



A universal label for the detection of PCR amplicons based on gold nanorods

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Abstract Owing to their optical versatility, gold nanorods represent an outstanding platform of plasmonic labels for a wide variety of photonic biosensors. Here, we address the feasibility of a universal system to label PCR amplicons with gold nanorods, based on their coupling to a nucleotidic tag of arbitrary choice and the use of primers modified with the complementary anti-tag sequence. This general concept is suitable for different formats of genosensors. For instance, when both the forward and the reverse primers are modified with the same anti-tag sequence, their daughter amplicons behave as homobifunctional cross-linkers for the polymerization of the gold nanorods and so trigger a specific discoloration of their suspension. This homogeneous approach seems

to be robust against a change of DNA target, but to require a careful choice of the hybridization buffer, and to exhibit a limited dynamic range in the order of 10 dB. Instead, when the amplicons are purified and immobilized on a solid substrate such as a paper strip, a single primer bearing the anti-tag sequence is enough to capture the gold nanorods and generate a visible stain that correlates with their concentration. This heterogeneous platform ensures a dynamic range exceeding 30 dB where it is suitable for quantitative readout, but it suffers from spurious interactions that may originate from the onset of denaturation of the spotted amplicons. Altogether, we found that the tested principles work as intended by design, but there may be practical limitations to their generic use in terms of the choice of both the analytical protocol and the nucleotidic tags, particularly in view of

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leveraging the optical versatility of gold nanorods in a multiplexable format.

Keywords Gold nanorods · Plasmonic labels · DNA amplicon detection · Particle aggregation test · Dot-blot assay · Photonic biosensors

Introduction

The so-called rapid antigen tests of various fingerprints of SARS-CoV-2 and COVID-19 [1–3] have brought gold nanoparticles into the spotlight as highly effective chromatographic labels. Owing to their bright color and antibody load, immuno-conjugated gold nanospheres ensure high sensitivity and specificity for their antigen target, while also being robust and inexpensive for large-scale deployment. However, at variance from other common labels such as polystyrene microspheres [4] or liposomes [5] stained with organic dyes, the resonance of gold nanospheres is bound to a small window, thus precluding future multicolor applications. As the size of the particles changes from a few to tens of nanometers, their color drifts a little from red to violet [6]. On the other hand, the last decades of lively research on the synthesis of morphological alternatives to gold nanospheres have laid a substantial groundwork to overcome this limitation [7–9]. Noble metal particles of many shapes such as rods, shells, cages, plates, and stars have been engineered to display all sorts of colors and enable cutting-edge applications based on multidimensionality and multiplexing.

In particular, so-called gold nanorods may represent an outstanding platform of multicolor labels for a broad variety of chromatographic sensors [10, 11]. Their asymmetry gives rise to an intense mode of longitudinal plasmonic oscillations that ranges between approximately 600 and 2000 nm, according to accessible variables such as their aspect ratio [12] or coating [13, 14]. There are a large number of well-established protocols for the synthesis of gold nanorods [15–17], and their bioconjugation to a plethora of units like small molecules [18, 19], antibodies [20, 21], and genetic probes [22, 23] for use in biosensors. The case of antibodies has reached such a level of readiness that current research focuses on fine parameters like the orientation of their Fab and

Fc fragments [24, 25] or the storability of the final batches [26].

The case of genetic probes has seen less progress. The approach of choice remains the method developed in the lab of Prof Chad Mirkin for gold nanospheres [27], which exploits the well-known thiol-Au chemistry within a procedure of salt aging that plays on a delicate balance of electrokinetic factors to combine colloidal stability and loading efficiency. However, at variance from gold nanospheres, by featuring quaternary ammonium compounds as a surfactant, the synthesis of nanorods yields a cationic rather than an anionic zeta potential, and it needs reversing before starting the addition of DNA. This step was the subject of specific investigation in the lab of Prof. Alexander Wei [28], who proposed a stepwise procedure for gradually replacing the cationic surfactant first with an anionic polymer and then with citrate, which is the main stabilizer of gold nanospheres. This procedure alone consumes at least 2 working days, and the same goes for salt aging according to Mirkin et al. [29], so that the full modification of each batch of gold nanorods with DNA becomes a week-long task. With such complexity and timing, the agile use of gold nanorods for bio applications, such as addressing contingent pools of genetic markers, becomes an unlikely scenario. Here, we describe a universal approach to make the same batch of gold nanorods responsive to any particular gene of interest, yet without compromising the eventual perspective to couple different particles to different targets.

This contribution aims to assess the working principles of a possible implementation of a universal label for plasmonic genosensing based on gold nanorods. In the recent past, we worked toward a similar solution for the immuno-conjugation of gold nanorods, and we proposed their modification with protein G in order to obtain a particulate that was suitable for storing at $-20\text{ }^{\circ}\text{C}$ and ready for thawing and binding to any antibody in few minutes [25]. Here, we cover the case of a nucleotidic target, and in particular, a double-stranded amplicon resulting from an ordinary run of end-point PCR. Compared to our previous work on immunoglobulins, instead of a generic DNA-binding ligand like Hoechst 33,342 or 33,258 and DAPI, we propose a scheme that may be multiplexed in future multicolor uses by utilizing complementary oligonucleotides: one immobilized as

a tag on the particles, and the other added as a modifier to the PCR primers.

As a main limitation, our current work relates to the detection of amplicons rather than a generic family of oligonucleotides. The detection of unamplified genes has become a hallmark of highest-end approaches to genosensing such as surface plasmon resonance (SPR) [30, 31] or even electrochemical methods [32]. However, for point-of-care applications, the integration of an amplification step is becoming an increasingly cost-effective solution, thanks to the rapid advancement of end-point PCR and its variants towards lower costs [33] and higher speeds [34]. Working with amplicons not only dramatically mitigates the requirements on the analytical platform in terms of sensitivity and specificity, but also operability in complex and real matrices. On the other hand, however, amplicon detection can be achieved through established technologies such as gel electrophoresis or real-time PCR, often in combination with probes such as ethidium bromide, SYBR Green, or specific markers designed for multiplexing such as TaqMan probes, which overall require a substantial degree of hardware and consumable complexity, cost, and management overhead. Our goal is to develop a system that works with the naked eye in combination with end-point PCR, without sacrificing its sustainability with the need for additional instrumentation and vulnerable or hazardous materials.

Our approach is similar, for instance, to the recent work by Joda et al. [35] or by Madhagi et al. [36], who developed standard sandwich assays with different reporters in electrochemical genosensors for the detection of amplicons originating from modified forward and reverse primers. Figure 1 illustrates the working principles of our system.

In practice, here, we couple a specific population of gold nanorods to a nucleotidic tag such as a poly-T strand, and we modify the 5' end of the desired primers with a stopper and the respective anti-tag sequence such as a poly-A moiety, in this example. This general scheme is suitable for multiple concepts of homo or heterogeneous assays made in a buffer or on a substrate and based for instance on the modification of the forward, reverse, or both primers. Here we focus on the functionality of a simple model of tag-anti-tag pair, we demonstrate practical examples of its use, and we discuss relevant perspectives and limitations. As an illustration, we show the implementation

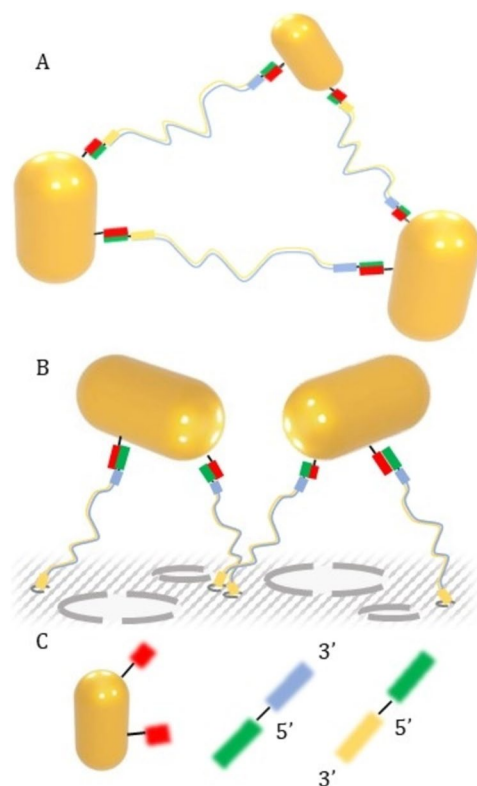


Fig. 1 Schemes of the setups for homogeneous (A) or heterogeneous (B) detection of amplicons obtained from a PCR reaction with the components shown in C, i.e., gold nanorods modified with poly-T (red) and forward (cyan) and reverse (yellow) primers both ending with a stopper and poly-A (green). In the homogeneous format, in theory, the working principle for discriminating between the amplicons and the unreacted primers rests on the hybridization between the filaments extended upon amplification. Instead, by design, notice that it requires a step of purification of double-stranded DNA in the heterogeneous setup

of the same universal label in complementary cases of genes of clinical (*mecA*) and agricultural (*rolC*) relevance.

The *mecA* gene encodes the penicillin-binding protein 2a (PBP2a), which shows a low affinity for β -lactam antibiotics, thus leading to resistance to the majority of this kind of antimicrobial agents, such as methicillin [37, 38]. In particular, *mecA* or PBP2a are considered fundamental targets for the detection of methicillin-resistant *Staphylococcus aureus* (MRSA) [39], which is a pathogenic bacterium responsible for many common surgical, cardiovascular, and lower respiratory tract infections. The *rolC* oncogene belongs to the family of *rol* genes of *Agrobacterium*

rhizogenes, which is a Gram-negative soil bacterium. It modulates cellular metabolism and differentiation and causes neoplastic growth in plants [40, 41]. Its expression also seems to correlate with the evolution and establishment of new botanical species [42, 43]. In both cases, the use of PCR is a typical step in the pipeline for the analysis of *mecA* and *rolC* genes in various applications.

Materials and methods

Materials

All chemicals for the synthesis of gold nanorods, cetyltrimethylammonium bromide (CTAB), tri-sodium citrate dihydrate ($\text{Na}^+_3\text{-citrate}$), Tween 20, tris(2-carboxyethyl)phosphine hydrochloride (TCEP), sodium chloride (NaCl), low EEO agarose, tris–borate EDTA (TBE) buffer, DirectLoad™ 50 bp DNA step ladder, 6×DNA loading buffer, Gel-Green® Nucleic Acid Stain, sodium dodecyl sulfate (SDS), phosphate-buffered saline (PBS), and all reagents needed for PCR amplification were purchased

from Merck (Merck KGaA, Germany). All unmodified and A_{18} -labelled PCR primers were obtained from Eurofins Genomics (Eurofins Scientific, Germany) and the T_{18} tag sequence from metabion (metabion international AG, Germany).

Synthesis and functionalization of gold nanorods

Cetrimonium-coated gold nanorods were prepared following the protocols described by Ye et al. [16]. Cetrimonium was then gradually replaced with citrate according to the procedure reported in our recent work [23].

Citrate-coated gold nanorods were bio-conjugated with a synthetic T_{18} oligonucleotide modified with a thiol group at its 3'-end (see Table 1), after treatment with 10 mM TCEP for 1 h at room temperature. The particles (0.8 mM Au) were incubated with the poly-T filament (about 4 μM) at 4 °C in 0.5 mM $\text{Na}^+_3\text{-citrate}$ and 0.005% Tween 20, with a slow addition of NaCl for 2 days until a concentration of 1 M. The suspensions were finally washed four times to remove any unbound oligonucleotide and reconstituted in a

Table 1 DNA sequences used as molecular probes and targets for the homo and heterogeneous systems and as PCR primers for *mecA* and *rolC* gene amplification

Name	Sequence 5' > 3'	Use
Poly-T tag	TTT TTT TTT TTT TTT TTT $(\text{CH}_2)_6\text{-SH}$	GNRs-probe, target
161-bp <i>mecA</i> sequence	CCT CTG CTC AAC AAG TTC CAG ATT ACA ACT TCA CCA GGT TCA ACT CAA AAA ATA TTA ACA GCA ATG ATT GGG TTA AAT AAC AAA ACA TTA GAC GAT AAA ACA AGT TAT AAA ATC GAT GGT AAA GGT TGG CAA AAA GAT AAA TCT TGG GGT GGT TAC AAC GT	Target
308-bp <i>rolC</i> sequence	ATG GCT GAA GAC GAC CTG TGT TCT CTC TTT TTC AAG CTC AAA GTG GAG GAT GTG ACA AGC AGC GAT GAG CTA GCT AGA CAC ATG AAG AAC GCC TCA AAT GAG CGT AAA CCC TTG ATC GAG CCG GGT GAG AAT CAA TCG ATG GAT ATT GAC GAA GAA GGA GGG TCG GTG GGC CAC GGG CTG CTG TAC CTC TAC GTC GAC TGC CCG ACG ATG ATG CTC TGC TTC TAT GGA GGG TCC TTG CCT TAC AAT TGG ATG CAA GGC GCA CTC CTC ACC AAC CTT CCC CCG TAC CAG CAT GAT GTG ACT CTC GAT GA	Target
<i>mecA</i> fw primer	CCT CTG CTC AAC AAG TTC CAG	PCR primer
<i>mecA</i> rev primer	ACG TTG TAA CCA CCC CAA GA	PCR primer
<i>mecA</i> anti-tag fw	AAA AAA AAA AAA AAA AAA $(\text{CH}_2)_{18}$ CCT CTG CTC AAC AAG TTC CAG	PCR primer, target
<i>mecA</i> anti-tag rev	AAA AAA AAA AAA AAA AAA $(\text{CH}_2)_{18}$ ACG TTG TAA CCA CCC CAA GA	PCR primer
<i>rolC</i> fw primer	ATG GCT GAA GAC GAC CTG TGT	PCR primer
<i>rolC</i> rev primer	TCA TCG AGA GTC ACA TCA TGC	PCR primer
<i>rolC</i> anti-tag fw	AAA AAA AAA AAA AAA AAA $(\text{CH}_2)_{18}$ ATG GCT GAA GAC GAC CTG TGT	PCR primer, target
<i>rolC</i> anti-tag rev	AAA AAA AAA AAA AAA AAA $(\text{CH}_2)_{18}$ TCA TCG AGA GTC ACA TCA TGC	PCR primer

ready-to-use hybridization buffer (DIG Easy Hyb™, Roche Diagnostics, Germany).

Preparation, amplification, and characterization of DNA targets

The cloning vectors used as templates for PCR amplification of the *mecA* and *rolC* genes were pUC57 (2710 bp, GenScript, USA) and pUC19 (2686 bp, New England BioLabs®, USA), respectively. In particular, the sequence deposited in GenBank with accession number KC243783.1, corresponding to the *mecA* gene coding for the PBP2a protein of *Staphylococcus aureus*, was used to design primers amplifying a discrete region of 161 bp (see Table 1). The amplified fragment was then cloned into the EcoRV site of the polylinker region of the pUC57 vector. A 1860-bp *Agrobacterium rhizogenes* (strain 1855) HindIII-EcoRI restriction fragment that encompassed both the promoter and the coding region of the *rolC* gene (ORF12, 308 bp) was cloned into the pUC19 polylinker restriction sites and ligated with T4 DNA ligase (New England BioLabs®, USA). The recombinant vectors were then transformed in *E. coli* One Shot Mach 1™-T1 (Thermo Fisher Scientific, USA) highly competent cells. Plasmids were extracted from the recombinant clones, checked by digestion with EcoRV and HindIII-EcoRI restriction enzymes, respectively, and then used as templates in PCR reactions.

In particular, templates (about 1 ng) were amplified by end-point PCR (T100™ Thermal Cycler, Bio-Rad Laboratories, USA) with the specific unmodified and A₁₈-tagged primers (200 nM, see Table 1) at an annealing temperature of 55 °C, in the presence of 0.8 mM dNTPs and 0.05 U/μl JumpStart™ Taq DNA Polymerase (Sigma-Aldrich®, Merck KGaA, Germany), for 45 cycles in several replicates.

The purification of the PCR products was performed by using MicroSpin columns (illustra™ GFX PCR DNA and gel band purification kit, GE Healthcare, UK), by eluting the samples with 10 mM Tris-HCl buffer (pH 8.0). Their concentration was finally quantified by a Qubit fluorometer (Invitrogen, Thermo Fisher Scientific, USA).

In order to verify the effective modification of the amplicons with the poly-A tails at the end of one or both strands, each sample was subjected to agarose gel electrophoresis (1.6% in 0.5×TBE buffer)

at 100 V for 90 min. The various DNA bands were detected with GelGreen® nucleic acid dye under a blue LED light transilluminator (MaestroGen Ultra-Slim LED Transilluminator, MaestroGen Inc., Taiwan), and their molecular weights were compared with each other against a 50 bp DNA marker (Direct-Load™ 50 bp DNA step ladder, Sigma-Aldrich®, Merck KGaA, Germany).

Homogeneous genosensor setup

A homogeneous genosensor assay was set up to observe the aggregation of the bioconjugated particles in solution in the presence of different nucleotidic filaments.

Experiments were carried out in a thermal cycler by incubating the T₁₈-tagged gold nanorods (1 or 2 mM Au) in DIG Easy Hyb™ buffer with decreasing concentrations (from 5 ppm to 0) of target amplicons modified with an A₁₈-tail at the 5'-end of one or both strands. The hybridization protocol consisted of a preliminary step at 53 °C for 5 min, which was useful to avoid potential dimers or any undesired folding of the oligonucleotides, followed by a gradual quenching (1 °C/min) until 23 °C to induce the correct base pairing. All samples were maintained at 23 °C for at least 1 h, and their changes in color and colloidal stability were recorded in the following hours.

In order to delve deeper into the dynamics involved in the color change, samples were also observed by spectrophotometry (BioSpectrometer basic from Eppendorf, Germany) and analyzed by dynamic light scattering (DLS, Zetasizer Nano ZS90 from Malvern, UK). These experiments were performed at least 48 h after the end of the heat treatment, in an attempt to compare the series of all samples under stationary conditions. Each experiment was repeated at least three times, and DLS scans were performed in triplicate.

Heterogeneous genosensor setup

A dot-blot assay was chosen to detect the hybridization between the bioconjugated gold nanorods and bare primers or purified amplicons immobilized on a solid substrate, as a representative model of heterogeneous system.

One microliter of each of several DNA samples were spotted onto nylon membranes and fixed

for 3 min under a UV-crosslinker (UVP HL-2000 HybriLinker™, Analytik Jena GmbH, Germany). The PCR products of the *mecA* and *rolC* genes were not thermally denatured before deposition. Decreasing concentrations (from 10 μ M to 0) of the T₁₈ sequence and the A₁₈-modified forward primers were used as negative and positive controls, respectively. After a pre-hybridization step, membranes were incubated at 25 °C with a fixed volume (3.5 ml/100 cm²) of suspensions of T₁₈-tagged gold nanorods at the nominal concentration of 600 μ M Au in DIG Easy Hyb™ buffer under gentle stirring in a hybridization oven for 24 h. Two low stringency washes in 2×saline-sodium citrate (SSC) solution with 0.1% sodium dodecyl sulfate (SDS) were finally performed for 5 min each at room temperature.

Also in this approach, each experiment was repeated at least three times.

Results

Modified particle and primer couples

As a model of tag-anti-tag pair, we chose a poly-T-poly-A couple. Specifically, the gold nanorods were modified with a poly-T tag strand, and the primers with its complementary poly-A anti-tag filament, which was interposed with an alkane cross-linker designed to stop the extension reaction at that position.

The upper panels of Fig. 2 display the electrophoretic runs of amplicons derived from *mecA* and *rolC* and obtained in a PCR reaction with standard primers as well as tagged forward and standard reverse primers or both tagged primers. The sharp bands demonstrate that the modified primers convey amplicons carrying 18 or 36 extra nucleotides as expected, i.e., genes with single-stranded poly-A moieties at one or both ends, respectively. Moreover, a comparison of band intensities suggests that primer modification does not compromise amplification efficiency. Similarly, the quantity of double-stranded material recovered after purification fluctuated between assays, but irrespective of the presence of poly-A.

The middle panel of Fig. 2 is a picture of suspensions of gold nanorods before and after immobilization with thiolated poly-T. The tagged gold nanorods displayed a consistent and bright red color after

modification and resuspension in a buffer containing as much as 1 M NaCl, which demonstrates their successful bioconjugation, by comparison with their citrate-capped precursors that undergo rapid and massive flocculation in the same saline buffer, as well as in common hybridization buffers such as DIG Easy Hyb™ [23]. As shown in Fig. 2C, the spectra of optical extinction confirm a certain sharpening of the plasmonic bands in the poly-T-modified sample compared to the original suspension. The transversal band is centered around 520 nm, while the longitudinal one peaks at 780 nm, in this case. Additionally, Fig. 2D depicts the morphology of the poly-T-modified gold nanorods used in this work, revealing an aspect ratio of approximately 4.

Homogeneous system

We first tested our platform in a homogeneous setup where the amplicons behave as a homobifunctional cross-linker for the specific polymerization of the gold nanorods, and thus, their presence alters the color of the colloidal system. In particular, we implemented a PCR reaction with both primers labeled with the poly-A tail, so that both ends of the resulting amplicons terminate with a ligand of the gold nanorods bearing the poly-T sequence and may trigger their aggregation below the melting temperature of the tag-anti-tag duplex (see Fig. 1A).

Figure 3 shows pictures of vials containing suspensions of tagged gold nanorods incubated with different concentrations of purified amplicons bearing the anti-tag moiety at one or both ends. In the cases of no (Fig. S2) or only one primer modified with the poly-A sequence, no visible interaction occurred as expected, because the resulting amplicons were inapt to cross-link the particles, thus suggesting that interactions between particles modified with a nonspecific oligonucleotide and a generic DNA strand are negligible. However, when both primers carried the poly-A tail, a clear change in the color of the colloidal suspension occurred, and it weakly depended on the concentration of the dosed amplicons. From lower to higher concentrations, the test tubes varied from red to purple, grey, and transparent, which is consistent with the onset of a process of polymerization, plasmonic coupling, and flocculative sedimentation of the gold nanorods. The lower panels of Fig. 3 illustrate these trends in

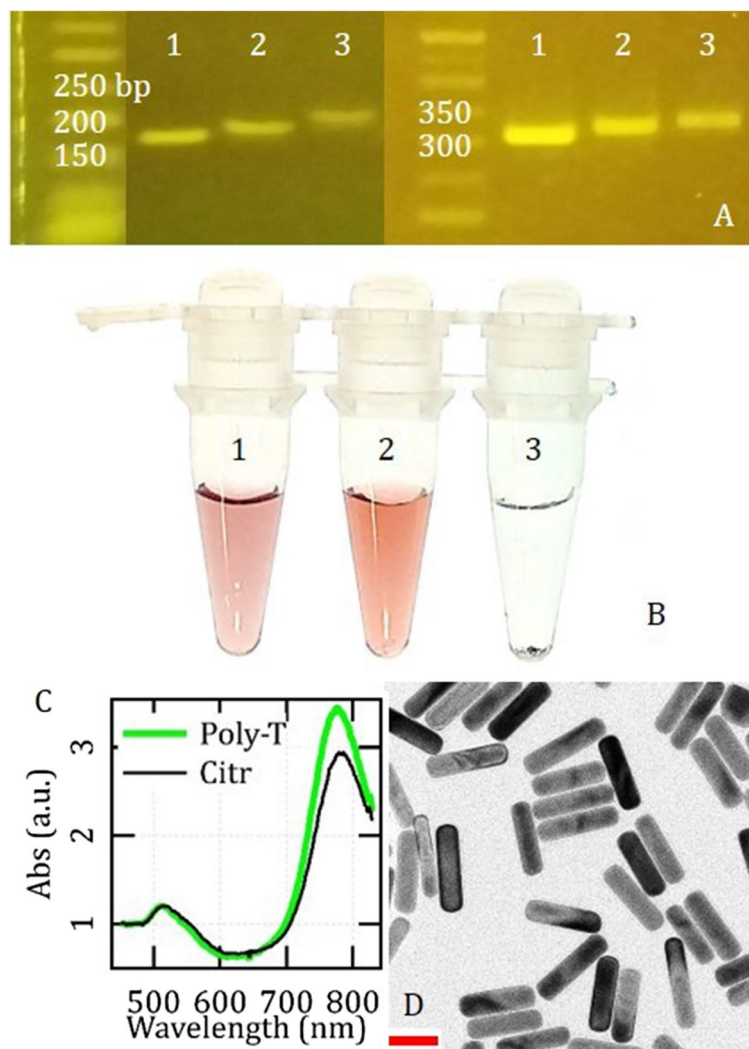


Fig. 2 **A** Amplicons of *mecA* (left) and *rolC* (right) obtained after 45 cycles of end-point PCR, with standard primers (1), or one (2) or both primers (3) modified with an anti-tag sequence made of a $(\text{CH}_2)_{18}$ (C_{18}) spacer and an A_{18} ligand (anti-tag-fw *mecA*: 5'- A_{18} - $[\text{C}_{18}]$ -CCTCTGCTCAACAAGTCCAG-3'; anti-tag-rev *mecA*: 5'- A_{18} - $[\text{C}_{18}]$ -ACGTTGTAACCAACCCCAA GA-3'; anti-tag-fw *rolC*: 5'- A_{18} - $[\text{C}_{18}]$ -ATGGCTGAAGAC GACCTGTGT-3'; anti-tag-rev *rolC*: 5'- A_{18} - $[\text{C}_{18}]$ -TCATCG AGAGTCACATCATGC-3'). The hydrogel contained 1.6% agarose in $0.5 \times$ Tris–Borate EDTA buffer. The electrophoretic run was done at 100 V for 90 min. The original images of the whole gels are reported in Supplementary Informa-

tion. **B** Picture of aqueous suspensions of as-synthesized gold nanorods (1), gold nanorods modified with a T_{18} tag and kept in a citrate buffer containing 1 M NaCl (2), and gold nanorods capped with citrate and incubated in the same buffer without oligonucleotides (3). All suspensions nominally contained 800 μM Au. **C** Spectra of optical extinction (BioSpectrometer basic from Eppendorf, Germany) of the same samples 1 (Citr) and 2 (poly-T) in **B**. **D** A 380 nm $\times$ 380 nm transmission electron micrograph (TEM Talos F200X STEM from Thermo Scientific, MA, USA) of gold nanorods modified with a T_{18} tag, transferred to ultrapure water, dried on a holey carbon TEM grid, and imaged at 200 kV (scale bar is 50 nm)

terms of color saturation. For each condition, the color saturation was assessed by sampling three different ROIs of 3×3 pixels within each vial and represented as mean and standard deviation. The thresholds seen with the naked eye were around

5 nM in the case of 161-bp *mecA* treated with 2 mM Au, and 2.5 nM for 308-bp *rolC* with 1 mM Au. The color change takes a few tens of minutes to develop and stabilize at the optimal hybridization temperature of 23 °C and then remains consistent

Fig. 3 **A** Suspensions of tagged gold nanorods at the nominal content of 2 mM Au incubated with different concentrations of purified amplicons of *mecA* with one (upper row) or both (lower row) ends modified with poly-A (DNA content was 50 (sample 1), 20 (2), 10 (3), 5 (4), or 2 (5) nM). **B** Same as **A** for a nominal content of 1 mM Au and amplicons of *rolC* (in this case, DNA content was 50 (1), 25 (2), 10 (3), 5 (4), 2.5 (5), 1 (6), or 0.5 (7) nM). All samples were incubated for 2 h at 23 °C in DIG Easy Hyb™ hybridization buffer after a gradual decrease of temperature from 53 °C in a thermocycler. **C** relates to an analysis of the lower rows of the above photographs in terms of color saturation plotted as a function of molar concentration of the different targets. Continuous lines are guides to the eye. Error bars refer to intra-assay variability

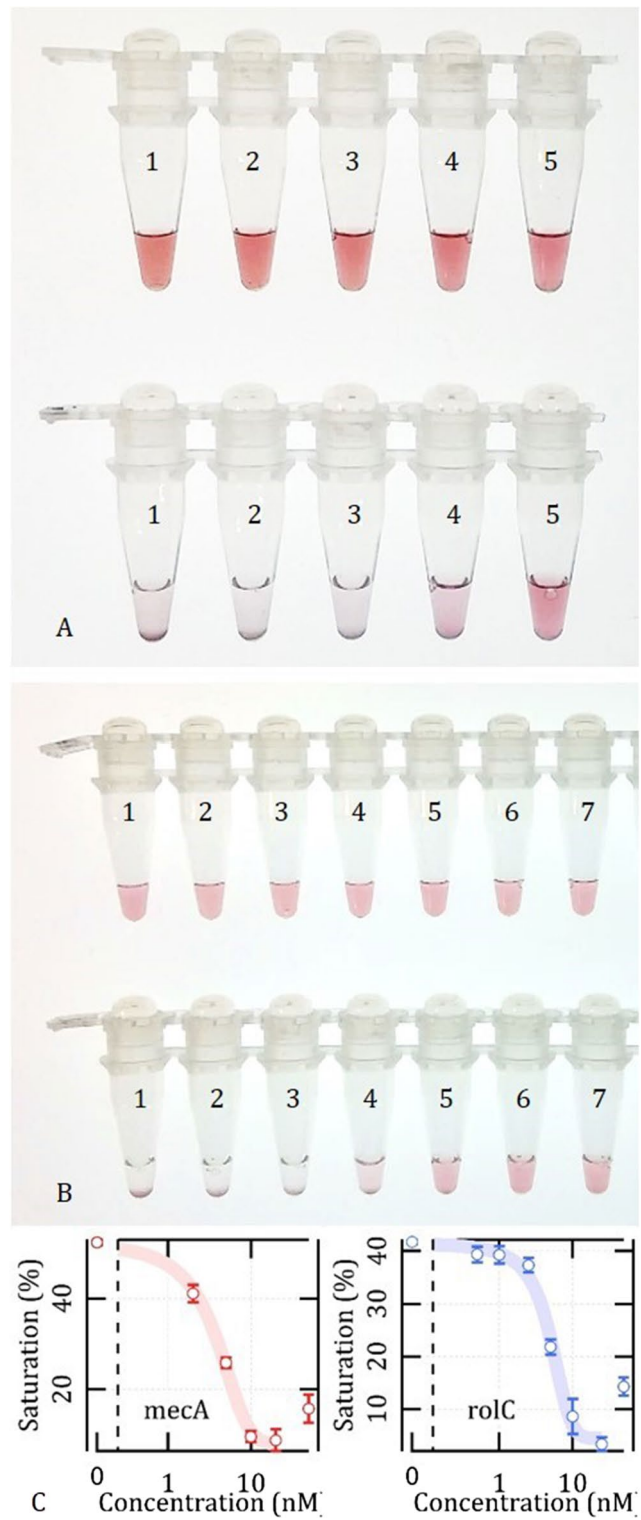
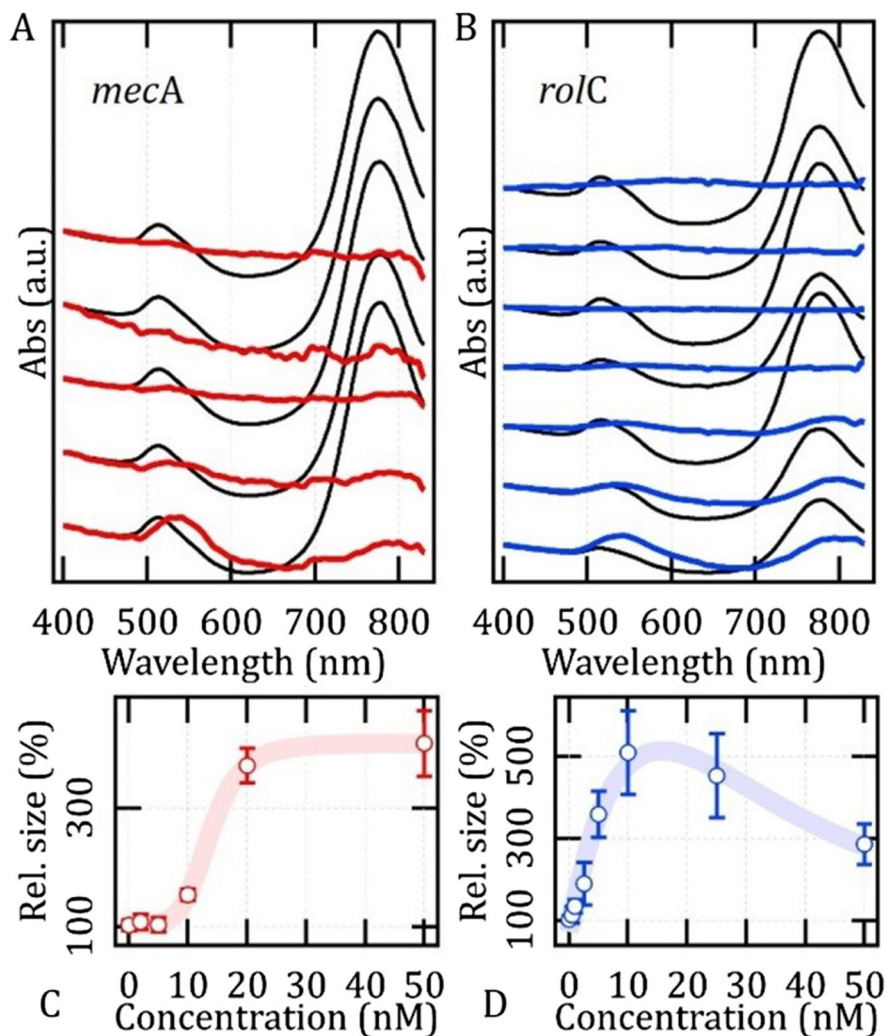


Fig. 4 **A** and **B** Spectra of optical extinction from suspensions of tagged gold nanorods at nominal Au contents of 2 mM (**A**) and 1 mM (**B**) incubated with different concentrations of purified *mecA* (**A**) and *rolC* (**B**) amplicons. One end (black lines) or both ends (red and blue lines) of the amplicons were modified with poly-A. DNA concentrations were 2, 5, 10, 20, and 50 nM for *mecA* and 0.5, 1, 2.5, 5, 10, 25, and 50 nM for *rolC* (bottom to top). Spectra were offset for clarity. **C** and **D** Hydrodynamic diameter of particles and aggregates at nominal Au contents of 2 mM (panel C) and 1 mM (panel D) treated with purified *mecA* (panel C) and *rolC* (panel D) amplicons, both ends modified with poly-A. Diameter is plotted as a function of target concentration. Continuous lines are guides to the eye. All samples were incubated for over 48 h in DIG Easy Hyb™ hybridization buffer before analysis. Error bars in **C** and **D** represent the standard deviation of three consecutive scans



for at least several hours or even days (see Supplementary information for details).

The upper panels of Fig. 4 confirm that particles incubated with amplicons containing a single poly-A sequence retain their characteristic transversal and longitudinal bands of plasmonic oscillations at approximately 520 and 780 nm, regardless of target concentration. In contrast, the use of both tagged primers red-shifts and then suppresses the visible feature of 2 or 1 mM Au above 5 nM *mecA* or 2.5 nM *rolC*, respectively. Notably, the NIR feature displays even greater sensitivity to low target concentrations than the visible one, further emphasizing the unique advantages of gold nanorods. The lower panels of Fig. 4 reveal that the loss of plasmonic features coincides with a sharp increase in hydrodynamic size,

which partially recovers at higher target concentrations, especially for 1 mM Au treated with *rolC*.

Heterogeneous system

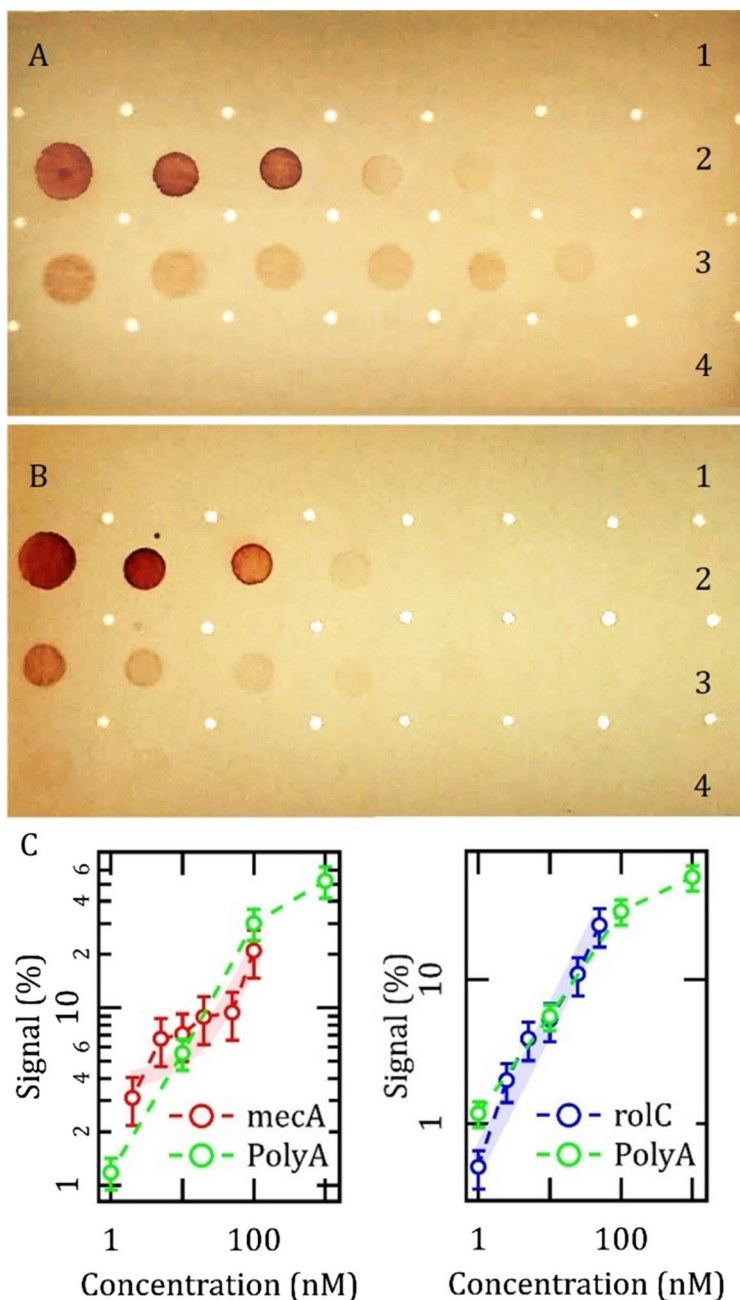
Another approach of special relevance in the context of biosensing with plasmonic particles is a heterogeneous setup where the genetic target mediates their interaction with a solid substrate, whether it be a paper-based strip for a dot-blot or a lateral-flow assay [44], or a chip for SPR [30, 31], surface enhanced Raman scattering [45], plasmon enhanced fluorescence [46], etc. This general scheme is also multipliable in many variants of direct or competitive assays. Here we tested the simplest case of a direct dot-blot model made by spotting the amplicons obtained from

one primer only modified with the anti-tag sequence, and then staining with the tagged gold nanorods (see Fig. 1B).

Figure 5 displays representative pictures of test strips spotted with different concentrations of poly-T (1) ($T_{18}-(CH_2)_6-SH$), poly-A ($A_{18}-(CH_2)_{18}$ -fw primer), and amplicons ending with poly-A or without modification and incubated with buffers containing gold

nanorods labeled with poly-T. All photographs were taken under nominally identical conditions, by using a regular smartphone and a light board in an otherwise dark room. A quantifiable signal was worked out from these pictures as the variation of image color from spot to background, and it is displayed in the lower panels of Fig. 5 as a function of the molar concentration of the different targets, together with

Fig. 5 Pictures of test strips spotted with different concentrations of poly-T (1) or poly-A (2) (10, 1, 0.1, 0.01, 0.001, and 0 μM from left to right) or decreasing concentrations of amplicons with one (3) or no (4) end modified with poly-A. **A** relates to *mecA* (100, 50, 20, 10, 5, 2, and 0 nM) and **B** to *rolC* (50, 25, 10, 5, 2.5, 1 and 0 nM). In both cases, the test strips were incubated with tagged gold nanorods suspended in DIG Easy Hyb™ hybridization buffer at a nominal content of 600 μM Au for 24 h at 25 °C and treated with only two low-stringency post-hybridization washes with 2 $\times$ SSC and 0.1% SDS for 5 min. **C** relates to an analysis of the above photographs in terms of a signal defined as relative distance in RGB space from spot to background and plotted as a function of molar concentration of the different targets. Error bars refer to intra-assay variability



relevant regression lines. For each condition, the color variation was assessed by sampling 20 different ROIs of 3×3 pixels inside and outside the spot, to estimate the intra-assay variability. The interaction between the bioconjugated gold nanorods and their same poly-T strand was negligible, whereas that with their complementary poly-A sequence was consistent from strip to strip, and depended on its concentration. In particular, visible signals were detectable by the naked eye until a concentration around 1 nM poly-A even without any protocol of enhancement with silver or gold [47, 48]. The intensity of the stain consistently increased with concentration until at least 1 μ M poly-A.

Dosing increasing amounts of amplicons bearing a poly-A chain also produced spots of growing visibility. The detection limits for *mecA* (2 nM) and *rolC* (2.5 nM) were comparable with those found with the homogeneous system. Instead, the case of amplicons without the anti-tag tail resulted in no or weak signals apparently depending on their particular nucleotidic composition.

Discussion

There are sporadic previous reports on genosensors based on coupling a universal reporter and a target-specific probe through respective complementary modifiers [5, 49], essentially as an analogue of a secondary antibody-detection antibody pair in an indirect ELISA assay. Also, the implementation of PCR primers modified with an oligonucleotidic probe for affinity capture is relatively uncommon with respect to other ligands, such as small molecules like biotin [50–53], digoxigenin [52, 53], or dinitrophenol [54] for use in pairs with avidin or respective antibodies. The case of a reporter of customizable color coupled to a PCR primer through a tag of customizable sequence is a prime novelty of our work, which conveys a universal probe that is also inherently relevant for future multicolor applications. Detecting different cases of amplified fragments from plasmid DNA is another point of topical significance. In this study, we investigated the fundamental operating principles of our system through a series of simple homo- and heterogeneous assays. These experiments aimed to verify the feasibility of naked-eye detection of amplicons with minimal additional resources and

instrumentation compared to conventional end-point PCR. Our goal was to identify key opportunities and potential limitations.

Starting from the experimental design, it may appear that some steps in our protocols unnecessarily limit their translational relevance. In particular, before evaluation, we extracted the amplicons in Tris–HCl buffer, at the cost of bottlenecking the entire workflow and preventing the analysis of the PCR mix as is, e.g., for the perspective of real-time use during reaction. In our basic model of heterogeneous assay, this preliminary step is the effective means to specificity, but we found that the purification of the amplicons and their redispersion in an appropriate buffer is important even in the homogeneous case. Various factors may concur with this need. A clear issue is the excess monofunctional primers left free to compete with the homobifunctional amplicons in the interaction with the tagged particles. We tested ways to optimize the consumption of primers but made no substantial progress (data not shown). In order to solve a similar issue, Zou et al. implemented folded anti-tags that became accessible only after the formation of the amplicons [55]. Although the same approach may also be adopted in our case, there remain concomitant factors that require the extraction of amplicons from the reaction mixture, such as optimizing the hybridization buffer. In particular, we will next demonstrate that the standard PCR mix performs poorly as a buffer for the polymerization of the gold nanorods. Other barriers to real-time use of the system include general concerns on the colloidal and morphological stability of the gold nanorods [23], and their potential to interfere with the biochemical process according to their shape and coating [56, 57]. For these reasons, we believe that introducing the tagged gold nanorods downstream of a standard step of purification and transferring them to a dedicated buffer may remain the best approach to a generic genosensor.

Apart from this limitation in scope, we obtained excellent results on the colloidal stability of the oligonucleotide-tagged gold nanorods [58] and a satisfactory response from the overall functioning of the analytical platform, with the homo- and heterogeneous formats revealing their own opportunities and pitfalls.

We found that the analytical sensitivity of the homogeneous scheme is about the same as the heterogeneous one, and it falls in the order of the density of gold nanorods, which is between 1 to 10 nM

for a concentration of Au of 1 or 2 mM. A similar conclusion was drawn in the case of gold nanospheres in a comparable platform [59] and agrees with an intuitive picture of the process of polymerization (see Fig. 1A). In the case of gold nanospheres and a similar hybridization scheme, Reynolds et al. observed that the use of fewer and larger particles may help enhance the sensitivity of the system without compromising too much on optical density [60]. At the highest concentrations of the amplicons, we observed a slight delay in the process of polymerization probably due to an effect of steric hindrance and saturation of the tagged particles, also in agreement with previous reports [61]. Particularly in the case of *rolC*, this effect appears as a decrease in hydrodynamic size at higher target concentrations. In practice, the working range of the genosensor may be between about one and ten times the density of the gold nanorods, or around 10 dB. In terms of lag time, it takes almost an hour for a visible response to develop at room temperature, which is another barrier against the feasibility of this system for real-time PCR monitoring. Thereafter, it remains fairly stable for at least a few hours or even days, thus leaving room for perspectives of quantitative exploitation. In this regard, we observed that spectrophotometric monitoring of the transversal band of the gold nanorods may serve as an especially sensitive and promising marker. When we tested different genes like *mecA* and *rolC*, we found a substantial consistency. The choice of assay buffer is critical. To assess the direct feasibility of the assay within the standard PCR mix, we evaluated a range of commercially available buffers optimized for DNA amplification, specifically solutions from Sigma-Aldrich and BioChain (Fig. S4). Additionally, we tested various common DNA hybridization buffers. Among the tested systems, only DIG Easy Hyb™ buffer, a proprietary formulation, yielded satisfactory and consistent results. It is well established that factors that may foster the process of particle polymerization include buffer ionic strength [62, 63] and the presence of additives like SDS, which may mitigate the electrostatic repulsion between the anionic tagged particles and reduce the incidence of non-specific binding [64]. While the exact composition of DIG Easy Hyb™ buffer remains undisclosed, our experimental characterization suggests the presence of surfactants and an

ionic strength comparable to other common hybridization buffers (see Supplementary Information for details).

With respect to the homogeneous case, the heterogeneous setup promises an easier and wider range of quantitative readout [65, 66], as it depends less on the stoichiometric ratio between particles and amplicons. Indeed, according to Fig. 5, its dynamic range of signal variation proved to exceed a factor of a thousand or 30 dB, with a model target of poly-A. However, the linearity of its response results from a greater complexity of factors, which may require a more careful design than a plain direct assay. In the cases of both 100 nM *mecA* and 50 nM *rolC*, we found that the signal from the tagged amplicons falls somewhere between that from 10 and 100 nM anti-tag sequence alone. However, the degree of correlation between blot intensity and concentration varies from target to target. In the representative cases shown in Fig. 5, we estimated a color distance from the background in RGB space, which changed from about 21 to 3.1% when the concentration of tagged *mecA* decreased from 100 to 2 nM, and from about 24 to 0.5% when that of tagged *rolC* went from 50 to 1 nM. The corresponding slopes and Pearson r coefficients were $(0.160 \pm 0.015)\% \text{nM}^{-1}$ and 0.95 ± 0.15 for *mecA* and $(0.52 \pm 0.03)\% \text{nM}^{-1}$ and 0.996 ± 0.042 for *rolC*. While specific fit parameters varied between assays, *rolC* consistently demonstrated superior sensitivity and linearity compared to *mecA* across identical concentration ranges and tag systems. The underlying reasons for this discrepancy remain elusive and may involve a complex interplay of steric or electrokinetic factors. When employing untagged primers, we observed significant differences in non-specific interactions. In contrast to *mecA*, *rolC* exhibited a propensity for non-specific binding at elevated concentrations. In the case illustrated in Fig. 5, at a *rolC* concentration of 50 nM, non-specific color change reached approximately 4.6%, equivalent to the specific signal generated by 10% or more of the tagged amplicon. This effect may arise from cross-reactivity between the poly-T chain and unintended denatured *rolC* fragments, which contain multiple adenine-rich regions, such as the 11-adenine sequence spanning positions 278 to 295 on the anti-sense strand. Intentional denaturation, as depicted in Fig. S6, further exacerbated non-specific binding relative to specific binding. To mitigate these undesirable signals in

cases like *rolC*, we anticipate that optimizing post-hybridization wash conditions with increased stringency will be necessary, albeit at the cost of added complexity.

These observations suggest that there may be limitations to quantitative exploitation of a paper-based approach and that the choice of the tag sequence may require a careful assessment of its cross-reactivity with the genetic target, especially in view of multiplexing in a future multicolor implementation.

In conclusion, we have successfully validated the feasibility of using gold nanorods functionalized with universal and customizable nucleotide linkers within both homogeneous and heterogeneous genosensor platforms. Our strategy focuses on the detection of amplicons generated by primers specifically tagged to recognize the linker bound to the gold nanorods. This approach allows direct identification of double-stranded DNA while ensuring specificity through primer rather than particle modification. This method offers substantial advantages over previous plasmonic particle-based genosensor systems, which commonly depend on the detection of single-stranded DNA or RNA filaments via specific linkers [67–71], or non-specific DNA structures [72, 73]. Primarily, it is essential to emphasize that, despite recognizing some limitations, our technology holds the potential for multiplexed sensing capabilities, thanks to the prospect of combining gold nanorods of different colors and nucleotide linkers of different compositions.

We anticipate that the heterogeneous setup presented here may recover some of the advantages of the homogeneous approach by designing alternative protocols. Examples may be a competitive assay with a poly-A ligand spotted on the substrate, as shown in Fig. 5, and the tagged particles left to interact in solution with amplicons labeled with a poly-A sequence, or a sandwich architecture where the amplicons may be made to carry a poly-A sequence on one end and another ligand of a receptor immobilized on the substrate on the opposite side, which may be ideal for a lateral-flow assay format.

Conclusions

We undertook a critical assessment of a universal platform for the optical detection of PCR products, based on the use of gold nanorods tagged with a

nucleotidic sequence and PCR primers labeled with the corresponding anti-tag filament. In the future, the same concept may be multiplexed by labeling particles of different colors with tags of different sequences, in order to color-encode as many amplicons bearing the due anti-tag strands. We tested both a simple homogeneous setup, where the use of modified forward and reverse primers provides amplicons that behave as homobifunctional cross-linkers for the polymerization of the gold nanorods in solution, and a heterogeneous setup that requires only one modified primer and a step of purification for a direct analysis of amplicons spotted on a substrate. Both setups work as intended by design and provide a detection limit in the range of low nM, which is satisfactory given that analyte amplification is a prerequisite of the system anyway. By comparing results from two different genes, we found that the homogeneous approach may be more robust to the particular choice of target, while the heterogeneous system provides approximately 100 times wider operational range and easier quantitative use.

In the future, we will pursue a technological development of our platform by implementing assay formats of translational relevance and by multiplexing the detection pattern in multicolor schemes. We will explore ways to combine the advantages of the homo and heterogeneous approaches, for instance by designing schemes where the amplicons interact with the tagged particles in solution and afterwards with a functionalized substrate for colorimetric readout. In this context, the lateral flow assay format may be ideal to schedule a chain of reactions in solution and on a substrate. The first case of a multicolor system may be a simplex lateral flow genoassay where target and control coexist in the same line. We believe that our work will help advance the development of innovative genosensors based on popular platforms such as paper-based test strips, in combination with PCR.

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Data availability Data is provided within the manuscript or supplementary information files.

Declarations

Competing interests The authors declare no competing interests.

References

- Li Z et al (2020) Development and clinical application of a rapid IgM-IgG combined antibody test for SARS-CoV-2 infection diagnosis. *J Med Virol* 92:1518–1524. <https://doi.org/10.1002/jmv.25727>
- Filchakova O, Dossym D, Ilyas A, Kuanysheva T, Abdizhamil A et al (2022) Review of COVID-19 testing and diagnostic methods. *Talanta* 244:123409. <https://doi.org/10.1016/j.talanta.2022.123409>
- Boehringer HR, O’Farrell BJ (2022) Lateral flow assays in infectious disease diagnosis. *Clin Chem* 68:52–58. <https://doi.org/10.1093/clinchem/hvab194>
- Carter DJ, Cary RB (2007) Lateral flow microarrays: a novel platform for rapid nucleic acid detection based on miniaturized lateral flow chromatography. *Nucleic Acids Res* 35:e74. <https://doi.org/10.1093/nar/gkm269>
- Baeumner AJ, Pretz J, Fang S (2004) A universal nucleic acid sequence biosensor with nanomolar detection limits. *Anal Chem* 76:888–894. <https://doi.org/10.1021/ac034945l>
- Byzova NA, Zherdev AV, Khlebtsov BN, Burov AM, Khlebtsov NG et al (2020) Advantages of highly spherical gold nanoparticles as labels for lateral flow immunoassay. *Sensors* 20:3608. <https://doi.org/10.3390/s20123608>
- Dreaden EC, Alkilany AM, Huang X, Murphy CJ, El-Sayed MA (2012) The golden age: gold nanoparticles for biomedicine. *Chem Soc Rev* 41:2740–2779. <https://doi.org/10.1039/c1cs15237h>
- Zeng J et al (2020) Anisotropic plasmonic nanostructures for colorimetric sensing. *Nano Today* 32:100855. <https://doi.org/10.1016/j.nantod.2020.100855>
- Kim EY, Kumar D, Khanga G, Lim DK (2015) Recent advances in gold nanoparticle-based bioengineering applications. *J Mater Chem B* 3:8433–8444. <https://doi.org/10.1039/C5TB01292A>
- Yu C, Irudayaraj J (2007) Multiplex biosensor using gold nanorods. *Anal Chem* 79:572–579. <https://doi.org/10.1021/ac061730d>
- Rao H, Xue X, Wang H, Xue Z (2019) Gold nanorod etching-based multicolorimetric sensors: strategies and applications. *J Mater Chem C* 7:4610–4621. <https://doi.org/10.1039/C9TC00757A>
- Pérez-Juste J, Pastoriza-Santos I, Liz-Marzán LM, Mulvaney P (2005) Gold nanorods: synthesis, characterization and applications. *Coord Chem Rev* 249:1870–1901. <https://doi.org/10.1016/j.ccr.2005.01.030>
- Lai S et al (2017) A multifunctional organosilica cross-linker for the bio-conjugation of gold nanorods. *Colloids Surf B Biointerfaces* 157:174–181. <https://doi.org/10.1016/j.colsurfb.2017.05.068>
- Milanesi A et al (2020) Optically activated and interrogated plasmonic hydrogels for applications in wound healing. *J Biophotonics* 13:e202000135. <https://doi.org/10.1002/jbio.202000135>
- Lohse SE, Murphy CJ (2013) The quest for shape control: a history of gold nanorod synthesis. *Chem Mater* 25:1250–1261. <https://doi.org/10.1021/cm303708p>
- Ye X, Zheng C, Chen J, Gao Y, Murray CB (2013) Using binary surfactant mixtures to simultaneously improve the dimensional tunability and monodispersity in the seeded growth of gold nanorods. *Nano Lett* 13:765–771. <https://doi.org/10.1021/nl304478h>
- Vigderman L, Zubarev ER (2013) High-yield synthesis of gold nanorods with longitudinal SPR peak greater than 1200 nm using hydroquinone as a reducing agent. *Chem Mater* 25:1450–1457. <https://doi.org/10.1021/cm303661d>
- Ratto F et al (2015) Plasmonic particles that hit hypoxic cells. *Adv Funct Mater* 25:316–323. <https://doi.org/10.1002/adfm.201402118>
- Huang S, Upputuri PK, Liu H, Pramanik M, Wang M (2016) A dual-functional benzobisthiadiazole derivative as an effective theranostic agent for near-infrared photoacoustic imaging and photothermal therapy. *J Mater Chem B* 4:1696–1703. <https://doi.org/10.1039/C5TB02367J>
- Manohar S, Ungureanu C, Van Leeuwen TG (2011) Gold nanorods as molecular contrast agents in photoacoustic imaging: the promises and the caveats. *Contrast Media Mol Imaging* 6:389–400. <https://doi.org/10.1002/cmmi.454>
- Centi S et al (2014) In vitro assessment of antibody-conjugated gold nanorods for systemic injections. *J Nanobiotechnol* 12:55. <https://doi.org/10.1186/s12951-014-0055-3>
- Parab HJ, Jung C, Lee JH, Park HG (2010) A gold nanorod-based optical DNA biosensor for the diagnosis of pathogens. *Biosens Bioelectron* 26:667–673. <https://doi.org/10.1016/j.bios.2010.06.067>
- Borri C et al (2022) Paper-based genetic assays with bioconjugated gold nanorods and an automated read-out pipeline. *Sci Rep* 12:6223. <https://doi.org/10.1038/s41598-022-10227-7>
- Joshi PP, Yoon SJ, Hardin WG, Emelianov S, Sokolov KV (2013) Conjugation of antibodies to gold nanorods through Fc portion: synthesis and molecular specific imaging. *Bioconjugate Chem* 24:878–888. <https://doi.org/10.1021/bc3004815>
- Centi S, Ratto F, Tatini F, Lai S, Pini R (2018) Ready-to-use protein G-conjugated gold nanorods for biosensing and biomedical applications. *J Nanobiotechnol* 16:5. <https://doi.org/10.1186/s12951-017-0329-7>
- Hamaly MA, Abulateefeh SR, Al-Qaoud KM, Alkilany AM (2018) Freeze-drying of monoclonal antibody-conjugated gold nanorods: colloidal stability and biological activity. *Int J Pharm* 550:269–277. <https://doi.org/10.1016/j.ijpharm.2018.08.045>

27. Hurst SJ, Lytton-Jean AKR, Mirkin CA (2006) Maximizing DNA loading on a range of gold nanoparticle sizes. *Anal Chem* 78:8313–8318. <https://doi.org/10.1021/ac0613582>
28. Mehtala JG, Zemlyanov DY, Max JP, Kadasala N, Zhao S, Wei A (2014) Citrate-stabilized gold nanorods. *Langmuir* 30:13727–13730. <https://doi.org/10.1021/la5029542>
29. Hill H, Mirkin C (2006) The bio-barcode assay for the detection of protein and nucleic acid targets using DTT-induced ligand exchange. *Nat Protoc* 1:324–336. <https://doi.org/10.1038/nprot.2006.51>
30. Mariani S, Scarano S, Spadavecchia J, Minunni M (2015) A reusable optical biosensor for the ultrasensitive and selective detection of unamplified human genomic DNA with gold nanostars. *Biosens Bioelectron* 74:981–988. <https://doi.org/10.1016/j.bios.2015.07.071>
31. Mariani S, Scarano S, Carra M, Barale R, Minunni M (2015) Direct genotyping of C3435T single nucleotide polymorphism in unamplified human MDR1 gene using a surface plasmon resonance imaging DNA sensor. *Anal Bioanal Chem* 407:4023–4028. <https://doi.org/10.1007/s00216-014-8424-1>
32. Hajian R et al (2019) Detection of unamplified target genes via CRISPR–Cas9 immobilized on a graphene field-effect transistor. *Nat Biomed Eng* 3:427–437. <https://doi.org/10.1038/s41551-019-0371-x>
33. Velders AH, Ossendrijver M, Keijser BJB, Saggiomo V (2022) T-Cup: a cheap, rapid, and simple home device for isothermal nucleic acid amplification. *Global Chall* 6:2100078. <https://doi.org/10.1002/gch2.202100078>
34. Roche PJR et al (2017) Real time plasmonic qPCR: how fast is ultra-fast? 30 cycles in 54 seconds. *Analyst* 142:1746–1755. <https://doi.org/10.1039/C7AN00304H>
35. Joda H et al (2015) Modified primers for rapid and direct electrochemical analysis of coeliac disease associated HLA alleles. *Biosens Bioelectron* 73:64–70. <https://doi.org/10.1016/j.bios.2015.05.048>
36. Al-Madhagi S, O'Sullivan CK, Prodromidis MI, Katakis I (2021) Combination of ferrocene decorated gold nanoparticles and engineered primers for the direct reagentless determination of isothermally amplified DNA. *Microchimica Acta* 188:117. <https://doi.org/10.1007/s00604-021-04771-8>
37. Hiramatsu K (1995) Molecular evolution of MRSA. *Microbiol Immunol* 39:531–543. <https://doi.org/10.1111/j.1348-0421.1995.tb02239.x>
38. Reynolds PE, Brown DF (1985) Penicillin-binding proteins of beta-lactam-resistant strains of *Staphylococcus aureus*. Effect of growth conditions *FEBS Lett* 192:28–32. [https://doi.org/10.1016/0014-5793\(85\)80036-3](https://doi.org/10.1016/0014-5793(85)80036-3)
39. Peacock SJ, Paterson GK (2015) Mechanisms of methicillin resistance in *Staphylococcus aureus*. *Annu Rev Biochem* 84:577–601. <https://doi.org/10.1146/annurev-biochem-060614-034516>
40. Costantino P, Capone I, Cardarelli M, De Paolis A, Mauro ML et al (1994) Bacterial plant oncogenes: the rol genes' saga. *Genetica* 94:203–211. <https://doi.org/10.1007/BF01443434>
41. Bulgakov VP (2008) Functions of rol genes in plant secondary metabolism. *Biotechnol Adv* 26:318–324. <https://doi.org/10.1016/j.biotechadv.2008.03.001>
42. Intrieri MC, Buiatti M (2001) The horizontal transfer of *Agrobacterium rhizogenes* genes and the evolution of the genus *Nicotiana*. *Mol Phylogenet Evol* 20:100–110. <https://doi.org/10.1006/mpev.2001.0927>
43. Aoki S, Syno K (1999) Horizontal gene transfer and mutation: ngrol genes in the genome of *Nicotiana glauca*. *Proc Natl Acad Sci USA* 96:13229–13234. <https://doi.org/10.1073/pnas.96.23.13229>
44. Liu R, McConnell EM, Li J, Li Y (2020) Advances in functional nucleic acid based paper sensors. *J Mater Chem B* 8:3213–3230. <https://doi.org/10.1039/C9TB02584G>
45. Ngo HT, Wang HN, Fales AM, Vo-Dinh T (2016) Plasmonic SERS biosensing nanochips for DNA detection. *Anal Bioanal Chem* 408:1773–1781. <https://doi.org/10.1007/s00216-015-9121-4>
46. Lechner B et al (2021) In situ monitoring of rolling circle amplification on a solid support by surface plasmon resonance and optical waveguide spectroscopy. *ACS Appl Mater Interfaces* 13:32352–32362. <https://doi.org/10.1021/acsami.1c03715>
47. Lytton-Jean AK, Han MS, Mirkin CA (2007) Microarray detection of duplex and triplex DNA binders with DNA-modified gold nanoparticles. *Anal Chem* 79:6037–6041. <https://doi.org/10.1021/ac070635h>
48. Ye H, Xia X (2018) Enhancing the sensitivity of colorimetric lateral flow assay (CLFA) through signal amplification techniques. *J Mater Chem B* 6:7102–7111. <https://doi.org/10.1039/C8TB01603H>
49. Glynou K, Ioannou PC, Christopoulos TK, Syriopoulou V (2003) Oligonucleotide-functionalized gold nanoparticles as probes in a dry-reagent strip biosensor for DNA analysis by hybridization. *Anal Chem* 75:4155–4160. <https://doi.org/10.1021/ac034256+>
50. Landgraf A, Reckmann B, Pingoud A (1991) Quantitative analysis of polymerase chain reaction (PCR) products using primers labeled with biotin and a fluorescent dye. *Anal Biochem* 193:231–235. [https://doi.org/10.1016/0003-2697\(91\)90014-K](https://doi.org/10.1016/0003-2697(91)90014-K)
51. Mao X, Gurung A, Xu H, Baloda M, He Y et al (2014) Simultaneous detection of nucleic acid and protein using gold nanoparticles and lateral flow device. *Anal Sci* 30:637–642. <https://doi.org/10.2116/analsci.30.637>
52. Rajendran VK, Bakthavathsalam P, Bergquist PL, Sunna A (2019) Smartphone detection of antibiotic resistance using convective PCR and a lateral flow assay. *Sens Actuators, B Chem* 298:126849. <https://doi.org/10.1016/j.snb.2019.126849>
53. Miller BS et al (2020) Spin-enhanced nanodiamond biosensing for ultrasensitive diagnostics. *Nature* 587:588–593. <https://doi.org/10.1038/s41586-020-2917-1>
54. Ubukata K et al (1992) Rapid detection of the mecA gene in methicillin-resistant staphylococci by enzymatic detection of polymerase chain reaction products. *J Clin Microbiol* 30:1728–1733. <https://doi.org/10.1128/jcm.30.7.1728-1733.1992>
55. Zou L, Shen R, Ling L, Li G (2018) Sensitive DNA detection by polymerase chain reaction with gold nanoparticles. *Anal Chim Acta* 1038:105–111. <https://doi.org/10.1016/j.aca.2018.07.006>
56. Vanzha E, Pylaev T, Khanadeev V, Konnova S, Fedorovab V et al (2016) Gold nanoparticle-assisted polymerase

- chain reaction: effects of surface ligands, nanoparticle shape and material. *RSC Adv* 6:110146–110154. <https://doi.org/10.1039/C6RA20472D>
57. Chan KP, Chao SH, Yong Kah JC (2018) Universal mRNA translation enhancement with gold nanoparticles conjugated to oligonucleotides with a poly(T) sequence. *ACS Appl Mater Interfaces* 10:5203–5212. <https://doi.org/10.1021/acsami.7b16390>
 58. Jung YL, Jung C, Parab H, Li T, Park HG (2010) Direct colorimetric diagnosis of pathogen infections by utilizing thiol-labeled PCR primers and unmodified gold nanoparticles. *Biosens Bioelectron* 25:1941–1946. <https://doi.org/10.1016/j.bios.2010.01.010>
 59. Thaxton CS, Georganopoulou DG, Mirkin CA (2006) Gold nanoparticle probes for the detection of nucleic acid targets. *Clin Chim Acta* 363:120–126. <https://doi.org/10.1016/j.cccn.2005.05.042>
 60. Reynolds RA, Mirkin CA, Letsinger RL (2000) Homogeneous, nanoparticle-based quantitative colorimetric detection of oligonucleotides. *J Am Chem Soc* 122:3795–3796. <https://doi.org/10.1021/ja000133k>
 61. Cordray MS, Amdahl M, Richards-Kortum RR (2012) Gold nanoparticle aggregation for quantification of oligonucleotides: optimization and increased dynamic range. *Anal Biochem* 431:99–105. <https://doi.org/10.1016/j.ab.2012.09.013>
 62. Lytton-Jean AKR, Gibbs-Davis JM, Long H, Schatz GC, Mirkin CA et al (2009) Highly cooperative behavior of peptide nucleic acid-linked DNA-modified gold-nanoparticle and comb-polymer aggregates. *Adv Mater* 21:706–709. <https://doi.org/10.1002/adma.200801724>
 63. Hurst SJ, Hill HD, Mirkin CA (2008) “Three-dimensional hybridization” with polyvalent DNA–gold nanoparticle conjugates. *J Am Chem Soc* 130:12192–12200. <https://doi.org/10.1021/ja804266j>
 64. Rose K, Mason JO, Lathe R (2002) Hybridization parameters revisited: solutions containing SDS. *Biotechniques* 33:54–58. <https://doi.org/10.2144/02331st01>
 65. Zhao W, Ali MM, Aguirre SD, Brook MA, Li Y (2008) Paper-based bioassays using gold nanoparticle colorimetric probes. *Anal Chem* 80:8431–8437. <https://doi.org/10.1021/ac801008q>
 66. Ruppert C, Phogat N, Laufer S, Kohl M, Deigner HP (2019) A smartphone readout system for gold nanoparticle-based lateral flow assays: application to monitoring of digoxigenin. *Microchim Acta* 186:119. <https://doi.org/10.1007/s00604-018-3195-6>
 67. Cordray MS, Amdahl M, Richards-Kortum RR (2012) Gold nanoparticle aggregation for quantification of oligonucleotides: optimization and increased dynamic range. *Anal Biochem* 431(2):99–105. <https://doi.org/10.1016/j.ab.2012.09.013>
 68. Liu Y et al (2012) Simple, rapid, homogeneous oligonucleotides colorimetric detection based on non-aggregated gold nanoparticles. *Chem Commun* 48:3164–3166. <https://doi.org/10.1039/C2CC16741G>
 69. Veigas B et al (2012) Gold on paper—paper platform for Au-nanoprobe TB detection. *Lab Chip* 12:4802–4808. <https://doi.org/10.1039/C2LC40739F>
 70. Storhoff JJ et al (2004) Gold nanoparticle-based detection of genomic DNA targets on microarrays using a novel optical detection system. *Biosens Bioelectron* 19(8):875–883. <https://doi.org/10.1016/j.bios.2003.08.014>
 71. Hunt A, Torati SR, Slaughter G (2024) Paper-based DNA biosensor for rapid and selective detection of miR-21. *Biosensors* 14(10):485. <https://doi.org/10.3390/bios14100485>
 72. Li H, Rothberg L (2004) Colorimetric detection of DNA sequences based on electrostatic interactions with unmodified gold nanoparticles. *Proc Natl Acad Sci USA* 101(39):14036–14039. <https://doi.org/10.1073/pnas.0406115101>
 73. Chen C, Song G, Yang X, Ren J, Qu X (2010) A gold nanoparticle-based strategy for label-free and colorimetric screening of DNA triplex binders. *Biochimie* 92(10):1416–1421. <https://doi.org/10.1016/j.biochi.2010.07.004>

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