²⁺ ions that act as a “glue” to link two**
 178 **neighboring NP-cysteine complexes results in NP cross-linking. Reproduced with permission from**
 179 **[3c].**

180

181 Enzymes, such as deoxyribozymes (DNAzymes) with high specificity for metal ions ~~and~~
 182 can form blue aggregates with AuNPs, have also been used for the detection of metal ions.
 183 Specifically, Pb²⁺ induces NPs aggregation, thus maintaining the color of the original solution
 184 [3a].

185 Peptide-based AuNPs also have been used as colorimetric sensors for metal ions. For
 186 example, in the Flg-A3 fusion peptide, the N-terminal Flg (-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-
 187 Lys-) includes charged and aromatic residues involved in metal ion complexation, whereas the

188 A3 peptide (-Ala-Tyr-Ser-Ser-Gly-Pro-Ala-Pro-Pro-Met-Pro-Pro-Phe-) binds to gold surfaces.
189 This led to an overall negative charge (pI=3.9) that prevents NPs aggregation by repulsive forces
190 [3d]. Interestingly, after addition into the solution of peptide-AuNPs, the colorimetric response of
191 each metal ion (Co^{2+} , Hg^{2+} , Pb^{2+} , Pd^{4+} and Pt^{2+}) was different and reproducible and occurs within
192 1 min [3d] (Fig. 4).



193
194
195 **Fig. 4. Colorimetric response of peptide-AuNPs to metal ions. A) Optical microscopy images of**
196 **peptide-AuNP solutions (10 mM in Au atoms) after exposure to 10mM of different metal salts. B)**
197 **Corresponding UV/Vis spectra of peptide-AuNPs in the presence of the different metal ions [3d].**
198 **PFNs, peptide-AuNPs without metal ions.**

199
200 This indicates that peptides are versatile ligands and their complexation with metal ions is driven
201 through interaction of the peptide backbone with their amino acid side chains. Modifications in
202 the peptide sequences could affect the metal speciation and coordination geometry [3a, 3d]. The
203 limitation of the peptide-NP colorimetric sensor is that DNAzyme activity in the presence of NPs
204 solution should be minimal.

205 4. Detection of small molecules: cysteine, dopamine

206 This section focuses on small molecules such as analytes and colorimetric substrates
207 (Table 2), particularly cysteine and dopamine, because they have been extensively studied.

208

Table 2: Biomolecule-sensing based on AuNPs or AgNPs aggregation.

Nanoparticle (size)	Analyte/s	Sample/s	Functionalization	Analytical performance	Ref
AuNPs Monodisperse	Bisphenol-A	Urine and water samples	Aptamer–AuNPs	LOD: 0.01 pg mL ⁻¹ LDR: 10 000 to 0.1 pg mL ⁻¹	[4ab]
AuNPs (40–50 nm) Monodisperse	Concanavalin A	Lectin (jack beans)	Thioglucose-AuNPs	LOD: 9 nM LDR: 10–100 nM (R ² = 0.983)	[25]
AuNPs (12.33 nm) Monodisperse	Maltose, mannose, glucose, lactose and D-mannopentaose	Carbohydrate-AuNPs	Carbohydrate–protein interactions	NR	[26]
AuNPs (15, 20, and 2.5 nm) Monodisperse	Cysteine, glutathione and glutathione disulfide	Blood/serum	Citrate-AuNPs: 15 nm CTAB-AuNPs: 20 nm NaBH ₄ -coated AuNPs: 2.5 nm	LDR: cysteine, NR LDR: glutathione: 10–100 and 200–800 μM LDR: glutathione disulfide 10–300 and 400–800 μM LOD: cysteine <0.5 LOD: glutathione 10 μM LOD: glutathione disulfide 10 μM	[11b]
AuNPs (~13 nm) Monodisperse	Dopamine	Interferent: ascorbic acid	AuNPs induced by copper ions	LOD: 30 nM LDR 1: 33 nM to 100 nM LDR 2: 0.1 μM to 4.5 μM	[4i]
AuNPs (15 nm) Monodisperse	Dopamine (DA)	Spiked serum	DA inhibits melamine-induced AuNP aggregation	LOD: 33 nM LDR: 33 nM to 3.33 mM	[12]
AuNPs (13 nm) Monodisperse	Dopamine	Common interferents, such as 3,4-dihydroxyphenylalanine (DOPA), catechol, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), epinephrine (EP) and ascorbic acid (AA).	The aptamer conformational change could facilitate salt-induced AuNP aggregation 58-mer dopamine-binding aptamer (DBA)	LOD: 360 nM LDR: 0.54 -5.4 μM	[27]

AuNPs ±15 nm Multi-dispersed	Dopamine (DA)	Human urine, human serum	DA-induced aggregation of 4-amino-3-hydrazino-5-mercapto-1,2,4-triazol (AHMT)–AuNPs through hydrogen-bonding interactions	LOD: 70 nM LDR: 0.2–1.1 μM	[28]
AuNPs Monodisperse	Dopamine (DA)	Human serum	DA colorimetric sensing based on AuNP aggregation induced by copper ions	LOD: 200 nM LDR: 0.5–10 μM	[29]
AgNPs (NR) Monodisperse	Coralyne	Selectivity test against intercalating ligands, ethidium bromide (EB) and daunomycin (DM)	Homoadenine-AgNPs, label-free colorimetric detection of small molecules using DNA oligonucleotides and AgNPs	LOD: 0.25 coralyne molecules/adenine base	[4f]
AgNPs (5–15 nm) Multi-disperse	4-nitroaniline (4-NA)	self-assembled AgNPs on DNA	4-NA reduction to para-phenylenediamine		[20]
AgNPs (12 ± 2 nm) Multi-disperse	Adenine, guanine, cytosine, thymine	NR	Strength of interactions between the fundamental chemical components of DNA and AgNP surfaces	NR	[13]
AuNPs (13 nm) Multi-disperse	Ampicillin	ssDNA aptamer-AuNPs	Colorimetric assay of ampicillin using specific aptamers	LOD: 5 ng mL ⁻¹	[4l]
AuNPs	Oxytetracycline	Tetracyclines (TCs) as counter targets	Ultrasensitive colorimetric detection of oxytetracycline using shortened aptamer	LOD: 0.1 nM	[4v]
AuNPs (13 nm)	Oxytetracycline	Aptamer-AuNPs	Aptamer-specific colorimetric assay	LOD: 25 nM LDR: 25 nM to 1 μM	[4g]
AuNPs (18 nm) Monodisperse	Bisphenol a	Water samples	AuNP aggregation by competitive binding of bisphenol A and aptamer	LOD: 0.1 ngmL ⁻¹	[4q]
AuNPs (13 nm) Multi-disperse	Ochratoxin A	Standards	Aggregation occurs as random coil structures to compact rigid antiparallel G-quadruplexes	LDR: 20 to 625 nM LOD: 20 nM	[4k]
AuNPs (13 nm) Monodisperse	Cysteine	Amino acids, glutathione, thioglycolic acid and mercaptoethyl alcohol	2:1 cysteine/Cu ²⁺ complex	LOD: 10 nM	[11c]
AuNPs (13 nm) Monodisperse	Ascorbic acid	Fruit juices	Alkyne–azide click reaction	LOD: 3.0 nM	[4i]

AuNPs (NR) Monodisperse	Cysteine	19 amino acids	ssDNA-AuNPs	LOD: 100 nM LDR: 0.1 to 5 μ M	[4e]
AuNPs (20.1 \pm 1.8 nm) Multi-disperse	Influenza B/Victoria, influenza B/Yamagata	Virus dilution (hemagglutination assay titer, 512)	Sialic acid-AuNPs	LOD: 0.09 vol%. upper limit of linearity 2.5 vol %	[10]
AuNPs (13 nm) Monodisperse	Tryptophan enantiomers	D/L enantiomers	AuNPs	LOD: 0.1 μ M	[4y]
AuNPs (~11.89 nm) Mono-disperse	Arginine, histidine, lysine	Urine samples	Quercetin-AuNPs	LOD: 0.04, 0.03, and 0.02 μ M. LDR: 2.5–1,250 μ M (Arg) and 1–1,000 μ M (His and Lys),	[30]
AuNPs-I (15 nm) AuNPs-II (30 nm) AuNPs-III (40 nm) Multi-disperse	Melamine	Pre-treated milk	Citrate-AuNPs	AuNPs-I (2.37 x 10 ⁻⁸ M) AuNPs-II (3.3 x 10 ⁻⁸ M) AuNPs-III (8.9 x 10 ⁻⁸ M)	[4z]
AgNPs (NR) Mono-disperse	Cysteine	Various metals	2:1 cysteine/Ca ²⁺ complex	LOD: 83 nM LDR: 0.25 – 10 μ M	[3c]
AgNPs (10 – 15 nm) Multi-disperse	Cysteine	10 mM of nine amino acids	2:1 cysteine/Cr ³⁺ complex	LOD: 1 nM	[3b]
AgNPs (10-20 nm) Multi-disperse	Dopamine, L-DOPA, noradrenaline adrenaline	Tyrosinase	AgNPs	LOD: dopamine, L- DOPA and noradrenaline 2.5 μ M adrenaline 20 μ M tyrosinase activity-10 units (-100 μ g mL ⁻¹)	[4b]
AuNPs (~ 13 nm) Multi-disperse	Kanamycin	Other antibiotics: streptomycin, sulfadimethoxine and ampicillin	ssDNA aptamer-AuNPs	LOD: 10 nM	[4j]
AuNPs (~ 13 nm) Mono-disperse	Sulfadimethoxine (SDM)	NR	AuNPs	LDR: 50 ng mL ⁻¹ to 1.0 μ g mL ⁻¹ LOD: 50 ng mL ⁻¹	[4n]
AuNPs (~15 nm)	Penicillin G	Different penicillins	CTAB-AuNPs	LOD: 0.007 mg mL ⁻¹	[7m]

Multi-disperse					
AuNPs (20 nm) Mono-disperse	Cysteine	19 essential amino acids	DNA-AuNPs	LOD: 100 nM LDR: 100 nM – 2 μM	[4d]
AuNPs (13 nm) Multi-disperse	Adenosine	inosine, guanosine, and cytosine	aptamer-OD-AuNPs	LOD: 10 μM	[31]
AuNPs (13 nm) Mono-disperse	Adenosine and caffeine	Other nucleosides	Aptamers	LOD: 0.3 mM	[4c]
AuNPs (15 nm) Mono-disperse	Digitoxin	Rat serum	AuNPs	LOD: 571 pM	[4aa]
AuNPs (10.8-13.1 nm) Multi-disperse	Cysteine	Interferents: Na ⁺ , Cu ²⁺ , Cl ⁻ and urea	AuNPs	LDR: 0.1 to 0.6 ppm LOD: 0.01ppm	[4u]
AgNPs (5 – 20 nm) Multi-disperse	Cysteine/cystine	Other amino acids	AgNPs	LDR: 25–250 μM LOD: 2.5 ppm	[4m]
AgNPs (8 nm) Mono-disperse	Cysteine and homocysteine	Human urine and plasma samples	Non-ionic fluorosurfactant-AuNPs,	LOD: 0.4 μM.	[4o]
AgNPs (8±1.6nm) Multi-disperse	Cysteine	Human urine and plasma samples	Fluorosurfactant-AgNPs	LOD: 0.05 μM. LDR: 1.5–6.0 μM	[4r]
AgNPs (9.5±2.0 nm) Multi-disperse	Cysteine	Serum and artificial cerebrospinal fluid	AgNPs and Ca ²⁺	LDR: 0.1–1000 μM LOD: 0.1 μM	[4t]
AuNPs (20.0±1.4) nm Multi-disperse	Cysteine	Human urine	Pectinase-protected AuNPs	LDR: 4.85x10 ⁻⁶ to 302 μM and 3.25 to 0.103 mM LOD: 4.6x10 ⁻⁹ M.	[4ac]
AuNPs (13 nm) Monodisperse	Cysteine	Brain microdialysate (sample), lactate, ascorbic acid and glucose (interferents)	Cysteine-AuNPs	LDR: 0.166 to 1.67 μM LOD: 0.1 μM	[23]
AuNPs (13 nm) Monodisperse	Pyruvic acid	Interferents: lactic acid (LA), ascorbic acid (AA) and glucose.	AuNPs	LDR: 5.6 μM to 168.0 μM LOD: 3.0 μM	[4w]
AuNPs (13 nm) Monodisperse	Sulfadimethoxine (SDM), kanamycin (KAN) and adenosine	Mixture of KAN, SDM and ADE	Aptamer of KAN, SDM and ADE (1:1:1 mixture).	NR	[4x]

	(ADE)				
AuNPs (13 nm) Monodisperse	17 β -estradiol	Interferents: methanol, diethylstilbestrol, bisphenol A, 19- nortestosterone, estriol, estrone	AuNPs	LOD: 0.1 ng mL ⁻¹	[4x]
AuNPs (20 nm) Monodisperse	Caffeine	ATP and target-doped blood serum	AuNPs	LOD: 1.25 μ M	[3f]
AuNPs (13 nm) Monodisperse	Parathion	Sea and tap water	Parathion inhibits AChE- induced aggregation of AuNPs	LDR: 15 to 65 ppb and 140 to 1000 ppb LOD: 0.7 ppb (2.4 nM)	[4ad]

210 CTAB: cetyl trimethyl ammonium bromide; OD: oligonucleotides; SDM: sulfadimethoxine; KAN: kanamycin; ADE: adenosine; AChE:
211 acetylcholinesterase, Cyt C: cytochrome c.; LDR: linear dynamic range; LOD: limit of detection.

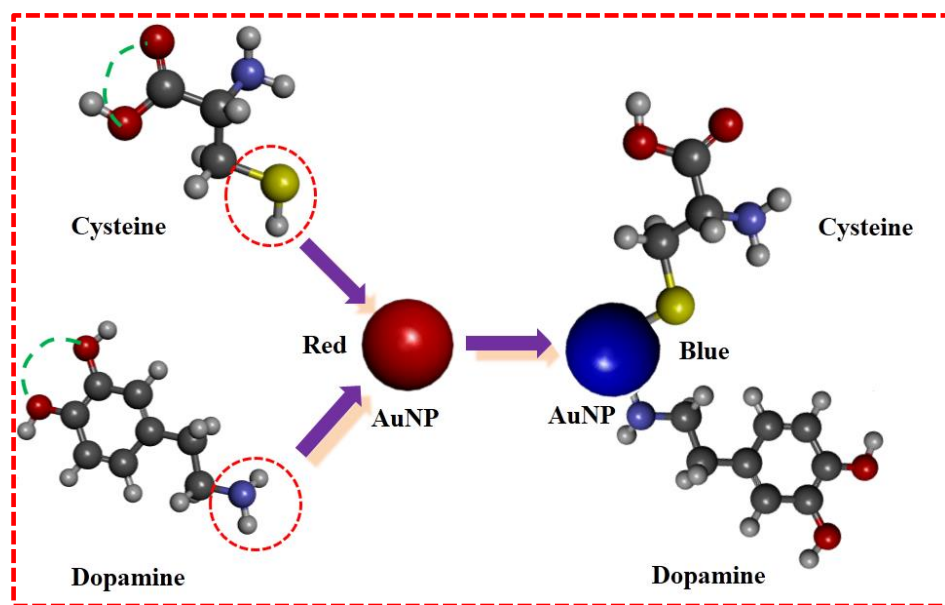
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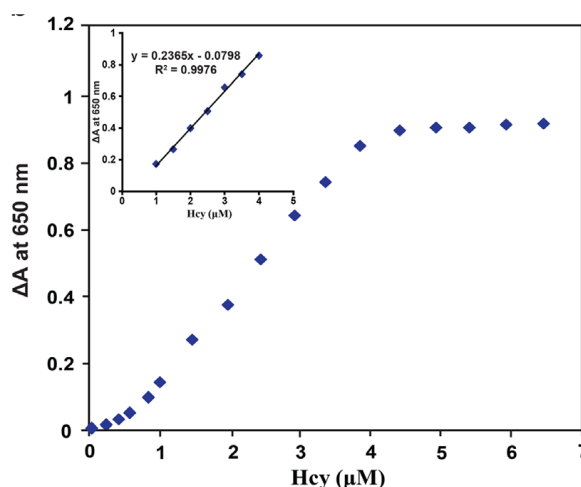
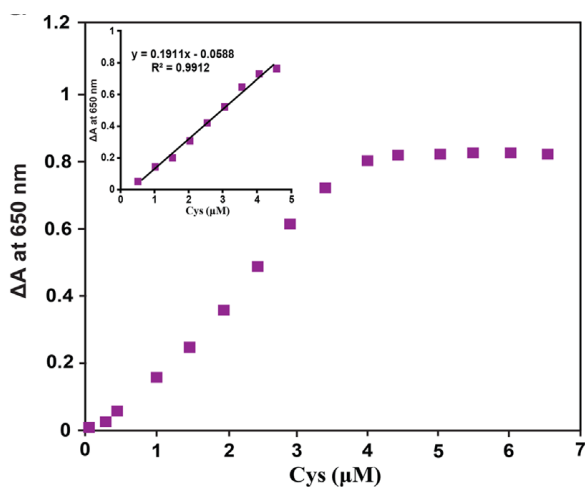
215 Cysteine has been employed as a common strategy for the detection of metals, but it also
216 can be quantified by colorimetric assays. Metal ions are used as cross-linking agents for
217 cysteine-AuNP or -AgNP pairs to induce NP aggregation and the consequent red to blue color
218 change of the NP solution is observed. The degree of aggregation depends on the cysteine
219 concentration and the average AuNPs diameter in the presence of different cysteine
220 concentrations. In such colorimetric assays, organic molecules bind to the Au/AgNPs surface
221 via their amine (-NH₂) (blue) or thiol (-SH) (yellow) terminal groups (Fig 5).

(A)



(B)

(C)



222

223 **Fig. 5A) Chemisorption model for cysteine and dopamine on the AuNP surface. The solution color**
224 **changes from red to blue in the presence of cysteine or dopamine. B and C shows linear**
225 **relationship of nonionic fluorosurfactant-capped gold nanoparticles versus Cys and Hcy 650 nm.**
226 **The linear range for Cys was 0.5–4.5 μM , and for Hcy was 1.0–4.0 μM . Conditions: pH 6.5, 100**
227 **mM NaCl, 30 s incubation period at 50 °C**
228

229 Ligands with the $\text{C}_6\text{H}_8\text{O}_6$ formula, without $-\text{NH}_2$ or $-\text{SH}$ functional groups, preferably
230 bind to the AuNPs surface, and are highly unlikely to induce AuNPs aggregation [4i, 4u, 32].

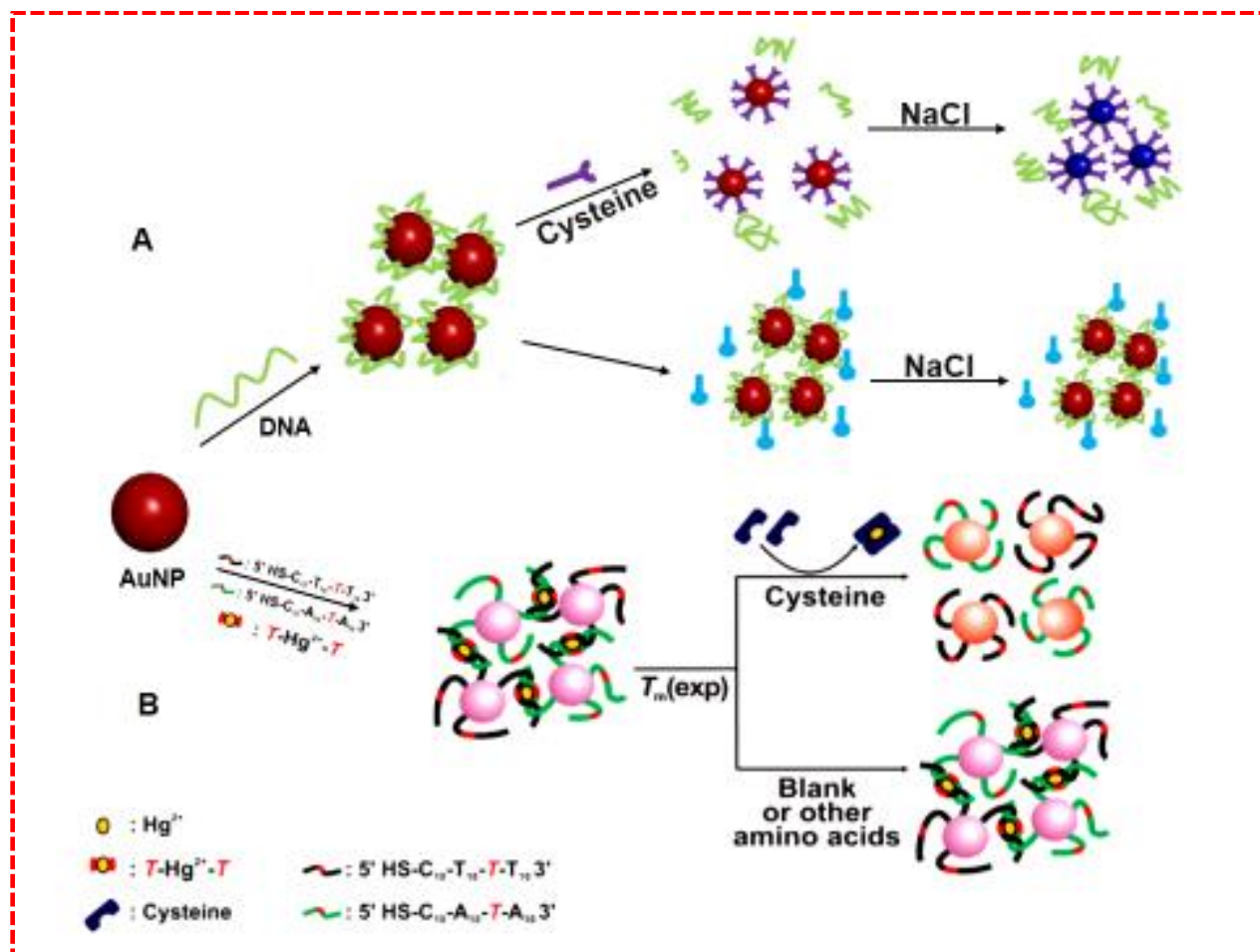
231 The thiol groups of cysteine interact with the surface of colloidal AuNPs or AgNPs via
232 chemisorption-type interactions; however, cysteine can complex with metal ions, such as Pb^{2+} ,
233 Zn^{2+} , Cu^{2+} [4i, 11c], Ca^{2+} [3c, 4t], Cr^{3+} [3b], at a ratio of two cysteines for each metal ion [10, 11c]. In the
234 presence of metal ions, cysteines can induce AgNP and AuNP aggregation with a color change
235 from yellow to purple and blue to red, respectively [3b, 3c, 4u, 11c]. These assays are based on the NP
236 distance-dependent optical properties after coordination, as confirmed by the change in the zeta
237 potential from -30.7 mV for pure AgNPs to -19.63 mV after interaction with cysteine and Cr^{3+}
238 [3b]. Specifically, Hajizadeh *et al.* reported that cysteine can rapidly induce AgNP aggregation
239 (yellow-to-red color change) in the presence of Ca^{2+} and 10 mM NaCl, leading to a decrease in
240 electrostatic repulsion and faster aggregation [3c]. Cysteine concentration can be determined also
241 by using AuNPs and a UV-Vis spectrometer with a LOD of 10 nM (1.2 ng ml^{-1}) [11c]. The ratio
242 between absorption at 524 and absorption at 396 nm (A_{524}/A_{396}) is linear with a cysteine
243 concentration range from 0.25 to 10 mM ($R^2 = 0.993$) with a LOD of 83 nM [3c]. Jongjinakool
244 and co-workers detected cysteine in a concentration range from 0.1 to 0.6 ppm with a LOD of
245 0.01 ppm [4u]. In Figure 5b-c, a multi-component mixtures demonstrated that cysteine and
246 homocysteine were identified based on the different SPR wavelengths induced by aggregation of
247 non-ionic fluoro-surfactant-functionalized AuNPs upon addition of a mixture of the amino acids.
248 The absorbance changes due to AuNPs aggregation induced by cysteine and homocysteine
249 increase the individual absorbance values (LDR from 0.5 to 4.5 μM for cysteine and LOD
250 ($S/N=3$) of 0.4 μM for homocysteine) [4o].

251 When dopamine (DA) and Cu^{2+} solutions are mixed, the amine group directly
252 coordinates with Cu^{2+} without nitrogen atoms bonded to the gold surface [4i]. The LOD for DA is
253 30 nM with, differently from cysteine-based methods, a linear calibration curve for two
254 concentration ranges (3.3×10^{-8} to 1.0×10^{-7} M and 3.0×10^{-7} to 4.5×10^{-6} M) with correlation
255 coefficients of 0.9981 and 0.9979, respectively [4i]. Likewise, addition of 5 mM Cu^{2+} improves

256 the colorimetric probe to a LOD of 200 nM ^[29]. AuNPs also can be used for the quantitative
257 colorimetric detection of neurotransmitters that mediate the generation and growth of AuNPs,
258 with a LOD of 2.5 μ M for dopamine, L-DOPA and noradrenaline, and of 20 μ M for adrenaline
259 ^[4b]. The metal ion-Au/AgNPs interaction is mainly a coulombic interaction and its strength is
260 directly related to the molecular structure and charged groups. Therefore, selectivity can be
261 improved by working on these two parameters ^[4r]. For instance, researchers highlighted the
262 excellent selectivity of AuNP-based colorimetric assays for cysteine compared with other
263 biomolecules, such as thioglycolic acid and mercaptoethyl alcohol ^[11c], glutathione ^[11b, 11c]
264 glutathione disulfide ^[11b], aspartic acid and glutamic acid ^[23]. Moreover, selectivity changes also
265 when using cysteine derivatives, namely glycine, dipeptide Cys-Gly, cysteamine,
266 mercaptopropionic acid, S-protected (S-methyl-L-cysteine), N-protected (N-acetylcysteine) and
267 O-protected cysteine (L-cysteine methylester hydrochloride), as indicated by the color change
268 from yellow to pink and peak broadening ^[4m]. Therefore, it is imperative to investigate the
269 detection of sulfur-containing amino acids compared with other standard amino acids ^[4m]. Chen
270 *et al.* used 19 naturally occurring amino acids, but they could not improve the colorimetric
271 response of cysteine ^[4e].

272 Another strategy for cysteine detection is based on the observation that when cysteine is
273 added to AuNPs/ssDNA, the ssDNA molecules that stabilize AuNPs against salt-induced
274 aggregation are displayed spontaneously by cysteine encapsulation on the AuNPs surfaces, via
275 an Au-S bond ^[4e]. The salt-induced aggregations result in a characteristic AuNPs color change
276 from red to blue ^[4e, 4m]. According to Chen *et al.* ^[4e] this approach is not feasible with other
277 amino acids (Fig 6).

278



279

280 **Fig. 6. In the presence of cysteine, the ssDNAs is displayed by cysteine on AuNPs surface resulting**
 281 **in AuNP aggregation and in a color change from red to blue upon addition of NaCl. Other amino**
 282 **acids do not lead to a color change due to the absence of thiol groups [4e]. (B) Cysteine colorimetric**
 283 **detection using AuNPs probes that contain T-T mismatches complexed with Hg²⁺: competitive approach in**
 284 **which cysteine can displace Hg²⁺[4d]**

285

286 In this system, the A₆₄₀/A₅₂₅ ratio is linearly dependent on the cysteine concentration (from 0.1 to
 287 5.0 μM with LOD of 100 nM) [4e]. Differently from methods that require AuNPs modification,
 288 this approach is simple and fast, but it requires specific links between the biomolecules and
 289 AuNPs to allow ssDNA displacement from the NPs surface. On the other hand, Mirkin's group
 290 developed a cysteine detection assay where two sets of AuNPs probes are functionalized with
 291 different oligonucleotide sequences (probe A: 5' HS-C₁₀-A₁₀-T₁₀-A₁₀ 3'; probe B: 5' HS-C₁₀-
 292 T₁₀-T₁₀-T₁₀-3') and rapidly form aggregates upon combination through the thymidine-thymidine
 293 (T-T) mismatches complexed with Hg²⁺ with LODs as low as a 100 nM [4d]. Comparison of the
 294 two methods (ssDNA-AuNPs and mismatch assay) highlights that they rely on the distance-
 295 dependent optical properties of AuNPs, the sharp melting transition of oligonucleotide-AuNPs

296 aggregates and the very selective coordination of Hg^{2+} with cysteine during which the purple-to-
297 red color change occurs ^[4d, 4e]. However proteins with one free cysteine residue, such as human
298 serum albumin, can spontaneously attach to AuNPs surfaces through Au-S bond formation ^[2].
299 These studies demonstrate that the presence of negatively-charged carboxyl groups in the cross-
300 linkers remains essential to induce AuNPs aggregation through ion pair interactions between
301 amino groups present in cysteines and carboxyl groups in the cross-linkers ^[4m, 23]. On
302 comparison of the dopamine- and cysteine-based methods it was evident that functional groups
303 present on the surface also plays a key role in metal detection.

304

305 4.1. Recognition of chiral molecules

306 Stereochemistry plays a central role in molecular recognition and interactions. Indeed, the
307 molecule's chemical and biological properties depends not only on the nature of their constituent
308 atoms, but also on their position in space [32b]. Currently, chiral molecules are mostly separated
309 with techniques like capillary electrophoresis, high-performance liquid and gas chromatography.
310 Nanoparticles allow for the easy detection of chiral molecules by the naked eye. For instance,
311 the color of the AuNPs solution changes from red to blue in the presence of D-tryptophan (LOD
312 of 0.1 μM and LDR of 0.2–10 μM), but not of L-tryptophan [4y]. Interestingly, AuNPs can
313 selectively adsorb D-tryptophan, and therefore, L-tryptophan molecules can easily be separated
314 by simple centrifugation of the tryptophan/AuNP solution [4y]. The infrared spectra confirms the
315 D-tryptophan absorption to AuNPs by disappearance of the NH (NH^{3+}) stretching absorption
316 peaks (3078 and 3038 cm^{-1}), leaving the carboxylic group (-COOH) and nitrogen atom of the
317 indole ring free for further coordination. This distinctive feature allows for binding of one Cu^{2+}
318 ion to two tryptophan molecules by coordination with the COOH and nitrogen atom of the indole
319 ring, hence allowing for recognition [4y]. Another reported visual differentiation is between the
320 D- and L-forms of mandelic acid (MA) was based on their chirality towards 13 nm l-tartaric
321 acid-capped AuNPs. The L-MA solution changes the red colour of l-TA-capped AuNPs to a
322 bare-eye observable blue, while d-MA does not trigger any color changes [33]. The AgNPs
323 capped with a novel chiral R-mandelic acid-derived calix[4]arene (R-MAC4), for its good optical
324 and structural properties. These self-assembled NPs were used to recognize the N-Fmoc-d/l-
325 aspartic acid (d/l-FAA)[34].

326

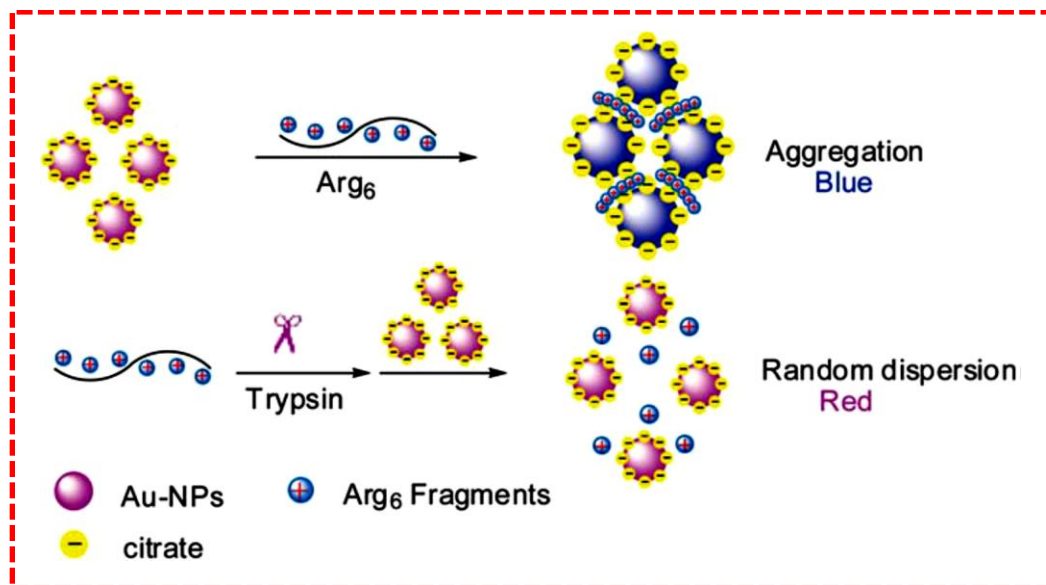
327 4.2. Detection of macromolecules

328 Nanoparticles can be easily modified by replacing surface-adsorbed weak ligands (e.g.,
329 negatively charged citrate ions) with thiolated macromolecules that are difficult to displace due
330 to their strong binding to the surface.

331 Xue and co-workers described citrate-AuNP-based assays for trypsin and arginine
332 residues screening (Fig. 8). The aggregation of negatively charged citrate-capped AuNPs in the
333 presence of a peptide composed of six arginine residues (Arg_6) occurred mainly through
334 electrostatic interactions, and led to a red-shift of the usual SPR profile [35]. However, when the
335 Arg_6 peptide was hydrolyzed into fragments upon trypsin addition in the solution, the

336 electrostatic interactions between AuNPs and arginine residues were weakened and, therefore,
337 neither AuNPs aggregation nor SPR shift was observed.

338

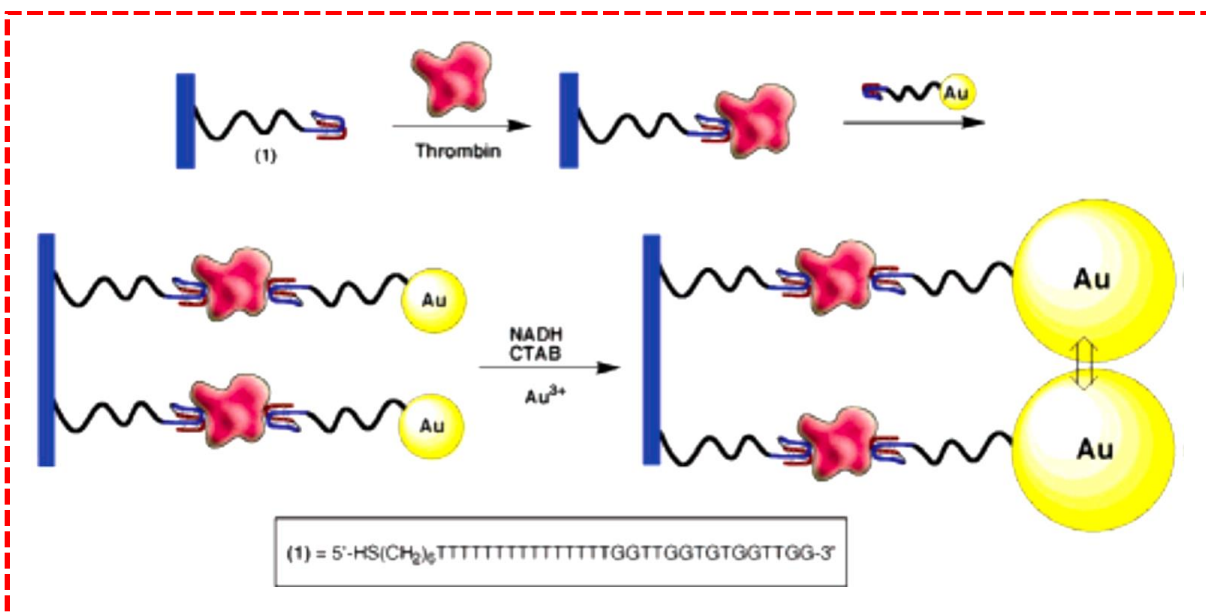


339

340 **Fig. 7. Colorimetric assay for trypsin by using AuNP crosslinking/aggregate based on trypsin-**
341 **catalyzed hydrolysis of Arg₆ for random dispersion of citrate capped AuNPs. Reproduced with**
342 **permission from [35].**

343

344 Moreover, Arg₆ hydrolysis catalyzed by trypsin is retarded if trypsin inhibitors are present in the
345 solution. This feature was used to develop a label-free assay for trypsin (LOD: 1.6 ng ml⁻¹) and
346 Arg₆ residues screening with AuNPs [35]. Similarly, interaction of citrate-AuNPs with fibrinogen
347 to form fibrinogen–AuNPs through electrostatic and hydrophobic interactions was used for the
348 detection of thrombin (LOD: 0.04 pM and LDR: 0.1–10 pM; R² = 0.96) [71]. Although the
349 mechanism of detection was the same, the molecular interactions was different. Indeed,
350 fibrinogen was adsorbed on NPs before the addition of thrombin [71]. Conversely, trypsin interacts
351 with Arg₆ before the NPs addition into the system [35]. Detection of thrombin has been improved
352 by the use of catalytic enlargement as shown in Fig. 9. [5a].



353
 354 **Fig. 8. Amplified thrombin detection on surfaces by catalytic enlargement of thrombin-aptamer-**
 355 **functionalized Au-NPs [5a]. As the concentration of thrombin increases, the surface loading of bound**
 356 **thrombin is higher, resulting in a number of Au NP seeds for enlargement.**

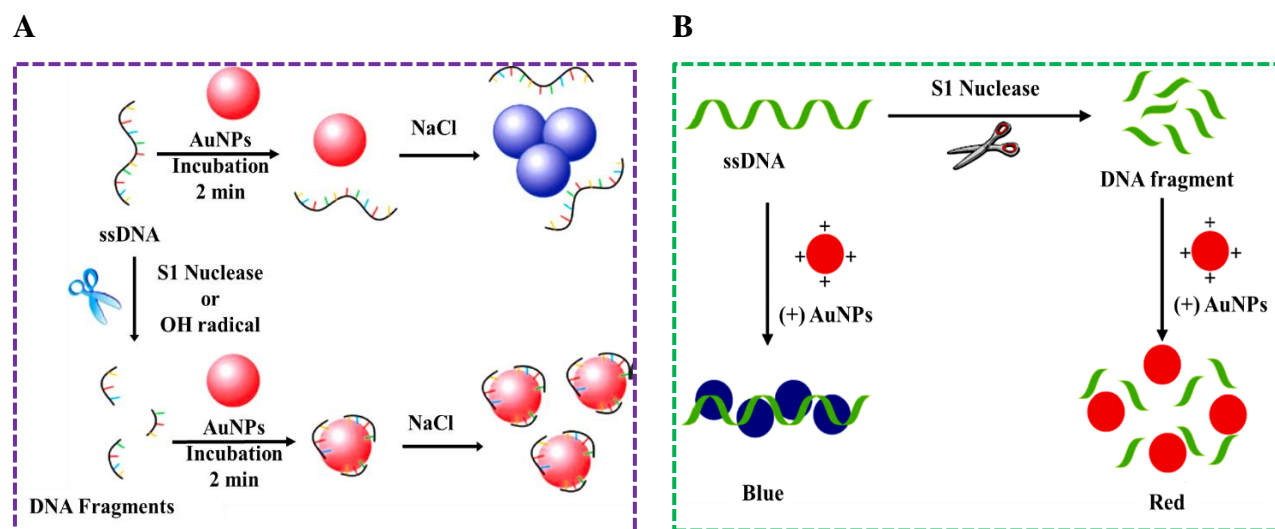
357 Specifically, upon aptamer-AuNPs reaction with thrombin, AuNPs aggregate. But in the
 358 presence of other proteins (200 nM BSA or human IgG antibodies), the aptamer-AuNPs does not
 359 precipitate implying that the precipitation originates from the specific interaction between
 360 aptamer and thrombin (LOD: 20 nM) [5a].

361 Similarly, Chen's group evaluated the possibility of using mannopyranoside-encapsulated
 362 AuNPs/concanavalin (Man-AuNPs/Con A) complexes for a competitive colorimetric assay for
 363 ten proteins. However, only thyroglobulin, *bandeiraea simplicifolia* lectin I (BS-I), soybean
 364 agglutinin (SBA) and *maackia amurensis* (MAL) significantly modified the absorption spectrum
 365 of Man-AuNPs/Con A complexes [5b]. In contrast to the method proposed by Xue *et al.* [35],
 366 where upon the introduction of thrombin in the fibrin-AuNP solutions, catalyzes the
 367 polymerization of the free and conjugated fibrinogen species to form insoluble fibrillar fibrin-
 368 AuNP agglutinates [71]. Finally, Guarise *et al.* exploited the fact that, compared with the native
 369 peptide substrates, protease-cleaved peptides do not induce NPs aggregation (and thus the color
 370 of the solution does not change) to detect two proteases (thrombin and lethal factor) [36].

371 Quercetin-AuNPs have been used as a colorimetric probe for the detection of amino
 372 acids, such as arginine (Arg), histidine (His) and lysine (Lys). Indeed, quercetin-AuNPs
 373 aggregation caused by amino acids leads to a color change from red to blue [30]. In optimal

374 conditions, a linear relationship exists between the absorption ratios at different wavelengths
 375 (A_{702}/A_{525} for Arg, A_{693}/A_{525} for His, and A_{745}/A_{525} nm for Lys) and the concentration ranges
 376 (from 1.25 to 2.50 μM for Arg; from 1 to 1,00 μM for His and Lys), with LOD values of 0.04,
 377 0.03, and 0.02 μM , respectively, at pH 5.0 [30]. Siddhartha and Debabrata reported that protein
 378 estimation is within a LOD of 10-80 $\mu\text{g mL}^{-1}$ using unmodified AgNPs [5h].

379 Lately, the sequence-length-dependent adsorption of ssDNA on AuNPs has been
 380 investigated for colorimetric nuclease assays and measurement of oxidative DNA damage [6g].
 381 Based on ssDNA adsorption rate on citrate-AuNPs, it can be hypothesized that incubation with
 382 AuNPs for a specific period of time can lead to differential adsorption of short and long ssDNA.
 383 Consequently, the stability of the ssDNA-AuNPs complex in the presence of salt could be
 384 influenced by the ssDNA length [6g]. The confirmation of this hypothesis led to the development
 385 of colorimetric assays taking advantage of ssDNA length to improve adsorption. For example,
 386 when ssDNA is cleaved by the S1 nuclease or $-\text{OH}$ radicals in small fragments, these shorter
 387 ssDNA can be rapidly adsorbed on AuNPs and significantly enhance the negative charge density
 388 on each AuNP surface for the same time of incubation (Fig. 9).



389 **Fig. 9. Schematic representation of AuNP-based colorimetric strategies for ssDNA cleavage assays.**
 390 **(a) Salt-induced NP aggregation before and after ssDNA cleavage by the S1 nuclease or OH**
 391 **radicals. Reproduced with permission from [6g]. (b) Nuclease activity assay using positively charged**
 392 **AuNPs and polyanionic ssDNA ((+)AuNPs). Reproduced with permission from [7n].**

393
 394 Basically, charging the NPs surface increases the electrostatic repulsion between the ssDNA-
 395 AuNPs complexes enough to inhibit NP aggregation at the same salt concentration, and the
 396 solution color does not change. Thus, DNA cleavage can be directly visualized by naked eye [6g].

397 Charge interaction between positively charged AuNPs and polyanionic ssDNA leads to AuNPs
398 aggregation that can be monitored by the color change from red to blue (Fig. 9) ^[7n]. However, in
399 the presence of the S1 nuclease, the ssDNA substrate is cleaved into small fragments, and the
400 AuNPs solution remains red. Thus, the nuclease activity can be easily monitored by naked eye or
401 with a simple colorimetric reader ^[7n]. In this case, the exocyclic amino group of nucleotides is
402 the main cause of nucleotide-dependent aggregation because cysteamine-capped AuNPs are
403 positively-charged at pH 3.6 (with a pKa of 10.75), leading to efficient electrostatic interactions
404 with the negatively charged DNA. These results complement each other because during S1
405 enzymatic activity, ssDNA is degraded into smaller fragments that cannot induce aggregation <sup>[6g-
406 7n]</sup>.

407 Additionally, the non-crosslinking AuNPs aggregation induced by the loss of surface charges
408 also is exploited for enzymatic activity testing and screening for potential inhibitors ^[7a]. This
409 approach has been extended to the serotype I-specific detection of dengue virus DNA. In the
410 absence of the DNA target solution, peptide nucleic acids (PNA) induce AuNPs aggregation with
411 a red-to-purple color change and the appearance of a second absorbance peak at 650 nm due to
412 the AuNPs surface coating by PNA ^[37]. Likewise, sialic acid-reduced and -stabilized AuNPs ($d=$
413 20.17 ± 1.8 nm) can be used for the colorimetric detection of influenza viruses ^[10]. The
414 discrimination of such molecules can also be standardized using chemometric techniques
415 including hierarchical cluster analysis and principal component analysis. This approach was used
416 to accurately classified and measure the array response of cysteine, glutathione, glutathione
417 disulfide and interferences without any misclassification ^[38].

418 5. DNA-functionalized nanoparticles (aptasensors)

419 This is the most fascinating area of NP-based colorimetric assays. Much attention has
 420 been focused on aptamers (i.e., ssDNAs and oligopeptides with high binding affinity and
 421 selectivity for target molecules) as powerful biological macromolecules (Table 3). Particularly,
 422 the aptamer advantages compared with antibodies, such as possibility of chemical synthesis and
 423 modification and lower immunogenic response, contribute to their potential [4j]. They are
 424 generally selected *in vitro* by using the systematic evolution of ligands by exponential
 425 enrichment (SELEX) technique and random-sequence nucleic acid libraries [4j, 4l, 4p, 4v, 4x, 39]. This
 426 allows selecting, the highest binding aptamer(s). Then, aptamers can be capped with thiol groups
 427 that bind to two AuNPs [6c, 9, 18]. The principle of colorimetric sensing based on aptamers to
 428 detect specific DNA sequences was introduced about a decade ago and is now a key tool in
 429 biodiagnostics [9, 18]. Since then, other aptamer-based sensors have been developed for the
 430 detection of metal ions, small molecules [4d, 4e] and proteins [5c, 5g, 7d].

431

432 **Table 3: DNA/protein sensing based on AuNP aggregation**

Nanoparticle (size)	Analyte/Target	Functionalization	Analytical application	Analytical performance	Ref.
AuNPs (13 nm) and AgNPs (10 -20 nm) Monodisperse	DNA3 complementary DNA	DNA1-AuNPs DNA2-AgNPs	Complementary DNA recognition	NR	[16]
AuNPs (12.33 nm) Monodisperse	Maltose > mannose > glucose > lactose > MAN5.	Carbohydrates-AuNPs	carbohydrate-lectin interactions	NR	[26]
AuNPs (13-nm) Monodisperse	Lysozyme	HSA-AuNPs	Colorimetric detection	LOD: 50 nM.	[7f]
AgNPs (10-15 nm) Monodisperse	Globular proteins (BSA and IgG)	AgNPs	Concentration-dependent particle agglutination	LOD: 10 to 80 $\mu\text{g mL}^{-1}$	[5h]
AuNPs and AgNPs Monodisperse	Specific DNA sequence	AuNPs and AgNPs	Detection of DNA through nucleic acids (PNA) hybridization	LOD: DNA/PNA ratio of 0.05	[6a]
AuNPs (50 nm) Multidisperse	Con A	p-Aminophenyl-D-mannose- AuNPs	Carbohydrate-lectin system	LOD: 9.0 nM ($R^2 = 0.983$) LDR: 10-100 nM	[25]
AuNPs Multidisperse	PDGFs and PDGFR	Apt-AuNPs	Protein analysis and cancer diagnosis	-2.5-10 and 10-20 nM, respectively, for 0.42 nM Apt-	[5c]

				AuNPs -25-75 and 75-200 nM, respectively, for 8.4 nM Apt- AuNPs.	
AuNPs (32 nm) Monodisperse	Con A binding partners	Mannopyranoside- encapsulated gold nanoparticles (Man-AuNPs)	Competitive colorimetric assay for ConA binding partners through protein–protein interactions	NR	[5b]
AuNPs (13 nm) Monodisperse	24-bp polynucleotide target	AuNPs capped with 3'- and 5' (alkanethiol) oligonucleotides	Hybridization of the target with the probes		[6c]
AuNPs (20 nm) Monodisperse	H ₂ N-Cys-Tyr(PO ₃ ²⁻)- Arg-OH	AuNPs	Sensing phosphatase activity of alkaline phosphatase	54: 3.4 uM of peptide : alkaline phosphate	[40]
AuNPs (15 nm) Monodisperse	Bla molecules	AuNPs	β-Lactamase activity Identification of Bla molecules and screening for Bla inhibitors	60 pM	[41]
AgNPs (31 nm) Multidisperse	AgNP-oligonucleotide conjugates	DNA-AgNPs	Hybridization of two complementary DNA-AgNPs	NR	[17a]
AuNPs (13 nm) Monodisperse	Single-stranded DNA cleavage	AuNPs	Enzymatic cleavage and oxidative damage of single-stranded DNA	NR	[6g]
AuNPs (14 nm) Monodisperse	HIV-1 ribonuclease H	AuNPs	Colorimetric detection of HIV- 1 ribonuclease H activity by AuNPs	27 units mL ⁻¹	[7o]
AuNPs (13 nm) Monodisperse	Tyrosin	AuNPs	Crosslinking/ aggregation of Au-NPs based on trypsin-catalyzed hydrolysis of Arg6	1.6 ng mL ⁻¹	[35]
AuNPs Monodisperse	Methyltransferase activity	DNA-AuNPs	Colorimetric assay for endonuclease/ methyltransferase activity and inhibition	NR	[7k]
AuNPs (13 nm) Monodisperse		DNA-modified AuNPs	Enzymatic cleavage of nucleic acids, colorimetric biosensors	0.5 units mL ⁻¹	[7i]
AuNPs (13 nm) Monodisperse	Thrombin	Aptamer-AuNPs	Colorimetric assay based on the aptamer folding and unfolding	LDR: 0 to 167 nM LOD: 0.83 nM	[5g]
AuNPs (20 nm) Monodisperse	Thrombin	AuNPs	G-quadruplex structure folding	LOD: 10 nM	[3f]
AuNPs (NR)	Triplex DNA binders: BePI or CORA	Aptamer-AuNPs	Screening triplex DNA binders	NR	[42]

AuNPs (13.3±1.2 nm) Multidisperse	Thrombin	Fibrinogen-AuNPs (56 nm)	Colorimetric assay for blood plasma	LOD: 0.04 pM LDR: 0.1 to 10 pM	[7i]
AgNPs (NR) Multidisperse	CIAP and PKA	AgNPs	Adenosine phosphorylation and dephosphorylation	LOD: CIAP: 1.0 unit mL ⁻¹ PKA: 0.022 unit mL ⁻¹	[7h]
AuNPs (~ 12.74 nm) Monodisperse	staphylococcal enterotoxin B	AuNPs	Colorimetric assay based on aggregation in the absence of the aptamer	LDR: 10 to 50 ng mL ⁻¹ LOD : 10 ng mL ⁻¹	[4p]
AuNPs (NR) Multidisperse	Aminopeptidase N	Gold nano-composites conjugated with a thermo-responsive copolymer	Activity based on inhibition of the disassembly of Gold nano-composites	AR: 20 to 50 U L ⁻¹	[43]
AuNPs (15.1 nm) Multidisperse	β-galactosidase and β-glucosidase	Gal-Lip-AuNPs and Glc-Lip-AuNPs	Glycosidase inhibitor screening	LODs: β-galactosidase 9.2 nM and β-glucosidase 22.3 nM at 20°C	[44]
AuNPs (26 nm) Monodisperse	ADA	AuNPs	Nucleotide-dependent aggregation	LOD: 0.8227 U L ⁻¹	[8]
AuNPs (13 nm) Monodisperse	Endonuclease	DNA-AuNPs with duplex interconnection	Endonuclease activity and inhibition	NR	[7e]
AuNPs (13 nm) Monodisperse	Biotin (biotinylation)	Peptide-AuNPs and avidin-AuNPs	Kinase-catalyzed substrate biotinylation	NR	[7d]
AuNPs (20 nm) Multidisperse	Protein kinase A,	AuNPs	Kinase activity based on the coagulating ability of a cationic substrate peptide and its phosphorylation	NR	[7g]
AuNPs (NR) Multidisperse	Acetylcholinesterase	Citrate-AuNPs	AChE-catalyzed hydrolysis of acetylthiocholine	0.6 mU mL ⁻¹	[45]
AuNPs (16 nm) AgNPs (16 nm) Monodisperse	Con A	Mannose AuNPs Mannose-AgNPs	Con A induced aggregation	Mannose -AuNPs LOD: 0.04 μM LDR: 0.04-0.10 μM Mannose-AgNPs: LDR: 0.08 – 0.26 μM LOD: 0.1μM	[2]

AuNPs (15 nm) AgNPs (20 nm) Monodisperse	Kaposi's sarcoma associated herpesvirus and Bartonella DNA	Oligonucleotides-AuNPs Oligonucleotides-AgNPs	Aggregation reaction with multi-color changes	LOD: 1 nM	[15]
AgNPs (10-15 nm) Multidisperse	BSA and immunoglobulins	AgNPs	Nanoparticle agglutination	LOD: 10 -80 $\mu\text{g mL}^{-1}$	[5b]
AuNPs (21 nm) Monodisperse	polyA, polyC, polyU, polyI, BSA, lysozyme, dsDNA,ssDNA	AuNPs	Aggregation due to self-assembly (discrimination and detection)	LOD: Protein ~100 pM Homopolynucleotide ~10 pM	[5i]
AuNPs (13.2 nm) Monodisperse	Dengue virus	AuNPs	PNA/DNA hybridization	LOD: 0.12 μM	[37]
AuNPs (~13 nm) Monodisperse	ssDNA and dsDNA	AuNPs	DNA sequences based on electrostatic interactions	AR: 100 fmol	[6c]
AuNPs (13 nm) Monodisperse	Abrin	Catalytic AuNPs	Peroxidase-like activity	LDR: 0.2 to 17.5 nM LOD: 0.05 nM	[46]
AuNPs (13 nm) Monodisperse	Lipase	Tween 20-GNPs	Enzyme-regulated AuNP aggregation	LOD: 0.028 mg mL^{-1} LDR: 0.15 to 1.80 mg mL^{-1}	[7p]
AuNPs (50 nm) Multidisperse	Native proteins	DNA-AuNPs	50% human urine	Cluster analysis	[5j]

433 NR: Not Reported; CIAP: Calf Intestine Alkaline Phosphatase; HSA-AuNPs : Human serum albumin-modified gold
434 nanoparticles, PKA: Protein Kinase A; SEB: Staphylococcal enterotoxin B; LOD: Limit of detection; AR: Activity Range;
435 PDGFs : Platelet-derived growth factors; PDGFR : platelet- derived growth factor receptors,, Con A: Concanavalin A; Man-
436 AuNPs: Mannopyranoside- encapsulated gold nanoparticles; Gal: β -galactosidase and Glc: β -glucosidase; ADA: Adenosine
437 Deaminase; AChE: Acetylcholinesterase, KSHV: Kaposi's sarcoma associated herpesvirus; BePI: benzo[e]pyridindole; CORA:
438 coralyne, BSA: Bovine serum albumin; PNA: peptide nucleic acid; LDR: linear dynamic range; LOD: limit of detection.

439
440 The development of DNA-aptamer-based colorimetric assays by Mirkin's group was inspired by
441 the fact that the steps necessary for NPs+ modification with ligands can be tedious or time-
442 consuming and relatively expensive^[6c, 18, 47]. NPs stabilized by ssDNA aptamers do not aggregate
443 with the addition of salt only ^[3e, 4c]. Conversely, in the presence of the target/analyte, the aptamer
444 is folded because it binds to the target while desorbing from the NPs surface, which leads to NPs
445 aggregation and colorimetric changes. Ideally, folded aptamers or dsDNAs should hardly adsorb
446 onto the NPs. This is related to the higher structure rigidity and high proton density inside

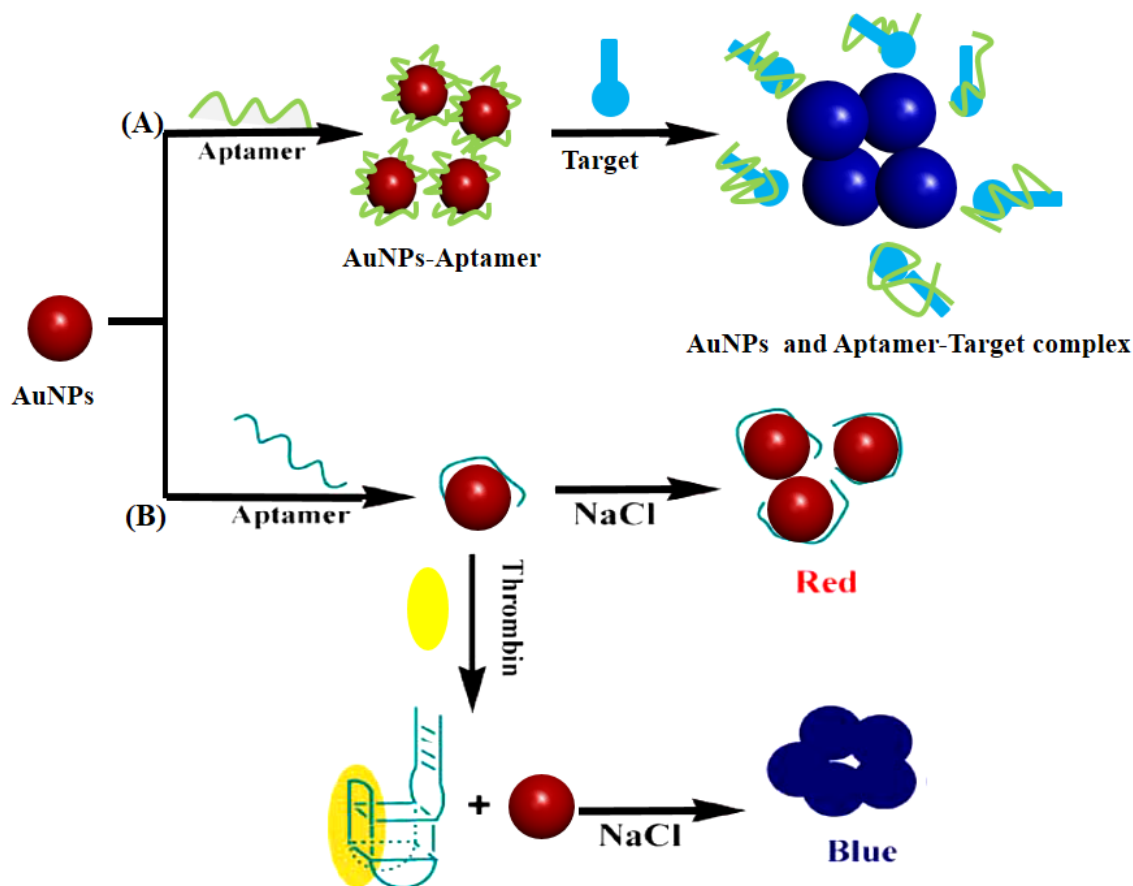
447 dsDNA [3e, 4g, 4x, 6e]. Indeed, ssDNAs cannot hybridize with each other to form dsDNAs. Thus,
448 their strategy is based on the observation that unmodified AuNPs and AgNPs can differentiate
449 between ssDNA and dsDNA, mainly due to the higher structure rigidity of the latter [3e, 4n, 6e].
450 However, not only the strands but also their lengths contribute to NP stabilization. For instance,
451 Chen and co-workers tested ssDNAs of different length (18nt: 5' TAG AAT ACT CCC
452 CCAGGT 3'); 24nt: 5' GGT TGG TCA GAT TCA GTG GGT TAG 3', and 30nt: 5' AAA CCC
453 CCC TGC TAAAAC CCC AAA CCC 3') for AuNP stabilization and consequently for detection
454 and sensitivity. They found that the longer ssDNAs have a better stabilization effect because at
455 the same molar concentration, longer ssDNAs have more monomeric deoxynucleotide units.
456 Moreover, it is difficult for cysteine to replace highly stable DNA-protected AuNPs [4e]. DNA
457 bases possess higher affinity towards gold than silver via coordination between Au and nitrogen
458 atoms (thus favoring DNA adsorption). However, the negatively charged surfaces of AuNPs
459 electrostatically repel DNA phosphate backbones (reducing DNA adsorption) [3e]. The key
460 challenge to their successful application is in transforming the aptamer-binding events into
461 physically detectable signals [4c].

462 The development of the nanotechnology for NPs functionalization with DNA and the
463 biotechnology for the *in vitro* selection of target-specific nucleic acids offer a unique opportunity
464 for designing colorimetric biosensors [7c, 17a]. The four types of DNA aptamer-based colorimetric
465 approaches are highlighted below:

466 5.1. TYPE I: Aptamers adsorbed on nanoparticles

467 Type I aptasensors includes two common steps: i) adsorption of the DNA unit onto the
468 NPs surface and ii) recognition of the target molecule by the DNA strands while serving as an
469 optical sensing element. DNA adsorption on NPs surface is favored by the high charge density
470 and stability provided by the aptamer [4f] (Fig. 10). This is a crucial step because the selectivity of
471 the targeting molecule must be retained during adsorption onto NPs, especially when the aptamer
472 is designed for qualitative assays [4f]. The aptamer conjugation constant is stronger than that of
473 antibodies and that of non-specific adsorption between aptamer and NPs [4q]. With these
474 conditions in mind, Xu and co-workers used unmodified DNA and AgNPs to detect ligands
475 binding to homoadenine, by monitoring the color change from yellow to brown due to AgNP
476 aggregation after salt addition. When coralyne binds to the homoadenine sequence in the
477 aptamer, the aptamer is removed from the AgNP surface and AgNP can aggregate [4f]. The

478 A_{550}/A_{397} ratio shows a good linear correlation with coralyne concentrations between 0.0 and 10
 479 mM with a LOD of 0.3 mM [4f]. Using a similar strategy, DNA-AuNPs/AgNPs were used to
 480 detect several targets, such as bisphenol A (LOD: 0.1 ng mL⁻¹ [4q] and LOD: 0.01 pg mL⁻¹ [4ab]),
 481 digoxin (LOD: 571 pM) [4aa], oxytetracycline (OTC) (LOD: 25 nM) [4g], thrombin, (LOD: 0.83
 482 nM) [5g], kanamycin (LOD: 25 nM) [4j], OTC (LOD: 0.1 nM), ampicillin (LOD: 5 ng mL⁻¹) [4i],
 483 staphylococcal enterotoxin B (LOD: 10 ng mL⁻¹) [4p], and pyruvic acid (LOD: 3.0 μM) [4w].



484
 485 **Fig. 10. Schematic representation of Type I colorimetric aptasensors for detection (A) small [4q, 4aa]**
 486 **and (b) large molecular targets [5g]**
 487

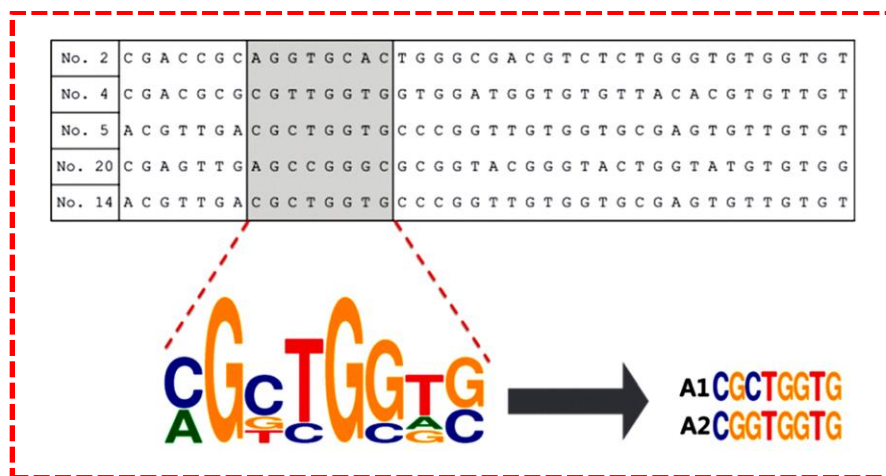
488 It is acknowledged that for many type I aptamers, addition of the target/ligand induces the
 489 aptamer release from the NP surface and consequently the color change as the salt tolerance
 490 decreased by NPs [4f, 4q, 4aa, 48]. Two groups reported an intriguing observation using a similar
 491 approach [4c, 49]. When aptamers are added to NPs, their interaction via hybridization leads to NP
 492 aggregation and consequently to the color change from red to blue. Addition of the target
 493 molecule (e.g., adenosine) to the aptamer-NP solution induces a dramatic conformational change

494 of the aptamer structure that leads to the dissociation of the NP network and to a new color
495 change (blue to red) ^[4c, 49].

496 AuNPs have also been used by Dong's group ^[5g] to understand the conformational
497 changes of thrombin-binding aptamers (TBA) when they are removed from the AuNP colloidal
498 solution in the presence or not of thrombin. Addition of 100 mL of 0.5 M NaCl causes a quick
499 color change (red to purple) in the solution with thrombin, but not in that with only TBA.. Due to
500 the color change, the TBA conformation modification from unfolded to G-quadruplex/duplex
501 formation could be directly monitored by naked eye, thus allowing the easy detection of
502 thrombin ^[5g]. Likewise, the addition of enough salt could be used to inhibit the repulsion
503 between unmodified negatively charged AuNPs and result in their aggregation and in the
504 corresponding red-to-blue color change. As previously reported, there is stronger coordination
505 interaction between the nitrogen atoms of unfolded ssDNA and AuNPs than electrostatic
506 repulsion between the negatively charged phosphate backbone and the negatively charged
507 AuNPs ^[5g]. Conversely, the relatively rigid structure of dsDNA or folded ssDNA (e.g., G-
508 quadruplexes) prevents the exposure of the DNA bases to AuNPs and the high density of
509 negative charges increases the repulsion between DNA and AuNPs. However aggregation of
510 DNA-functionalized AuNPs can be induced also by hybridization of target DNA that does not
511 cross-link the NPs. A conceivable disadvantage of this non-crosslinking system, compared with
512 the crosslinking system, is the consumption of target DNA ^[48].

513 Recently, a viable approach to overcome the limitations of type I aptamers due to the
514 DNA length was reported. Briefly, the design of shortened aptamers is mainly based on selecting
515 nucleotide bases characterized by high homogeneity in accordance with their conserved regions
516 ^[4i, 4v]. Shortened aptamers that contain common regions have approximately the same binding
517 affinity as the original. For instance, based on the conserved sequences with high homogeneity of
518 the original five 76-mer aptamers, A1 and A2 (8-mer sequences) were successfully obtained and
519 still exhibited high affinity and specificity for tetracycline (TC) ^[4v] (Fig. 11).

520



521
 522 **Fig. 11: Truncation process after analysis of the sequences of the original five 76-mer aptamers that**
 523 **bind to oxytetracycline, 20 to 8 mer ^[4v].**
 524

525 Although only the original stacking pocket and six additional specific bases are present in A1
 526 and A2, they display higher binding affinity (K_d 1.067 nM for TC). The LOD of A2 for
 527 oxytetracycline (OTC) was 0.1 nM, which is about 500-fold better than that of the original 76-
 528 mer aptamer, and the color change can be detected in the presence of 10 nM OTC ^[4v]. Similarly,
 529 based on their common sequence and predicted structure, Changill Ban's group ^[41] shortened
 530 three 90-mer ssDNA aptamers that specifically bind to ampicillin to obtain AMP4 (21-mer 5'-
 531 CACGGCATGGTGGGCGTCGTG-3'), AMP17 (19-mer 5'-GCGGGCGGTTGTATAGCGG-
 532 3'), and AMP18 (21-mer 5' TTAGTTGGGGTTCAGTTGG-3') ^[41]. Comparison of AMP17,
 533 AMP4 and AMP18 (at concentrations of 100 mM, 150 mM, and 200 mM, respectively) showed
 534 that ampicillin can be detected at concentrations as low as 5 ng mL⁻¹ using the AuNP-based dual
 535 fluorescence–colorimetric method and in a milk sample at 10 ng mL⁻¹ ^[41].

536
 537
 538 Thus, the results by Song *et al.* ^[4v] and Kwon *et al.* ^[41] illustrate and confirm that using aptamers
 539 harboring only the binding site/active site sequence can further improve their selective features.

540 Importantly, the target must not react or crosslink with NPs. Moreover, the ratio between
 541 NPs and aptamer could affect the final sensitivity. Too many aptamers in the sensing system
 542 reduce the sensitivity, while too few decrease the stability of the sensing systems ^[4q]. One of the
 543 primary challenges of Type I approaches relies on the different binding affinities of ssDNA and
 544 dsDNA towards unmodified NPs. However, the important feature is that negatively charged

545 ssDNA sequences can effectively stabilize NPs against salt-induced aggregation, providing a
546 convenient route for colorimetric assays without NP surface biomodification.

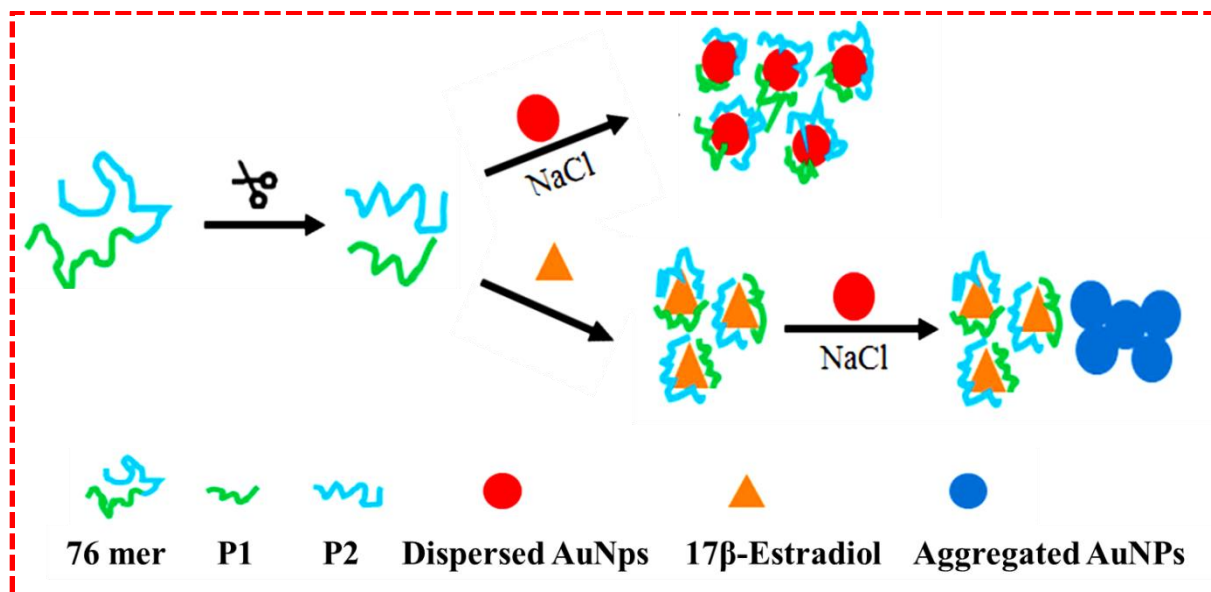
547

548 **5.2. TYPE II: Aptamer-target adsorbed on nanoparticles**

549 Type II aptamers also include two common steps: (i) aptamer linkage to the target
550 molecule to form a complex, and (ii) aptamer-target complex adsorption to the NP surface. With
551 Type II approaches, it is always wise to check the interaction of the pure aptamer with NPs
552 (Type I) because the system may follow a similar mechanism ^[4c, 50]. Ideally, aptamer adsorption
553 onto the NP surface should not lead to NP aggregation (and thus color change) after addition of
554 high salt concentration. In the presence of the target, the aptamers should bind in competition
555 with AuNPs, resulting in a color change in the presence of salts. A typical Type II system has
556 been used by Chen *et al.* for sulfadimethoxine (SDM) detection using unmodified AuNPs. In
557 optimal conditions (pH 8, 0.2 mM of aptamer and 2 M of salt), the LDR was 50 ng mL⁻¹ to 1 mg
558 mL⁻¹ and the LOD was 50 ng mL⁻¹ ^[4n].

559 Upon addition of SDM, the conformation of the SDM-binding aptamer changes from a random
560 coil structure to a more folded rigid structure that promotes the detachment of the adsorbed
561 aptamers from AuNPs and results in the subsequent AuNP aggregation after salt addition (Fig.
562 15). This leads to a color change from red to purple-blue that can be easily observed by naked
563 eye ^[4n]. Recently, Liu *et al.* assessed whether aptamer truncation could improve the sensitivity
564 also in Type II aptamers ^[4x] (Fig. 1).

565

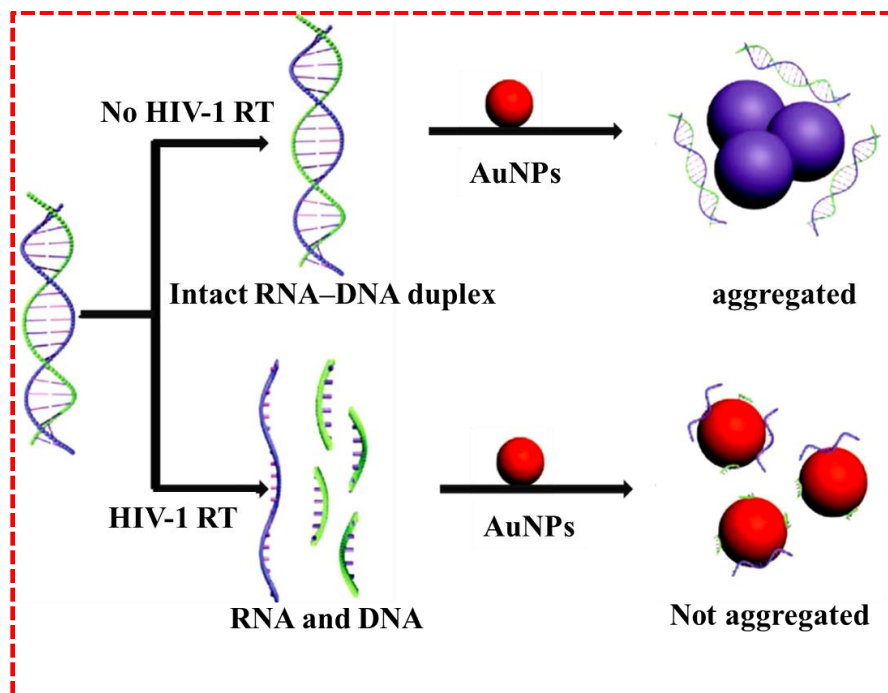


566

567 **Fig. 12. Schematic illustration of an AuNP-based colorimetric aptasensor to detect 17β-estradiol**
 568 **using split aptamers. Reproduced from [4x]. Split aptamers binds irreversibly to the target analyte**
 569

570 Briefly, the long (76-mer) aptamer specific for 17β-estradiol was split in two shorter sequences
 571 (P1 and P2) that still retain the original aptamer affinity and specificity, but with 10-fold higher
 572 LODs. Indeed, 17 β-estradiol could be detected with a LDR from 0.1 ng mL⁻¹ to 105 ng mL⁻¹ [4x].
 573 The authors hypothesized that this increased sensitivity is caused by the lower aptamer
 574 adsorption concentration and lower affinity for AuNPs of the shorter ssDNA sequences [4v, 4x].
 575 Likewise, Xie *et al.* developed an assay in which incubation of a RNA-DNA duplex with the
 576 HIV-1 reverse transcriptase (RT) leads to the production of ssDNAs and ssRNAs that can form a
 577 charged protecting layer on the AuNPs surface and consequently, to NP stabilization at a
 578 precisely defined salt concentration (Fig. 17). In the absence of RT, the selected RNA-DNA
 579 duplex remains intact, and the unprotected AuNPs aggregate in the presence of salt with a
 580 concomitant change in color [7o].

581



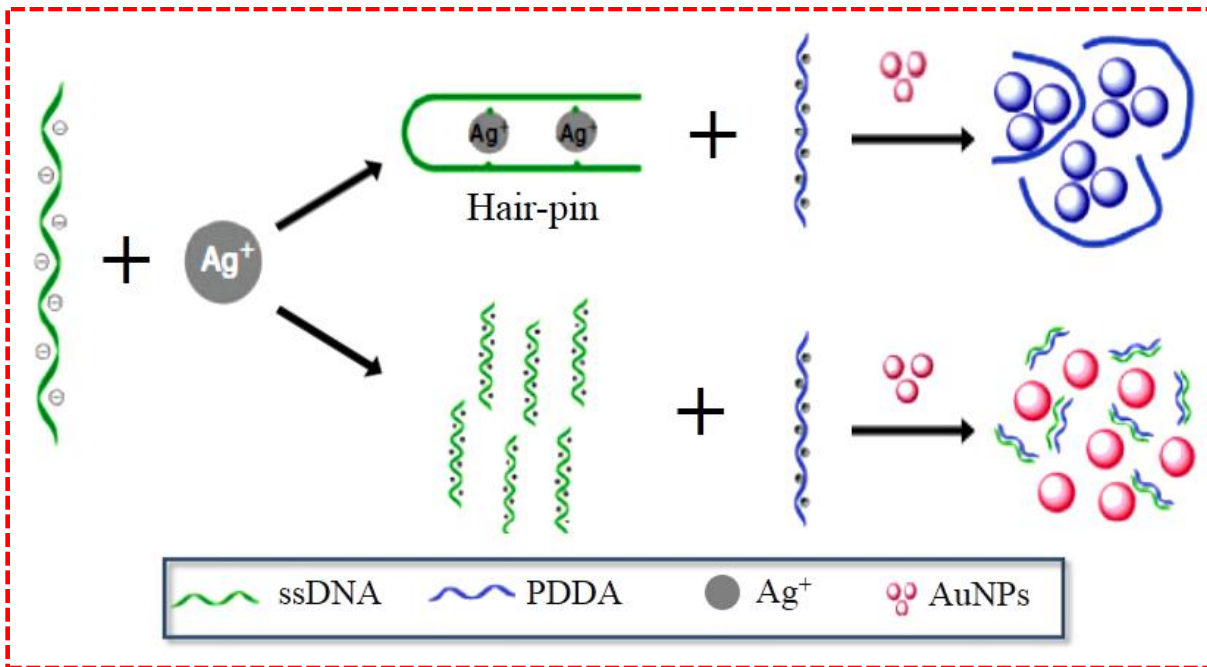
582

583 **Fig. 13. Schematic of the approach by Xie *et al.* A synthetic RNA–DNA duplex substrate is first**
 584 **incubated or not with HIV-1 RT. HIV-1 RT should cleave the RNA into fragments, resulting in the**
 585 **dissociation of ssDNA and ssRNA probes at room temperature ($\approx 28\text{ }^{\circ}\text{C}$). Reproduced with**
 586 **permission from [70]. GNPs, AuNPs.**
 587

588 Wang *et. al* [3e] used K^+ as a target because it stabilizes ssDNA, thus facilitating the formation of
 589 G-tetrads within 4 min. AuNPs incubated with G-tetrads changes color (red-to-purple) like
 590 unmodified AuNPs, suggesting that the G-tetrad structure is not significantly adsorbed onto
 591 AuNPs [3e]. The presented assay, which uses C-rich (5'-CCTCCCTCCTTTTCC ACCCACC-3')
 592 oligonucleotide aptamers, cationic polymers and AuNPs, provides a platform for the detection of
 593 other ions and molecules [3g] (Fig. 18). For instance, in the presence of Ag^+ , the two
 594 oligonucleotide form a tightly bound complex with a C- Ag^+ -C notation and change
 595 conformation, from a random coil to a hairpin structure with a stronger π - π^* transition of the
 596 bases with deoxyribose. The resulting C- Ag^+ -C complex poorly interacts with a cationic polymer
 597 known as Poly (diallyldimethylammonium chloride) (PDDA) and subsequently the polymer
 598 aggregates AuNPs through electrostatic interactions, with a color change from wine red to blue
 599 [3g]. In the absence of Ag^+ , the positively charged polymer can electrostatically interact with
 600 ssDNA and destroy the charge balance, leading to induction of AuNP aggregation (LOD of 48.6
 601 nM and LDR from 100 to 1000 nM for Ag^+). Together, the results by Wang *et al.* [3e, 3g] are in

602 agreement with the hypothesis that unstructured DNA oligonucleotides strongly adsorb onto the
603 NP surface and prevent salt-induced NP aggregation.

604
605



606
607 **Fig. 14. Schematic description of the colorimetric lead biosensor for Ag⁺ detection based on AuNP**
608 **aggregation induced by PDDA and Ag⁺ aptamers [3g].**

609
610 Recently, Zhang *et al.* [7p] demonstrated that AuNPs possess peroxidase-like activity that can
611 catalyze 3, 3, 5, 5-tetramethylbenzidine (TMB) in the presence of H₂O₂. AuNP peroxidase-like
612 activity can be improved by surface activation with target-specific aptamers. However, by
613 increasing the concentration of abrin (i.e., the target), AuNP peroxidase-like activity decreases
614 and the aptamer is desorbed from the AuNP surface, resulting in a decrease of AuNP catalytic
615 activity. The LDR for the current analytical system ranges from 0.2 nM to 17.5 nM with LOD of
616 0.05 nM [7p].

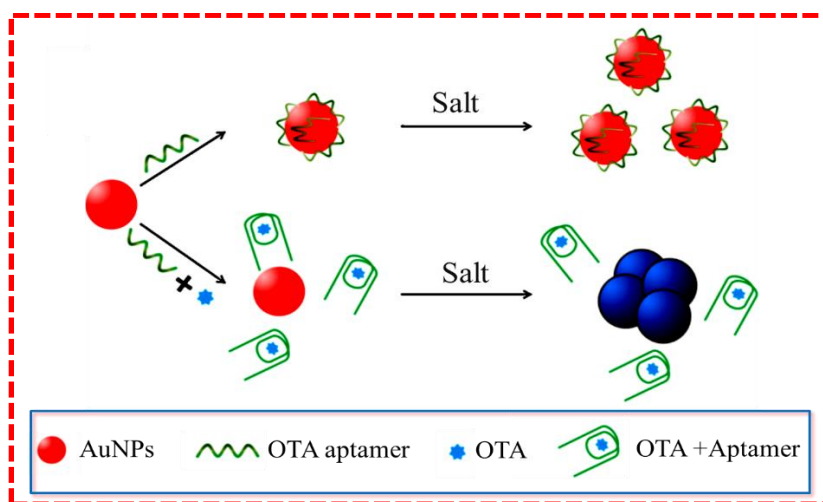
617 Comparison of Type I and Type II systems shows that aptamer-NP complexes are preferentially
618 formed in Type I and aptamer-target complexes in type II systems. It is reasonable to expect less
619 sensitivity from Type I systems because the colorimetric changes are related to the aptamer
620 detachment from the NP surface. The amount of aptamer removed will depend on the amount of
621 target. On the other hand, Type II systems are limited by the fact that the aptamer is expected to
622 retain its adsorption properties after complexation with the target. This suggests that if it is

623 folded during target binding, it should be flexible enough to facilitate colorimetric changes.
624 Overall it all depends on the aptamer capability because we would prefer induced aggregation in
625 the absence of the aptamer.

626 5.3. TYPE III: Competition in “One-pot detection systems”

627 Type III approaches are an intermediate between Type I and Type II systems because the
628 aptamer and the target (or targets) interacts in the presence of NPs, and therefore, this approach
629 is often referred to as “one-pot detection systems”. Sometimes, more than one class of aptamers
630 are used to stabilize NPs [4x]. Yang and co-workers [4k] described a “one-pot detection system”
631 for ochratoxin A (OTA) where phosphate buffered saline (PBS), Mg^{2+} , OTA and the aptamer are
632 mixed with AuNPs that can undergo salt-induced aggregation within 5 min [4k] (Fig. 15).

633



634

635 **Fig. 15. One-pot detection system of ochratoxin A (OTA). The target is bound to the aptamer and**
636 **upon salt addition, AuNP aggregation can be detected by the solution color change.**

637

638 Although the method is different, the authors hypothesized that “the duly formed G-quadruplex
639 structure could not protect AuNPs against salt-induced aggregation, and thus the color change
640 from red to blue could be observed by the naked eye”, as previously proposed by Wei *et al.* [5g]
641 and Wang *et al.* [3e] for Type I and Type II detection systems. Interestingly the LOD is 20 nM,
642 while the LDR from 20 to 625 nM [4k]. The major limitation of the Type III approach is that there
643 is more than one source of electrostatic interactions that could change the solution color. For
644 instance, if the metal is in excess, it will also contribute to the electrostatic interactions, and this

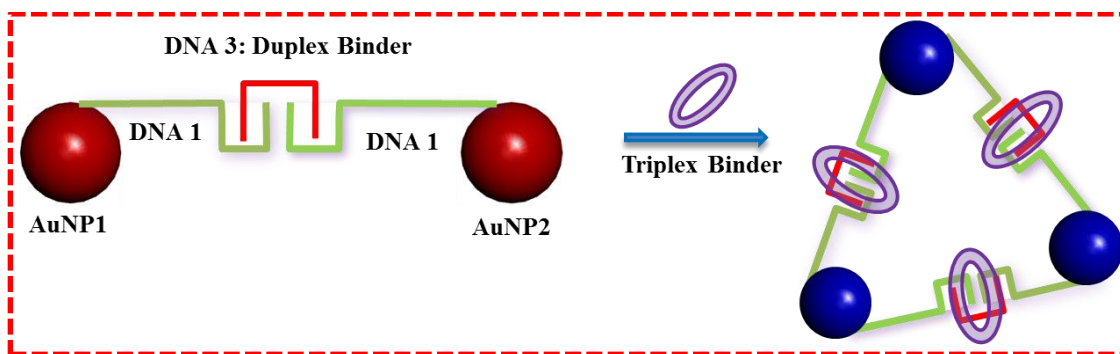
645 can only be prevented by having ssDNA in excess. Therefore selectivity is a major limiting
646 factor for this approach.

647

648 5.4. TYPE IV: Multiplex Aptasensors

649 In this section, the systems that use more than one aptamer based on the previously
650 described structure-switching strategies are described. In homogeneous multiplex aptasensors,
651 more than one class of aptamer is used to stabilize NPs for detection of single or several targets
652 [4x]. Several approaches are based on the likelihood that the target DNA molecules with one
653 nucleotide mismatch have different melting temperatures, and therefore they can be
654 distinguished by NPs disassociation based on temperature [6b, 6c, 6f, 7e, 17a, 50]. A typical example of
655 such a DNA sensor was reported by Mirkin and co-workers, in which the target DNA molecules
656 triggered AuNPs aggregation by hybridizing two complementary DNA strands on the AuNPs
657 [18].

658



659

660 **Fig. 16. Representation of the structure and color change of nano-assemblies in the presence of a**
661 **triplex binding agent at room temperature [42].**

662

663 Another assay includes AuNPs of different sizes (AuNP1 and AuNP2) that are functionalized
664 with non-complementary DNAs (3' or 5' pyrimidine-rich thiol-modified oligonucleotides) (Fig.
665 16). Functionalized AuNP1 and AuNP2 are then cross-linked with another complementary DNA
666 to form non-aggregating duplexes [42]. Introduction of a triplex binding agent induces triplex
667 formation through base hydrogen bonds and consequently, reversible NP aggregation that result
668 in a red-to-blue color change [42]. Analysis of the aggregate melting properties in terms of
669 cooperative binding theories suggests a lower DNA surface coverage on AgNPs functionalized
670 with 12 mer-thiolated homo-oligonucleotides containing only adenine (AgNPs/ST) than that on

671 AuNPs functionalized with 12mer-thiolated homo-oligonucleotides containing only thymine
672 (AuNPs/ST), while exhibiting changes that are significantly different from those of AuNPs upon
673 hybridization ^[51].

674 Interestingly, Sato *et al.* ^[48] demonstrated that ssDNA-AuNPs have different stability against
675 salt-induced aggregation in the presence of complementary DNA, although there is no triplex
676 binder ^[48]. Using a similar assay format, Zhao *et al.* configured oligonucleotide-modified AuNPs
677 duplexes with a short complementary oligonucleotide. Upon addition of adenosine (the target),
678 the aptamer switches its structure from a DNA duplex to an aptamer/target complex, because the
679 aptamer preferentially binds to the target molecule ^[7b]. Importantly, the aptamer on NP surfaces
680 must retain its switching capability ^[7b, 18, 42, 48]. Erickson's group ^[15] developed a multiplexed
681 one-pot detection system for Kaposi's sarcoma-associated herpesvirus (KSHV) and Bartonella
682 using both AuNPs and AgNPs. Specifically, when the Bartonella-targeted DNA (BA-DNA) is
683 introduced in the solution, AgNPs aggregate and the solution turns pink, more dependent on the
684 SRP characteristics of non-aggregated AuNPs. When KSHV-DNA was introduced, AuNP
685 aggregate and the solution changes to a murky yellow-orange color, more dependent on AgNP
686 aggregation ^[15]. The multi-color change tuning of AuNPs and AgNPs gave LODs down to 1 nM
687 and 2 nM, respectively ^[15].

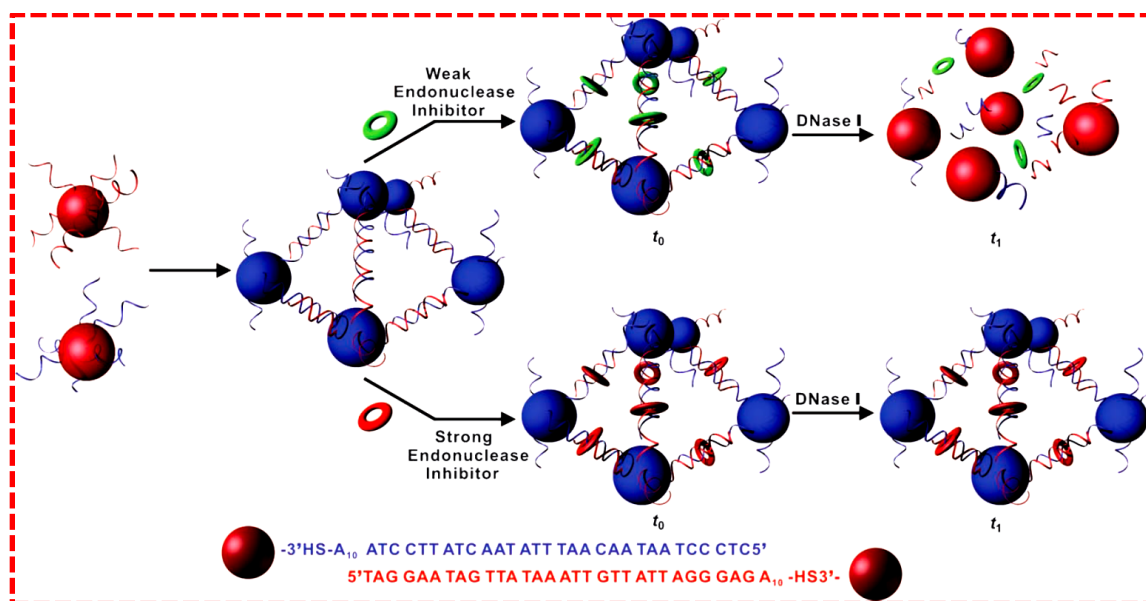
688 Niu *et al.* ^[4x] used more than one class of aptamers to stabilize AuNPs (Type I). Specifically, a
689 kanamycin-specific aptamer (750 nM), a sulfadimethoxine-specific aptamer (250 nM) and an
690 adenosine-specific aptamer (500 nM) are mixed (1:1:1 volume ratio) and adsorbed directly onto
691 the surface of unmodified AuNPs by electrostatic interaction. Upon addition of any of the three
692 targets, the conformation of the corresponding aptamer changes from a random coil structure to a
693 rigid folded structure that cannot adsorb and stabilize AuNPs ^[4x]. Although this looks more like a
694 type III system, more than one aptamer is present and the reaction does not proceed sequentially.

695 Ultimately, multiplex systems are not straight forward because multiplex detection
696 largely depends on the concentration of each aptamer and the buffer used for the aptamer
697 reaction with its target ^[4x]. Moreover, all the aptamers in solution can be adsorbed onto the NP
698 surface; however, the level of adsorption also depends on the neighboring aptamers. For this
699 reason, the use of aptamers with short sequences gives better adsorption yields. It has been
700 shown that changing the length of the ssDNA sequences yields different particle dispersion
701 profiles on unmodified AuNPs, and that short DNA sequences might improve the colloidal

702 stability against salt-induced aggregation [4l, 4v, 6e]. Most importantly, the aptamer on the NP
 703 surface must retain its switching capability and for this reason the switching capability of the
 704 structures with aptamers is a key factor that determine the LOD of the assay [7b].
 705 The multiplex type of aptamers has proven to be useful for enzyme activity and inhibitor assays.

706 5.5. Nanoparticle-based enzyme assays

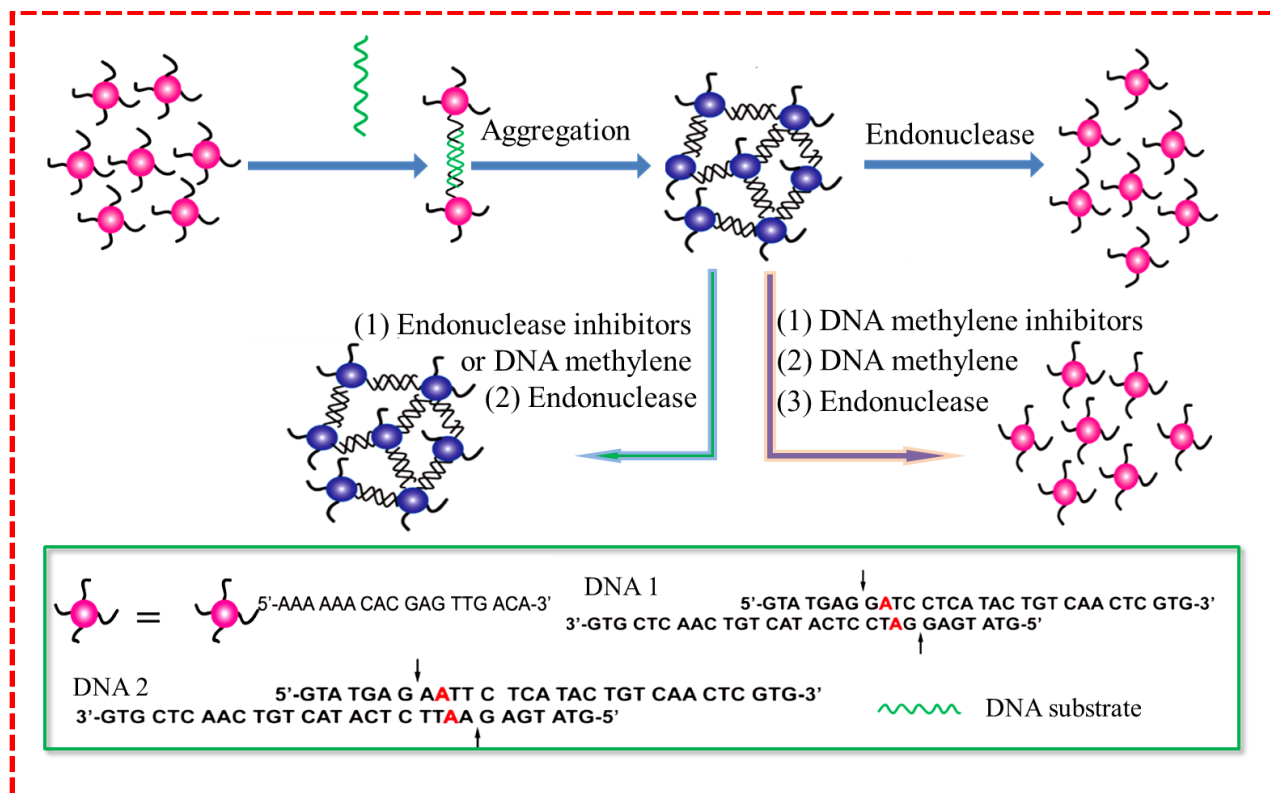
707 NPs can be used also to improve colorimetric assays of enzymes [52]. In these systems, the
 708 substrate for the target enzyme should also be a suitable NP stabilizer. For instance, it should be
 709 stable at high salt concentration (approximately 100 mM). Moreover, a charged molecule that
 710 can provide electrostatic and steric stabilization is likely to yield better selectivity. The strong
 711 interaction between amino groups and AuNPs surfaces has been well confirmed and the bond
 712 energy is comparable to that of a thiol–Au bond. The rapid aggregation induced by the non-
 713 crosslinking process is a useful approach for enzyme inhibition-based colorimetric screening, as
 714 shown by several studies using assays that rely on polymeric aggregates of DNA-functionalized
 715 AuNPs (DNA-AuNPs) with DNA-duplex interconnections [7e, 7j, 7k]. Mirkin’s group
 716 functionalized two separate batches of 13-nm AuNPs with two different thiol-modified
 717 oligonucleotide strands (DNA-1: 5’-CTCCCTAATAACAATTTATAACTATTCCTA-A10-SH-
 718 3’, and DNA-2: 5’-TAGGAATAGTTATAAATTGTTATTAGGGAG-A10- SH-3’) (blue and
 719 red ribbons, respectively, in Fig. 17) [7e].



720
 721 **Fig. 17. Aggregation and dissociation of the DNA-AuNPs probe used in the colorimetric screening**
 722 **of endonuclease inhibitors. The probe consists of spherical AuNPs functionalized with two**
 723 **complimentary oligonucleotides (blue and red ribbons). Individual NPs (red) aggregate into a cross-**

724 **linked network of NPs (blue) through hybridization of their oligonucleotide chains. Upon addition**
725 **of DNase I, the aggregates remain intact longer in the presence of a strong endonuclease inhibitor**
726 ^[7e].
727

728 The endonuclease (DNase I) degrades the DNA-duplex interconnections and NPs are released,
729 thus generating a red color ^[7e] (Fig 17). In the presence of inhibitors, the DNase 1 activity is
730 decreased and the aggregates are strongly hydrolyzed (T_H) . Consequently, the time required for
731 the color change is much longer. Most importantly, strong inhibitors (in contrast to weak
732 inhibitors) hinder DNase I activity to such an extent that the color change is no longer possible
733 ^[7e]. In their method, endonucleases cleaves dsDNA in the absence of inhibitors and cross-linked
734 AuNPs can separate into single AuNP molecules, as indicated by the instant color change, from
735 blue to red. With this approach, the inhibitor performance can be directly evaluated. Similar
736 observations were made using a system that includes a single type of DNA-AuNPs probe and an
737 appropriate oligonucleotide linker that can hybridize with the DNA probe. The linker was
738 designed to contain a self-complementary region that can form a duplex structure with a base-
739 pair overlap that contains the recognition sites and overhanging 3'-ends ^[7k] (Fig. 18). Significant
740 color change is observed when the endonuclease (DNA methyltransferase, DNA MTase)
741 degrades the DNA duplex ^[7k].
742

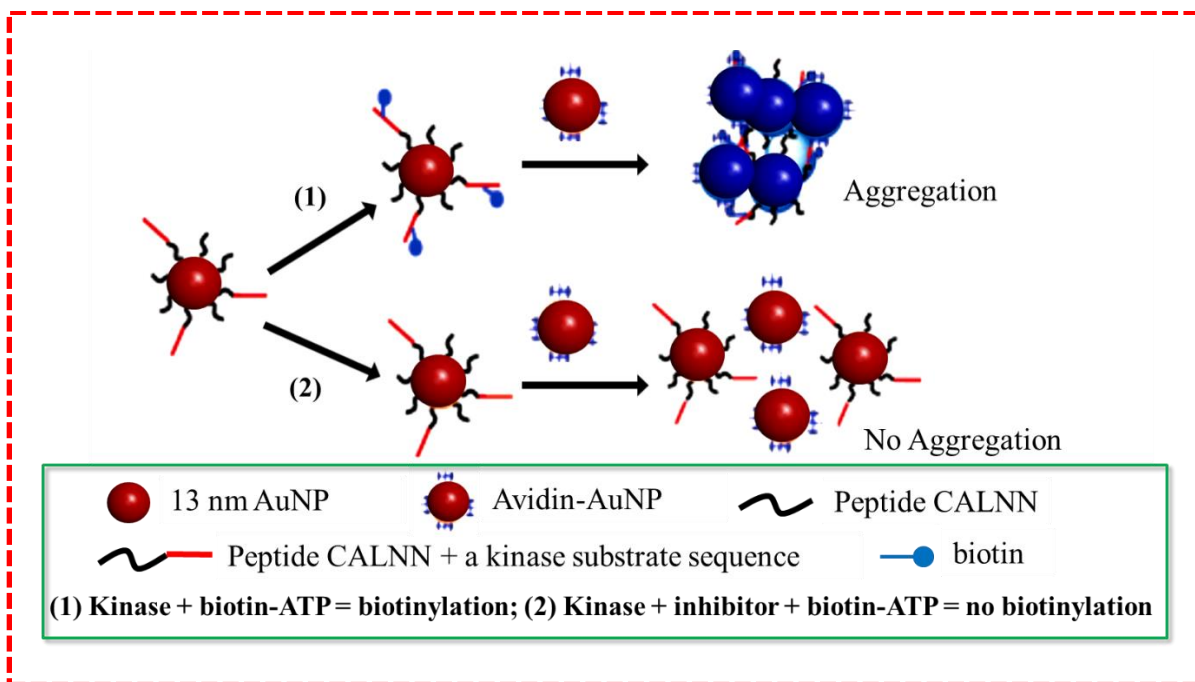


743
744 **Fig. 18. (A) Schematic representation of the assay to assess endonuclease and methyltransferase**
745 **activity and inhibition. (B) Sequences of the DNA probe, DNA-1 (recognition site for the**
746 **DpnII/Dam MTase) and DNA-2 (recognition site for the EcoRI/EcoRI MTase). The arrows show**
747 **the cleavage sites, and the red letters indicate the methylation sites** ^[7k].
748

749 Although highly selective and more sensitive than conventional methods, this visual inspection
750 assay is limited for the preparation of probes by functionalizing two separate AuNP batches with
751 two different thiol-modified oligonucleotide strands. On the other hand, this approach can be
752 used for most endonucleases by simply changing the recognition sequence in the linker DNA ^[7k].
753 For instance, similar assays were used for assessing adenosine triphosphate (ATP)
754 dephosphorylation by calf intestine alkaline phosphatase (CIAP) and peptide phosphorylation by
755 protein kinase A (PKA). ATP can protect AgNPs from salt-induced aggregation only in the
756 absence of enzymes. Phosphorylation and dephosphorylation can be readily detected by the color
757 change of AgNPs (CIAP LOD: 1 unit mL⁻¹, and PKA LOD: 0.022 unit mL⁻¹) ^[7h]. Zhao and co-
758 workers took advantage of the non-crosslinking AuNPs aggregation phenomenon to develop a
759 simple colorimetric assay for monitoring an enzymatic dephosphorylation reaction, where ATP
760 is converted into adenosine by CIAP ^[7a]. AuNPs capped by adenosine 5'-monophosphate
761 (AMP), adenosine 5'-diphosphate (ADP), or adenosine 5'-triphosphate (ATP) are progressively

762 more stable than bare AuNPs, but their stability gradually decreases (and thus the color of the
763 solution) with the dephosphorylation process ^[7a]. Likewise, Choi *et al.* ^[40] described an alkaline
764 phosphatase assay based on AuNPs aggregation ^[40]. To develop an adenosine deaminase assay,
765 Zhang and co-workers hypothesized that the interaction between adenosine amino group and
766 AuNPs surface will displace the weakly bound citrate ions from the AuNPs surface and diminish
767 the stability of citrate-capped AuNPs, resulting in the aggregation of AuNPs in the presence of
768 NaCl and a corresponding red to blue color change. Adenosine, guanosine and cytidine
769 (molecules that contain amino groups) strongly interact with AuNPs, causing aggregation.
770 Conversely, inosine, thymidine and uridine have negligible effects on AuNPs stability, therefore
771 the solution remains red because of the stronger electrostatic repulsion between negatively-
772 charged AuNPs ^[8]. Xinhui *et al.* described a suitable method for nucleases, such as the S1
773 nuclease ^[7j]. In the presence of nucleases and their substrates, unmodified AuNPs are stabilized
774 by dNMPs at high salt concentration and the solution remains red. Conversely, in the absence of
775 nucleases or substrates, the unmodified AuNP solution turns blue at high salt concentration due
776 to aggregate formation ^[7j].

777 Xu and co-workers developed a colorimetric assay to screen for inhibitors of several
778 kinases with the same type of NPs ^[7d] (Fig. 19). The method takes advantage of peptide-capped
779 NPs, in which 10% of peptide ligands carry an extension that is the substrate for a specific kinase
780 (PKA or calmodulin-dependent kinase II, CaM KII). Using γ -biotin-ATP as a co-substrate, the
781 kinase reaction results in substrate-AuNP biotinylation.



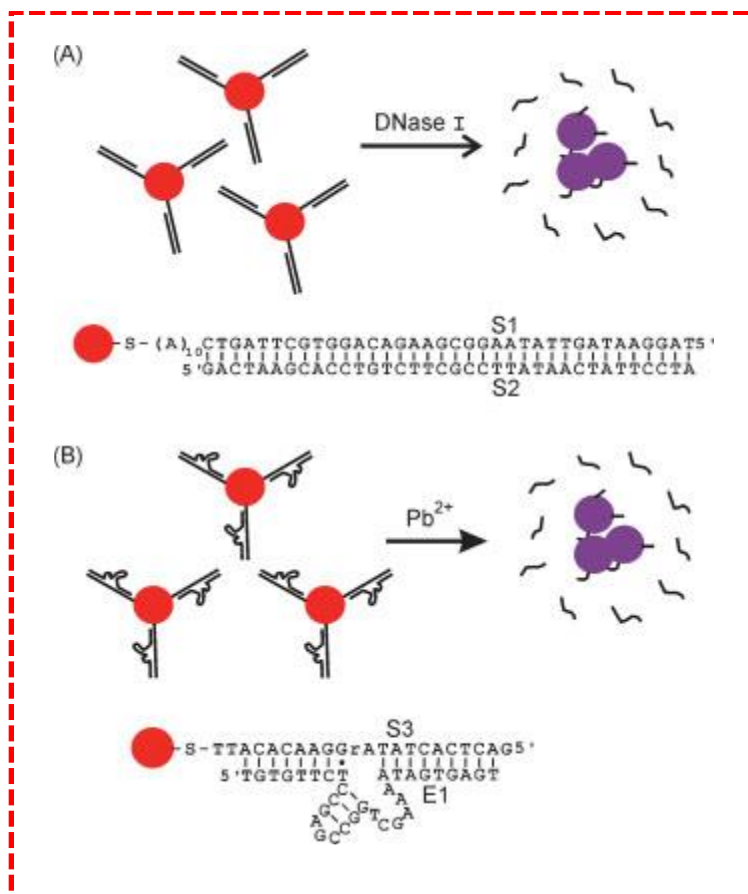
782

783 **Fig. 19. Schematic representation of phosphorylation/biotinylation of substrate-NPs followed by**
 784 **addition of avidin-modified NPs, in the presence and absence of a kinase inhibitor** ^[7d]
 785

786 When biotinylated substrate-AuNPs are mixed with avidin-AuNPs, they immediately aggregate
 787 due to the specific binding between avidin and biotin ^[7d]. Similarly, Wei *et al.* ^[7h] reported that in
 788 the absence of the kinase, or in the presence of an efficient inhibitor, no observable color change
 789 occurs after addition of avidin-modified NPs, and the solutions are indefinitely stable without
 790 showing signs of aggregation ^[7d]. Furthermore, a hydrolysis-based colorimetric assay for
 791 acetylcholinesterase (AChE) was developed based on the finding that AChE can catalyze
 792 acetylthiocholine hydrolysis into thiocholine ^[45]. AChE and acetylthiocholine are added in the
 793 AuNP solution, the generated thiocholine can take the place of citrate on the AuNP surface,
 794 promoting NP aggregation and a change of color from red to gray. Addition also of the AChE
 795 inhibitor tacrine (1,2,3,4-tetrahydroacridin-9-amine) leads to less AuNP aggregation and a
 796 slower color change ^[45]. Uehara *et al.* ^[43] reported that gold nanocomposites conjugated with a
 797 thermo-responsive copolymer can be used in a colorimetric assay to quantify the activity of
 798 aminopeptidase N (APN). By heating the solution, the assembled gold nanocomposites
 799 disassemble and the solution color change from blue, purple to red. This process is inhibited by
 800 cysteine, therefore the enzymatic decomposition of cysteinylglycine into cysteine and glycine by
 801 APN can be monitored ^[43].

802 Tiwari *et al.* hypothesized that particles could be used for the detection of the hydrolytic
803 activity of penicillin G acylase (PGA) on penicillin G. This hydrolysis reaction leads to a shift in
804 the surface plasmon band of AuNPs from 527 to 545 nm accompanied by a solution color change
805 from red to blue. The presence of 0.007 mg ml⁻¹ PGA can be detected [7m]. The enzyme is known
806 to hinder the salt-induced NP aggregation. Xie and co-workers found that DNA-RNA duplexes
807 cannot stabilize unmodified NPs at a certain salt concentration, a typical type II approach.
808 However, addition of the active HIV-1 RNase H enzyme leads to the specific cleavage of RNA
809 strands into RNA fragments and ssDNAs that can stabilize NPs against salt-induced aggregation
810 [7o]. In an assay for glycosidases based on self-immolative elimination to release amines,
811 functionalized trigger-AuNPs aggregate by electrostatic attraction upon cleavage of the trigger.
812 The assay gives LODs for β -galactosidase (Gal) and β -glucosidase (Glc) of 9.2 and 22.3 nM,
813 respectively, at 20 min, and they improve slightly over time [44]. The functionalized AuNPs (2.0
814 nM), which were capped with the enzyme substrate ligand Gal-Lip (or Glc-Lip) and lipoic acid
815 at a ratio of 1:1, showed a typical SPR peak at 521 nm (i.e., a red shift of 2 nm compared with
816 citrate-AuNPs) and good stability in PBS. The respective addition of Gal or Glc caused a time-
817 dependent decrease of the peak absorbance induced by NP aggregation that could be observed by
818 naked eye.

819 Two different systems (the DNA endonuclease DNase I and the Pb²⁺-dependent RNA-
820 cleaving DNA enzyme 8-17) were chosen to demonstrate the utility of an assay for the detection
821 of metal ions and enzyme activities based on rapid NP aggregation driven by van der Waals
822 attraction [7i] (Fig. 20).



823
 824 **Fig. 20. Schematic illustration of AuNP aggregation and color change triggered by the enzymatic**
 825 **cleavage of DNA on AuNPs. Before enzymatic cleavage, DNA-modified AuNPs are stable at a**
 826 **relatively high salt concentration, due to their electrostatic and steric stabilization. DNA removal**
 827 **from the AuNP surface by enzymatic cleavage destabilizes AuNPs and results in their rapid**
 828 **aggregation. A) Cleavage of a DNA duplex by DNase I. B) Pb²⁺-mediated cleavage of an RNA-**
 829 **containing DNA substrate by the 8-17 DNA enzyme [7i].**

830
 831 The authors reasoned that the removal from the NP surface of DNA strands, which serve as
 832 electrostatic and steric stabilizers at relatively high salt concentrations (e.g., 40 mM MgCl₂),
 833 should result in AuNPs destabilization and aggregation, a process driven by van der Waals
 834 attraction [7i]. Indeed, addition of 100 mM Pb²⁺ generated a rapid red-to-purple color change at
 835 room temperature [7i].

836 AuNPs generation induced by neurotransmitters can be used to analyze the activity of
 837 tyrosinase, an enzyme that catalyzes the O₂-induced hydroxylation of tyrosine to L-DOPA [4b].
 838 As tyrosinase concentration increases, the SPR bands of NPs are intensified and slightly blue-
 839 shifted, suggesting that larger particles are formed, and small Au nanoclusters enlarged [4b]. This
 840 system can be used for the sensitive detection of dopamine, L-DOPA and noradrenaline (LOD of

841 2.5 μM) and adrenaline (0.2 μM) that act as active reducing agents for Au-NP generation of Au-
842 NPs [4b].

843 Colorimetric aptasensors for the quantitative analysis of abrin using catalytic AuNPs
844 were reported for the first time by Zhang *et al.* and Wu *et al.* The AuNP peroxidase-like activity
845 can catalyze 3, 3', 5, 5'-tetramethylbenzidine (TMB) formation in the presence of H_2O_2 , leading to
846 a color change [4a, 7p]. Particularly, Song *et al.* [7k] quote the work by Mirkin's group [7e] and say
847 that the rest of the methods eliminate the binder use of different DNAs to obtain functionalized-
848 NP1 and functionalized-NP2. However, such method requires cumbersome preparation of
849 modified NPs and the formation of crosslinked NPs. Thus, it is not suitable for high-throughput
850 screening.

851 Crosslinking also offers a unique selectivity in reversibility of NP aggregation, where
852 analytes dissociate the cross-linker and re-disperse the NP aggregates to their original color. For
853 instance, Lu and co-workers described detection assays for Pb^{2+} , adenosine and cocaine where
854 DNA molecules with a single RNA linkage serve as cross-linkers that bring complementary
855 DNA-AuNPs into aggregates. The DNA enzyme catalyzes the specific hydrolytic cleavage of the
856 substrate strand that disrupts the NP assembly, changing the color from purple to red and thus
857 indicating the presence of Pb^{2+} [3a, 7c].

858 Zhao and co-workers speculated that if the substrate and product of an enzymatic reaction affect
859 differently AuNP stability by changing their electrophoretic properties, such a reaction can be
860 monitored colorimetrically and the enzymatic activity can therefore be determined [7a].

861

862 **6. Core-Shell Nanoparticles and Ratios**

863 Core-shell nanoparticles (CSNs) are a class of nanostructured materials that have
864 recently received increased attention owing to their interesting properties and broad range of
865 applications in catalysis, biology, materials chemistry and sensors. By rationally tuning the cores
866 as well as the shells of such materials (ratios), a range of core-shell nanoparticles can be
867 produced. In this review, the combination of gold and silver enhancement, an electron-dense
868 deposits that can be read by a simple colorimetric array workstation [6d]. However, Cao *et al.*
869 stated that AgNPs cannot be effectively passivated by alkylthiol-modified-oligonucleotides using
870 the protocols for modifying AuNPs, because they irreversibly aggregate when heated in a
871 solution with 0.05M NaCl, the concentration needed for DNA hybridization [17b]. For this reason,

872 an Au shell can be grown on AgNP, forming a particle with an Au outer surface ($3.1 \pm 0.6 \text{ \AA}$).
873 This surface can be easily modified with alkylthiol-oligonucleotides and indefinitely suspended
874 in high salt solutions. Ag/Au core-shell NPs retain the optical properties of the silver core, but
875 have optical properties different from pure gold NPs, thus providing another “color” option for
876 30-mer DNA target-directed colorimetric detection ^[6f, 17b].

877 By taking advantage of their reversible aggregation and melting nature, oligonucleotide-
878 modified Ag@SiO₂ nano-probes can be prepared by using 5' TCT-CAA-CTC-GTA-(CH₂)₇-NH₂
879 3' and non-complementary oligonucleotides with a 5' TAC-GAG-TTG-AGA-GAG-TGC-CCA-
880 CAT3' sequence in which no hybridization product was reported ^[50]. The high stability of oligo-
881 modified Ag@SiO₂ nano-probes at elevated temperatures (30-70°C) was confirmed by the
882 absence of peak shift and of broadening of well-dispersed nano-probes in their UV-Vis spectra
883 after long heating. The fast hybridization kinetics of the resulting Ag@SiO₂ nano-probes with
884 complementary target oligonucleotides render them very useful for fast colorimetric detection
885 based on the sequence-specific hybridization properties of DNA ^[50].

886 CSNs can also be used to introduce a second colorimetric change, distinct from the gold
887 system, for monitoring two different oligonucleotide targets in one sample ^[17b]. For instance,
888 when lactose-stabilized AuNPs are mixed with mannose (2-mercaptoethyl R-D-
889 mannopyranoside)-stabilized AgNPs ^[2], addition of concanavalin A (Con A) leads to the
890 aggregation only of mannose-stabilized AgNPs, thereby demonstrating the system selectivity.
891 Limited non-specific interaction occurs with lectin Con A, thereby enabling the subsequent
892 specific interaction with the lectin from *Ricinus Communis* Agglutinin (RCA120). Although
893 mannose-stabilized AgNPs show a longer LDR and faster reaction kinetics for the target lectin,
894 mannose-stabilized AuNPs provide the most sensitive bioassay ^[2].

895 Nevertheless, the synthesis of the CSNs can be a daunting task for quick assays ^[4f].

896

897 **7. Summary and outlook**

898 The detection of metals and molecules based on bare-eye observable colorimetric changes
899 depends on various parameters that are even more complex for macromolecules. The functional
900 groups on the NP surface are very influential in various areas of research, such as sensor arrays
901 and biosensor detection. Overall in any given condition, both thiols and nucleotides compete for
902 adsorption sites, and the equilibrium shifts towards thiols adsorption as the salt concentration

903 increases. Therefore, nucleotide adsorption could be completely eliminated after being ‘crowded
904 out’ (steric hindrance) as more thiols are adsorbed onto the metal. In one-pot detection approach
905 (where nanoparticles, analyte, DNA or enzyme are mixed together) qualitative analyses is
906 deemed imprecise because the color changes are not only due to the analyte, as they could be
907 triggered also by DNA or enzyme adsorption to the NP surface.

908 Also, the multiplex aptasensors have some limitations. For instance, it is not possible to
909 determine which target is detected when the sample gives a positive result (change of color).
910 While enzyme-assisted assays have a huge potential for many applications in biomedicine and
911 bio-imaging, the application of the described *in vivo* techniques faces formidable challenges.
912 Indeed, the interactions of NPs with enzyme molecules are not yet fully understood.

913 Through the review, there are viable approaches that have been introduced, Firstly, aptamer
914 truncation to overcome the limitations due to the DNA length, while maintaining the original
915 binding affinity. Secondly, overcoming the limitation imposed by the stability of AgNPs by
916 coating them with a thin layer of gold to produce a core–shell structure that retains the
917 spectrophotometric signature of the silver core. Thirdly, use of chemometric approaches provides
918 synergy in colorimetric discrimination or classification of small molecules and macromolecules.

919 Overall, the AuNP-based colorimetric aptasensors are currently used for many analytes, largely
920 because of the ease of detection, high sensitivity and potential for high-throughput analysis.
921 Moreover, the synergy between chemometrics and biotechnology selectivity narrows the gap for
922 the development of smart apta-based colorimetric sensing devices. In any case, careful
923 interpretation of the findings is critical because the colorimetric changes are not general or
924 universal, suggesting that they are highly dependent on the analytes and matrices.

925

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