

Multilayer Plasmon Hybridization in Au–Ag–Au Nanoparticles Enables Extinction Amplification in Transport-Limited One-Step Confirmatory Syphilis Biosensing

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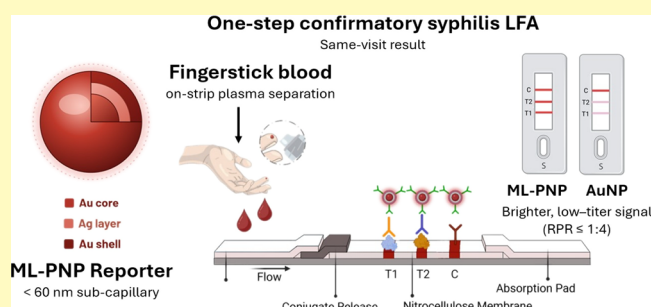
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ABSTRACT: Syphilis diagnosis requires pairing treponemal and non-treponemal antibody results, yet rapid tests combining both markers lose sensitivity at the low RPR titers ($\leq 1:4$) most critical in early infection and pregnancy. The limitation lies with the reporter: conventional gold nanoparticles (AuNPs) cannot generate high-contrast signals at low antibody concentrations without exceeding the diameter that allows capillary transport through a nitrocellulose membrane. Here, we report gold–silver–gold (Au–Ag–Au) multilayer plasmonic nanoparticles (ML-PNPs) that exploit interfacial plasmon hybridization to overcome this trade-off. Polyhedral 50–58 nm particles were prepared by seed-mediated growth followed by galvanic replacement, and their layered structure was confirmed by electron microscopy, elemental mapping, and X-ray photoelectron spectroscopy. A silver interlayer between chemically robust gold domains couples across two metal–dielectric interfaces, while the outer gold shell preserves colloidal stability and thiol-based bioconjugation at hydrodynamic diameters below 60 nm. Finite-difference time-domain (FDTD) simulations and hyperspectral imaging showed a broadened, red-shifted resonance (scattering maximum 658 nm vs 555 nm for AuNPs) with 18-fold enhanced absorption. In a dual-marker colorimetric lateral flow immunoassay (CLFIA), ML-PNPs improved the visual detection limit by 3-fold and required 6-fold fewer particles per visible test spot. Across 36 clinical specimens spanning RPR titers of 1:1 to 1:128, treponemal and nontreponemal test-line intensities increased by 19–46%, and low-titer 1:1 samples missed by AuNP strips were reliably detected, with full agreement with reference methods and no cross-reactivity with HIV-1 or HCV sera. Coupled with fingerstick sampling and on-strip plasma separation, this supports same-visit execution of the reverse testing algorithm in an instrument-free format.

KEYWORDS: plasmonic nanoparticles, syphilis, point-of-care diagnostics, lateral flow immunoassay, global health



Syphilis is a multistage infection caused by *Treponema pallidum* subsp. *pallidum*, that progresses from infectious primary and secondary disease through asymptomatic latency to tertiary stages associated with cardiovascular and neurologic sequelae.^{1–4} Global incidence, particularly of congenital syphilis, has increased sharply over the past decade; in pregnancy, untreated infection is associated with stillbirth, prematurity, low birth weight, and vertical transmission, highlighting the need for rapid, same-visit diagnosis and treatment.^{2–8} Diagnosis, therefore, relies on serologic testing that integrates treponemal tests (TTs), which detect anti-*T. pallidum* antibodies that typically remain positive lifelong, with non-treponemal tests (NTTs) such as rapid plasma reagin (RPR) and Venereal Disease Research Laboratory (VDRL), whose titers correlate with disease activity and treatment response.^{9–12} Because neither marker class alone reliably discriminates active, past, and treated infection, contemporary algorithms employ a reverse sequence in which a treponemal immunoassay is

followed by quantitative NTT and, when discordant, confirmatory TT.¹² This multistep laboratory workflow introduces delays and is vulnerable to loss to follow-up. Furthermore, no FDA-approved PCR assay is available for routine use, and direct detection methods such as darkfield microscopy remain limited to specialized settings and early symptomatic lesions.^{4,12–16}

Existing POC platforms inadequately address this diagnostic gap. FDA-cleared treponemal-only CLFIAs provide qualitative detection but cannot distinguish active from treated infection, necessitating laboratory-based NTT follow-up that reintroduces

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the delays the rapid format was intended to eliminate.^{17–21} Dual-marker rapid assays available in limited markets incorporate a non-treponemal component but exhibit reduced sensitivity for low-titer infections ($RPR \leq 1:4$), the clinical range most critical in early disease and pregnancy (Figure 1a).^{17–22} This performance limitation arises in part because conventional AuNP reporters lack the extinction and scattering cross-

sections required to generate high-contrast signals at low antibody concentrations on nitrocellulose membranes. The stage-dependent kinetics of treponemal and non-treponemal antibodies (Figure 1b) further complicate detection: IgM antibodies appear early during primary infection but decline rapidly following treatment or disease progression, rendering IgM-only tests prone to false negatives in latent, secondary,

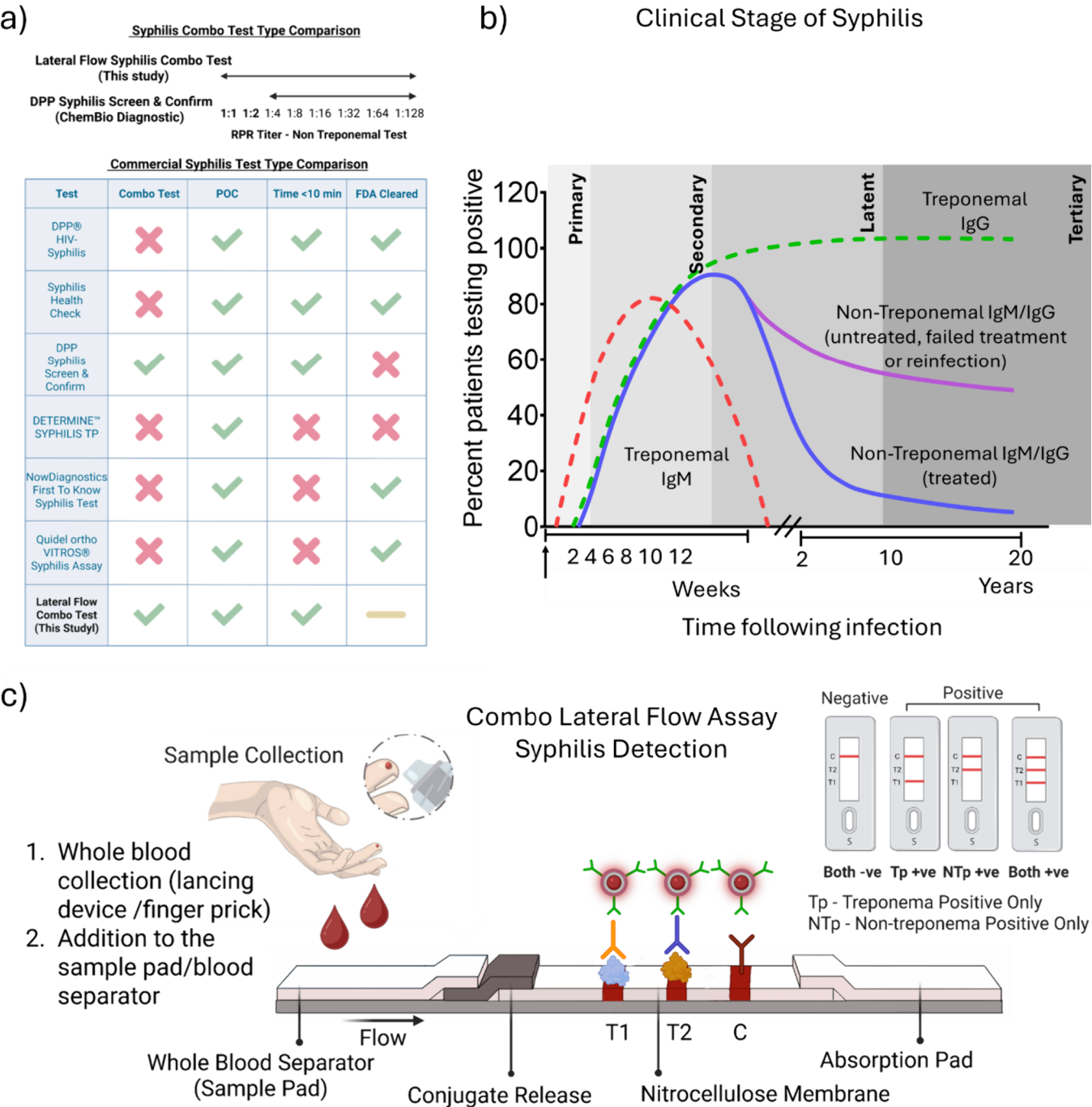


Figure 1. a) Comparison of the lateral flow combo syphilis test developed in this study with the ChemBio Diagnostic test regarding non-treponemal titer detection. Additionally, the comparison of the developed lateral flow combo test with the current landscape of syphilis POC diagnostic tests available in the United States (U.S.) market. b) Schematic representation of treponemal and non-treponemal antibody kinetics across the clinical stages of syphilis, highlighting windows of detectability for each marker class. c) Workflow for finger-prick whole blood collection, plasma separation on the sample pad, and subsequent multiplex detection of treponemal (T1) and non-treponemal (T2) antibodies on the lateral flow strip, with example result interpretation for negative, dual-positive, and single-marker positive test outcomes.

and reinfection cases, whereas IgG antibodies persist and extend diagnostic coverage across disease stages. A combined IgM/IgG treponemal detection strategy paired with a quantitative non-treponemal readout on a single strip would therefore enable same-visit implementation of the reverse algorithm, facilitating real-time disease staging without laboratory referral. This requires reporters to simultaneously deliver high optical output at low analyte concentrations, maintain sub-60 nm hydrodynamic diameters for capillary transport, meet robust bioconjugation requirements, and enable interfacial plasmon hybridization.

Here, we report Au–Ag–Au multilayer plasmonic nanoparticles (ML–PNPs) as high-performance colorimetric reporters for a dual-marker syphilis CLFIA that enables simultaneous detection of treponemal (IgG + IgM) and non-treponemal antibodies on a single lateral flow strip (Figure 1c). ML–PNPs implement this design strategy by incorporating an optically active Ag interlayer between inner and outer Au shells in a core–shell–shell architecture.^{23–25} The inner Au core serves as a structurally defined plasmonic scaffold; the intermediate Ag shell enhances the local electromagnetic field and shifts the localized surface plasmon resonance (LSPR) to longer wavelengths; and the outer Au shell protects the Ag domain from oxidative degradation while maintaining compatibility with thiol-based surface functionalization.^{23–25} The optical response of these nanostructures arises from plasmon hybridization, in which closely spaced metal–dielectric interfaces support coupled dipolar modes that give rise to bonding and antibonding plasmon states, analogous to molecular orbital formation. This coupling leads to spectral broadening and red-shifting of the extinction profile, accompanied by enhanced electromagnetic field confinement at both interfaces.^{23–25} In the Au–Ag–Au configuration, Ag's higher bulk plasma frequency and lower intrinsic losses promote stronger interfacial charge oscillations, while the surrounding Au layers stabilize the structure and influence radiative damping. As a result, ML–PNPs exhibit increased absorption and scattering cross-sections without a proportional increase in particle volume, effectively decoupling optical enhancement from geometric scaling. Furthermore, shell thickness and interfacial spacing are tunable parameters that govern the balance between nonradiative dissipation and far-field scattering, enabling precise control of the extinction line shape in the visible region while maintaining hydrodynamic diameters below 60 nm, which are compatible with capillary-driven transport in porous membranes. Although Au–Ag–Au core–shell–shell nanoparticles have been previously explored as LFA reporters,²⁶ prior studies did not systematically examine plasmon hybridization as a governing design principle for extinction enhancement (Table S1). In contrast, the present work leverages interfacial electromagnetic coupling across multiple metal–dielectric boundaries as a deliberate design framework to engineer high-brightness, transport-compatible colorimetric reporters for lateral flow biosensing.

Polyhedral Au–Ag–Au nanostructures (50–58 nm) were synthesized via seed-mediated growth followed by galvanic replacement, and their multilayer composition was verified by high-resolution TEM, STEM–EDS elemental mapping, XPS, FTIR, and hyperspectral dark-field imaging. Finite-difference time-domain (FDTD) simulations, corroborated by UV–vis spectroscopy, reveal that plasmon hybridization across the Au–Ag and Ag–Au interfaces produces a broadened, red-shifted LSPR with up to ~18-fold higher absorption and markedly enhanced scattering relative to AuNPs of comparable size (~50 nm).

When deployed in a dual-marker CLFIA, ML–PNPs deliver clinically meaningful performance gains: a threefold improvement in visual detection limit (25 vs 75 pg/mL for streptavidin), a 6-fold reduction in the particle count required for a visible test spot, and 19–46% higher treponemal and non-treponemal test-line intensities compared with standard AuNP reporters. Furthermore, these advancements were validated on 36 clinical serum specimens spanning RPR titers from 1:1 to 1:128, including reliable identification of low-titer 1:1 samples that AuNP-based strips failed to detect, with full concordance to reference RPR and TP-PA laboratory methods and no cross-reactivity with HIV-1 or HCV antibodies. Moreover, the entire workflow shown in Figure 1c highlights that the assay can be used with fingerstick sampling, on-strip plasma separation, and simple visual interpretation of T1/T2 patterns. By demonstrating that interfacial plasmon hybridization within a sub-60 nm colloidal particle can substantially enhance colorimetric signal output without compromising membrane transport, bioconjugation compatibility, or assay simplicity, this work establishes a generalizable nanomaterials design principle for overcoming sensitivity barriers in transport-limited POC diagnostics.

RESULTS AND DISCUSSION

Principle of Detection

In CLFIA, plasmonic nanoparticles serve as optical reporters, and the intensity of the visible test line scales with the reporter's optical extinction cross-section, the sum of its absorption and scattering cross-sections.^{26,27} Conventional AuNPs smaller than 60 nm are absorption-dominated, with an LSPR near 520 nm that produces the characteristic red test line. However, their extinction coefficient is only 10^8 – 10^9 M^{−1} cm^{−1}, limiting the attainable signal, and the narrow LSPR band provides limited color contrast against the nitrocellulose membrane. Enlarging the particle toward 100 nm raises the extinction coefficient to 10^{11} M^{−1} cm^{−1}, as scattering overtakes absorption and brightens the test line.^{26,27} Beyond this diameter, however, sedimentation and aggregation degrade colloidal stability, causing uneven membrane migration and poor reproducibility. Size therefore offers no further headroom, and enhancement must come from composition instead (Table S1).

In the quasi-static limit, the extinction cross-section of a sphere depends on the radius R , the wavelength λ , the dielectric constant of the surrounding medium (ϵ_m), and the real (ϵ_r) and imaginary (ϵ_i) components of the metal dielectric function:^{26–29}

$$\sigma_{\text{ext}} = \frac{(24\pi^2 R^3 \epsilon_m^{3/2})}{\lambda} \left[\frac{\epsilon_i}{(\epsilon_r + 2\epsilon_m)^2 + \epsilon_i^2} \right] \quad (1)$$

Because (ϵ_i) sets the optical loss, metals with small (ϵ_i) sustain stronger plasmon oscillations and correspondingly larger absorption and scattering. Silver (Ag) meets this condition better than gold (Au): its lower (ϵ_i) and higher bulk plasma frequency place the silver nanoparticles (AgNP) resonance near 400 nm and yield molar extinction coefficients of 10^{10} to 10^{11} M^{−1} cm^{−1}, one to two orders of magnitude above Au of equal diameter, tunable across a broader region of the visible spectrum.^{30,31} Ag alone, however, oxidizes readily and presents a less inert surface that complicates bioconjugation, whereas Au offers colloidal stability and reliable Au–thiol functionalization

at lower brightness. Whether the Au–Ag–Au architecture resolves this trade-off at diameters compatible with membrane transport is addressed below, beginning with simulations of the hybridized plasmon modes.

Computational Modeling of Multilayer Plasmon Coupling

To elucidate the optical mechanisms underlying ML-PNP performance, finite-difference time-domain (FDTD) simulations were performed using Ansys Lumerical under X-polarized plane-wave illumination with the propagation vector (k) oriented along the Z-axis. The model geometry (Figure 2a) consisted of a 13 nm radius Au core, a 10 nm thick Ag shell, and a 10 nm thick outer Au shell embedded in a homogeneous dielectric medium (refractive index $n = 1.33$). The computational model employs an idealized concentric core-shell-shell geometry to isolate the fundamental electromagnetic mechanisms governing multilayer plasmon coupling and parametric trends in shell thickness. This concentric core-shell-shell model was constructed prior to synthesis to establish the electromagnetic basis of multilayer plasmon coupling and to identify the Ag shell-thickness regime of interest. The experimentally synthesized nanoparticles exhibit faceted polyhedral morphologies resulting from galvanic replacement, with local variations in shell thickness and edge curvature that are not represented in the model. Thus, simulations are intended only to provide mechanistic understanding of the origin of extinction enhancement rather than predictive optimization of the experimental particle geometry. The qualitative agreement between simulation and experiment, including spectral red-shifting, resonance broadening, and enhanced optical response, supports the validity of this simplified physical framework. Simulated extinction spectra (Figure 2b) showed a progressive

redshift and increased intensity for ML-PNPs relative to monometallic Au and core-shell (Ag–Au) structures, confirming strong plasmonic coupling between the Au and Ag layers. Electric field intensity maps at 527 nm (Figure S1) revealed pronounced near-field confinement at both the inner (Au–Ag) and outer (Ag–Au) metal–dielectric interfaces. This dual localization, absent in single and bimetallic particles, reflects efficient coupling between core and shell plasmons and yields up to 18-fold higher absorption than size-matched AuNPs, amplification obtained without a proportional increase in particle volume. Magnetic field distributions (Figure S2) track the same progression from Au to Ag@Au to ML-PNP, with the multilayer structure again localizing field at both interfaces. These are relative intensities for comparison across the three systems rather than absolute values. The pattern is that expected of hybridized modes: in-phase oscillation at the two interfaces produces a strongly coupled, red-shifted bonding state, while out-of-phase oscillation gives the higher-energy antibonding state (Figure S3).

Simulated optical spectra (Figure 2c) showed a ~ 1000 -fold increase in radiative output for ML-PNPs over monometallic Au particles. Varying Ag shell thickness from 2 to 14 nm (Figure 2d) increased extinction up to an optimum, beyond which radiative damping and retardation broadened and attenuated the peak. Decomposition into scattering, absorption, and extinction components (Figure 2e) confirmed high radiative efficiency and identified the Ag interlayer as the primary driver of extinction amplification. The shoulder near 600 nm arises where the dominant dipolar bonding mode overlaps higher-order hybrid modes; in the Au–Ag–Au geometry, coupling between the inner and outer Au interfaces at finite size generates additional multipolar resonances that broaden the

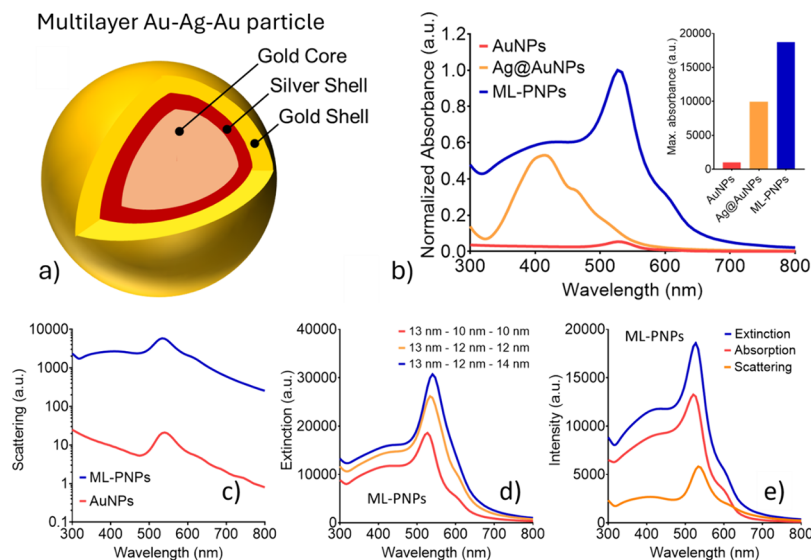


Figure 2. Finite-difference time-domain (FDTD) simulations showing the optical response of Au–Ag–Au multilayered plasmonic nanoparticles (ML-PNPs) designed for enhanced colorimetric biosensing. All simulations were performed using an idealized geometry consisting of a gold core (radius = 13 nm), a silver inner shell (thickness = 10 nm), and a gold outer shell (thickness = 10 nm) embedded in a homogeneous dielectric medium ($n = 1.33$). a) Schematic of the nanoparticle architecture consisting of a gold core, a silver inner shell, and a gold outer shell. b) Simulated absorbance spectra for Au, Au@Ag, and ML-PNP (Au–Ag–Au) nanoparticles showing a blue shift for Au@AgNP compared to AuNP; ML-PNP showed a progressive red shift and intensified localized surface plasmon resonance (LSPR) for the multilayered configuration compared to both Au and Ag@Au NPs, indicative of plasmonic coupling between the gold and silver layers. c) Simulated scattering spectra comparing Au and multilayered (ML-PNP) nanoparticles. d) Extinction spectra of ML-PNPs with varying layer dimensions, showing tunable plasmon resonance and red-shifted spectral response. e) Decomposition of the total optical response of ML-PNPs into scattering, absorption, and extinction components.

spectrum in this region. Collectively, these simulations establish that ML-PNPs achieve optical amplification through interfacial plasmon hybridization at dual metal–dielectric boundaries rather than through size-dependent geometric scaling. The qualitative agreement between simulation and experiment, spectral red-shifting, resonance broadening, and enhanced optical response support the validity of this simplified physical framework and identify Ag interlayer thickness as the parameter governing brightness in transport-compatible reporters.

Synthesis and Dimensional Characterization of ML-PNPs

ML-PNPs comprising an Au core, an intermediate Ag shell, and an outer Au shell were synthesized by seed-mediated growth followed by galvanic replacement (Figure S4a,b).^{32–41} Citrate-stabilized Au seeds with an approximate diameter of 15 nm were synthesized as nucleation templates using our previously reported methods.^{42–51} These seeds were then enlarged by hydroquinone-mediated reduction of HAuCl_4 in the presence of sodium citrate, yielding citrate-stabilized Au cores with a hydrodynamic diameter of approximately 50 nm (Figure S4a). Hydroquinone served as a mild reducing agent that promoted controlled Au growth (converting Au (III) ions to Au (0) atoms), whereas citrate acted as a stabilizing ligand to suppress aggregation during enlargement. An intermediate Ag shell was subsequently deposited onto the Au cores by reduction of silver nitrate (AgNO_3) with L-ascorbic acid under vigorous stirring. Briefly, 50 mL of the ~ 50 nm AuNP suspension was mixed with 0.25 mL of 0.1 M L-ascorbic acid (reducing agent to convert Ag(I) ions into metallic silver), followed by dropwise addition of 1.25 mL of 10 mM AgNO_3 , and the mixture was stirred for 1 h at room temperature to obtain Au–Ag core–shell intermediates. Controlled Ag precursor addition promoted uniform shell growth while maintaining colloidal stability (Figure S4b). The outer Au shell was then formed by galvanic replacement of the Ag-coated particles. Specifically, 50 mL of the Au–Ag intermediate suspension was heated to a boil, and 8 mL of 1 mM HAuCl_4 was added dropwise via a syringe pump under continuous stirring (Figure S4b). During this step, partial oxidation of the less-noble Ag layer, coupled with Au deposition, generated the final Au–Ag–Au multilayer architecture. The detailed procedure is described in the Materials and Methods section.

Structural and Optical Characterization of ML-PNPs

The structural morphology, elemental composition, and optical properties of the synthesized ML-PNPs were characterized using UV–vis spectroscopy, transmission electron microscopy (TEM), high-angle annular dark-field scanning TEM (HAADF-STEM), energy-dispersive X-ray spectroscopy (EDS), dynamic light scattering (DLS), X-ray photoelectron spectroscopy (XPS), and hyperspectral dark-field imaging. Initially, approximately 15 nm Au seeds were synthesized for ML-PNP formation, and successful synthesis was confirmed by a characteristic absorption peak at 520 nm in the UV–vis spectrum (Figure S5). DLS and TEM confirmed narrow particle-size distributions, indicating mono-dispersity (Figures S6 and S7). For optimal optical performance in conventional CLFIAs, the ideal AuNP diameter is 33–60 nm. Therefore, the initially synthesized 15 nm AuNPs were enlarged to ~ 50 nm (hydrodynamic diameter by DLS) to serve as cores for subsequent ML-PNP synthesis (Figure S8) and were coated with a silver shell, as validated by HAADF-STEM (Figure S9). Optical images of the as-synthesized colloidal solutions

demonstrate visible color changes: the ~ 50 nm AuNPs appear red, Ag@AuNPs (silver shell) appear yellow, and the final ML-PNPs exhibit a deeper red, consistent with enhanced plasmonic coupling (Figure S10a). Upon spectral analysis, the extinction and absorbance profiles revealed a systematic shift: the plasmon resonance of AuNPs at 520 nm blue shifts to approximately 420 nm following silver shell growth, then broadens and red shifts again after depositing the outer gold shell (Figure S5). We note that the normalized experimental absorbance spectra (Figure S5) are not expected to be identical to the simulated spectra in Figure 2b. The FDTD calculations assume monodisperse, idealized spherical particles in a uniform dielectric medium, whereas the experimental colloids exhibit finite size and shape dispersion, and are measured in aqueous suspension. In addition, the experimental spectra are normalized at the maximum absorbance of each sample, while the simulations are plotted as absolute extinction cross-sections. Despite these differences, both datasets show the same qualitative trends: a blue shift upon Ag shell growth, followed by broadening and red shifting of the plasmon band after deposition of the outer Au shell. ML-PNPs thus display a broad extinction profile, attributed to a broadened extinction cross-section arising from multilayer plasmonic coupling (Figure S10b). DLS measurements confirmed that the hydrodynamic diameter of the ML-PNPs remained below 60 nm, an ideal size for LFAs (Figure S10c). Zeta potential measurements further showed that the surface charge of ML-PNPs (-28.4 ± 2.5 mV) was less negative than citrate-stabilized AuNPs (-39.4 ± 2.0 mV), attributed to the lower ionization of the ascorbic acid ligand (AA^{-1}) in contrast to citrate (Cit^{-3}) (Figure S10d). Nanoparticle tracking analysis (NTA) measurements indicated that the number concentration of ML-PNPs in the prepared colloidal suspensions was approximately 1.2×10^{11} particles/mL, consistent with expected batch yields. This high particle concentration ensures that during conjugate pad preparation and strip assembly, a sufficient and consistent number of reporter particles are deposited per unit area of the conjugate pad, thereby minimizing batch-to-batch variability in signal intensity and improving assay reproducibility.

The structural morphology and elemental distribution of ML-PNPs were analyzed using TEM, HAADF-STEM, and EDS mapping. Low-magnification TEM (Figure S11a; scale bar: 500 nm) revealed that ML-PNPs exhibited a distinct polyhedral morphology, with particle diameters predominantly in the 40–60 nm range, indicating reasonable batch homogeneity. Some clustering observed on TEM grids was attributed to drying artifacts during sample preparation rather than colloidal instability, as confirmed by narrow DLS size distributions and zeta potential measurements (-28.4 ± 2.5 mV) indicating sufficient electrostatic stabilization. Medium-magnification imaging revealed moderate shape variation between faceted polyhedral and quasi-spherical geometries, reflecting local differences in facet-selective Ag deposition and galvanic replacement kinetics, a degree of polydispersity that does not compromise LFA performance, as capillary transport in nitrocellulose membranes tolerates modest size distributions (Figure 3a) (Figure S11b). This polyhedral morphology likely results from the complex interaction of crystallographic facet-selective growth, controlled shell deposition, and galvanic replacement mechanisms during synthesis. During seed-mediated growth, citrate-stabilized gold cores serve as smooth templates. Silver deposits preferentially at high-energy facets and edges, creating an initial anisotropy

and a faceted shell.^{25,33–37} When the outer gold shell forms via galvanic replacement, gold atoms replace silver atoms at higher-energy crystal facets, thereby promoting further development toward frame-like polyhedral structures.^{25,33–37} These processes are also supported by thermal diffusion and surface ligand effects, which drive atomic reorganization along specific facet directions, stabilizing the entire multilayer structure with prominent edges and corners.^{25,33–37} High-resolution TEM (Figure 3b) further demonstrates the crystalline nature of these structures and allows direct visualization and measurement of shell thicknesses, which consistently range from 2–4 nm, confirming nanoscale layer control. HAADF-STEM imaging (Figure 3c) offers contrast primarily based on atomic number (Z-contrast), enabling multilayer architecture to be readily observed as bright and dark regions corresponding to Au and Ag, respectively. Elemental mapping via STEM-EDS (Figure 3d–f) provides spatially resolved distributions of Au

and Ag within the nanoparticles. The overlay image (Figure 3d) and separate Au (Figure 3e) and Ag (Figure 3f) maps confirm an Au-rich core and shell flanking a sandwiched Ag layer, verifying the intended multilayer (Au–Ag–Au) composition. The apparent difference in visual prominence between the Ag shell (Figure 3f) and outer Au shell (Figure 3e) in the color-scaled EDS maps reflects differences in color scaling and spatial distribution. Ag is concentrated in a narrow intermediate shell, whereas Au is distributed between the core and outer shell, rather than the absence of a substantial Au overlayer. Moreover, line profile EDS analysis (Figure 3g) further quantifies the intensity distribution across the particle, showing higher Au counts at the center and outer edges, and a distinct Ag intensity localized within the shell, confirming the presence of the outer Au shell as expected from the intended design. Next, the surface properties and elemental composition of ML-PNPs were studied using high-resolution XPS, providing

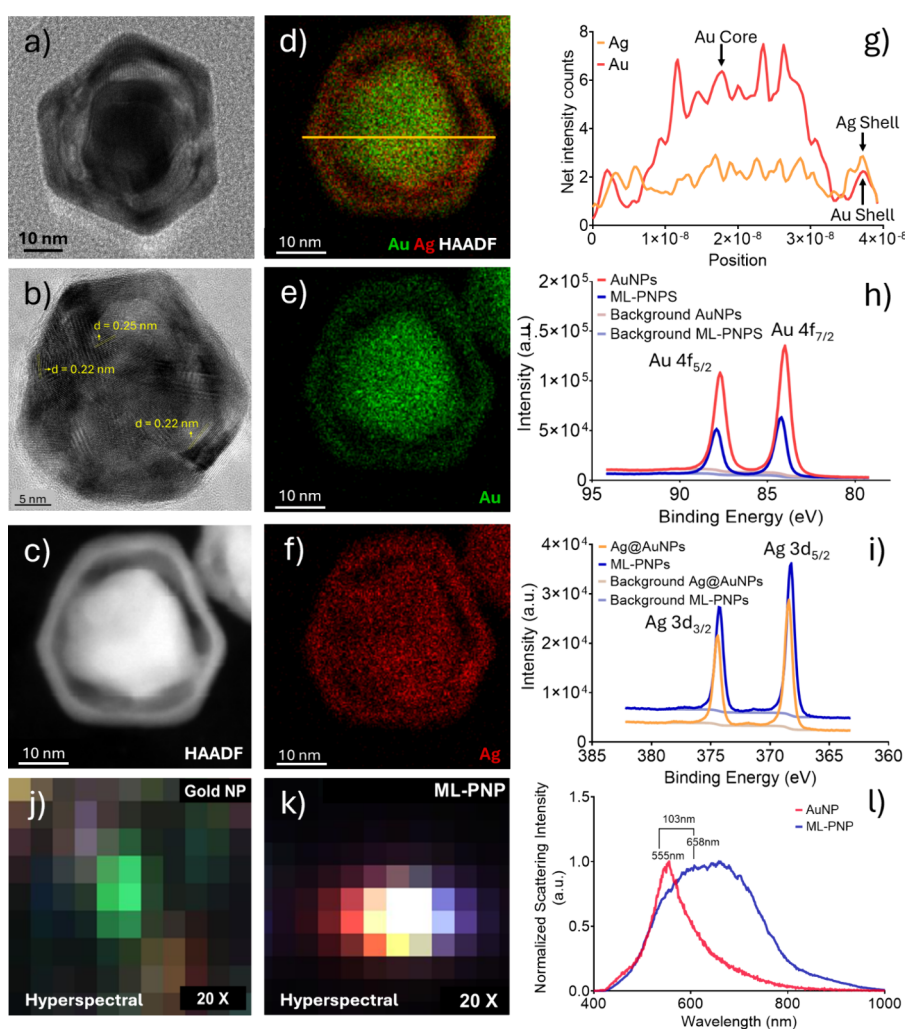


Figure 3. a) Low-magnification TEM image showing the polyhedral morphology of a representative ML-PNP. b) High-resolution TEM of the ML-PNP illustrating crystalline domains and shell thickness (2–4 nm). c) HAADF-STEM image enables visualization of atomic number contrast within the multilayer structure. STEM-EDS elemental maps: d) overlay of Au (green) and Ag (red), e) Au distribution, f) Ag distribution in the ML-PNP. g) Line profile of EDS intensity across the particle, confirming localization of gold in the core and shell, and silver in the intermediate layer. h) XPS spectra of the Au 4f region for AuNPs and ML-PNPs, resolving Au 4f_{7/2} and Au 4f_{5/2} peaks. i) XPS spectra of the Ag 3d region for Ag@AuNPs and ML-PNPs, highlighting Ag 3d_{5/2} and Ag 3d_{3/2} peaks and confirming the persistence of silver within the multilayered structure. Hyperspectral darkfield imaging maps for conventional AuNPs j) and ML-PNPs k), illustrating differences in scattering color profile. l) Normalized scattering intensity spectra showing a broadened and red-shifted peak for ML-PNPs (658 nm) versus AuNPs (555 nm), supporting enhanced and tunable optical properties of the ML-PNPs.

clear evidence of their multilayer structure. The XPS spectra of the ML-PNPs closely resembled those of the initial AuNPs, with characteristic signals for elemental Au. However, in ML-PNPs, additional peaks corresponding to Ag were observed, confirming the successful incorporation of an Ag shell within the multilayer structure. Furthermore, detailed analysis of the Au 4f region revealed two distinct peaks, $4f_{7/2}$ and $4f_{5/2}$, for both pure AuNPs and ML-PNPs (Figure 3h). The binding energies and sharpness of these peaks indicate that the Au component primarily exists in the metallic state (Au^0) in both sample types. Notably, the gold peak intensities in the ML-PNPs were lower compared to those of pure AuNPs, suggesting that the Au is partially covered by the overlying Ag layer in the multilayer composite. The presence and characteristics of Ag layers were evaluated using the Ag 3d region.⁵² Both core-shell Ag@AuNPs and ML-PNPs exhibited strong Ag $3d_{5/2}$ and Ag $3d_{3/2}$ peaks, aligned with typical binding energies for metallic silver (Ag^0) (Figure 3i).⁵² In Ag@AuNPs, the Ag peaks were the most prominent, and the Au signal was weaker, indicating a thin (2–4 nm) Ag coating on the Au core. In ML-PNPs, the Au peaks remain visible but are attenuated compared to pure AuNPs, while strong Ag signals continue, confirming that the Ag shell still exists and is covered by an additional Au shell. Finally, hyperspectral imaging was employed to study the scattering properties of the ML-PNPs (Figure 3j,k).⁵¹ Hyperspectral dark-field imaging showed that the ML-PNPs exhibited a broad and distinct color profile compared to the conventional AuNPs (Figure 3j,k). Figure 3l demonstrates that the ML-PNPs exhibit not only a red-shifted scattering peak (658 nm vs 555 nm for AuNPs), but also a noticeably broader scattering band. This combination of peak shift and spectral broadening results from plasmonic coupling within the multilayered structure, yielding higher, more tunable optical signal intensity suitable for sensitive LFA detection.

ML-PNPs as Enhanced Colorimetric Reporters in Lateral Flow Assays

Conventional CLFIAs using 30–40 nm AuNPs as reporter probes have fundamental limitations, including low capture rates (<5%) and weak signal-to-background ratios, resulting in relatively low sensitivity.^{53–55} To determine whether ML-PNPs could be used as reporter probes in CLFIA and overcome the limitations of AuNPs, we compared their performance against size-matched ~50 nm AuNPs on nitrocellulose membranes. For all comparative assays, the reference AuNPs were the same ~50 nm citrate-stabilized Au cores used as precursors for ML-PNP synthesis, prior to Ag and outer Au shell deposition. Comparisons between AuNPs and ML-PNPs were therefore conducted using particles with closely matched overall size (~50 nm versus ~54–58 nm), thereby allowing the observed differences in optical and assay performance to be attributed primarily to multilayer architecture rather than particle size. We first established the minimum number of particles required to generate a visibly detectable signal by drop-casting serial dilutions of AuNPs (Figure S12) and ML-PNPs (Figure S13) at specific concentrations onto a nitrocellulose membrane. Nominal calculations indicated that approximately 1.25×10^6 ML-PNPs were sufficient to produce a visible spot, compared to 7.25×10^6 AuNPs, a 6-fold reduction in particle number, demonstrating a substantially lower detection threshold for ML-PNPs. Next, to directly compare the performance of ML-PNPs and AuNPs in the CLFIA format, we used the

well-characterized biotin-streptavidin conjugate system, which exhibits extremely high binding affinity. Both reporters (AuNPs and ML-PNPs) were functionalized with biotin; streptavidin served as the target analyte, and biotinylated bovine serum albumin (biotin-BSA) was immobilized as the capture ligand (Figures S14 and S15). The optimal biotin concentration of 3 $\mu\text{g/mL}$ was selected for surface functionalization of both ML-PNPs and AuNPs based on concentration screening 1–8 $\mu\text{g/mL}$, which showed that lower concentrations (1–2 $\mu\text{g/mL}$) resulted in insufficient surface coverage and weaker binding, while higher concentrations ($\geq 4 \mu\text{g/mL}$) did not improve performance and may have caused surface crowding (Figures S17 and S18). Test spots on the nitrocellulose membrane were prepared by applying 0.3 μL of 1 mg/mL biotinylated BSA, and LFA strips were assembled as described in the Materials and Methods. LFA strips were then challenged with streptavidin standards ranging from 0.1 pg/mL to 100 $\mu\text{g/mL}$, while a fixed input of each reporter (1 OD, biotin-conjugated AuNPs or ML-PNPs) was applied (Figure S16). Colorimetric reporters flow along the nitrocellulose membrane after binding to the target analyte (streptavidin) via capillary action and are then captured by the capture ligand (biotin-BSA), resulting in nanoparticle accumulation at the test spot. Accumulation of a sufficient number of reporters produces a red signal in the case of AuNPs and a dark red/brownish signal in the case of ML-PNPs, indicating a positive result for the target analyte. In both cases, 1 OD input generated readily visible test spots, with signal intensity decreasing as streptavidin concentration decreased. Notably, ML-PNPs enabled visual detection of streptavidin down to 25 pg/mL, whereas AuNPs were limited to 75 pg/mL, representing a 3-fold improvement in visual detection limit (Figures S14 and S15). These results establish that ML-PNPs serve as brighter, more sensitive colorimetric reporters for lateral flow immunoassays, enabling lower limits of detection without increasing particle size or input.

Antibody Conjugation and Surface Characterization of ML-PNPs

Having established that ML-PNPs function as highly sensitive colorimetric reporters, they were then functionalized with goat anti-human IgG + IgM (H&L) detector antibodies (Abs) via noncovalent physisorption. Bioconjugation was performed at an optical density of 1 (OD = 1) for all reporter batches, with OD measured at each particle's localized plasmon resonance (LSPR) maximum. OD was used as the normalization parameter because it reflects the bulk optical reporter input responsible for the visible colorimetric signal in the LFIA; matching OD therefore provides a practical basis for comparing AuNP and ML-PNP performance under equivalent optical input. Because AuNPs and ML-PNPs have different extinction coefficients and spectral profiles, OD-matched suspensions are not expected to contain equal particle numbers or equal total geometric surface area. Accordingly, the comparison between AuNPs and ML-PNPs should be interpreted as a matched optical-input comparison, rather than a particle-number or surface-area-normalized comparison. This normalization strategy is appropriate for the objective of this study, which was to compare assay sensitivity and visual signal strength under equivalent reporter signal input. UV-vis spectra of all bioconjugated ML-PNPs exhibited sharp and intense LSPR bands, indicating that no aggregation occurred during the bioconjugation step (Figure S19). To further confirm the presence of Abs on the

ML-PNP surface, XPS and FT-IR analysis were employed. High-resolution C 1s XPS spectra were deconvoluted using consistent peak assignments for both unmodified and antibody-conjugated ML-PNPs. Before conjugation, the C 1s spectrum of the ML-PNPs (Figure 4a) was deconvoluted into components assigned to aliphatic C–H/C–C (285 eV), C–O (287 eV), and a minor N–C=O (~288 eV) contribution, consistent with adsorbed citrate and adventitious carbon on the nanoparticle surface. After antibody attachment, the overall C 1s envelope (Figure 4b) broadened with increased

contributions from C–O/C–N (286 eV) and prominent amide (N–C=O)/C=O (287.8–288.2 eV) and carboxyl O–C=O (288.8–289.0 eV) components, reflecting the introduction of protein-like material on the nanoparticle surface. The relative increase of heteroatom-containing carbon peaks compared with the C–H/C–C component is typical of antibody layers rich in peptide backbones and amino acid side chains. The S 2p region of unmodified ML-PNPs (Figure 4c) showed a low-intensity $S_{2p_{3/2}}$ peak attributed to residual sulfur-containing species from synthesis or trace contaminants. Following conjugation, the S 2p

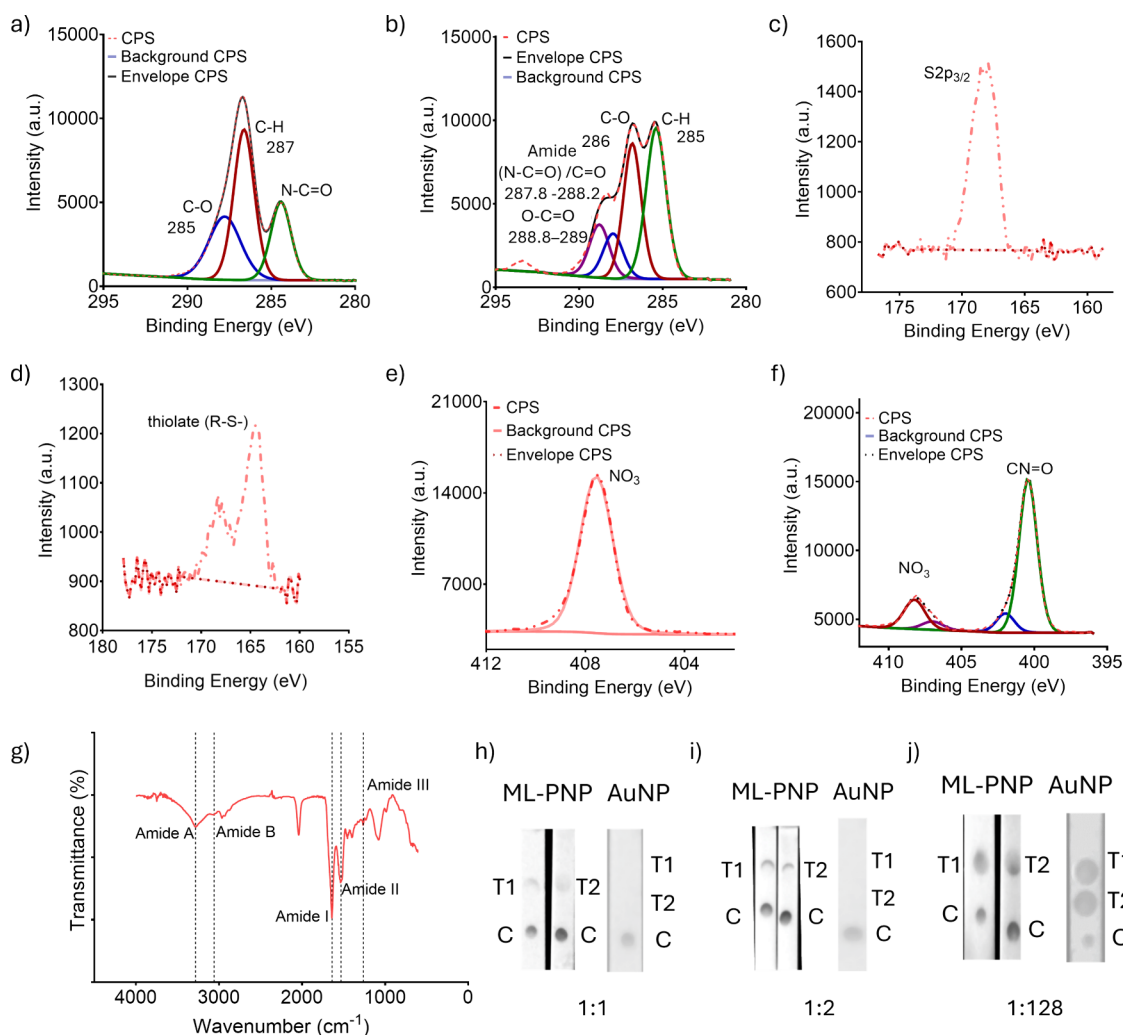


Figure 4. XPS characterization of ML-PNPs and Abs conjugates. High-resolution C 1s spectra of a) unmodified ML-PNPs and b) antibody-conjugated ML-PNPs. Spectra were deconvoluted using consistent peak assignments: C–C/C–H (adventitious and aliphatic carbon), C–O/C–N (hydroxyl, ether, and amine environments), C=O (carbonyl), and N–C=O (amide). The unmodified ML-PNPs show a simpler carbon envelope dominated by C–C/C–H and C–O contributions from residual citrate and adventitious carbon. Following antibody conjugation, the spectrum broadens with increased contributions from C–O/C–N, C=O, and a prominent N–C=O amide component, consistent with the introduction of peptide backbone and amino acid side-chain functional groups from the antibody layer. c) The S 2p region of ML-PNPs conjugated with Abs reveals the emergence of a lower binding energy thiolate (R–S–) component, indicating chemisorption of the Abs via Au–S bonds. d) The S 2p spectrum of ML-PNPs conjugated with Abs reveals the emergence of a lower binding energy thiolate (R–S–) component, indicating chemisorption of the Abs via Au–S bonds. e) N 1s spectrum of plain ML-PNPs is dominated by a nitrate-related signal from residual synthesis reagents, while f) antibody-conjugated ML-PNPs show an additional higher-binding-energy CN=O component attributed to amide groups in the antibody layer. g) FTIR spectrum of antibody-conjugated ML-PNPs. Characteristic protein absorption bands are observed, including the Amide I band (~1650 cm⁻¹), arising primarily from C=O stretching vibrations of peptide bonds, and the Amide II band (~1540 cm⁻¹), attributed to N–H bending and C–N stretching modes in the antibody backbone. Additional features include broad O–H/N–H stretching (~3000–3500 cm⁻¹) and C–H stretching (~2900 cm⁻¹) contributions. h–j) compare LFIA strips prepared with ML-PNP–Ab conjugates (left) or conventional citrate-stabilized AuNP–Ab conjugates (right). TT and NT antibodies were detected on separate strips for ML-PNPs and tested with three TT-positive samples having different NT titers: h) 1:1, i) 1:2, and j) 1:128. Under these conditions, ML-PNP–Ab conjugates consistently yield strong, well-defined signals at both test lines (T1, T2) and the control line (C), whereas conventional AuNPs show weaker or absent test line development.

spectrum (Figure 4d) exhibited a well-defined thiolate (R-S^-) signal at lower binding energy (~ 163 eV), consistent with chemisorption of cysteine residues or thiol-containing linkers from the antibody layer onto the outer Au shell via Au-S bonds. The emergence and enhancement of this thiolate peak support the formation of strong coordination bonds that anchor the antibodies to the nanoparticle surface. Finally, the N 1s region of unmodified ML-PNPs (Figure 4e) was dominated by a single peak assigned to nitrate (NO_3^-) at ~ 407 eV, arising from residual ionic species associated with synthesis. After antibody immobilization, the N 1s spectrum (Figure 4f) broadened and was fitted with multiple components corresponding to different nitrogen environments within the protein, such as amide N in peptide bonds (CN=O , ~ 400 eV) and amine groups, while the nitrate contribution became comparatively less prominent. This increase in chemically diverse nitrogen species is consistent with the presence of a protein layer coating the nanoparticle surface. Collectively, the emergence of amide contributions in the C 1s and N 1s spectra, the appearance of a pronounced thiolate S 2p peak, and the redistribution of nitrogen environments from nitrate to protein-like functionalities confirm successful antibody immobilization on the ML-PNP surface. Additionally, FT-IR spectroscopy confirmed antibody conjugation onto NPs and assessed protein integrity. The spectrum (Figure 4g) shows characteristic protein amide vibrations. A broad Amide A band (~ 3283 cm^{-1}) from N-H stretching and a weaker Amide B band (~ 3059 cm^{-1}) indicate peptide bonds and hydrogen bonding typical of antibodies on nanomaterial surfaces. A strong Amide I band (~ 1639 cm^{-1}), associated with C=O stretching, shows a slight red shift, suggesting β -sheet formation or partial unfolding due to adsorption onto ML-PNP. The presence of the Amide II (~ 1531 cm^{-1}) and Amide III (~ 1262 cm^{-1}) bands confirms the preservation of the peptide backbone structure. Overall, the clear Amide I–III features confirm successful antibody immobilization and indicate that the protein retains its essential structural framework, thereby supporting its use in downstream lateral flow assay applications.

Detection of TT Antibodies and Clinical Rationale for Dual IgM/IgG Recognition

Having established that ML-PNPs can be effectively conjugated with antibodies and used as sensitive colorimetric reporters, we assessed their ability to detect treponemal (TT) antibodies in a lateral flow assay format. TT antibodies include IgM and IgG produced against *T. pallidum*-specific membrane lipoproteins, primarily TpN15, TpN17, and TpN47.^{32,52,55–59} During natural infection, seroconversion to these antigens generally occurs within 2–3 weeks and, in most cases, persists for life, even after antimicrobial therapy, unless treatment is administered very early.^{2–8} The stage-dependent kinetics of TT antibodies present a diagnostic challenge: IgM antibodies appear early during primary infection but decline rapidly following treatment or disease progression, whereas IgG antibodies appear slightly later but persist over longer time frames (Figure 1b). Traditional rapid tests relying solely on IgM detection therefore yield false negatives in latent, secondary, and reinfection cases, limiting their clinical and epidemiological utility. In contrast, combined IgM/IgG detection extends diagnostic coverage across the full immunological spectrum, capturing early seroconversion ($\text{IgM}^+/\text{IgG}^-$), past or treated infection ($\text{IgM}^-/\text{IgG}^+$), and seronegative status, thereby reducing misclassification and enhancing sensitivity across disease stages.

To implement this dual-antibody recognition strategy, two reporter conjugates were prepared for both ML-PNPs and AuNPs: a goat anti-human IgG/IgM (H&L) detector conjugate (6 $\mu\text{g/mL}$, 1 OD) for signal development at the test lines, and a goat anti-rabbit IgG conjugate for control-line signal development. The goat anti-human IgG + IgM (H&L) detector was selected to provide broad isotype coverage across infection stages, with IgM predominating in early infection and IgG in later and post-treatment stages, and functions as a generic signal-generating element for antigen-bound human immunoglobulin rather than as a source of pathogen specificity. The test line (T1) was prepared by applying 0.3 μL of 0.5 mg/mL Tp15–Tp17–Tp47 recombinant fusion antigen onto a nitrocellulose membrane. This multiepitope construct integrates three major immunodominant *T. pallidum* antigens, offering broad reactivity with antibodies generated during both primary and latent infection and improving diagnostic sensitivity compared to single-antigen formats. The control line (C) was prepared with 0.3 μL of 0.25 mg/mL rabbit IgG, which is captured by the goat anti-rabbit IgG conjugated reporters present in the conjugate mixture. Species compatibility was considered explicitly in the design: the detector is goat-hosted and directed against human immunoglobulin, whereas the control channel is an orthogonal species pair in which immobilized rabbit IgG is bound by goat anti-rabbit IgG-conjugated reporters. Rabbit IgG is absent from human serum, and the control reporter does not react with human IgG, so the control channel reports strip function independently of syphilis status without competing with the analyte channel. LFA strips were challenged with 20 μL of syphilis antibody-positive reference sera obtained from the CDC repository. Across the panel, ML-PNP reporters consistently produced higher T1 intensities than AuNPs, with clear visual differentiation at both low (Figure 4h,i) and high (Figure 4j) clinical titers. Notably, AuNP-based strips did not show visible T1 signals for two samples, whereas ML-PNP strips maintained reliable reactivity across all samples, indicating superior analytical sensitivity for TT antibody detection. This combined IgM/IgG detection capability, enabled by the enhanced optical output of ML-PNPs, recapitulates the CDC-endorsed reverse sequence algorithm in a portable, field-deployable format, connecting the precision and stage-resolving ability of laboratory-based immunoassays with the speed, simplicity, and instrument-free operation required for POC diagnostics.

Detection of NT Antibodies for Disease Activity Monitoring

Having confirmed superior performance for TT antibody detection, we expanded the assessment to NT antibodies to enable comprehensive disease characterization. Unlike TT antibodies, which remain detectable lifelong, NT antibodies are directed against cardiolipin–lecithin–cholesterol complexes released during *T. pallidum* infection and tissue damage.^{32,52,55–59} Their titers correlate with disease activity and typically decline following successful antimicrobial therapy, serving as quantitative markers for treatment monitoring and reinfection detection.^{32,52,55–59} Evaluating NT antibodies alongside TT antibodies, therefore, differentiates active from past infection and facilitates clinical staging, critical functions for implementing the reverse algorithm at the POC.^{32,52,55–59} The test line (T2) was prepared by applying 0.3 μL of 0.5 mg/mL Venereal Disease Research Laboratory (VDRL) antigen (Arlington Scientific) onto a nitrocellulose membrane. VDRL antigen is a standardized suspension containing cardiolipin, cholesterol, and lecithin that detects reagin antibodies

produced in response to cellular damage caused by *T. pallidum* infection. The control line and conjugate configurations were identical to those described for TT detection. LFA strips were challenged with 20 μL of syphilis reference sera from the CDC repository spanning RPR titers from 1:1 to 1:128. ML-PNP reporters consistently produced higher T2 intensities than AuNPs across all titers. Critically, AuNP-based strips did not show visible T2 signals at low titers (1:1, Figure 4h,i; 1:2), whereas ML-PNP strips maintained reliable reactivity at these concentrations, which are clinically relevant for monitoring treatment response in late latent or previously treated cases. At higher titers (1:128, Figure 4j), both reporters generated visible signals, though ML-PNPs remained visibly brighter. By integrating both TT and NT antibody detection onto a single strip using ML-PNP reporters, this platform enables the same-visit implementation of the reverse-sequence algorithm without laboratory instrumentation. The enhanced sensitivity at low NT titers, where conventional AuNP-based tests fail, addresses a critical gap in POC syphilis diagnostics and provides a scalable, affordable solution for syphilis surveillance and screening in resource-limited settings.

Sample Volume Optimization and Multiplexed Detection of TT and NT Antibodies

Having validated ML-PNP performance for individual detection of TT and NT antibodies, the assay was configured for simultaneous dual-marker detection on a single strip, enabling same-visit implementation of the reverse-sequence algorithm. The

multiplexed CLFIA incorporated three lines on the nitrocellulose membrane: a treponemal test line (T1) spotted with *Treponema pallidum* Tp15–Tp17–Tp47 recombinant antigen, a non-treponemal test line (T2) spotted with VDRL antigen, and a control line (C) spotted with rabbit IgG (Figure 5a). Sample volume was optimized using a preliminary dot assay format. Serial dilutions of serum volume (10–30 μL) combined with chase buffer (50–150 μL) were tested on spotted nitrocellulose membranes. A 20 μL serum input, followed by 100 μL of chase buffer, produced the most uniform signal distribution without membrane oversaturation or edge effects (Figure S20). After establishing the optimal sample volume, the assay was transitioned to a line-based format and tested with ML-PNP and AuNP conjugates. Using the optimized 20 μL serum input, well-defined signals were observed at T1, T2, and C lines with ML-PNP reporters, confirming that simultaneous detection of both antibody classes on a single strip was operationally feasible (Figure S20). Scanning electron microscopy (SEM) of the nitrocellulose membrane before (Figure S21a) and after (Figure S21b) sample flow revealed the typical porous fibrous network that supports capillary transport. Post-assay imaging showed a dense accumulation of ML-PNPs in the test-line regions (Figure S21c, indicated by arrows), confirming effective particle capture and stable line formation under standard lateral flow conditions. These experiments established operational parameters for multiplexed detection and provided a foundation for subsequent clinical validation.

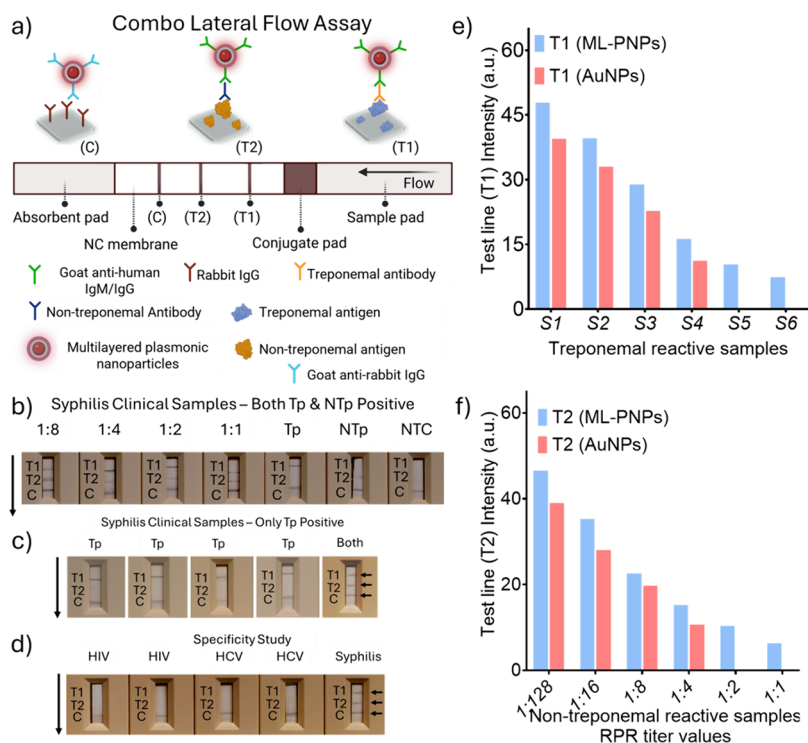


Figure 5. a) Schematic representation of the proposed combo lateral flow strip based on the novel multilayered plasmonic nanoparticles. b) Results were obtained from 36 serum samples using the proposed combo LFA. Sample numbers are indicated in Table 1. The figure shows results from 7 samples, in which the assay successfully detected low RPR titers (1:1 and 1:2). c) Results from 4 additional samples that were reactive only toward the Abbott treponemal test (Tp) and non-reactive in the non-treponemal test. d) Specificity study of the proposed LFA using the two most common blood-borne pathogens, HIV and HCV. The LFA showed a signal only for syphilis-positive samples, with no signal detected for the other cases. T1: treponema, T2: non-treponema and C: control. Test line (T1 and T2) intensity comparison between multilayered plasmonic NP and standard 50 nm gold nanoparticles. (The arrow indicates the direction of the flow). For treponemal e) and non-treponemal f) assay. These nanoparticles can deliver 19–46% greater sensitivity.

Clinical Validation with De-Identified Patient Specimens

Finally, to evaluate the clinical performance of the multiplexed ML-PNPs CLFIA, the assay was validated using de-identified patient serum samples obtained from the CDC and Boca Biolistics. A total of 36 ($N = 36$) serum samples, including syphilis-positive cases ($N = 32$) confirmed by reference methods and healthy controls ($N = 4$), were analyzed. Sample positivity was established by standard treponemal (Abbott TP-PA) and non-treponemal (RPR) serological tests (Table 1 and Table S2). The ML-PNP CLFIA successfully detected TT antibodies across the full range of clinical titers. Representative strips from syphilis-reactive samples showed well-defined signals at both T1 (treponemal) and T2 (non-treponemal) lines, clearly distinguishable from non-reactive controls (Figure 5b). Critically, the assay detected samples with RPR titers as low as 1:1 and 1:2 (Figure 5b), which represent early-stage or low-activity infections that are challenging for conventional AuNP-based rapid tests. The ability to reliably identify low-titer specimens addresses a known limitation of current POC serodiagnostics and is essential for early diagnosis and treatment monitoring. To assess the assay's ability to differentiate infection stages, strips were challenged with clinical specimens that were exclusively treponemal-reactive (TP-PA-positive, RPR-nonreactive; $N = 4$, Table 1). These samples yielded signals only at T1, with no reactivity at T2 (Figure 5c), demonstrating that the multiplexed format distinguishes past or successfully treated infection from active disease, a critical advantage over treponemal-only rapid tests that cannot provide staging information. Analytical specificity was evaluated against human immunodeficiency virus HIV-1 ($N = 2$) and hepatitis C virus (HCV) ($N = 2$), which were selected as cross-reactivity controls because they represent the most clinically relevant co-infections in syphilis-affected populations. Sexually transmitted and bloodborne infections frequently co-occur due to shared transmission routes and risk factors (injection drug use, sexual contact), and serological cross-reactivity is a recognized concern, particularly because polyclonal immune activation during HIV infection can produce false-positive results on non-treponemal tests. No false-positive signals were observed at T1 or T2 for HIV or HCV samples, whereas a syphilis-positive control produced strong signals at both test lines (Figure 5d). This lack of cross-reactivity indicates that the selected treponemal and cardiolipin antigens, together with the assay blocking conditions, limit nonspecific binding despite the known immunological interference associated with these co-infections, and is consistent with reported specificity values above 95% for dual-marker syphilis rapid tests.

Because a pan-specific anti-human IgG + IgM detector is used, we further considered whether immunoglobulins raised against

concurrent infections could competitively saturate the reporter and suppress true-positive signals. This is unlikely on quantitative grounds: human serum contains approximately 10 mg/mL of total polyclonal IgG and IgM, exceeding any pathogen-specific antibody population by several orders of magnitude, and the reporter conjugate is formulated in excess to accommodate this background. The immunoglobulin contributed by a concurrent infection is therefore small relative to the bulk immunoglobulin load already present in every specimen. Consistent with this, the assay retained sensitivity at the lowest clinically relevant antibody concentrations, generating clear non-treponemal signals from RPR 1:1 specimens that size-matched AuNP reporters failed to detect, indicating that signal generation is not detector-limited even when specific antibody is scarce against a full physiological immunoglobulin background.

Quantitative signal enhancement was assessed using an ESEQuant Lateral Flow Reader. Across the clinical panel, ML-PNP-based strips exhibited 19–46% higher test-line intensities compared to AuNP-based strips for both TT and NT antibody detection (Figure 5e,f), with the greatest improvements observed at low antibody concentrations. This quantitative advantage translated directly to improved visual detection limits, enabling reliable same-visit diagnosis without instrumentation. Overall, the multiplexed ML-PNP CLFIA demonstrated 100% concordance with reference RPR and Abbott TP-PA methods across all 36 clinical specimens (Table 1), with enhanced sensitivity for low-titer samples (RPR 1:1–1:2) that are frequently missed by conventional lateral flow assays. By enabling simultaneous detection of treponemal and non-treponemal antibodies with performance comparable to laboratory gold standards, this platform provides a practical solution for implementing the CDC-endorsed reverse sequence algorithm at the POC.

CONCLUSIONS

In summary, we have demonstrated that Au–Ag–Au multilayer plasmonic nanoparticles, rationally designed through interfacial plasmon hybridization, serve as high-performance colorimetric reporters that overcome the sensitivity limitations of conventional AuNP-based lateral flow immunoassays. By embedding an optically active Ag interlayer between chemically stable Au domains, the ML-PNP architecture achieves up to ~18-fold higher absorption and markedly enhanced scattering while maintaining sub-60 nm hydrodynamic diameters fully compatible with capillary-driven membrane transport. Applied to a dual-marker syphilis CLFIA, the platform enables simultaneous detection of TT and NT antibodies on a single strip, operationalizing the CDC-endorsed reverse testing algorithm at the POC with a 3-

Table 1. Clinical Characterization of De-Identified Serum Panels from Boca Biolistics and the CDC Was Used to Evaluate the ML-PNPs Based on the Combo LFIA^a

sample source	samples (N)	Abbott Syphilis (treponemal)	RPR (rapid plasma reagin) (non-treponemal)	LFIA treponemal (T1)	LFIA non-treponemal (T2)
Boca Bio	21	reactive - 19 non-reactive - 2	reactive - 1:1, 1:2, 1:4, 1:8 non-reactive - 8	reactive - 19 non-reactive - 2	reactive - 13 non-reactive - 8
CDC	15	reactive - 9 non-reactive - 6	reactive - 1:1, 1:2, 1:4, 1:16, 1:128 non-reactive - 8	reactive - 9 non-reactive - 6	reactive - 9 non-reactive - 8

^aThe table summarizes sample numbers; reference treponemal results (Abbott syphilis TP); corresponding non-treponemal RPR reactivity and titer distributions; and concordant treponemal (T1) and non-treponemal (T2) outcomes obtained with the developed LFIA.

fold improvement in visual detection limit, a 6-fold reduction in the required particle count, and 19–46% higher test-line intensities relative to standard AuNP (~50 nm) reporters across 36 clinical serum specimens spanning RPR titers from 1:1 to 1:128. The assay reliably identified low-titer samples that conventional reporters failed to detect, showed concordance with reference RPR and TP-PA methods, and exhibited no cross-reactivity with HIV-1 or HCV antibodies. Analytical specificity was assessed using HIV-1 ($N = 2$) and HCV ($N = 2$) specimens, which establishes the absence of false-positive treponemal or non-treponemal signals from these co-infection-relevant sera but does not directly test for signal suppression in specimens harboring concurrent infections. A dedicated interference study using serologically confirmed co-infected specimens, or syphilis-positive sera spiked with heterologous antibody, would provide direct confirmation that positive detection is retained under co-infection, and is a priority for future validation. Beyond syphilis serodiagnosis, these results establish interfacial plasmon hybridization in sub-60 nm colloidal particles as a generalizable nanomaterials design principle for transport-limited colorimetric biosensing. Future work will focus on multi-site clinical validation in whole-blood matrices from diverse populations, integration of on-strip plasma separation for true fingerstick operation, and expansion of the multiplex panel to additional infectious disease markers, with the aim of translating this plasmonically engineered reporter platform into a scalable, field-deployable diagnostic tool.

MATERIAL AND METHODS

Materials

All the chemicals and biological materials were purchased from reputable vendors and used without further purification. Gold (III) chloride trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$), trisodium citrate dihydrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$), silver nitrate (AgNO_3), hydroquinone, L-ascorbic acid and molecular grade water were purchased from Sigma Aldrich (Saint Louis, MO). Antigen T1 (recombinant *Treponema pallidum* p15/p17/p47) was purchased from Meridian Life Science, Inc. (Cincinnati, OH; Catalog No. R01682). Antigen T2 (non-*Treponema pallidum*, VDRL; Catalog No. 950010) was obtained from Arlington Scientific (Springville, UT). The capture antibody (CP), rabbit anti-human IgG + IgM (H&L), and the control line antibody (CL), anti-rabbit IgG, were purchased from Abcam (Cambridge, MA; Catalog Nos. ab102411 and ab190492, respectively). The detection antibody, goat anti-human IgG + IgM (H&L), and the control-line reporter antibody, goat anti-rabbit IgG, were purchased from Abcam (Cambridge, MA; Catalog Nos. ab102411 and ab190492, respectively). Rabbit IgG (Catalog No. I5006), immobilized as the control-line ligand, was purchased from Sigma-Aldrich (Saint Louis, MO). Biotin-labeled bovine serum albumin (Catalog No. A8549) and streptavidin from Streptomyces avidinii (Catalog No. 9013-20-1) were purchased from Sigma-Aldrich (Saint Louis, MO). Lateral flow assay components, including sample pads (Catalog No. 8114-2250), conjugation pads (Standard 17; Catalog No. 8134-10078), and absorbent pads (CF6; Catalog No. 8116-6621), were obtained from Cytiva (Marlborough, MA). The nitrocellulose membrane (HF135) was sourced from Sigma-Aldrich (Saint Louis, MO). Syphilis clinical samples were partially sourced from the Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA) repository under a material transfer agreement (MTA). Additional samples were obtained from Boca Biolistics (Deerfield Beach, FL, USA). The Institutional Review Board approval (STUDY00022757) and oversight governed the collection and transfer of these clinical materials, ensuring compliance with ethical and safety guidelines for handling potentially infectious specimens.

Synthesis of Au@Ag@Au Multilayer Plasmonic Nanoparticles (ML-PNPs)

Preparation of 15 nm Au Nanoparticle (AuNP). 97 ml of deionized (DI) H_2O and 1 ml of 25 mM HAuCl_4 were added to a 250 ml round-bottom (RB) flask and heated to 100 °C under vigorous stirring (500 rpm). Once boiling, 3 ml of 1% (w/v) trisodium citrate solution was added in a single portion. The reaction mixture was maintained at 100 °C with continuous stirring for 30 min, during which the solution turned red, indicating AuNP formation. The heat source was then removed, and the colloidal suspension was cooled to room temperature (RT).^{23–25,33–49}

Preparation of 40 nm AuNPs. To obtain 40 nm AuNPs, 92.8 ml of DI H_2O and 5 ml of 1% (w/v) trisodium citrate solution were placed in a separate 250 ml RB flask and heated to 90 °C under stirring. Subsequently, 1 ml of 25 mM HAuCl_4 was added. Under vigorous stirring, 1 ml of 25 mM hydroquinone was added, causing the solution to turn blue. Immediately, 4.2 ml of the previously prepared 15 nm AuNP seed solution was added to the reaction mixture, which produced a reddish-purple color, consistent with the formation of ~50 nm AuNPs. The dispersion was stirred at RT for 2 h.^{23–25,33–49}

Synthesis of Au@Ag Core–Shell Nanoparticles (Au@Ag NP). Au@Ag nanoparticles were prepared by depositing a silver shell on the 50 nm Au cores. 50 ml of the 50 nm AuNP dispersion was transferred to a 250 ml RB flask and equilibrated at 30 °C under stirring. 0.25 ml of 0.1 M L-ascorbic acid was added, and the mixture was allowed to age for 20 min. 1.25 ml of 10 mM silver nitrate (AgNO_3) solution was then added dropwise. The reaction was maintained at 30 °C with continuous stirring for 1 h to complete Ag shell growth.^{23–25,33–49}

Synthesis of Au@Ag@Au Multilayer Plasmonic Nanoparticles (ML-PNP). For the outer Au shell, 50 ml of the Au@Ag NP dispersion was placed in a 250 ml RB flask and heated to boiling under vigorous stirring. After 10 min of boiling, 8 ml of 1 mM HAuCl_4 was injected into the flask via a syringe pump at 20 ml/h using a 10 ml BD plastic syringe, with constant stirring. Following completion of the addition, the reaction mixture was allowed to cool to RT, yielding the final ML-NP.^{23–25,33–49}

Electromagnetic FDTD Simulation

The electromagnetic field of the ML-PNPs was simulated using Ansys Lumerical FDTD. The complex refractive indices of gold (Au) and silver (Ag) were incorporated using Johnson and Christy's optical data.⁶⁰ A uniform mesh size of 1 nm was applied across the simulation domain. The total-field scattered-field (TFSF) source was employed to study the scattering behavior of the microparticles, with perfectly matched layer (PML) boundary conditions to ensure accurate computation of absorption cross-sections. The excitation wavelength was varied from 300 nm to 800 nm. A frequency-domain field monitor was used to capture the local electric field distribution within the plasmonic structures.

For the core–shell–shell configuration, mesh ordering was used to assign material priorities in overlapping regions: a gold core (radius = 13 nm), a silver inner shell (thickness = 10 nm), and a gold outer shell (thickness = 10 nm). The simulation domain dimensions were 50 nm × 50 nm × 50 nm for Au and Au–Ag (core–shell) systems, and 100 nm × 100 nm × 100 nm for the Au–Ag–Au (core–shell–shell) structure.

UV–Vis Spectroscopy

For absorbance measurements, 100 μL of the synthesized nanoparticle suspension was transferred into a 96-well plate, and endpoint as well as spectral scans were recorded using a BioTek Synergy Neo2 microplate reader. Each experiment was performed at least three times, and the resulting spectra was averaged. The absorbance spectra were then normalized on a 0–1 scale using min–max normalization,

$A_{\text{norm}} = \frac{A - A_{\text{min}}}{A_{\text{max}} - A_{\text{min}}}$ where A is the measured absorbance, and A_{min} and A_{max} are the minimum and maximum absorbance values for each spectrum, respectively. The normalized data were subsequently compared to standardize assay parameters across independent experiments.^{42–49,61–71}

Dynamic Light Scattering

The synthesized nanoparticle suspension (500 μL) was diluted with 500 μL DI H_2O to obtain a 1 mL solution, which was loaded into a disposable quartz cuvette. DLS measurements were performed on a Zetasizer Nano series instrument (Malvern Instruments) equipped with a 633 nm laser. Each sample was measured in triplicate, and the hydrodynamic size distributions were averaged using the Zetasizer software. For analysis, the refractive index and viscosity of water were set to 1.33 and 0.8872 cP, respectively (at 25 $^\circ\text{C}$).^{42–49,61–71}

Nanoparticle Tracking Analysis

NTA measurements were performed using a ZetaView instrument (Particle Metrix, Germany) equipped with a 488 nm laser and scatter detection, controlled by ZetaView software (version 8.05.12 SP2). Nanoparticle suspensions were diluted 1:4000 in Milli-Q water (electrolyte “MQ”; pH 7.4; conductivity $\approx 12.3 \mu\text{S}/\text{cm}$) to obtain an appropriate particle count per frame. Measurements were carried out at 25 $^\circ\text{C}$ in stationary layer mode (stationary, 1 cycle) with a sensed electric field of 3.33 V/cm. Videos were acquired using a camera calibration of 0.712 $\mu\text{m}/\text{px}$, and analysis thresholds were set to a minimum area of 10 pixels, maximum area of 1000 pixels, and minimum brightness of 20. For each sample, multiple videos were recorded, and particle trajectories were tracked to determine particle concentration and zeta potential using the instrument’s Brownian motion and electrophoretic mobility algorithms with the Smoluchowski approximation. Average particle concentration in the diluted samples was converted to the original stock concentration using the known dilution factor, and all values are reported as the mean of at least three independent experiments.

Zeta Potential

The zeta potentials of the particles were measured in triplicates using the Malvern Zetasizer Nano Series by injecting optimally diluted concentrations of the water-dispersed nanoparticle solutions.^{42–49,61–71}

Transmission Electron Microscopy

10 μL of NP dispersion was drop cast onto freshly glow-discharged carbon-coated copper grids and blotted to dry. Imaging, SAED, and EDS were performed on a Talos F200X (Thermo Fisher Scientific) TEM operated at 200 kV and equipped with a Ceta CMOS CCD camera and four in-column SDD Super-X detectors. For EDS, elemental maps were acquired for approximately 5 min (beam current = 0.12 nA) and analyzed using Velox software. ImageJ was used for additional image analysis. D-spacing measurements were made using the plot profile tool and measuring the distance between extrema. Three measurements were taken and averaged.^{42–49,61–71}

Hyperspectral Imaging

Samples for hyperspectral enhanced dark-field microscopy were prepared by diluting each suspension 1:7 (v/v) in water; 10 μL was drop-cast onto glass slides, overlaid with a coverslip, and edge-sealed with clear nail polish to prevent evaporation and oil ingress. Imaging used a CytoViva enhanced dark-field system (Auburn, AL, USA) on an Olympus microscope with a 60 $\times$ oil-immersion objective and a tungsten-halogen illuminator. Hyperspectral data were acquired in line-scan mode with a transmission-grating spectrograph operating in the VNIR range (400–1000 nm) and recorded on a CCD (PIXIS-400BR, Princeton Instruments), producing an x - y - λ datacube. Datacubes were processed in ENVI 4.8 (CytoViva): one-dimensional scattering spectra were extracted from 15 user-defined regions of

interest (ROIs) per sample to build a spectral library, and whole-image classification used the Spectral Angle Mapper (SAM) algorithm to identify pixels sharing those spectral signatures. Spectra were normalized before quantifying peak positions and spectral shifts, which were used to assess the formation of core-shell particles.^{42,51}

X-ray Photoelectron Spectroscopy

XPS experiments were performed using a Physical Electronics VersaProbe III instrument equipped with a monochromatic Al $K\alpha$ X-ray source ($h\nu = 1486.6 \text{ eV}$) and a concentric hemispherical analyzer. Charge neutralization was performed using both low energy electrons ($<5 \text{ eV}$) and argon ions. The binding energy axis was calibrated using sputter cleaned Cu (Cu $2p_{3/2} = 932.62 \text{ eV}$, Cu $3p_{3/2} = 75.1 \text{ eV}$) and Au foils (Au $4f_{7/2} = 83.96 \text{ eV}$). $\dagger$ Peaks were charge referenced to CH_x band in the carbon 1s spectra at 284.8 eV. Measurements were made at a takeoff angle of 45 $^\circ$ with respect to the sample surface plane. This resulted in a typical sampling depth of 3–6 nm (95% of the signal originated from this depth or shallower). Quantification was done using instrumental relative sensitivity factors (RSFs) that account for the X-ray cross section and inelastic mean free path of the electrons. On homogeneous samples major elements ($>5 \text{ atom } \%$) tend to have standard deviations of $<3\%$ while minor elements can be significantly higher. The analysis size was $\sim 200 \mu\text{m}$ in diameter.^{42–49,61–71}

Fourier Transform Infrared (FTIR) Spectroscopy

Infrared spectroscopy was performed in attenuated total reflection (ATR) mode on a Bruker Hyperion 3000 microscope equipped with a liquid nitrogen cooled mercury cadmium telluride (MCT) detector and a 20X germanium ATR objective with a 150 μm germanium tip. S. A total of 500 scans was averaged at a resolution of 6 cm^{-1} , and absorbance was calculated by referencing the clean bare Germanium tip.

ML-PNPs and Streptavidin Conjugation

Biotin Streptavidin LFA test spots were created by depositing 0.5 μL of 5 mg/mL biotinylated BSA onto the nitrocellulose membrane. The LFA strips were prepared as previously outlined. For biotin-streptavidin LFAs utilizing AuNP and ML-PNPs, 1 μL of biotinylated AuNPs or ML-PNPs was mixed with 99 μL of streptavidin standard solutions ranging from 0.1 pg/mL to 100 $\mu\text{g}/\text{mL}$ in 96-well plates. This allowed streptavidin to bind with the biotinylated nanolabels. Duplicate LFA strips were exposed to both sample and standard solutions for 20 min.

To functionalize nano labels with streptavidin, 1 μL of 3 $\mu\text{g}/\text{mL}$ streptavidin (or BSA-biotin or detection antibody) was added to 1 mL of OD1 nano labels. The mixture was incubated for 1 h on a shaker at room temperature. To stabilize the particles, 1 μL of 10 mg/mL BSA was added, and the mixture was further incubated for 20 min. Unbound protein was removed by washing the solution four times with pH 10 DI water (prepared with 1 μL NaOH in 10 mL water). Finally, nanolabels were redispersed in 1% BSA in 1 $\times$ PBS solution for use in the LFAs. For functionalizing nanolabels with antibodies (goat anti-Human IgM/IgG detection antibody and goat anti-rabbit IgG control line reporter antibody), a similar process was employed with different concentrations.

ML-PNPs and IgG/IgM Conjugation

Antibodies were bound to the surface of AuNPs using passive adsorption. Stable conjugation requires careful control of conditions, such as pH and antibody concentration. To determine the optimal pH, the pH of ML-PNPs and AuNPs was adjusted to different levels (6, 7, 8, 9) by adding 0.2 M K_2CO_3 . Subsequently, 100 μL of goat anti-human IgG/IgM antibody solution (1 $\mu\text{g}/\text{mL}$) was added to microwell plate tubes containing 200 μL of ML-PNPs and AuNPs, which were rotated for 60 min at 200 rpm. Then, 20 μL of NaCl (1.5 M) was added to each tube and incubated for 15 min, with careful observation of the resulting color change. The absorbance of the solutions before and after conjugation was measured using UV-vis spectrophotometry. To determine

the optimal antibody concentration, 100 μL of different antibody concentrations (2, 4, 6, and 8 $\mu\text{g}/\text{mL}$) were added to 200 μL of ML-PNPs at the optimized pH and incubated for 30 min. Subsequently, 20 μL of NaCl (1.5 M) was added to each solution and incubated for an additional 15 min. The absorbance of the solutions before and after conjugation was measured using UV–vis spectrophotometry.

Lateral Flow Test Strip Preparation and Sample Testing

The lateral flow strip consisted of a sample pad, a glass-fiber conjugate pad (Standard 17, Cytiva), an HF135 nitrocellulose (NC) membrane, and a CF6 absorbent pad, all assembled on a polystyrene adhesive backing. The biorecognition elements, treponemal antigen as test line 1 (T1), non-treponemal antigen as test line 2 (T2), and rabbit IgG as the control line (C), were immobilized on the nitrocellulose membrane using a Claremont stripping machine and then dried at 37 $^{\circ}\text{C}$ for 2 h. Stripped membranes were held in a vacuum desiccator at room temperature for 1 h prior to assembly. The sample pad was pretreated with 1 $\times$ PBS containing 0.5% BSA and 0.05% Tween-20, then dried before assembly. In all experiments, nanoparticle–antibody reporter conjugates were dried onto the conjugate pad; conjugates were not premixed with the sample. The glass-fiber conjugate pad was first pretreated by immersion in conjugate pad buffer consisting of 1 $\times$ PBS with 10% sucrose, 0.5% BSA, and 0.1% Tween-20, then dried overnight at 37 $^{\circ}\text{C}$. Sucrose was included as a lyoprotectant to preserve conjugate integrity during drying and to promote reporter release during assay flow; BSA served as a blocking agent to minimize non-specific binding and improve flow consistency; and Tween-20 was included to enhance wetting and support uniform migration through the strip. Both reporter populations, ML-PNPs conjugated with goat anti-human IgG + IgM (H&L) for test-line signal development and ML-PNPs conjugated with goat anti-rabbit IgG for control-line signal development, were dispensed onto the pretreated pads at 1 OD using a Claremont sprayer. Loaded conjugate pads were dried at 37 $^{\circ}\text{C}$ for 2 h and stored with desiccant until strip assembly. The absorbent pad, nitrocellulose membrane, conjugate pad, and sample pad were affixed sequentially onto the polystyrene adhesive backing, with a 2 mm overlap maintained between each pair of adjacent components to ensure effective fluid transfer and continuous capillary flow from the sample pad through the conjugate pad and nitrocellulose membrane to the absorbent pad. Assays were performed by applying 20 μL of serum to the sample pad, followed by 100 μL of chase buffer. Strips were allowed to develop for 10 min at room temperature before analysis.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acssensors.6c02500>.

Supplementary computational and experimental data, including additional FDTD electromagnetic simulations and near-field maps; the seed-mediated synthesis process flow; supplementary TEM, DLS, zeta-potential, and UV–vis characterization of Au cores, Au–Ag intermediates, and ML-PNPs; optimization studies and development of lateral flow assay; and comparison of nanoparticle reporters for lateral flow signal enhancement (Table S1); and the clinical specimen panel results (Table S2) (DOCX)

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Notes

The authors declare the following competing financial interest(s): The Pennsylvania State University have jointly filed a Patent Cooperation Treaty (PCT) international patent application (PCT/US2025/052770) on behalf of D.P., K.D., and S.N., for the concept. The application titled “Platform for Direct Treponemal and Nontreponemal Lateral Flow Assay for Syphilis” was filed on 28 October 2025 and is still pending at the time of publication. D.P. is also the founder or co-founder of three university-based startups. However, none of these entities supported this work.

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