

High-efficiency upconversion luminescent quenching via defect-engineered Cu_{3-x}P for dual-aptamers-mediated tri-modal H1N1 lateral flow immunoassay

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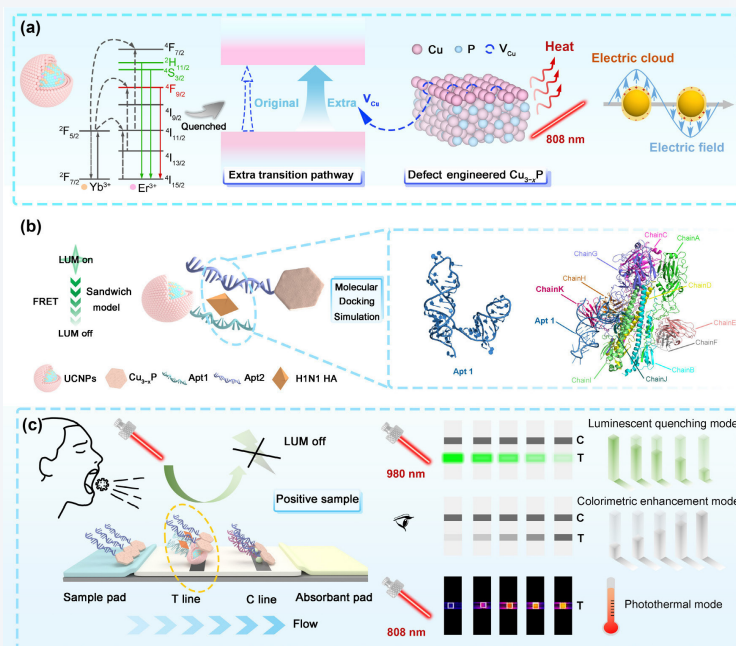
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ABSTRACT: Early diagnosis of Influenza A virus is critical for influenza prevention and control by reducing severe illness and mortality. However, conventional lateral flow immunoassay (LFIA) is limited by low sensitivity, and luminescent LFIA is short of significant signal changes at low target concentrations. Herein, a luminescence quenching LFIA platform is developed by integrating upconversion nanoparticles, defect engineered Cu_{3-x}P , and dual aptamers to address above bottleneck via a synergistic mechanism. Density functional theory calculations indicate that in the new type Cu_{3-x}P quencher, Cu vacancies induce new electronic states at the valence band edge and create extra electron transition pathway, which boost Förster Resonance Energy Transfer and upconversion luminescence quenching efficiency. Therefore, the test line luminescence signal is effectively suppressed to establish a sensitive luminescence quenching LFIA, which is sensitive to signal changes. Cu_{3-x}P also exhibits broad ultraviolet–visible (UV–vis) absorption for colorimetric and photothermal signals. The synergy luminescence-colorimetry-photothermal tri-modal analytical platform achieves a limit of detection of 0.907 ng/mL for H1N1 hemagglutinin. Moreover, two selected aptamers with stable and non-overlapping binding sites verified by molecular docking are critical for forming a sandwich structure, enabling high sensitivity toward H1N1 over H7N9, H9N2, H6N1, H7N7, and H5N1, along with good anti-interference ability. This work provides a scalable point of care influenza detection strategy balancing sensitivity and specificity, optimizing LFIA for on site viral diagnostics.

KEYWORDS: lateral flow immunoassay, Cu_{3-x}P , luminescent quenching, dual-aptamer, H1N1 detection



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1 Introduction

Influenza viruses are highly contagious, easily transmissible, and diversely pathogenic, leading to frequent outbreaks worldwide [1]. As the pathogens of infectious respiratory diseases, the infection characteristics manifest as symptoms such as fever, headache, and pneumonia, even lead to death in severe cases [2]. Therefore, early diagnosis is crucial for the prevention and control of influenza to reduce the risk of death [3]. Lateral flow immunoassay (LFIA) technology, with its ease of operation (no specialized equipment required), rapidity (< 20 min), and low cost, has become the preferred platform for home-based, simple, and rapid detection [4, 5]. Traditional LFIA employed antibodies as recognition elements and gold nanoparticles as the colorimetric signal source [6, 7]. However, single-color detection often suffered from low detection sensitivity and the need for additional signal amplification steps [8–10].

Given the inherent limitation of low detection sensitivity in conventional LFIA, luminescent LFIA has thus emerged as an advanced alternative [11]. Luminescent markers such as organic dyes [12–14], quantum dots [15, 16], and upconversion nanoparticles [17] have been widely used. Among them, rare-earth upconversion nanoparticles as a new type of luminescent labeling material have attracted considerable attention due to their high luminescence purity [18] and strong tunability of emission [19, 20]. Moreover, compared with traditional luminescent organic dyes or quantum dots, rare-earth upconversion nanoparticles use near-infrared light as the excitation source, which has lower autoluminescence background, narrower emission bandwidth, lower photobleaching, and longer luminescence lifetime [21, 22]. Their “anti-Stokes” luminescence mechanism can effectively avoid interference from biological autoluminescence, laying the foundation for high signal-to-noise ratio detection [23]. However, test strips that utilized only upconversion nanoparticles as the signal source often faced issues such as insufficient signal changes and limited signal intensity at low target concentrations.

Considering the limitations of upconversion nanoparticles-only test strips, luminescent turn-off test strips which coupled upconversion nanoparticles with quenchers have emerged as a potential solution [24–27]. Currently, quenchers of organic small molecules, noble metals, graphene oxide, MXene, and other layered nanostructured materials have been reported [28–31]. However, due to the non-specific adsorption of the materials themselves, electrostatic interference, and tendency to aggregate, the challenges such as large batch-to-batch differences, background signal drift, unstable chromatographic flow rates, and poor reproducibility still need to be solved [30, 32]. Based on the above unresolved bottlenecks of conventional quenchers, this study first proposed Cu_{3-x}P semiconductor nanocrystals containing Cu vacancies as an efficient quencher for upconversion luminescence. On the one hand, Cu_3P has a broad absorption spectrum, strong absorption capacity, and good stability, solving the problems of poor photostability and narrow absorption spectrum of organic quenchers [33, 34]. Moreover, compared with commonly used noble metal inorganic quenchers of gold nanoparticles, Cu_3P has a lower cost and can achieve controllable energy level structure through the introduction of vacancies, providing a new idea for the development of new quenchers.

In addition to optimizing signal sources and quenchers, the selection of recognition elements also plays a pivotal role in improving LFIA efficiency. Aptamers, as chemically synthesized

single-stranded DNA/RNA, can fold into specific three-dimensional (3D) structures to bind to targets with high affinity [35–37]. Their affinity and specificity for targets can be comparable to, and even superior to, antibodies in terms of magnitude [38]. When it comes to size, aptamers (1–2 nm) are much smaller than antibodies (10–15 nm), thus they can be immobilized on material surfaces at a higher density, thereby enhancing the sensitivity of the biosensor and reducing the detection limit [39]. In addition, aptamers also have the advantages of rapid and inexpensive synthesis methods, lack of immunogenicity, no batch-to-batch variability, and ease of chemical modification [40].

Leveraging the aforementioned advantages, this study constructed a novel luminescent quenching-based LFIA system by integrating the ultra-sensitive signal output of upconversion nanoparticles, high specificity recognition of aptamers, and the portability of LFIA. In this study, Cu_{3-x}P nanoparticles acted as the quencher due to their broad UV–vis absorption properties and vacancy-induced enhancement of upconversion emission utilization. To realize specific target recognition and Förster Resonance Energy Transfer (FRET) signal regulation, the system employed two aptamers (Apt1 and Apt2) with distinct loading strategies. Apt1 was conjugated to $\text{NaLuF}_4:\text{Yb},\text{Er}@\text{NaLuF}_4$ (UCNPs) and pre-immobilized on the T-line of the LFIA strip, while Apt2 was coupled with Cu_{3-x}P nanoparticles that undergo chromatographic flow along the strip. When the Cu_{3-x}P -Apt2 conjugates flow to the T-line, they specifically bind to H1N1 hemagglutinin (HA) and further form the Apt1-HA-Apt2 sandwich structure. Thus, the FRET process between UCNPs and Cu_{3-x}P switched the luminescent signal on the T-line from “on” to “off”. Moreover, Cu_{3-x}P functioned as both a colorimetric and a photothermal signal source in the LFIA platform, thereby enabling a tri-modal detection of H1N1 HA through luminescence, colorimetry, and photothermal sensing. The detection limit of the LFIA platform was 0.907 ng/mL, which paved the way for more accurately assessing the H1N1 HA levels in potential influenza patients.

2 Experimental section

2.1 Synthesis of $\text{NaLuF}_4:\text{Yb},\text{Er}$

Specifically, 0.8 mmol of lutetium acetate, 0.18 mmol of ytterbium acetate (III) hydrate, 0.02 mmol of erbium acetate tetrahydrate, and 2 mmol of anhydrous sodium acetate were precisely weighed using an electronic balance and transferred into a three-way flask. Subsequently, 10 mL of oleic acid and 10 mL of 1-octadecene were added to the flask. Under N_2 atmosphere, the mixture was heated to 180 °C at a ramping rate of 10 °C/min and maintained at the temperature for 20 min. The reaction mixture was then cooled to room temperature within the heating sleeve. After that, 2 mmol of sodium fluoride powder was accurately weighed and added to the flask. Under N_2 atmosphere, the mixture was heated to 250 °C and vigorously stirred for 30 min. The temperature was then further increased to 310 °C at a ramping rate of 5 °C/min, held for 60 min, and subsequently cooled to room temperature. The cooled reaction mixture was washed sequentially with ethanol, cyclohexane, and ethanol. Finally, the supernatant was decanted, and the pure $\text{NaLuF}_4:\text{Yb},\text{Er}$ nanoparticles were collected. The nanoparticles were ultimately dissolved in 5 mL of cyclohexane for further use.

2.2 Synthesis of $\text{NaLuF}_4\text{:Yb,Er@NaLuF}_4$ (UCNPs)

After cooling the reaction mixture containing $\text{NaLuF}_4\text{:Yb,Er}$ nanoparticles to room temperature, 0.25 mmol of acetic acid was weighed and transferred to a three-way flask. The reactants were then rapidly heated to 180 °C at a ramping rate of 10 °C/min under N_2 atmosphere and maintained at the temperature for 20 min. Subsequently, the mixture was cooled to room temperature within the heating sleeve. Thereafter, 0.5 mmol of sodium fluoride was weighed using an electronic balance and added to the flask. The mixture was heated to 310 °C at a ramping rate of 5 °C/min under N_2 atmosphere and held at the temperature for 60 min, followed by cooling to room temperature. The reaction mixture was then transferred to a centrifuge tube and washed sequentially with ethanol, cyclohexane, and ethanol. Finally, the supernatant was decanted, and the clean UCNPs were collected and dissolved in 5 mL of cyclohexane for storage. A consistent stirring speed of 600 rpm was maintained throughout the entire synthesis process. A stable N_2 flow rate of 50 mL/min was applied to provide an inert atmosphere during the reaction. After the high-temperature reaction at 310 °C, the system was naturally cooled to room temperature under continuous N_2 protection.

2.3 Hydrophilic modification of $\text{NaLuF}_4\text{:Yb,Er@NaLuF}_4$

One milliliter of $\text{NaLuF}_4\text{:Yb,Er@NaLuF}_4$ dispersed in cyclohexane was mixed with 4 mL of cyclohexane and 5 mL of N,N-dimethylformamide, followed by the addition of 0.030 g of nitrosyl tetrafluoroborate. After stirring at room temperature for 15 min, an excess of toluene was added. The mixture was then centrifuged at 12,000 rpm for 10 min using a high-speed centrifuge. The resulting pellet was dissolved in 10 mL of N,N-dimethylformamide containing 0.02 g of polyethyleneimine and stirred for 24 h. Subsequently, an excess of acetone was added to precipitate the product, and the mixture was centrifuged again at 12,000 rpm for 10 min using a high-speed centrifuge. After decanting the supernatant, the pellet was washed three times with a 50% ethanol solution. The product was stored in MES buffer solution (0.1 M, pH = 5.6).

2.4 Synthesis of Cu_3P and Cu_{3-x}P

Specifically, for the synthesis of Cu_3P , 0.09 g of $\text{CuCl}_2\cdot\text{H}_2\text{O}$, 1.21 g of hexadecylamine and 1.55 g of triphenyl phosphite were precisely weighed and transferred to a three-way flask. Using a pipette, 12.7 mL of 1-octadecene was subsequently added to the flask. The reactants were stirred for 30 min under N_2 atmosphere. The mixture was then rapidly heated to 150 °C and maintained for 1 h, after which the reaction temperature was further increased to 300 °C and held for 30 min. Upon completion of the heating process, the temperature was first cooled to 80 °C within the heating sleeve. The reaction mixture was then centrifuged and washed three times alternately with isopropyl alcohol and chloroform. Finally, the washed Cu_3P was dissolved in 5 mL of chloroform for further use. For the preparation of Cu_{3-x}P , the other steps were the same as described above, except that the cooling step was replaced by a rapid cooling to 80 °C after first cooling to 200 °C in a heating sleeve.

2.5 Hydrophilic modification of Cu_3P and Cu_{3-x}P

Typically, 0.075 g of polyethylenimine was weighed and placed into a 50 mL beaker, followed by the addition of 2 mL of chloroform. Using a pipette, 3 mL of chloroform-dispersed Cu_3P or Cu_{3-x}P was

then added to the beaker. The mixture was stirred at room temperature in the dark for 6 h. The product was subsequently washed three times with ethanol, dispersed in 5 mL of MES buffer (0.1 M, pH = 5.6).

2.6 Synthesis of aptamer functionalized probes

The aptamer dry powder was dissolved in the corresponding volume of PBS buffer solution to obtain the aptamer solution, which was heated at 95 °C for 10 min and then quickly cooled to 4 °C in an ice water bath to complete the activation of the aptamer. One milliliter nanomaterial solution with hydrophilic modification and 10 mL activated aptamer were added into a 2.5 mL centrifugal tube and shaken for 18 h at 4 °C. After washing three times, the obtained product was dispersed in 1 mL of MES buffer solution (0.1 M, pH = 5.6). Subsequently, different volumes of blocking agent were added to the solution and vibrated for 2 h at 4 °C. The product was dispersed in the preservation solution after centrifugal washing, and the probe solution after sealing was obtained.

2.7 Spiked recovery test

Gradient concentrations of HA standard analyte were spiked into blank artificial saliva samples without endogenous HA interference. The prepared saliva samples were mixed and diluted with the running buffer with final concentrations gradient of 1, 3, 5, 7, and 9 ng/mL, respectively. Each prepared sample solution was subsequently analyzed by the as-established UCNPs- Cu_{3-x}P -based lateral flow immunochromatographic assay platform. The corresponding luminescence signal and colorimetric signal intensities of all test strips were systematically recorded, and the standard calibration curves were further established by fitting the signal values against corresponding HA concentrations. Subsequently, the determined sample concentrations, spiked recovery rates as well as relative standard deviation (RSD) were statistically calculated.

2.8 Theoretical calculation

Density functional theory (DFT) calculations were carried out with the Vienna *ab initio* simulation package (VASP). The exchange-correlation interaction was described by the Perdew–Burke–Ernzerhof (PBE) functional within the generalized gradient approximation, with a cut-off energy of 550 eV. Projector augmented-wave pseudopotentials from the official VASP database were employed. For Brillouin-zone integration, the Gaussian smearing method was used with a smearing width of 0.05 eV. All calculations were performed based on a periodic model. First, the unit cell of Cu_3P was optimized, resulting in lattice constants of $a = b = 6.99$ Å and $c = 7.17$ Å. A dense $15 \times 15 \times 1$ k-point mesh was adopted for static self-consistent field calculations, followed by non-self-consistent calculations to acquire the band structure. The convergence criteria for structure optimization were set to 0.03 eV/Å (force) and 10^{-4} eV (energy), respectively.

3 Results and discussion

3.1 Dual aptamer-mediated FRET-LFIA using UCNPs and Cu_{3-x}P for H1N1 HA detection

An LFIA system was developed in the study, which took advantage of the unique upconversion luminescence properties of upconversion nanoparticles, the quenching ability of Cu_{3-x}P , the

high specificity of dual aptamer recognition, and the portability of LFIA. This system represented an innovative three-modality detection platform integrating luminescence, colorimetry, and photothermal detection. As shown in Fig. 1, the scheme illustrates the innovative aspects of the LFIA system.

In the system, UCNP acted as luminescent donors, capable of emitting green light upon excitation by a 980 nm laser, while the Cu_{3-x}P nanoparticles served as efficient quenchers. The energy level diagram elucidated that new electronic state induced by Cu vacancies within Cu_{3-x}P constructs an extra transition pathway, which enhances FRET between the UCNP and Cu_{3-x}P (Fig. 1(a)). This process involved the initial absorption of energy from 980 nm laser by UCNP, which excited electrons to higher energy states, followed by radiative emission of green light that promoted electron transition via the extra electron transition pathway of Cu_{3-x}P . Consequently, the electron migration and energy transfer process substantially augmented the FRET efficiency, leading to the effective quenching of upconversion luminescence. Additionally, collective oscillations of free electrons could be induced in Cu_{3-x}P nanoparticles under 808 nm laser irradiation, corresponding to the typical localized surface plasmon resonance (LSPR). The resonant heating of free electrons is induced by the LSPR effect, and localized temperature increases is caused because of the accumulated heat

that generate observable photothermal signals. The photothermal conversion characteristic of Cu_{3-x}P was subsequently utilized as the source of photothermal detection signals within the LFIA system. This application provided an additional signal output, thereby enriching the multimodal detection capabilities of the system.

When the Apt1-H1N1 HA-Apt2 sandwich structure was formed (Fig. 1(b)), the distance between the UCNP and Cu_{3-x}P nanoparticles was reduced (< 10 nm), triggering the FRET process that switched the luminescence signal on the T line from “on” to “off”. Molecular docking simulations were conducted to confirm the formation of the sandwich structure between the dual aptamers and the target protein H1N1 HA, as depicted in the molecular docking simulation section.

The operational mechanism of the LFIA system was meticulously detailed in Fig. 1(c). Under the influence of capillary action, the working fluid containing the target antigen was drawn into the test strip, progressing along its length. Upon reaching the T line, the presence of the H1N1 HA antigen facilitated the formation of a sandwich model with the aptamers Apt1 and Apt2 that were immobilized. The formation of the sandwich complex initiated FRET between the UCNP and Cu_{3-x}P , thereby quenching the luminescence signal at the T line. Concurrently, due to the colorimetric and photothermal properties of Cu_{3-x}P , the T line

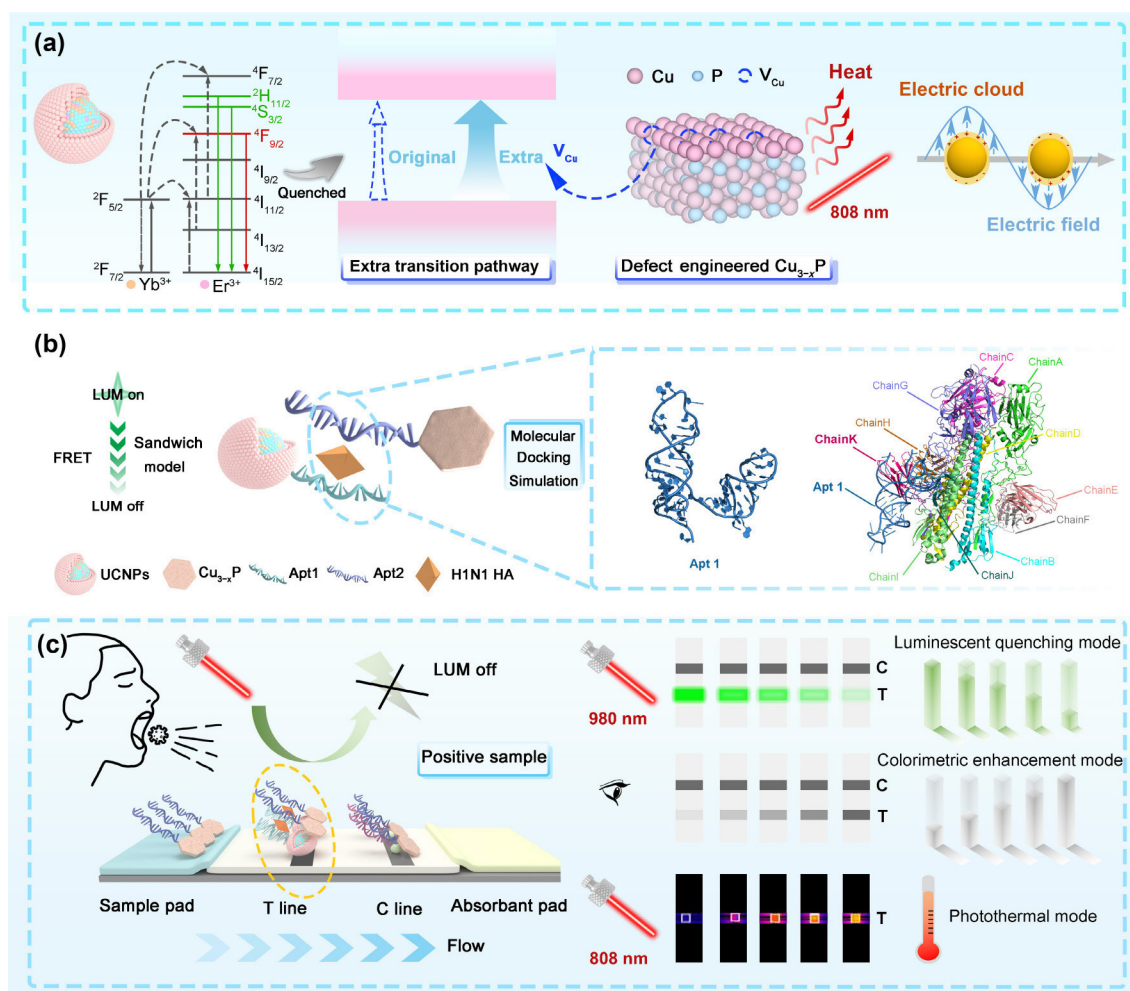


Figure 1 (a) Schematic diagrams of the quenching mechanism of UCNP- Cu_{3-x}P and the photothermal mechanism of Cu_{3-x}P . (b) Schematic diagram of the aptamer-based LFIA sandwich model and the molecular docking simulation results between nucleic acid aptamers and H1N1 HA protein. (c) Triple signals for the detection of H1N1 HA protein.

exhibited both color development and an enhancement in photothermal signals. The integration of multiple detection modes provides a robust and versatile tool for biological and clinical analysis.

3.2 Structural validation of synthesized upconversion nanoparticles and Cu_{3-x}P

In this study, UCNPs and defect-engineered Cu_{3-x}P were used as luminescent material and quencher for luminescence LFIA of H1N1 HA. The basic properties of luminescent and quencher materials are shown in Fig. 2. Figure 2(a) is the transmission electron microscopy (TEM) image of $\text{NaLuF}_4:\text{Yb},\text{Er}$. As can be seen, $\text{NaLuF}_4:\text{Yb},\text{Er}$ was composed of regular hexagonal nanoparticles with good dispersity and narrow size distribution, and the average diameter was 45.7 nm. After coating with NaLuF_4 shell (Fig. 2(b)), $\text{NaLuF}_4:\text{Yb},\text{Er}@\text{NaLuF}_4$ still showed apparently uniform size distributions and good dispersion in cyclohexane. Compared with $\text{NaLuF}_4:\text{Yb},\text{Er}$, the average particle size of UCNPs increased to 53.5 nm. As shown in Figs. 2(c) and 2(d), both Yb and

Er elements were concentrated near the core of UCNPs, while Na, Lu, and F elements were uniformly distributed throughout the nanoparticles, confirming the core-shell structure of the sample. Figure 2(e) shows the high-resolution TEM (HRTEM) image of UCNPs. The lattice spacing values of the sample were measured to be 0.286, 0.224, and 0.169 nm, which can be indexed to the (101), (111) and (211) lattice planes of hexagonal-phase NaLuF_4 , indicating the successful synthesis of hexagonal UCNPs crystal with high crystallinity. Figure 2(f) shows the X-ray diffraction (XRD) patterns of as-prepared samples and the standard cards. For the XRD pattern of $\text{NaLuF}_4:\text{Yb},\text{Er}$, the diffraction peaks at 17.1° , 29.9° , and 31.0° were respectively indexed to the (100), (110), and (101) crystal planes of the hexagonal structured NaLuF_4 (JCPDS#27-0726) with no impurity peaks. After the coating of the NaLuF_4 shell, the position of the diffraction peak remained unchanged, indicating that the UCNPs still maintained hexagonal phase and the crystal structure did not change. The hexagonal phase, thermodynamically favorable for upconversion luminescence, was retained to demonstrate that the shell-coating process preserves the core's crystalline order, which was a prerequisite for reproducible

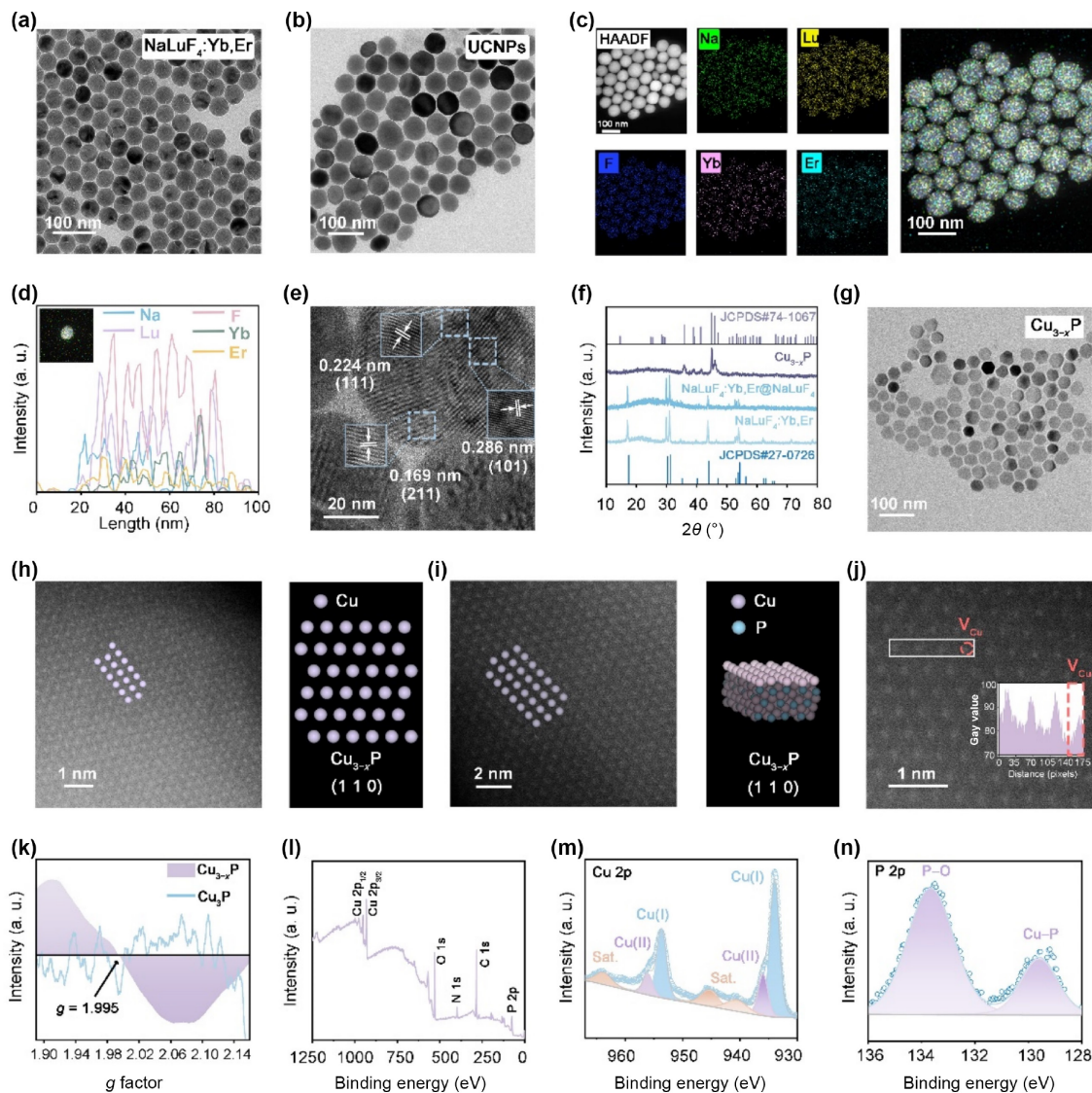


Figure 2 Basic characterizations. (a) TEM image of $\text{NaLuF}_4:\text{Yb},\text{Er}$. (b)–(e) TEM image, elemental mapping images, HRTEM image, and cross-sectional compositional line profiles of UCNPs. (f) XRD patterns. (g) TEM image of Cu_{3-x}P . (h)–(j) Cs-corrected HAADF-STEM images of Cu_{3-x}P . (k) EPR spectra of Cu_{3-x}P and Cu_3P . (l) The survey XPS spectrum of Cu_{3-x}P -PEI. (m) and (n) High-resolution XPS spectra of Cu 2p and P 2p in Cu_{3-x}P -PEI.

luminescent signals. Crystal defects or phase transitions introduce uncontrollable signal variations, which undermine the quantitative accuracy of H1N1 HA detection. Direct comparison of upconversion emission spectra between bare-core and core-shell UCNPs (Fig. S2 in the Electronic Supplementary Material (ESM)) reveals the pronounced luminescence enhancement induced by the NaLuF₄ shell. Under identical excitation conditions, the integrated luminescence intensity of core-shell UCNPs is approximately 21.25 times higher than that of the bare core, providing clear quantitative evidence that the shell effectively boosts upconversion luminescence.

The TEM image of Cu_{3-x}P quencher is shown in Fig. 2(g). It can be observed that the prepared Cu_{3-x}P nanoparticles were hexagonal sheet with a particle size about 29 nm. As shown in the XRD pattern of Cu_{3-x}P (Fig. 2(f)), the diffraction peaks at 25.8, 35.7, and 45.2° correspond to the (110), (112), and (300) crystal planes of hexagonal crystal system Cu₃P (JCPDS #74-1067), demonstrating the high purity and crystalline structure. According to Cs-corrected high angle annular dark field-scanning transmission electron microscopy (HADF-STEM) images (Fig. 2(h)), the atomic-scale structure of the (110) crystal plane of Cu_{3-x}P was revealed, which exhibited the periodic distribution of bright and dark spots adhering to the Z-contrast effect. Typically, the Cu atoms (purple) exhibited a bright spot, while the vacancies where Cu atoms were missing were represented as dark spots. The theoretical structure model was matched with image. Figures 2(i) and 2(j) show the Cs-corrected high angle annular dark field-scanning transmission electron microscopy (HADF-STEM) image of (110) crystal plane of Cu_{3-x}P. The cationic Cu vacancies (V_{Cu}) can be directly observed (Fig. 2(j)), which was marked with red circles. Moreover, the intensity profile recorded of corresponding region demonstrates atomic arrangement of Cu atoms with someone missing, which verified the presence of V_{Cu}. To further confirm the presence of vacancies, electron spin resonance (EPR) spectra of Cu₃P and Cu_{3-x}P were characterized. Figure 2(k) shows a strong EPR signal at $g = 1.995$, which was associated with the Cu vacancies in Cu_{3-x}P sample. For comparison, Cu₃P with no vacancies was synthesized. The difference in the synthesis methods lies in the fact that the cooling step of Cu₃P was slow cooling to 80 °C in a heating sleeve. As shown in Fig. 2(k), EPR spectrum failed to detect the presence of V_{Cu} in the Cu₃P sample. EPR spectroscopy provided complementary electronic evidence for V_{Cu}, as the $g = 1.995$ signal originated from unpaired electrons localized at vacancy sites. The absence of this signal in Cu₃P confirmed that controlled synthesis successfully introduces V_{Cu} into Cu_{3-x}P. In addition, the morphology (Fig. S1(a) in the ESM) and crystal structure (Fig. S1(b) in the ESM) of the Cu₃P were also examined, and the results were consistent with those of Cu_{3-x}P. The high resolution X-ray photoelectron spectroscopy (XPS) spectrum for the Cu₃P sample (Fig. S1(c) in the ESM) showed peaks at 952.5 eV (2p_{1/2}) and 932.6 eV (2p_{3/2}) assigned to Cu(I), indicating that the Cu element in the Cu₃P sample existed in the form of monovalent copper. Figure S1(d) in the ESM showed the high resolution XPS spectrum of P 2p in Cu₃P, with a peak at 134.4 eV attributed to phosphorus oxide.

In order to improve the hydrophilicity of the material to facilitate subsequent coupling of aptamers, the samples were modified with polyethyleneimine (PEI) for hydrophilicity. The XPS spectra of Cu_{3-x}P-PEI nanoparticles were used to reveal the surface composition and chemical state (Figs. 2(l)–2(n)). According to the survey XPS spectrum (Fig. 2(l)), Cu, P, O, C, and N were the primary elements in Cu_{3-x}P-PEI nanoparticles. The N element was attributed to the PEI coating during the hydrophilic modification

process. Figure 2(m) shows the high-resolution XPS spectrum of Cu. The peaks at 952.7 eV (2p_{1/2}) and 933.9 eV (2p_{3/2}) were attributed to Cu(I) in Cu_{3-x}P, while the peaks at 956.1 eV (2p_{1/2}) and 936.0 eV (2p_{3/2}) were attributed to Cu(II). The peaks located at 963.8, 945.4, and 940.7 eV were satellites peaks, which further confirmed the presence of Cu(II) in the Cu_{3-x}P sample. The coexistence of Cu(I) and Cu(II) in Cu_{3-x}P, confirmed by XPS, was a direct consequence of V_{Cu}. Vacancy-induced charge redistribution modulated the oxidation state of adjacent Cu atoms. As observed in the high resolution XPS spectrum of P (Fig. 2(n)) in the Cu_{3-x}P sample, two peaks at 129.6 and 133.6 eV were assigned to metal phosphides and oxidized P species, respectively.

The Fourier transform infrared (FTIR) spectra of Cu_{3-x}P before and after hydrophilic modification are shown in Fig. S3 in the ESM. For Cu_{3-x}P sample, the absorption peak at 2914 cm⁻¹ was attributed to the stretching vibration of C–H derived from the hexadecylamine used for synthesis. The absorption peak at 1000 cm⁻¹ was attributed to the stretching vibration of the P=O bond from triphenyl phosphite during the synthesis. For the Cu_{3-x}P-PEI sample, the strong absorption peak at 1640 cm⁻¹ was attributed to the deformation vibration of N–H, and the peak at 3415 cm⁻¹ is attributed to the stretching vibration of N–H. These two distinct absorption peaks originated from –NH– group in PEI, indicating that PEI has been successfully coated on the Cu_{3-x}P surface. Furthermore, the oleic acid ligand modified UCNPs (OA-UCNPs) prepared by high temperature thermal decomposition method were also hydrophilically modified by PEI (Fig. S4 in the ESM), the appearance of N–H peaks located at 1664 and 3426 cm⁻¹ also demonstrated the successful modification.

3.3 Quenching capability of Cu_{3-x}P and FRET efficiency improvement mechanism

Luminescence properties, aptamer conjugation, and FRET mechanism were characterized after the basic characterization of both luminescent material and quencher (Fig. 3). Figure 3(a) presents the upconversion luminescence spectra of UCNPs before and after conjugation with Apt1 under 980 nm laser excitation. For UCNPs-PEI sample, two primary green emissions with maxima at 521 and 543 nm could be assigned to ²H_{11/2} → ⁴I_{15/2} and ⁴S_{3/2} → ⁴I_{15/2} transitions of the Er³⁺ ions. After Apt1 conjugation, the emission peaks of UCNPs-Apt1 remained unchanged, indicating that the intrinsic luminescent properties were preserved. Besides, the luminescence intensity of UCNPs-Apt1 was reduced compared to that of UCNPs-PEI, because of the inevitable losses in the synthesis and washing processes. For Apt1 conjugation, glutaraldehyde cross-linking method was used.

Figure 3(b) shows the UV-vis absorption spectra of UCNPs-Apt1 at various glutaraldehyde amounts. It could be seen that when the amount of glutaraldehyde was 1.75 mL, the characteristic absorption peak at 260 nm representing Apt1 was the strongest, indicating that the coupling efficiency of Apt1 was the highest. By optimizing glutaraldehyde dosage, maximum aptamer loading was ensured to directly enhance the binding capacity of UCNPs-Apt1 for H1N1 HA and improve the LFIA strip detection sensitivity.

Figure 3(c) shows the UV-vis absorption spectra of UCNPs before and after coupling of Apt1. Clearly, UCNPs had no characteristic absorption peak in the range of 200–500 nm. The apparent absorption peak of Apt1 at 260 nm was due to the conjugated double bonds of the bases in the nucleic acid molecule. After successful coupling, UCNPs-Apt1 showed a new absorption

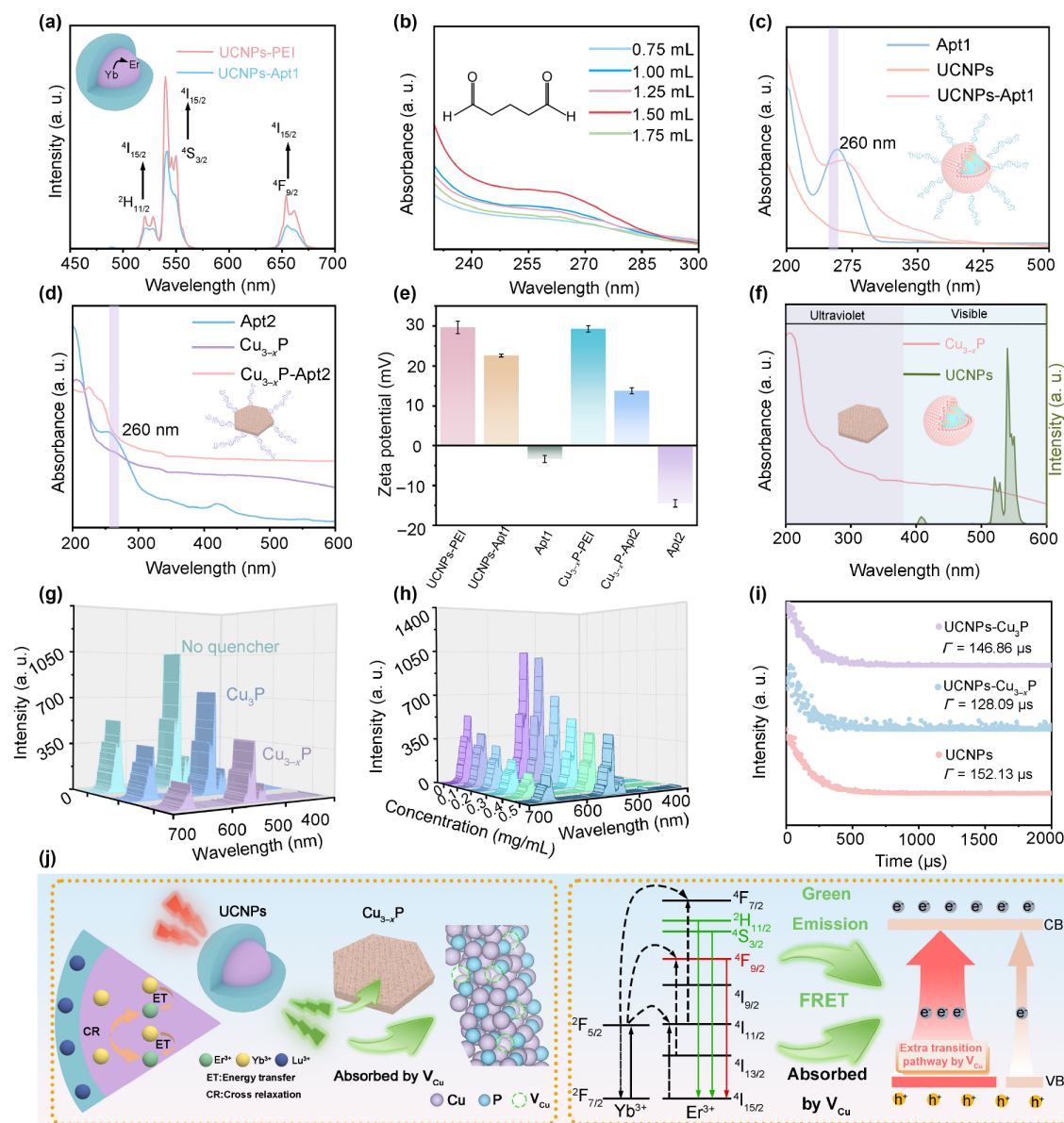


Figure 3 Luminescence properties, aptamer conjugation, FRET mechanism, and characterization. (a) Upconversion luminescence spectra of UCNPs-PEI and UCNPs-Apt1. (b) UV-vis absorption spectra of UCNPs-Apt1 at different glutaraldehyde contents. (c) and (d) UV-vis absorption spectra. (e) Zeta potential. (f) UV-vis absorption spectra of Cu_{3-x}P in chloroform and upconversion luminescence spectra of UCNPs in cyclohexane. (g) Upconversion luminescence spectra of UCNPs, UCNPs quenched by Cu₃P and Cu_{3-x}P in solution. (h) Upconversion luminescence spectra of UCNPs solution quenched by different concentration of Cu_{3-x}P. (i) Luminescence lifetime decay curves of UCNPs, UCNPs quenched by Cu₃P and Cu_{3-x}P at 540 nm. (j) Schematic illustration of FRET mechanism from UCNPs to Cu_{3-x}P.

peak around 260 nm, with the peak located at 265 nm, slightly offset from Apt1. This might be due to the formation of new chemical bonds during the cross-linking process, thus, the electronic transition characteristics of Apt1 were affected. The comparison of UV-vis absorption spectra shows that Apt1 had been successfully coupled to the surface of UCNPs. Similarly, the prominent UV-vis absorbance peak of Cu_{3-x}P at 206 nm underwent a red shift and exhibited enhanced intensity in the range of 220–250 nm (Fig. 3(d)), demonstrating the surface conjugation of the Apt2 to Cu_{3-x}P nanoparticles.

The zeta potentials of different samples are shown in Fig. 3(e). Both UCNPs-PEI and Cu_{3-x}P-PEI exhibited positive potentials of 29.63 and 29.27 mV, respectively. Because PEI existed in the form of polycationics in water. The potentials for Apt1 and Apt2 were −3.4 and −14.5 mV, respectively, which was caused by the large

amount of free phosphate in the nucleic acid aptamer. After coupling aptamers, the potential of UCNPs-PEI-Apt1 decreased to 22.6 mV, and the potential of Cu_{3-x}P-PEI-Apt2 also decreased to 13.8 mV. The reduction in positive potential arised from the electrostatic interaction between cationic PEI and anionic aptamers, providing complementary evidence for successful conjugation. Collectively, these findings suggested that the aptamers had been successfully immobilized onto the nanomaterial surfaces.

When the UV-vis absorption spectra of Cu_{3-x}P and upconversion luminescence spectra of UCNPs were drawn in the same Fig. 3(f), the Cu_{3-x}P sample demonstrated strong absorbance across the wavelength of 200–600 nm, which matched well with the emission peaks of UCNPs. Spectral overlap was fundamental for FRET. It allowed efficient energy transfer from UCNPs to Cu_{3-x}P, which induced upconversion luminescence quenching. To

investigate the influence of V_{Cu} on the luminescence quenching, solutions with Cu_3P and $Cu_{3-x}P$ quenchers at identical concentrations were added to UCNPs solutions (Fig. 3(g)). Notably, the UCNPs surface was functionalized with biotin, while the quencher surface was modified with streptavidin. The specific biotin-streptavidin interaction was employed to precisely control the donor-acceptor distance, a key parameter regulating FRET efficiency, and eliminate non-specific interactions that might interfere with quenching evaluation. The specific biotin-streptavidin interaction effectively reduces the donor-acceptor distance, thereby facilitating FRET. After thorough mixing, the luminescence intensity of the resulting mixtures was measured and compared. The luminescence intensity of UCNPs was highest in the absence of quenchers. The addition of Cu_3P induced a slight decrease in luminescence intensity. In contrast, mixing V_{Cu} -rich $Cu_{3-x}P$ with UCNPs resulted in 59.2% luminescence quenching, indicating that FRET was the dominant mechanism in the process. The significantly higher quenching efficiency (QE) of $Cu_{3-x}P$ compared to Cu_3P directly validated the regulatory role of V_{Cu} in enhancing FRET, which aligned with the defect engineering strategy designed for the quencher material. The luminescence intensity of the mixture was measured after adding varying concentrations of $Cu_{3-x}P$ to explore the relationship between quenching efficiency and $Cu_{3-x}P$ amount (Fig. 3(h)). As the $Cu_{3-x}P$ concentration increased from 0.1 to 0.5 mg/mL, the luminescence intensity of the solution decreased progressively, indicating enhanced quenching effect. The quenching efficiency approaches a saturation value at 0.4 mg/mL. To further elucidate the energy transfer mechanism between UCNPs and $Cu_{3-x}P$, time-resolved luminescence decay measurements were conducted. The luminescence decay curves of UCNPs, UCNPs- Cu_3P , and UCNPs- $Cu_{3-x}P$ solutions for $^4S_{3/2} \rightarrow ^4I_{15/2}$ transition are shown in Fig. 3(i). The luminescence lifetime of UCNPs- Cu_3P decreased by only 5.27 μs , whereas that of UCNPs- $Cu_{3-x}P$ decreased by 24.04 μs . The significant reduction in luminescence lifetime for UCNPs- $Cu_{3-x}P$ provided further evidence of efficient FRET. Because in the FRET process, the excited Er^{3+} in the donor returned to the ground state not only emitted luminescence, but also transferred energy to the acceptor of $Cu_{3-x}P$ by non-radiation mechanism, so that the acceptor was excited. The above energy transfer process was competitive with the luminescence emission process of the donor. Due to the addition of an energy dissipation pathway, the time that the donor was in the excited state was shortened, which led to the decrease of its luminescence lifetime ($^4S_{3/2} \rightarrow ^4I_{15/2}$).

Figure 3(j) shows a schematic illustration of the energy transfer process from UCNPs to $Cu_{3-x}P$, revealing the FRET mechanism. As can be seen, when excited by 980 nm laser, Yb^{3+} ions in UCNPs initially absorb photonic energy, thereby undergoing a transition from their ground state ($^2F_{7/2}$) to the excited state ($^2F_{5/2}$). Then, the energy is transferred from high-energy Yb^{3+} to Er^{3+} ions by two successive energy transfer processes, thus, the ground state ($^4I_{15/2}$) Er^{3+} is excited to high-energy states ($^4I_{15/2}$, $^4F_{9/2}$ and $^2H_{11/2}$). Finally, both the green and red emissions occur via $^4F_{9/2} \rightarrow ^4I_{15/2}$ and $^2H_{11/2}/^4S_{3/2} \rightarrow ^4I_{15/2}$ transitions. Next, due to the wide UV-vis absorption characteristics of $Cu_{3-x}P$, the green light can be absorbed. For $Cu_{3-x}P$, the presence of V_{Cu} improves the utilization of UCNPs emission, because V_{Cu} induces new electronic states at the valence band (VB) edge and creates an extra electron transition pathway, as shown in the right part of Fig. 3(j). Originally, for Cu_3P without V_{Cu} , electrons can only complete direct transition from the VB to the

conduction band (CB) through a single pathway. However, the introduction of V_{Cu} constructs an additional K to M indirect transition pathway on the basis of the intrinsic direct transition.

According to the DFT band structure calculation results, the specific energy levels are as follows: For Cu_3P , the valence band maximum (VBM) is -0.09 eV, the conduction band minimum (CBM) is 0.15 eV, and the band gap is 0.24 eV. For $Cu_{3-x}P$, the VBM is -0.08 eV, the CBM is 0.14 eV, the band gap is 0.22 eV, and V_{Cu} induces an additional electron band (-0.09 eV) in the VB region (Fig. S5 in the ESM). Notably, the PBE functional employed in the present calculation underestimates the material band gap [41]. The theoretical calculation results reveal that the introduction of V_{Cu} enables the electron band to distribute at -0.09 eV, forming a new electronic state at the valence band edge of the K point. The generated K to M transition serves as an extra electron transition pathway apart from the original direct transition at the M point, which facilitates the electron transition processes.

According to the photon energy conversion formula

$$E = 1239.8/\lambda \quad (1)$$

where E represents the photon energy, λ is the corresponding wavelength of light. The 521 nm green light of Er^{3+} corresponds to 2.38 eV, and the 543 nm green light corresponds to 2.28 eV. The comparison shows that the calculated band gap value is smaller than the photon energy of Er^{3+} green light, indicating that the electron transition from the VB to the CB is physically allowed. These results further confirm the FRET enhancement mechanism.

3.4 Photothermal performance of $Cu_{3-x}P$

The photothermal performance of $Cu_{3-x}P$ was also investigated (Fig. 4). The temperature change of the $Cu_{3-x}P$ solution after irradiated by 808 nm laser was measured by an infrared thermal imager as shown in Fig. 4(a).

It is well known that the photothermal properties of materials mainly depend on two factors of mass extinction coefficient (ϵ) and photothermal conversion efficiency (η). In order to reveal the photothermal properties, the ϵ of $Cu_{3-x}P$ was firstly calculated. Figure 4(b) shows the UV-vis absorption spectra of $Cu_{3-x}P$ with different concentrations. Typically, $Cu_{3-x}P$ exhibited strong absorption throughout the 500–850 nm wavelength. Thus, 808 nm near-infrared laser was chosen as the light source because the strong extinction and anti-interference ability were taken into comprehensive consideration [42]. Furthermore, a corresponding increase in absorption was observed at 808 nm with increasing $Cu_{3-x}P$ concentrations (Fig. 4(c)). According to the Lambert-Beer law, a linear relationship between the concentration and the absorption of $Cu_{3-x}P$ was obtained. Therefore, ϵ was calculated to be 7.63 L/(g-cm), indicating that $Cu_{3-x}P$ has a good absorption for 808 nm light.

Heating rate and highest temperature of $Cu_{3-x}P$ solutions irradiated by 808 nm laser with different power densities were measured (Fig. 4(d)). The temperature of the $Cu_{3-x}P$ solution (500 $\mu g/mL$) was increased to 71.9 $^{\circ}C$ in only 5 min when exposed to a laser with a power density of 1 W/cm². Even with a low power density of 0.2 W/cm², the solution could still be heated to 36.5 $^{\circ}C$, indicating that $Cu_{3-x}P$ is efficient and fast in converting 808 nm light into thermal energy. When the laser power density was maintained at 1 W/cm², the temperature and heating rate of the $Cu_{3-x}P$ solution were then investigated as the concentration increased (Fig. 4(e)). The $Cu_{3-x}P$ solution at the concentration of

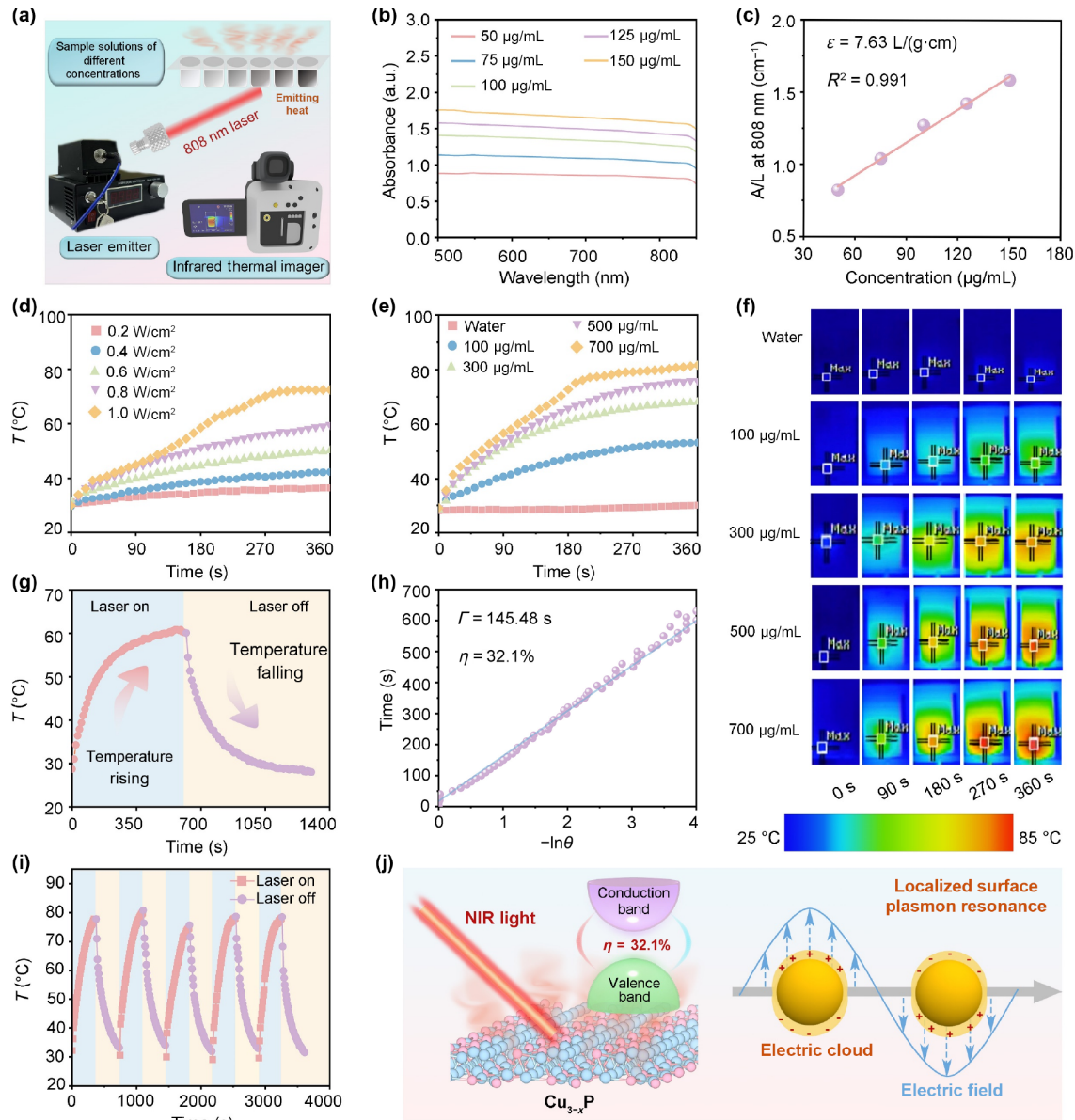


Figure 4 Photothermal performance of Cu_{3-x}P . (a) Schematic diagram of the photothermal property characterization device for Cu_{3-x}P samples. (b) UV-vis absorption spectra of Cu_{3-x}P at varied concentrations. (c) Mass extinction coefficient fitting curve of Cu_{3-x}P at 808 nm. (d) Power-dependent and (e) Concentration-dependent heating curves of Cu_{3-x}P solution irradiated by 808 nm laser for 5 min. (f) Infrared thermal images of Cu_{3-x}P solutions with different concentrations. (g) Photothermal heating and cooling curve of Cu_{3-x}P dispersed in water. (h) Linear relationship between the $-\ln\theta$ and time during the cooling period. (i) Heating curves of Cu_{3-x}P solution for laser on/off cycles under 808 nm irradiation. (j) Schematic diagram of the photothermal mechanism.

700 $\mu\text{g/mL}$ could reach a maximum temperature of 81.2 $^{\circ}\text{C}$ in 6 min. Even at a reduced concentration of 100 $\mu\text{g/mL}$, the temperature of the Cu_{3-x}P solution can reach up to 53.0 $^{\circ}\text{C}$. However, as control group, water experienced only negligible increase in temperature. Figure 4(f) shows thermal imager images of Cu_{3-x}P solutions at different irradiation times. In addition, the η was measured using the previously reported method. The heating and cooling curve of the Cu_{3-x}P solution was depicted in Fig. 4(g). After processing with Eq. (1), the variable θ at different times was able to be obtained by the cooling process of the Cu_{3-x}P solution. By using Eq. (2), the time constant (τ_s) for heat transfer and the maximum steady-state temperature were calculated to be 145.48 s and 60.8 $^{\circ}\text{C}$, respectively. According to the above result, the η was calculated to be 32.1% using Eq. (3) (Fig. 4(h)).

$$\theta = \frac{T - T_{\text{surr}}}{T_{\text{max}} - T_{\text{surr}}} \quad (2)$$

$$t = -\tau_s \ln\theta \quad (3)$$

$$\eta = \frac{(T_{\text{max}} - T_{\text{surr}})M_D C_D}{\tau_s I (1 - 10^{-A})} - Q_{\text{loss}} \quad (4)$$

where T represents the temperature of solution surface, T_{surr} represents the surrounding temperature, t represents corresponding time, M_D and C_D are the quality and specific heat capacity of dispersant separately, Q_{loss} means thermal loss from laser assimilated by the vessel, I is laser power density, and A is absorbance of Cu_{3-x}P solution at 808 nm.

Additionally, a laser on/off cycling test was conducted to reveal the photothermal stability of Cu_{3-x}P (Fig. 4(i)). The temperature changes of Cu_{3-x}P solution were recorded while the laser was turned on for 5 min, followed by natural cooling to room temperature once the laser was turned off. This process was repeated for 5 cycles, during which no decline in the photothermal performance of Cu_{3-x}P was observed, demonstrating the robust and stable photothermal properties of the material. This characteristic was particularly advantageous for its role as a photothermal signal source in the LFIA strip, ensuring reliable and repeatable detection performance. Deionized water was replenished to the original graduated scale between each laser on/off cycle, and the dispersion was fully mixed to ensure uniformity before each irradiation. This practice kept the solution concentration consistent at the beginning of every cycle. Given that the photothermal stability assessment focuses on the peak temperature and its cyclic variation trend, the approach effectively eliminated the disturbance from solvent evaporation and ensured the reliability of the results. The schematic diagram of the photothermal mechanism is summarized in Fig. 4(j). The photothermal effect of Cu_{3-x}P results from its LSPR in the near-infrared region [43]. When excited by near-infrared light, the free carriers in Cu_{3-x}P nanocrystals oscillate collectively. These high energy carriers transfer their energy to the lattice rapidly through the electron-phonon interaction, which leads to the increase of the lattice temperature and the photothermal effect. Above all, Cu_{3-x}P can be used as a photothermal signal probe with sufficient photothermal response, meanwhile, 808 nm laser can be chosen as the excitation source.

3.5 Molecular simulation docking and optimization of LFIA key parameters

The 3D structures of the H1N1 HA protein, necessary for investigating the binding site and interaction mode with Apt1, were sourced from the RCSB database, as illustrated in Fig. S6 in the ESM. The secondary structure of Apt1 was predicted using the UNAFold Web site. As shown in Fig. 5(a), Apt1 demonstrated a classic hairpin secondary structure. The hairpin loop was constructed from non-complementary nucleotides, whereas the hairpin stem constituted a double-stranded region formed by complementary nucleotide pairing. The intricate secondary structure of Apt1 featuring with multiple hairpin loops and stems created diverse binding pockets and surfaces. The architecture may enable more effective complementarity with the shape and chemical properties of target protein, thereby enhancing their interaction. With a Gibbs free energy of -16.57 kJ/mol, it is evident that the aptamer attains remarkable thermodynamic stability. The 3D structure of Apt1 was predicted using RNA composer on the base of the secondary structure (Fig. 5(b)). For the molecular docking simulation, the combination of Apt1 as ligand and H1N1 HA protein as the receptor was used and evaluated by HDock (Fig. 5(c)). PyMOL software was used to present the results. The molecular docking scores were estimated by an interactive scoring function of the HDock server that allowed the ranking of possible binding models based on negative docking score values. By analyzing the docking results, the minimum docking score of Apt1 and H1N1 HA protein binding conformation was -358 . A lower molecular docking score value indicated a stronger interaction between the aptamer and the protein, as well as a more stable binding [44]. The results showed that the combination was stable and the interaction was strong. Figure 5(d) presents the binding

conformation of the H1N1 HA protein-Apt1 complex. Apt1 was bound on the protein surface with 9 base sites forming hydrogen bonds with amino acid residues of the protein. For instance, base sites G-53 of Apt1 formed hydrogen bonds with SER-114 of the HA protein. C-59, A-61, and C-62 of Apt1 also established hydrogen bonds with ARG139 of the HA protein on Chain K. Besides, as shown in Figs. S7(a) and S7(b) in the ESM, base sites G-34 and G-35 of Apt1 formed hydrogen bonds with SER-424 and GLN-419 on Chain I, meanwhile, base sites A-19, A-21, and G-22 established hydrogen bonds with ASP-101, THR-81, and SER-95 on Chain G, respectively. The above numerous hydrogen bonds contributed to the stable binding of Apt1 and the H1N1 HA protein.

The secondary and 3D structures of Apt2 were predicted using the same approach as that for Apt1, with the results displayed in Fig. 5(e) and Fig. S8 in the ESM. Figure 5(e) reveals that secondary structure of Apt2 is primarily composed of a single-stranded region and a hairpin structure. The Gibbs free energy of Apt2, as calculated by UNAFold, is -3.99 kJ/mol. Figure S7 in the ESM illustrates the 3D structure of Apt2 as simulated by RNAComposer based on the secondary structure. For molecular docking simulations, the combination of Apt2 as the ligand and H1N1 HA protein as the receptor was employed and evaluated using HDock, with the results rendered using PyMOL software (Fig. S9 in the ESM). The minimum docking score for the Apt2 and H1N1 HA protein-binding conformations was -384.74 . This low docking score indicates stable binding conformations and strong interaction between Apt2 and the H1N1 HA protein. Figures 5(f)–5(h) show that Apt2 binds to the protein surface, with seven base sites forming hydrogen bonds with the amino acid residues in protein. These base sites are distributed across the hairpin structure of Apt2. Specifically, the base sites A-24, C-25 and C-26 of Apt2 form hydrogen bonds with THR-133, ARG-264, SER-247 and HIS-248 of the HA protein. Additionally, the base sites A-18 and C-19 of Apt2 interact with THR-310, ASN-312 and GLN-326 of the HA protein through hydrogen bonds. C-32 and T-33 form hydrogen bonds with ARG-351 and TYR-349 of the HA protein. These extensive hydrogen bonds contribute to the stable binding of Apt2 and the H1N1 HA protein. In conclusion, the high-stability binding of both Apt1 and Apt2 to the H1N1 HA protein theoretically confirms the feasibility of sandwich structure formation, providing a solid theoretical foundation for the development of lateral flow immunochromatographic platform based on the sandwich structure.

To achieve a more sensitive LFIA, several key test conditions were optimized. First, the amount of Apt1 conjugated on UCNPs and Apt2 conjugated on Cu_{3-x}P played a crucial role in the detection process. Too few aptamers may lead to low detection sensitivity, while too many aptamers may cause space obstruction and unnecessary waste. Thus, the amount of aptamer was explored. As shown in Figs. 5(i) and 5(j), the luminescence QE was maximized when the concentration of Apt1 and Apt2 was $2 \mu\text{mol/L}$ in the solution used for coupling, and remained stable thereafter. At the same time, the colorimetric signal intensity of the test strip was observed as a function of aptamer dosage (Figs. 5(m) and 5(n)). As can be seen from the graph, the colorimetric signal grew and the signal-to-noise ratio (S/N) rose as the aptamer dosage increases. At Apt1 and Apt2 concentrations of $2 \mu\text{mol/L}$, the value of S/N was 4.6. As Apt1 and Apt2 increased further to $2.5 \mu\text{mol/L}$, S/N only grew slightly to 4.8 and 5.0, respectively. Therefore, based on the

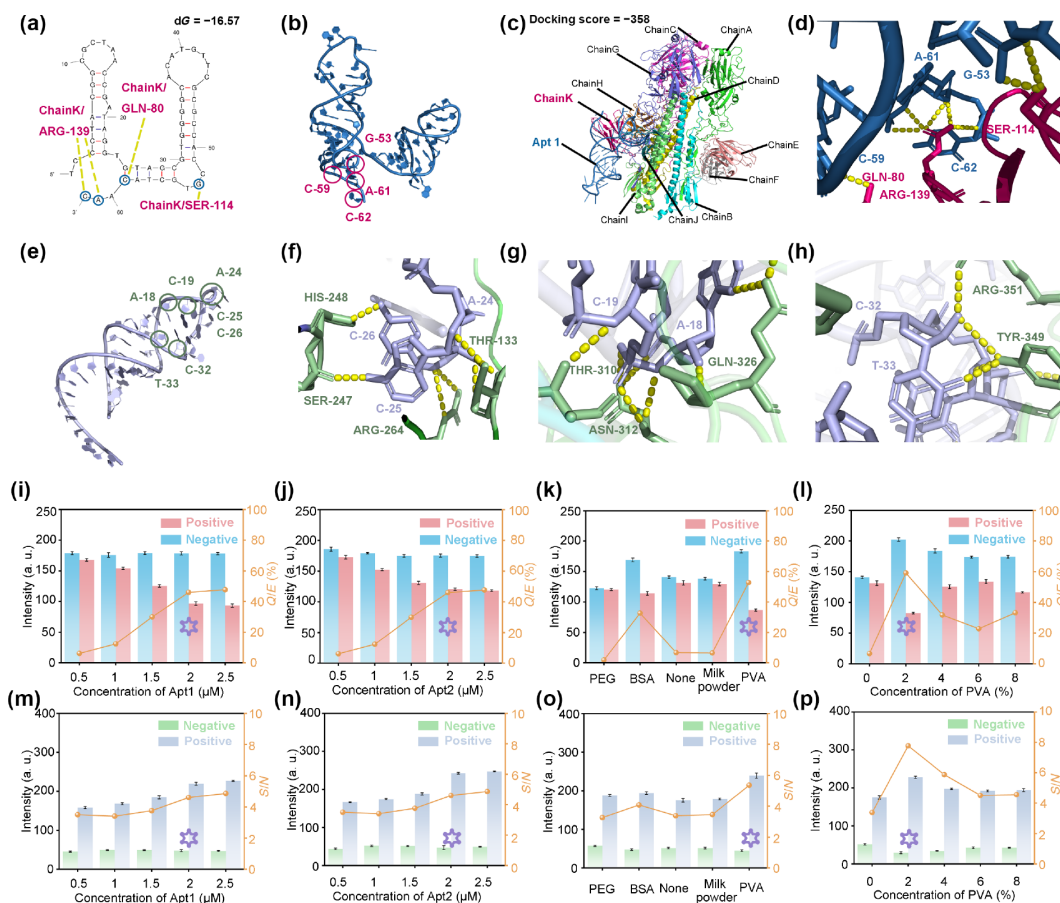


Figure 5 Molecular simulation docking results and optimization of critical elements for the signal intensity of UCNPs-Cu₃P-based LFIA. (a) Predicted secondary structures of Apt1. (b) Predicted tertiary structure of Apt1. (c) Result of molecular docking between Apt1 and H1N1 HA protein. (d) Local details of molecular docking of Apt1 with H1N1 HA protein. (e) Predicted tertiary structure of Apt2. (f)–(h) Local details of molecular docking of Apt2 with H1N1 HA protein. (i)–(l) The luminescence intensity of the test strip T line and the quenching efficiency vary with the concentration of Apt1, the concentration of Apt2, the type of blocking agent, and the concentration of PVA in the blocking agent during the conjugation process. (m)–(p) The colorimetric signal intensity and signal-to-noise ratio of the test strip vary with the concentration of Apt1, the concentration of Apt2, the type of blocking agent, and the concentration of PVA in the blocking agent during the conjugation process.

changes of luminescence signal and colorimetric signal, 2 $\mu\text{mol/L}$ was selected as the optimal aptamer dosage in the principle of cost saving. In addition, the amount of surfactant in the binding buffer also affected the chromatographic process. In this study, tween was used as surfactant, which could increase the dispersity of the system and improve the chromatography speed. However, too low tween content may cause problems such as insufficient liquid fluidity and increased non-specific adsorption. In contrast, too high tween content may make the chromatography speed too fast, so that the aptamers cannot fully react with antigens, which makes the signal less obvious. The volume fraction of tween was adjusted in the range of 0–0.05%. As shown in Fig. S10(a) in the ESM, QE reached its maximum at a tween volume fraction of 0.01%. As can be seen from Fig. S10(b) in the ESM, S/N also reached a maximum at 0.01% tween volume fraction. The colorimetric signal of the negative sample was enhanced with increasing tween volume fraction. This might be due to the fact that the high concentration of tween did not allow the sample to flow evenly across the T line, thus enhancing the colorimetric signal of the negative sample. As a final result, the volume fraction of 0.01% was determined as the best dosage of tween. Thirdly, in the LFIA process, the blocking agent can cover the redundant active sites on the surface of the nanomaterials, preventing these sites from non-specific binding to non-target molecules in the sample.

Meanwhile, the blocking agent can also provide a relatively stable environment for the aptamers coated on the surface of nanomaterials to prevent denaturation and inactivation during storage and detection. In this study, polyethylene glycol (PEG), bovine serum albumin (BSA), milk powder, and polyvinyl alcohol (PVA) were selected as blocking agents, and the chromatographic results were compared with those of the materials without blocking agents (Fig. 5(k)). The group using PVA as blocking agent exhibited the highest QE. Similarly, in Fig. 5(o), the group with PVA as the blocking agent showed the highest S/N. So PVA was used as blocking agent in the subsequent experimental procedures of this study. After PVA was selected as the blocking agent, the effect of PVA dosage in the UCNPs blocking solution was further investigated. It can be seen from Fig. 5(l) that when the volume fraction of PVA was 2%, the luminescence signal QE of the test strip reached the highest value of 59.2%. With the further increase of PVA volume fraction, the luminescence signal QE did not improve. Figure 5(p) shows the colorimetric signal of the test strip as a function of PVA volume fraction. The S/N reached a maximum at 2% PVA, in good agreement with the results in Fig. 5(p). Thereafter, the PVA volume fraction of 2% was selected as the optimal dosage of blocking agent. In addition, the content of KCl in the binding buffer also has an important influence on the chromatographic results. For single-stranded aptamers, KCl mainly

plays a role in stabilizing the structure and enhancing stability. Appropriate KCl concentration can keep the aptamer in conformation, which is beneficial to the interaction between the aptamer and the target analyte. However, excessive KCl concentration will reduce the binding affinity. As shown in Fig. S11(a) in the ESM, with the increase of KCl concentrations, the quenching efficiency of the luminescence signal of the test strip first increased slightly, reaching a maximum of 56.7% at a KCl concentration of 5 mM, and then decreased. In Fig. S11(b) in the ESM, the S/N of the colorimetric signal of the strip at KCl concentration of 5 mM was significantly higher than that of the other groups. Therefore, 5 mM was selected as the best concentration of KCl in the binding buffer. The composition of the sample pad treatment solution was a crucial factor influencing the sensitivity, stability, and chromatographic performance of test strips. In this study, nine distinct formulations of sample pad treatment solutions were prepared and their chromatographic outcomes were examined, with the results illustrated in Fig. S12 in the ESM. The analysis of variance indicated that the seventh formulation exhibited the highest Q/E and S/N values compared to the other groups. Consisting of 2% sucrose, 0.05% Tween, 2% BSA, and 2% Tetronic 1307, the formulation stood out as the optimal choice for enhancing the performance of the test strips. Subsequently, the flow time, a key parameter governing the completeness of the immunoreaction and the stability of dual-mode signals, was further optimized to balance reaction efficiency and detection speed. All tests were conducted at a representative HA concentration of 20 ng/mL, with flow time set from 0 to 10 min, and the corresponding luminescence and colorimetric signal intensities were recorded (Fig. S13 in the ESM). For the luminescence mode, the signal intensity gradually decreased with increasing flow time and stabilized after 8 min, indicating that the competitive immunoreaction was nearly complete at the stage. Meanwhile, the colorimetric signal intensity increased continuously with reaction time and reached a plateau at 8 min, reflecting the gradual accumulation of probes on the test line. Considering the stable signals of both modes, 8 min was selected as the optimal flow time to ensure sufficient immunoreaction. Combined with the subsequent signal readout step, the total assay time from sample loading to signal acquisition was approximately 10 min.

3.6 Triple-signal quantitative and specific detection of H1N1 HA

After confirming the possibility of aptamers binding to H1N1 HA protein by molecular docking, we employed the strips for quantitative detection of H1N1 HA under the optimized parameters. Figure 6 shows the luminescent, colorimetric, and photothermal triple-signals detection of H1N1 HA by using UCNPs-Cu_{3-x}P-based LFIA. The sensitivity of UCNPs-Cu_{3-x}P-based LFIA was determined using different concentrations of H1N1 HA as standards. Figure 6(a) shows the intensity of the green luminescent signal on the T-line of LFIA. As the H1N1 HA concentration increased, the luminescent signal intensity on the T-line decreased because UCNPs-Apt1 captured more H1N1 HA and formed a sandwich complex with Cu_{3-x}P-Apt2. The luminescent signal intensity on the T-line showed a decreasing trend due to the FRET process between Cu_{3-x}P and UCNPs. It was found that the intensity of luminescent signal was the lowest when the concentration of HA was 30 ng/mL.

Intensity-concentration curve was plotted for HA concentrations

in the range of 1–10 ng/mL (Fig. 6(b)). In this concentration range, there was a strong linear relationship between the luminescent signal intensity and the HA concentration. The linear fitting formula is $y = -11.03x + 106.118$, where y is the T-line luminescent signal intensity and x is the HA concentration, resulting in a cLOD of 0.907 ng/mL.

The luminescent spectrum of the T-line of the test strip at different HA concentrations measured by a luminescence spectrometer was shown in Fig. 6(c), the change trend was consistent with Fig. 6(a). As the HA concentration increases, the test strip turned from luminescence on to off. In addition to the decrease in the luminescence signal, an increase in the colorimetric signal was also observed on the T-line with increasing HA concentrations, as shown in Fig. 6(d). Intensity-concentration linear fitting curve was plotted for HA concentrations in the range of 1–10 ng/mL, as shown in Fig. 6(e). The linear fitting formula is $y = 5.75x + 111.510$. The cLOD was calculated to be 6.730 ng/mL. The cLOD value was determined using the widely accepted formula [45, 46]

$$\text{LOD} = 3\sigma/k \quad (5)$$

where σ represents the standard deviation of the blank measurements, and k stands for the slope of the calibration curve. Besides, the photothermal signal generated by LFIA was collected by a thermal imager for quantitative testing of HA. HA detection relies on the photothermal signal obtained following 20 s of laser irradiation. The photothermal signal from UCNPs-Cu_{3-x}P-based LFIA is shown in Fig. 6(f). Obviously, the photothermal signal and colorimetric signal exhibited a similar trend within the HA concentration range of 0–30 ng/mL. The temperature at the T-line of the strip increased from 28 to 54.2 °C with increasing HA concentrations. The photothermal photograph is shown in Fig. 6(f) inset.

Systematic comparison of key performance parameters—including LOD, linear range, detection time, and signal modalities—was performed to contextualize analytical performance against state-of-the-art H1N1 detection methods, with results detailed in Table S2 in the ESM. Luminescence-based LFIA achieved a low LOD of 0.907 ng/mL, outperforming most conventional colorimetric and fluorescent LFIA methods, with sensitivity comparable to standard ELISA-based assays. Notably, the optimized total detection time of approximately 10 min is shorter than many competing LFIA, microfluidic biosensors, and electrochemical sensors, enabling rapid on-site testing without compromising analytical performance.

To further evaluate the practical applicability of the developed LFIA in complex biological matrices and assess potential matrix effects, spike-and-recovery experiments were conducted using artificial saliva as a representative simulated sample. As shown in the revised Fig. S14 and Table S3 in the ESM, we established calibration curves for HA detection in artificial saliva, and tested a series of spiked HA concentrations. For the luminescence mode, the calculated concentrations were in good agreement with the spiked values, with recovery rates ranging from 96.58% to 100.31% and RSD values below 7.17%. For the colorimetric mode, recovery rates were between 97.70% and 103.88%, with all RSD values below 4.19%. The results are comparable to the average level of existing studies [47, 48]. These results demonstrate that our dual-mode LFIA maintains good accuracy and precision even in a simulated biological environment, indicating minimal matrix interference and satisfactory anti-interference capability.

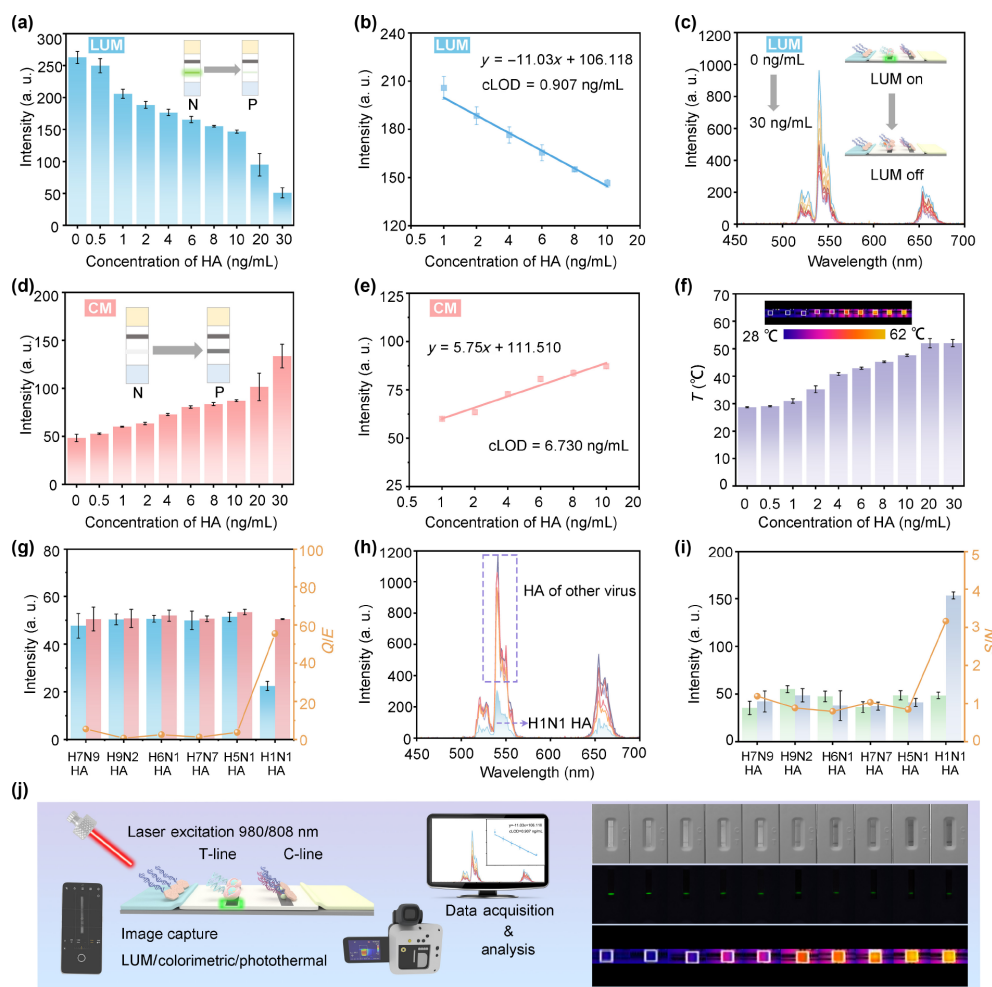


Figure 6 The luminescent, colorimetric and photothermal triple-signals detection of H1N1 HA by using UCNPs-Cu_{3-x}P-based LFIA. (a)–(c) Luminescent signal, dynamic linear range, and upconversion luminescence spectra of LFIA with H1N1 HA concentration varied from 0 to 30 ng/mL. (d) and (e) Colorimetric signal and dynamic linear range of LFIA with H1N1 HA concentration varied from 0 to 30 ng/mL. (f) Photothermal signal and pictures of LFIA with H1N1 HA concentration varied from 0 to 30 ng/mL. (g)–(i) Luminescent signal, upconversion luminescence spectra, and colorimetric signal of LFIA with HA of different type virus at a concentration of 30 ng/mL. (j) Colorimetric, luminescent, and photothermal photos of UCNPs-Cu_{3-x}P-based LFIA with different concentration of H1N1 HA and the schematic diagram of the signal reading process of LFIA.

In addition to precise quantitative testing, the specificity of the strip was explored. The results of the specificity detection of the luminescent signal are shown in Fig. 6(g). It can be seen that the luminescence intensity at the T-line of the strip exhibited nearly 60% quenching only in the presence of H1N1 HA. However, the other five subtypes of influenza virus HA had no significant quenching. The colorimetric signal results also showed that the strip had a distinct T-line only in the presence of H1N1 HA, as shown in Fig. 6(h). Luminescence spectral tests at the T-line of the strip further confirmed that among the six selected subtypes of influenza HA, only H1N1 HA caused T-line luminescence quenching (Fig. 6(i)). The above results showed that the test strip had strong specificity. The stability tests of the LFIA stored at 4 °C for 4 consecutive weeks were also carried out. As shown in Fig. S15 in the ESM, the signal intensities of both the luminescent and colorimetric readouts remained stable over the 4-week test period, with no significant degradation observed, indicating good storage stability of the prepared test strips under the given conditions. The intra-assay and inter-assay RSD at three different HA concentrations (1, 5, and 10 ng/mL) were summarized in Table S4 in the ESM. All RSD values for both luminescent and colorimetric

readouts were below 7%, demonstrating satisfactory reproducibility and precision of the developed LFIA.

The left part of Fig. 6(j) shows the acquisition and analysis process of the test strip signal. The right part shows the colorimetric, luminescent, and photothermal signals of the strip as the H1N1 HA concentration changes. The colorimetric signal of the strip could be directly observed by the naked eyes and recorded using a smartphone. A laser with 980 nm wavelength was used to irradiate the strip's T-line, which emitted a green light that could be captured by a smartphone camera. The T-line of the test strip was irradiated by a laser with a wavelength of 808 nm, and the Cu_{3-x}P accumulated at the T-line produced a photothermal signal after irradiation, which could be captured by an infrared thermal imager.

4 Conclusions

This study constructed a novel luminescent quenching type LFIA system by integrating the high specificity recognition of aptamers, the ultra-sensitive signal output of UCNPs, and the portability advantage of LFIA. In this study, UCNPs were used as the luminescent signal source, and Cu_{3-x}P nanoparticles were employed

as the quencher. With the formation of the Apt1-HA-Apt2 sandwich structure, the FRET process between UCNPs and Cu_{3-x}P turned the luminescent signal on the T-line from “on” to “off”. Moreover, Cu_{3-x}P also served as the chromogenic and photothermal signal source in this LFIA platform, thereby enabling the rapid and convenient detection of H1N1 HA in a tri-modal manner, including luminescence, colorimetry, and photothermal sensing. The detection limit of this LFIA platform was calculated to be as low as 0.907 ng/mL, which paved new possibilities for more accurately assessing the H1N1 HA levels in potential influenza patients. This study provides new insights for the design and development of efficient luminescent biosensing platforms.

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Data availability

All data needed to support the conclusions in the paper are presented in the manuscript and the Electronic Supplementary Material. Additional data related to this paper may be requested from the corresponding author upon request.

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Declaration of competing interest

All the contributing authors report no conflict of interests in this work.

Author contribution statement

Q. L.: Methodology, investigation, formal analysis, writing – original draft. S. L. G.: Supervision, writing – review & editing. H. J. G.: Investigation, visualization. Z. W.: Investigation, visualization. J. C.: Investigation, visualization. Q. Q. W.: Supervision. H. D.: Supervision, writing – review & editing. Y. M. R.: Supervision. F. N. X.: Supervision. P. P. Y.: Supervision, writing – review & editing. All the authors have approved the final manuscript.

Use of AI statement

None.

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