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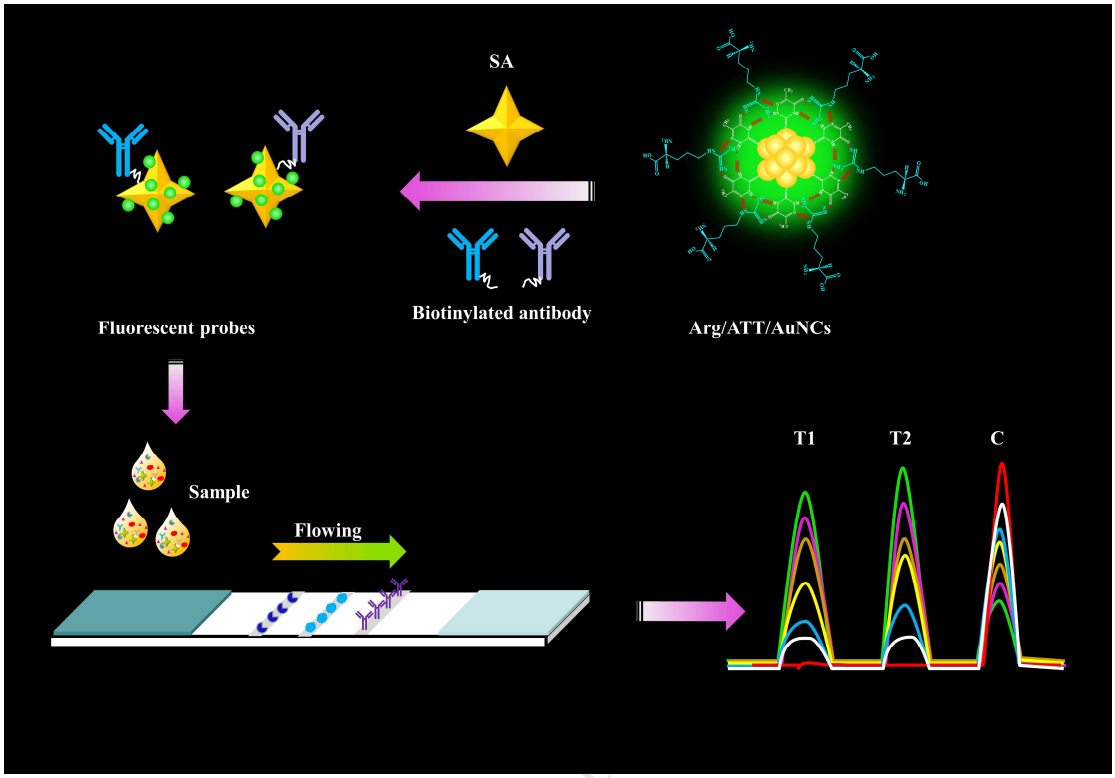
Graphic Abstract



Multiplex LFIA sensor based on highly luminescent green-emitting gold nanoclusters for simultaneous and quantitative determination of Clen and RAC in swine urine.

Highly luminescent green-emitting Au nanocluster-based multiplex lateral flow immunoassay for ultrasensitive detection of clenbuterol and ractopamine

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Abstract

A multiplex lateral flow immunoassay sensor based on highly luminescent green-emitting Au nanoclusters (AuNCs-MLFIA sensor) was successfully established for the simultaneous and quantitative determination of clenbuterol (Clen) and ractopamine (RAC) in swine urine. The antigens of Clen and RAC were dispersed on a nitrocellulose membrane as two test lines, and the Au nanoclusters were synthesized from 6-aza-2-thiothymine and L-arginine to obtain highly green luminescence and ultra-small nanoparticles (Arg/ATT/AuNCs). Free carboxyl groups on Arg/ATT/AuNCs enabled conjugation with biomolecules to afford an indicator for the biosensor. The AuNCs-MLFIA sensor is based on the indirect competition assay and could successfully detect samples within 18 min without sample pretreatment, qualitative results can be obtained by visual inspection under a UV lamp. The limits of detection of Clen and RAC by the naked eye were both 0.25 μg L^{-1}. In addition, the AuNCs-MLFIA sensor allowed quantitative detection combined with a portable fluorescence reader. The half-maximal inhibitory concentrations of Clen and RAC were 0.06 and 0.32 μg L^{-1}, respectively, with detection limits of 0.003 and 0.023 μg L^{-1}. Thirty blind-spiked swine urine samples were analyzed by the AuNCs-MLFIA sensor and liquid chromatography–tandem mass spectrometry, and the results of the two methods showed a significant correlation. The newly developed AuNCs-MLFIA sensor overcomes several limitations of conventional LFIA sensors, including their low sensitivity, limitation to quantify analytes, and single-analyte detection.

Keywords: AuNCs-MLFIA sensor; Arg/ATT/AuNCs; clenbuterol; ractopamine; multiplex detection.

1. Introduction

Food contamination from chemicals such as pesticides, veterinary drugs, and illegal additives increases the morbidity, mortality, and suffering of humans and exerts a heavy economic burden [1]. Enhanced monitoring of food safety is of great importance and a significant task. Point-of-care testing (POCT) has recently emerged as a powerful and effective method in disease diagnosis, food safety, and environmental monitoring; the technique allows portable, easy-operation, rapid, and cost-effective detection of targets in complex samples [2,3]. Lateral flow immunoassay (LFIA) is the most common and successful assay format of POCT [4,5]. LFIA based on colloidal Au nanoparticles (CG-LFIA) is a classical and extensively applied sensor with advantages of rapid detection, low cost, robustness, user friendliness, and easy operation [6,7]. The major limitations of traditional CG-LFIA sensors are lack of sensitivity, poor quantification capability, and single-analyte detection [8-10]. Thus, to improve the performances of LFIA sensors, novel nanomaterials, signal amplification strategies, multiplex detection, and optical strip readers have been employed.

Luminescent materials, such as quantum dots (QDs), fluorescent microspheres (FMs), up-converting phosphorus nanoparticles, and europium chelate-doped nanoparticles, among others [11-15], have been employed as labels to develop sensitive LFIA sensors. LFIA sensors based on noble metal nanoclusters (NMNCs) have not yet been reported. NMNCs have attracted considerable attention and emerged as promising fluorescence labels for detecting chemical and biological analytes due to their facile synthesis, large Stokes shift, good photostability, excellent biocompatibility, low toxicity, and high catalytic ability [16]. The fluorescence behaviors of NMNCs present important potential applications in the design of sensors [17], and these NCs are only limited by their lower quantum yield (QY) (≤10%) relative to those of QDs or FMs [18].

Great efforts have been made to prepare highly luminescent NMNCs. Rigidifying of the ligand or shell for fluorescence enhancement has recently been reported [17]. Deng et al. [19] introduced a novel synthetic route to obtain a highly luminescent Au

nanocluster (AuNCs) based on host–guest recognition; here, 6-aza-2-thiothymine (ATT) was used to synthesize ATT-stabilized AuNCs (ATT/AuNCs) with QYs of about 1.8%, and the fluorescence intensity observed was apparently enhanced after addition of L-arginine (Arg). Since the guanidine group of Arg binds with ATT through hydrogen bonding and significantly rigidifies the ligand shell, the resultant Arg/ATT/AuNCs exhibited good water solubility and strong green luminescence (QY = 65%) under UV irradiation. Arg/ATT/AuNCs have been used to detect arginase activity based on fluorescence alterations caused by arginase-catalyzed hydrolysis [20].

The potential applications of Arg/ATT/AuNCs should be investigated because of their notable advantages. The guanidine groups of Arg, for example, are bound to ATT, which means carboxyl groups at the hydrophilic end of Arg/ATT/AuNCs are free to conjugate with biomolecules. Thus, Arg/ATT/AuNCs can be employed as detection labels for immunoassays. In this research, highly luminescent green-emitting Arg/ATT/AuNCs were prepared and used as labels in a multiplex LFIA sensor for the simultaneous and ultrasensitive detection of clenbuterol (Clen) and ractopamine (RAC) in swine urine.

Clen and RAC, the most common synthetic β-adrenergic agonists currently available, can promote growth and improve muscle mass in livestock [21]. However, excess uptake of these agents might result in accumulation in animal edible tissues and exert harmful effects on human health through the food chain; in severe cases, such accumulation may even cause death [22]. As such, most β-adrenergic agonists have been prohibited as growth promoters in food animal breeding in China and the European Union, although RAC is still allowed as a feed additive in the United States [14]. Unfortunately, the illegal use of β-adrenergic agonists has not been completely eradicated due to the enormous economic benefits of their trade [23]. Over the last few decades, several analytical techniques have been developed to detect β-adrenergic agonist residues, including high performance liquid chromatography, gas chromatography–mass spectrometry, liquid chromatography–tandem mass spectrometry (LC-MS/MS), and enzyme-linked immunosorbent assay, but these

methods are time-consuming [24]. To reduce costs and improve detection speeds, multi-residue analysis based on immunoassays, especially LFIA sensors, have become a prominent research focus.

In this work, we design a multiplex LFIA sensor based on Arg/ATT/AuNCs (AuNCs-MLFIA sensor) with two test lines for the simultaneous detection of trace amounts of Clen and RAC residues in swine urine; a portable fluorescence reader is then combined with the proposed AuNCs-MLFIA sensor for quantitative detection (Scheme 1). In the proposed system, the ratio of the fluorescence intensity of the test lines to that of the control line is used as a quantitative signal to offset the inherent heterogeneity of AuNCs-MLFIA sensors [25]. All experiments, including parameter optimizations and measurement estimations, were carried out using swine urine samples. The developed AuNCs-MLFIA sensor for Clen and RAC determination features merits such as rapidity, facility, multiplex and ultrasensitive detection, user friendliness, and low cost.

2. Experimental section

2.1 Materials and reagents

Chloroauric acid ($HAuCl_4 \cdot 3H_2O$) and Arg were obtained from Sigma-Aldrich Chemical Corporation (St. Louis, Mo, USA). ATT was purchased from Alfa Aesar (China) Chemicals Co. Ltd (Shanghai, China). *N*-(3-(Dimethylamino)propyl)-*N*-ethylcarbodiimide (EDC·HCl), streptavidin (SA), sulfo-NHS-biotin, and *N*-hydroxysulfosuccinimide (NHSS) were acquired from Aladdin Reagent Co. (Shanghai, China). Sodium bicarbonate ($NaHCO_3$) and sodium hydroxide (NaOH) were bought from Sinopharm Chemical Reagent Beijing Co., Ltd. (Beijing, China). Monoclonal antibodies (mAb) of Clen and RAC and artificial antigens of Clen (Clen-BSA) and RAC (RAC-BSA) were provided by Beijing WDWK Biotechnology Co. (Beijing, China). Clen, RAC, salbutamol (SAL), cimaterol (CIM), terbutaline (TER), zilpaterol (ZIL), phenylethanolamine A (PHEA), and epinephrine (EPI) were obtained from the National Institutes for Food and Drug

Control (Beijing, China). Sample, PVC, and absorbent pads were obtained from Kinbio Tech Co., Ltd. (Shanghai, China). Nitrocellulose (NC) membrane was purchased from Sartorius (Göttingen, Germany), and dialysis bags (3500 Da) were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). All solvents and other chemicals used in this work were of analytical-reagent grade.

2.2 Apparatus

A SpectraMax M5 multifunctional microplate reader was purchased from Molecular Devices Co., Ltd. (Sunnyvale, CA, USA), and a UV spectrometer was obtained from Qiangyun Co., Ltd. (Shanghai, China). ZX1000 Dispensing Platform and CM4000 Guillotine Cutting Module were supplied by Kinbio Tech Co., Ltd (Shanghai, China). An ESE Quant LFR reader was purchased from QIAGEN (Dusseldorf, Germany). Ultrapure water was purified with a Milli-Q system from Millipore Corp. (Bedford, MA, USA).

2.3 Synthesis of Arg/ATT/AuNCs

Water-soluble, green-emitting AuNCs were synthesized according to a previously reported method [19]. ATT (171.8 mg) was dissolved in 15 mL of NaOH (0.2 M) and then added to 15 mL of $HAuCl_4$ solution (10 mg mL^{-1}). After continuous agitation for 1 h, the mixed solution was treated by ultrafiltration (Millipore, 50 kDa). The obtained ATT/AuNCs were resuspended in 30 mL of ultrapure water, and the pH of solution was adjusted to 10. Afterward, 2.0 mL of Arg (40 mM, pH 10) was added to 18 mL of the as-prepared ATT/AuNCs solution. The mixture was stirred in the dark at 37 °C for 24 h. Finally, the as-synthesized Arg/ATT/AuNCs solution was stored at 4 °C until use.

2.4 Preparation of SA@Arg/ATT/AuNCs and fluorescent probes

The Arg/ATT/AuNCs were coupled to SA via the active ester method. Arg/ATT/AuNCs solution (300 μL) and 100 μg of SA were added to 3.0 mL of Tris-HCl buffer (0.01 M, pH 8.0), and the mixed solution was allowed to react in the

presence of EDC and NHSS at room temperature for 2 h. After blocking with 50 μL of 20% BSA for 30 min, the as-prepared SA@Arg/ATT/AuNCs mixture was purified by ultrafiltration (Millipore, 100 kDa) and then resuspended in 2.0 mL of Tris-HCl buffer (0.01 M, pH 8.0). The obtained SA@Arg/ATT/AuNCs solution was stored at 4 °C until use.

Sulfo-NHS-biotin was directly conjugated to Clen and RAC mAbs in $NaHCO_3$ solution (pH 8.0). The obtained biotinylated mAbs were dialyzed using a dialysis bag (3500 Da) in PBS buffer (0.01 M, pH 7.0). To prepare the fluorescent probes, a certain proportion of the biotinylated mAbs was added to the obtained SA@Arg/ATT/AuNCs solution. The mixture was reacted in the dark place at room temperature for 2 h, and then stored at 4 °C until use.

2.5 Fabrication of the AuNCs-MLFIA sensor

The structure of the AuNCs-MLFIA sensor is presented in Scheme 1B. The sample pad was treated with 50 mM borate buffer (pH 7.4, containing 1% BSA, 0.5% Tween-20, and 0.05% sodium azide) and dried at 60 °C for 2 h. Clen-BSA, RAC-BSA, and rabbit anti-mouse antibody were dispensed onto the NC membrane as the test one (T1), test two (T2), and control (C) lines and then dried at 45 °C for 2 h. The AuNCs-MLFIA sensor was assembled by stacking and attaching the sample pad, NC membrane, and absorbent pad to the PVC pad, one on top of each other, with an overlap of 2.0 mm. The fabricated AuNCs-MLFIA sensor was cut into 4.0 mm-wide strips, kept dry, and stored at room temperature.

2.6 Qualitative and quantitative procedures of AuNCs-MLFIA sensor

Untreated swine urine (100 μL) and 2.0 μL of the fluorescent probes were mixed together, incubated for 3 min, and then added to the sample pad of the AuNCs-MLFIA sensor. After 15 min, the AuNCs-MLFIA sensor can be inspected to qualitatively detect the analytes under a UV lamp. It is based on the indirect competitive immunoassay, and the fluorescence intensity of the T line decreases with increasing amount of analytes present in the swine urine sample. The portable fluorescence

reader can be used to record the fluorescence intensity of the T and C lines for quantitative detection. A series of analytes concentrations (0, 0.0005, 0.0025, 0.005, 0.025, 0.05, 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 μg L^{-1}) was prepared by spiking the appropriate amounts of Clen and RAC standard solutions into blank swine urine samples. A quantitative standard curve was established by plotting the ratio of fluorescence intensity of the test line to that of the control line against the logarithmic concentrations of the analytes.

3. Results and discussion

3.1 Characterization of Arg/ATT/AuNCs and SA@Arg/ATT/AuNCs

ATT/AuNCs and Arg/ATT/AuNCs were synthesized through a facile method. The emission spectra of the AuNCs are shown in Fig. 1A, the fluorescence intensity of the Arg/ATT/AuNCs was about 100-fold higher than that of the ATT/AuNCs with the maximum emission wavelength (530 nm) had no change. Under a UV lamp, the Arg/ATT/AuNCs showed brighter green fluorescence than the ATT/AuNCs (Figs. 1Aa and 1Ab). This finding can be interpreted as follows: Insensitive host–guest recognition between the guanidine group of Arg and the ATT/AuNCs causes the ligands to become rigid, which suppresses the intramolecular vibration and rotation of ATT and reduces the nonradiative relaxation of excited states. Consequently, the fluorescence QYs of ATT/AuNCs drastically increase upon addition of Arg [19].

The fluorescence intensity of the Arg/ATT/AuNCs was barely altered over 4 months of storage (Fig. 1B), which shows that the Arg/ATT/AuNCs possess favorable stability. High-resolution transmission electron microscopy (HRTEM) showed that the as-synthesized Arg/ATT/AuNCs are monodispersed with a diameter of approximately 2.0 nm (Fig. 1C). Dynamic light scattering (DLS) analysis also indicated that the Arg/ATT/AuNCs are dispersed well with no aggregates (Fig. 1D). The valence states of Au atoms in the Arg/ATT/AuNCs were determined by X-ray photoelectron spectroscopy (XPS), and identical binding energies of $Au4f_{7/2}$ and $Au4f_{5/2}$ electrons, at 84.5 eV and 87.9 eV, respectively (Fig. 1E), were obtained. This result suggests that

Au(0) and Au(I) species coexist in the prepared Arg/ATT/AuNCs. The results thus far are consistent with those in a previous report [19] and demonstrate that Arg/ATT/AuNCs were synthesized successfully. The high luminescence, ultrasmall nanoparticles, and good stability of the Arg/ATT/AuNCs provide a promising strategy to improve the sensitivity of conventional LFIA sensors.

The free carboxyl groups of Arg/ATT/AuNCs were conjugated to the amino group of SA through the active ester method to prepare SA@Arg/ATT/AuNCs for antibody coupling. The fluorescence probes were obtained by coupling SA@Arg/ATT/AuNCs to biotinylated antibodies according to the general SA-biotin linking interaction. To confirm the conjugation of SA and Arg/ATT/AuNCs, FTIR analysis was conducted, and the FTIR spectra of SA, Arg/ATT/AuNCs, and SA@Arg/ATT/AuNCs are shown in Fig. 2A. Compared with that of the free Arg/ATT/AuNCs, the FTIR spectra of SA and SA@Arg/ATT/AuNCs showed the characteristic peaks of peptide bonds at 1645 cm^{-1} (C=O amide I) and 1539 cm^{-1} (N−C=O amide II). DLS measurement (Fig. 2B) illustrated that the hydrodynamic diameter of the NCs is markedly changed after they are conjugated with SA. The zeta potential of the SA@Arg/ATT/AuNCs was lower than that of the Arg/ATT/AuNCs due to addition of SA (Fig. 2C). These results demonstrate that SA is successfully covalently bound to the Arg/ATT/AuNCs.

An immunochromatographic test strip with the biotinylated antibody and antigen as T lines on the NC membrane was employed to evaluate the SA@Arg/ATT/AuNCs and fluorescence probes; here, the free Arg/ATT/AuNCs were set as the control. As shown in Fig. 2D, the two T lines have no fluorescence when the free Arg/ATT/AuNCs are flowed onto the NC membrane. By contrast, the SA@Arg/ATT/AuNCs and fluorescence probes turned on fluorescence of the biotinylated antibody and antigen lines, respectively. These results demonstrate that the antibody was successfully conjugated to the SA@Arg/ATT/AuNCs.

3.2 Optimization of the parameters of the AuNCs-MLFIA sensor

In a conventional LFIA test strip, the labeled detection probes are sprayed and distributed on the conjugated pad, which may decrease the reproducibility and

sensitivity of the assay [26]. In the present study, the AuNCs-MLFIA sensor only comprises a sample pad, a NC membrane, an absorbent pad, and a PVC pad. The prepared fluorescence probes were premixed with the samples in a microwell before testing. Compared with that of the conventional LFIA with immobilized fluorescence probes, the fluorescence intensities and inhibition rates of the AuNCs-MLFIA sensor are higher (Fig. S1).

pH is a key factor in the fluorescence signal. To explore the influence of pH on the fluorescence intensities of the three lines, the pH of swine urine samples was adjusted to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10. As shown in Figs. 3A and 3B, as pH increased from 6.0 to 10.0, the fluorescence intensities of the three lines gradually decreased. However, the fluorescence intensities of T2 and C lines decreased sharply when pH fluctuated from 6.0 to 4.0. This interesting phenomenon could be explained as follows: Arg and ATT on the AuNCs combine through hydrogen bonding. As the pH changes, the bond lengths and angles of hydrogen bonds between the guanidine group of Arg and ATT are changed, and Arg dissociates from the ATT/AuNCs, resulting in a decrease in fluorescence. Therefore, pH = 6.0 was set as the optimal pH for detecting swine urine samples.

The amount of antibodies and antigens were optimized to obtain a stronger fluorescence signal and better sensitivity. Swine urine samples spiked with 1.0 μg L^{-1} of Clen and 5.0 μg L^{-1} of RAC were set as positive samples. Comprehensive consideration of the fluorescence intensity, positive inhibition, and cost performances, 0.6 mg mL^{-1} of Clen-BSA (Fig. S2A) and 0.2 mg mL^{-1} (Fig. S2B) of RAC-BSA were selected as the appropriate parameters. Clen-BSA and RAC-BSA were dispensed onto the NC membrane as the T1 and T2 lines.

The fluorescent probes posed simultaneous recognition of Clen and RAC were prepared with different ratios of antibodies (anti-Clen mAb:anti-RAC mAb = 2:2, 2:1, 4:1). As shown in Fig. S3A, the AuNCs-MLFIA sensor exhibited high fluorescence signals in negative samples and high inhibition of fluorescence in positive samples with a 2:1 ratio of anti-Clen and anti-RAC mAbs. The volume of the fluorescence probe used in one strip was optimized, and the fluorescence intensity of the T lines in

negative samples increased with increasing volume of probes while those in positive samples declined. A volume of 2.0 μL of the fluorescence probes was selected in the subsequent experiments (Fig. S3B). Alterations in fluorescence intensities on the T and C lines indirectly reflect the interaction between the fluorescent probes and antigens or goat anti-mouse IgG on the developed AuNCs-MLFIA sensor. After addition, the mixed solution flows onto the NC membrane within 2 min and constant fluorescence intensities are achieved by the T1 and C lines within 10 min and by the T2 line within 15 min (Fig. S3C). This result indicates that measurement of fluorescence intensities could be accomplished within 18 min.

3.3 Performance of the AuNCs-MLFIA sensor for Clen and RAC detection

The AuNCs-MLFIA sensor was used to test swine urine samples spiked with standard solutions of Clen and RAC, and a calibration curve was constructed by plotting the fluorescence ratio (T1/C and T2/C) against the logarithm of Clen and RAC concentrations. Fig. 4A shows that the fluorescence intensities of the T1 and T2 lines gradually weakened with increasing concentrations of Clen and RAC while that of the C line improved. When the Clen and RAC concentrations were 0.25 μg L^{-1}, the fluorescence intensities of the two T lines exhibited a significant difference compared with those of the negative sample. The limit of detection (LOD) for qualitative detection by the naked eye was 0.25 μg L^{-1}.

The portable fluorescence reader was used to record fluorescence intensities, and fluorescence ratio was calculated, as shown in Fig. S4A, the fluorescence intensity of T lines decreased while that of the C line increased. Calibration curves were constructed by plotting the fluorescence ratio against the logarithmic concentrations of the analytes (Fig. S4B). The data (Fig. 4B) showed good linear ranges for Clen (0.005–4.0 μg L^{-1}) and RAC (0.025–4.0 μg L^{-1}) with half-maximal inhibitory concentrations of 0.06 and 0.32 μg L^{-1}, respectively. The regression equations for Clen and RAC determination were $y = -0.732 \lg(x) + 0.3697$ ($R^2 = 0.9913$) and $y = -1.203 \lg(x) + 0.8474$ ($R^2 = 0.9920$), respectively. The LOD for quantitative detection was calculated from the mean value of 11 blank samples plus three times the standard

deviation and found to be 0.003 μg L^{-1} for Clen and 0.023 μg L^{-1} for RAC. For comparison, the antigens and antibodies used in this study were employed to develop a CG-LFIA sensor with 40 nm colloidal Au nanoparticles as labels. As shown in Fig. S5, the LOD of the CG-LFIA sensor for qualitative detection of Clen and RAC was 2.0 μg L^{-1}, and its LODs for quantitative detection were 0.40 and 0.48 μg L^{-1}, respectively; these LODs are significantly higher than those of the AuNCs-MLFIA sensor.

The performance of different label-based LFIA sensors is summarized in Table 1. The developed AuNCs-MLFIA sensor revealed superior sensitivity compared with those of other LFIA sensors. Three factors are considered to improve the sensitivity of the AuNCs-MLFIA sensor: (i) its ultrasmall nanoparticles (~2.0 nm) of Arg/ATT/AuNCs allow more Arg/ATT/AuNCs to conjugate on a molecule of SA; (ii) introduction of the SA-biotin system promotes the coupling efficiency between Arg/ATT/AuNCs and antibodies; and (iii) mixing of the fluorescence probes with the sample before testing allows adequate bonding of the analyte to the fluorescent probes and leads to the competitive advantage of analytes in the samples.

The specificity of the AuNCs-MLFIA sensor for Clen and RAC was evaluated by fortifying blank swine urine with Clen, RAC, and six structurally related analogs (SAL, CIM, TER, ZIL, PHEA, and EPI) at a concentration of 50 μg L^{-1} and then measuring with the AuNCs-MLFIA sensor. Cross-reaction (CR) is defined as the ratio of measured value to spiked concentration. Results showed no CR between anti-Clen mAb and RAC nor between anti-RAC mAb and Clen. However, anti-Clen mAb showed CR with SAL, CIM, and TER (Fig. S6). The established sensor demonstrated 0.58%, 0.47%, and 0.12% CR with SAL, CIM, and TER, respectively, but negligible (<0.01%) CR with ZIL, PHEA, and EPI (Table S1).

The performance of the developed AuNCs-MLFIA sensor was evaluated by analyzing swine urine samples fortified with known concentrations of Clen and RAC (0.05, 0.5, and 1.0 μg L^{-1}). Table 2 shows that the average recoveries for intra-assay of Clen and RAC were 89.2%–111.4% and 93.3%–105.6%, respectively (coefficient of variation CV, <8.58%). The table also shows that the ranges of recovery for

inter-assay of Clen and RAC were 93.6%–12.3% and 91.4%–114.4%, respectively (CV, <16.9%). These results suggest that the accuracy and precision of the AuNCs-MLFIA sensor are acceptable for Clen and RAC detection.

3.4 Comparative evaluation

Ten swine urine samples collected from different areas and tested by LC-MS/MS and the AuNCs-MLFIA sensor revealed no traces of Clen or RAC. The acceptability of the new AuNCs-MLFIA sensor was evaluated by comparing results with those of LC-MS/MS. Thirty samples were blind spiked with Clen and RAC and then analyzed using the two methods. While the results of both methods (Fig. S7) showed good correlation (R^2 = 0.96), the proposed AuNCs-MLFIA sensor required only 18 min to analyze all samples. These findings suggest that the AuNCs-MLFIA sensor exhibits better performance than other immunoassays.

4. Conclusions

The highly luminescent Arg/ATT/AuNCs exhibited ultrasmall particles, favorable biocompatibility, good water solubility, and unique properties, all of which indicate their promising potential use as fluorescence labels for bioassay. A multiplex lateral flow immunochromatographic sensor based on Arg/ATT/AuNCs was thus developed for the simultaneous detection of Clen and RAC in swine urine samples. By integrating the advantages of Arg/ATT/AuNCs and LFIA, the proposed AuNCs-MLFIA sensor could simultaneously complete Clen and RAC screening within 18 min. Fluorescence signals were recorded by a portable fluorescence reader, thereby enabling quantitative detection. Under optimal conditions, the AuNCs-MLFIA sensor presented superior performance compared with other label-based LFIA sensors. Hence, the newly developed AuNCs-MLFIA sensor could be adapted for determining other chemical contaminants to ensure food safety.

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Table 1. Comparison of the published other LFIA sensor for Clen and RAC detection.

Analytes	Labels	Multiplex detection	Quantitative detection	IC_{50} Value ($\mu g\ L^{-1}$)	LOD ($\mu g\ L^{-1}$)	Time (min)	Samples	References
Clen	Fluorescent beads	Yes	Yes	---	0.1	10	Swine urine Tissue Feed	[14]
RAC				---	0.1			
Clen	Colloidal gold	Yes	Yes	0.56	0.1	5	Swine urine	[21]
RAC				0.71	0.1			
Clen	Time-resolved chemiluminescence	Yes	Yes	---	0.067	20	Swine urine	[22]
RAC				---	0.17			
Clen	Au-Ag nanoparticles	No	No	---	2.0	15	---	[24]
Clen	Colloidal gold	No	Yes	0.46	0.22	10	Swine urine	[25]
RAC	Colloidal gold	No	Yes	0.59	0.13	15	Swine urine	[27]
RAC	Quantum dots	No	Yes	---	0.1	---	Swine urine	[28]
Clen	Colloidal gold	Yes	Yes	0.73	0.4	10	Swine urine	This Work
RAC				1.09	0.48			
Clen	Arg/ATT/AuNCs	Yes	Yes	0.06	0.003	18	Swine urine	
RAC				0.32	0.023			

Table 2. Recovery and precision of the developed AuNCs-MLFIA sensors in spiked swine urine samples (n=3)

Spiked Level ($\mu g\ L^{-1}$)	Intra-assay				Inter-assay			
	Clen		RAC		Clen		RAC	
	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)
0.05	100.8	8.58	105.6	1.52	112.3	6.75	95.4	2.93
0.5	111.4	3.41	93.3	3.39	111.5	8.44	114.4	10.7
1.0	89.2	3.46	97.2	7.71	93.6	3.57	91.4	16.9

Figure captions

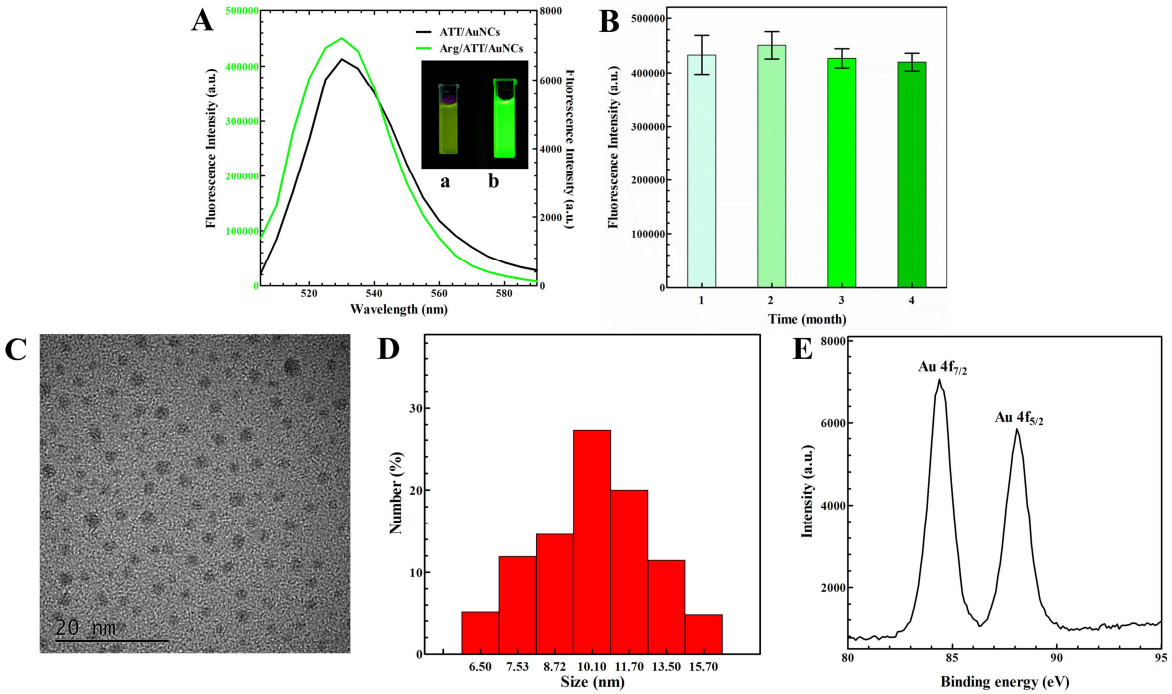
Scheme 1 (A) Schematic of the preparation of highly luminescent Arg/ATT/AuNCs and fluorescent probes. (B) Schematic diagram of the AuNCs-MLFIA sensor for simultaneous detection of Clen and RAC.

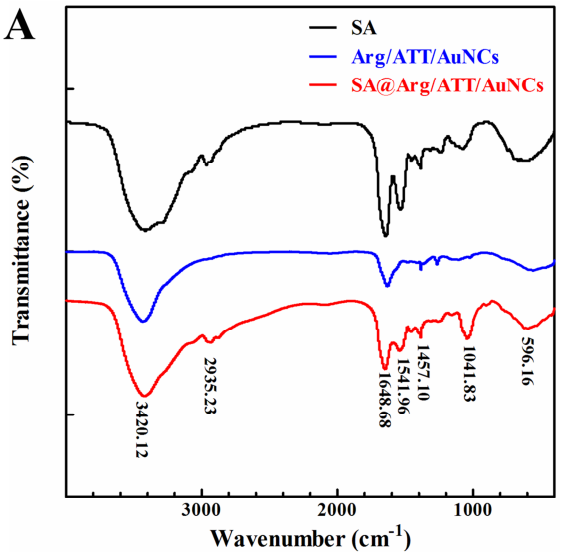
Fig. 1 Characterization of the Arg/ATT/AuNCs. (A) Fluorescence spectra of the ATT/AuNCs (black line) and Arg/ATT/AuNCs (green line). Inset: Photographs of (a) ATT/AuNCs and (b) Arg/ATT/AuNCs under a UV-vis lamp. (B) Stability of the prepared Arg/ATT/AuNCs over 4 months. (C–E) HRTEM image, hydrodynamic diameter, and XPS spectra of Arg/ATT/AuNCs.

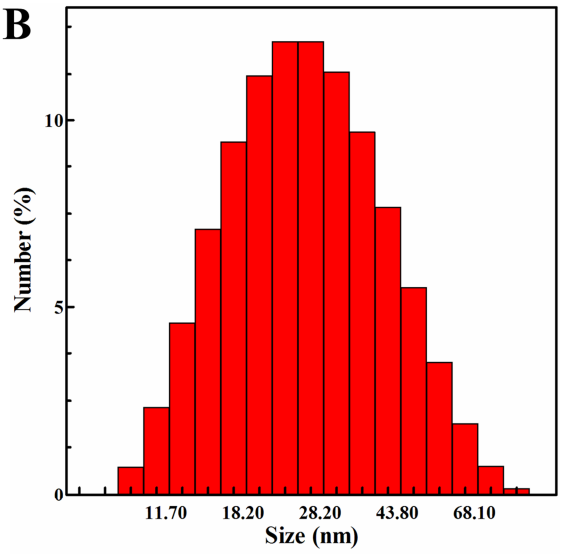
Fig. 2 (A) FTIR spectra of SA (black), Arg/ATT/AuNCs (blue), and SA@Arg/ATT/AuNCs (red). (B) Hydrodynamic diameter of SA@Arg/ATT/AuNCs. (C) Zeta potential of Arg/ATT/AuNCs and SA@Arg/ATT/AuNCs. (D) Results of immunochromatographic testing to evaluate the SA@Arg/ATT/AuNCs and fluorescent probes.

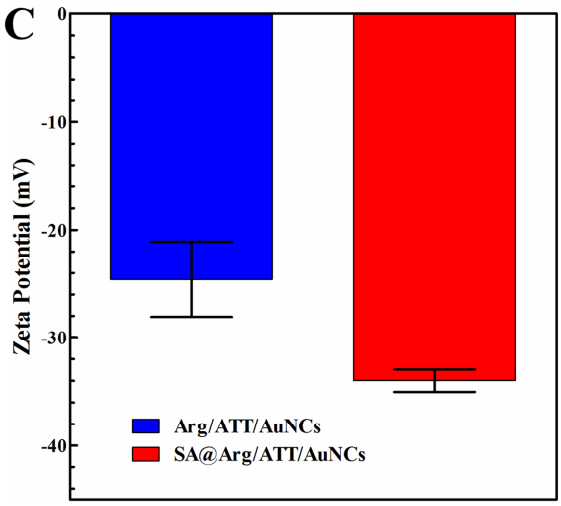
Fig. 3 Results of pH effects on T1, T2, and C lines observed by naked eyes (A), and the fluorescence intensities obtained by protable fluorescence reader (B).

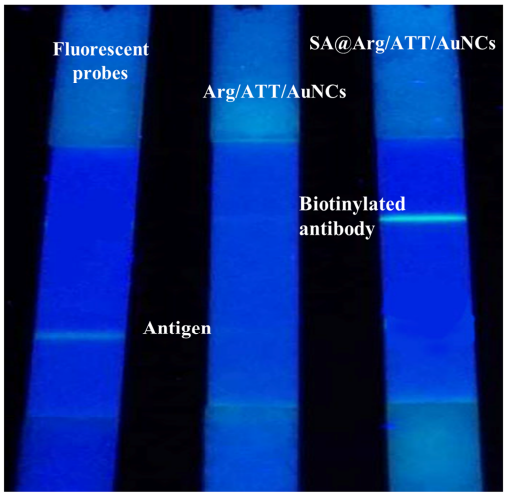
Fig. 4 Qualitative and quantitative detection results of the AuNCs-MLIFA sensor for Clen and RAC. (A) Photograph of AuNCs-MLIFA sensors with different concentrations of analytes. (B) Linear ranges for quantitative detection of Clen and RAC constructed by plotting the fluorescence ratio against the logarithmic concentration of the analyte.

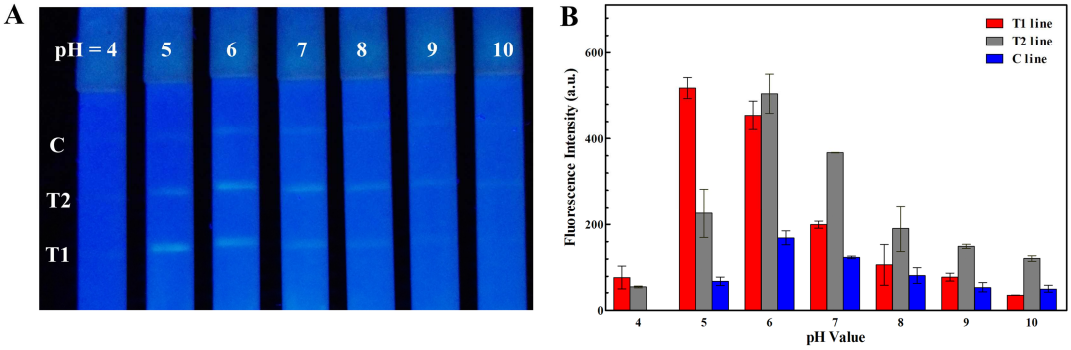


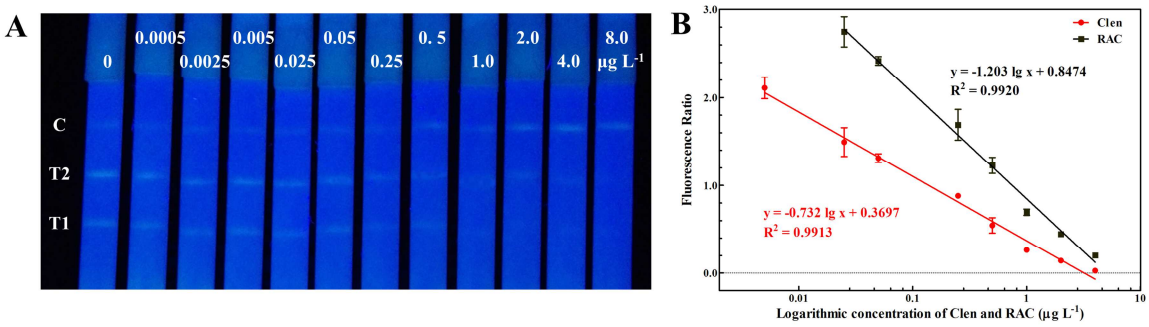


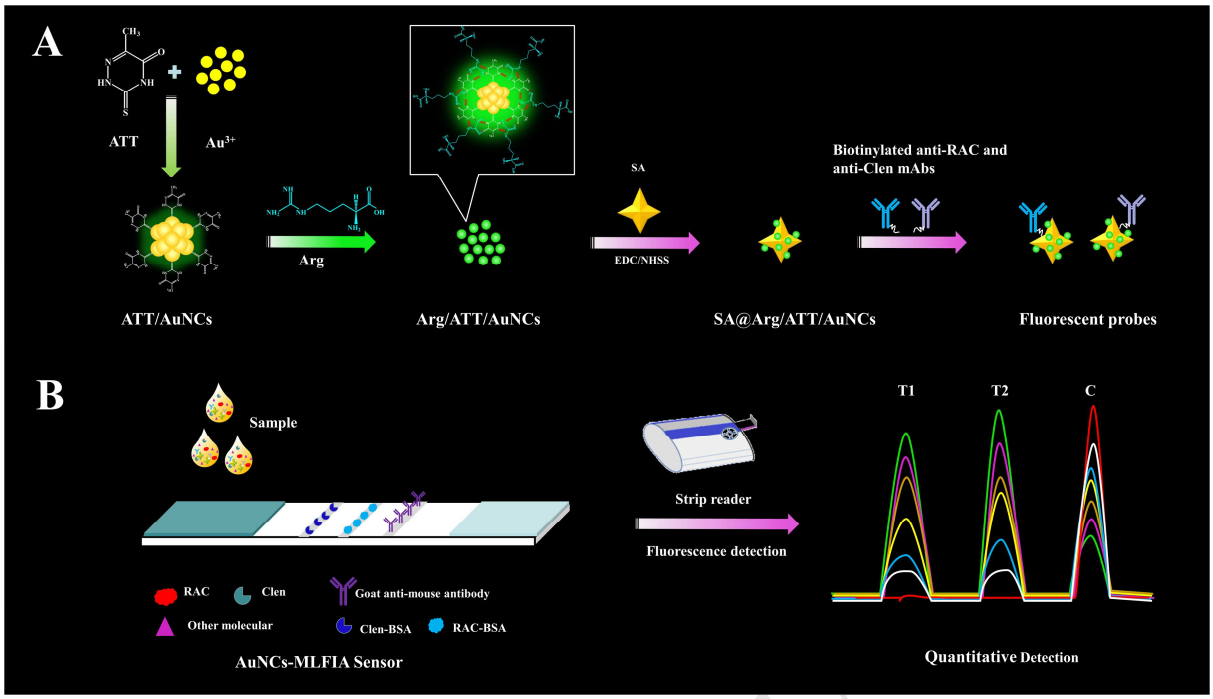












1. Highly luminescent green-emitting gold nanoclusters were successfully applied to lateral flow immunoassay for the first time.

2. Multiplex lateral flow immunoassay based on gold nanoclusters for simultaneous and quantitative determination of clenbuterol and ractopamine in swine urine.

3. The developed AuNCs-MLFIA sensor revealed superior in sensitivity than other LFIA sensors.