

Article

A Ratiometric Fluorescence Sensor Based on BSA Assembled Gold–Silver Bimetallic Nanoclusters for Highly Selective Detection of Chlortetracycline in Water

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Abstract

This study reports the precise synthesis of red-emitting gold–silver bimetallic nanoclusters (Au–AgNCs) via a one-pot hydrothermal method using thiolactic acid as both the ligand and reducing agent. The Au–AgNCs possess an average diameter of 1.85 nm and exhibit strong fluorescence emission at 687 nm. Furthermore, they display notable assembly-induced emission enhancement (AIEE) properties. Upon assembly with bovine serum albumin (BSA), their fluorescence quantum yield significantly increases from 2.50% to 7.78%. Then Au–AgNCs@BSA assembly was employed as a ratiometric fluorescence sensor for the detection of chlortetracycline (CTC). In the presence of CTC, the original red emission of the assembly at 687 nm remained stable, while a new blue emission emerged at 420 nm and intensified progressively with CTC concentration. The ratio of the two emission intensities (I_{420}/I_{687}) exhibited an excellent linear correlation with CTC concentration over the range of 0.10 to 15 μ M, with a limit of detection (LOD) of 20 nM. Notably, the sensor demonstrated exceptional selectivity for CTC, showing negligible response to common interfering substances such as metal ions, anions, amino acids, and crucially, other tetracycline antibiotics (tetracycline, oxytetracycline, and doxycycline). The practical applicability of the sensor was validated through the determination of spiked CTC in real water samples, achieving satisfactory recovery rates. In conclusion, this work accomplishes two key objectives: the development of novel AIEE-active Au–Ag bimetallic nanoclusters and the design of an efficient ratiometric sensing strategy. This approach enables the highly selective and sensitive detection of CTC, offering a promising tool for environmental monitoring.

Keywords: gold–silver bimetallic nanoclusters; assembly; chlorotetracycline; BSA; fluorescence sensor



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1. Introduction

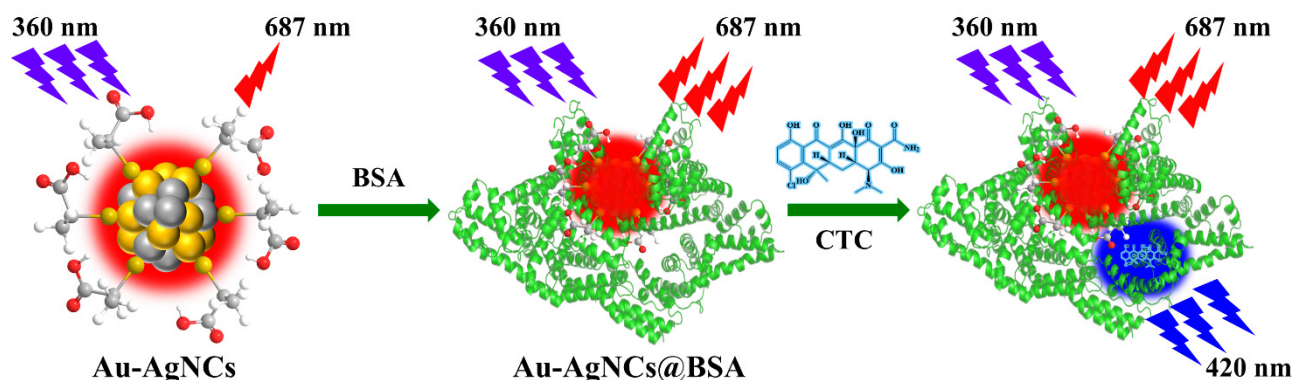
Tetracycline antibiotics (TCA), characterized by a four-ring core structure with various substituents (e.g., methyl, hydroxyl groups), represent a major class of broad-spectrum antibiotics. Common members include chlortetracycline (CTC), oxytetracycline (OTC), tetracycline (TC), and doxycycline (DC) [1,2]. Among them, CTC is of particular significance due to its extensive use as a low-cost, broad-spectrum agent in veterinary medicine and

livestock farming for both therapeutic purposes and growth promotion [3,4]. However, CTC exhibits relatively low bioavailability and environmental degradability, leading to its persistent accumulation in animal-derived products and, notably, in water bodies due to its water solubility [5,6]. This persistence poses distinct monitoring challenges and specific health risks, including potential hepatotoxicity and allergic reactions upon human exposure via the food chain or contaminated water [7,8]. Therefore, developing selective detection methods for CTC, alongside general TCA monitoring, is of high practical importance.

Currently, common techniques for TCA detection encompass high-performance liquid chromatography (HPLC) [9], capillary electrophoresis (CE) [10], immunochromatography [11], and electrochemical methods [12]. While HPLC and CE offer high sensitivity and accuracy, they often involve complex sample pretreatment, require expensive instrumentation, and demand skilled laboratory personnel [13,14]. Immunochromatographic assays provide rapid, on-site testing but may face challenges in achieving high sensitivity and multiplexed quantification. Electrochemical methods are promising for portability but can be susceptible to electrode fouling in complex matrices. In recent years, fluorescence sensing has garnered significant attention in detection research due to its potential to overcome these limitations, enabling real-time, in situ, rapid, and highly sensitive detection of target analytes [5,15]. The integration of nanomaterials can further enhance detection performance. For instance, Li et al. synthesized NH₂-MIL-53(Al) for the fluorescence quenching detection of CTC via an inner filter effect; however, the system could not differentiate among various TCA [16]. Similarly, Hui et al. reported a two-photon-excited fluorescent carbon dot (TP-CQDs) nanoprobe for detecting CTC in food, which also exhibited analogous fluorescence responses to OTC and TC [17]. The fundamental challenge lies in the high structural similarity among TCA, which share an identical tetracycline skeleton with only minor variations in substituents. This structural homology makes it exceedingly difficult for fluorescence sensors to achieve selective discrimination [18,19]. Most reported fluorescence sensors for CTC suffer from cross-sensitivity to other TCA, indicating poor selectivity. Therefore, developing a fluorescence sensor capable of highly selective CTC detection while effectively eliminating interference from other TCA remains a formidable challenge.

In our prior work, we observed that near-infrared-emitting (805 nm) thiolactic acid-protected gold nanoclusters (AuNCs) could interact with bovine serum albumin (BSA), resulting in an approximately three-fold enhancement in emission intensity [20]. Inspired by this finding, we introduced silver nitrate during synthesis to prepare thiolactic acid-protected gold–silver bimetallic nanoclusters (Au–AgNCs). We subsequently investigated their interaction with BSA and the consequent effects on fluorescence properties. The as-prepared Au–AgNCs exhibited strong red emission at 678 nm under 465 nm excitation. Notably, in the presence of BSA, the emission intensity increased by 3.2-fold. Previous studies report that TCA possess intrinsic but weak fluorescence, which can be significantly enhanced upon interaction with BSA [21,22]. This enhancement is primarily attributed to the encapsulation of TCA molecules within the hydrophobic cavity of BSA, which restricts molecular vibration and rotation, leading to a more intense fluorescence emission in the hydrophobic microenvironment. Building on this rationale, we applied the Au–AgNCs and BSA assembly (Au–AgNCs@BSA) to TCA detection (Scheme 1). Interestingly, the addition of CTC induced the emergence of a new blue fluorescence emission peak centered at 420 nm, and the intensity increased gradually with CTC concentration. Meanwhile, the original red emission of the nanoclusters remained unchanged. Crucially, this specific fluorescence response was not induced by common metal ions, anions, or amino acids typically found in natural water systems, or the other three tetracycline antibiotics, demonstrating exceptional recognition selectivity. Therefore, by introducing BSA to the system, we have not only

significantly improved the fluorescence quantum efficiency of Au-AgNCs but also achieved highly sensitive and specific detection of CTC. This work provides a novel and promising strategy for constructing novel tetracycline antibiotic sensors.



Scheme 1. Schematic illustration of the AIEE-enhanced Au-AgNCs@BSA assembly and its application in ratiometric fluorescence detection of CTC.

2. Experimental Details

2.1. Reagents and Instruments

Chloroauric acid hydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, $\geq 99\%$) and thiolactic acid ($\geq 99\%$) were purchased from Aladdin Chemical Co., Ltd. (Shanghai, China). Silver nitrate (AgNO_3 , $\geq 99\%$) was obtained from Beijing Chemical Reagent Company (Beijing, China). Chlortetracycline hydrochloride (CTC), doxycycline (DC), oxytetracycline hydrochloride (OTC), tetracycline (TC), and diclofenac sodium (DS) were supplied by Shanghai McLean Biochemical Co., Ltd. (Shanghai, China). The following amino acids arginine (Arg), methionine (Met), tryptophan (Trp), threonine (Thr), histidine (His), serine (Ser), glutamic acid (Glu), glycine (Gly), leucine (Leu), asparagine (Asn), lysine (Lys), phenylalanine (Phe), and aspartic acid (Asp) were all acquired from Beijing Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China). Inorganic salts, including zinc nitrate hexahydrate ($\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$), sodium carbonate (Na_2CO_3), potassium bromide (KBr), sodium chloride (NaCl), sodium nitrate (NaNO_3), potassium chloride (KCl), calcium chloride (CaCl_2), and magnesium chloride (MgCl_2), were procured from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). All chemicals were of analytical grade and used as received without further purification. Ultrapure water (resistivity $\geq 18 \text{ M}\Omega \cdot \text{cm}$) was obtained from a Milli-Q water purification system and used throughout the experiments.

Fluorescence spectra were recorded using an RF-5301PC spectrophotometer (Shimadzu Corporation, Kyoto, Japan). UV-Vis absorption spectra were measured with a UV-3600 spectrophotometer (PerkinElmer Inc., Waltham, MA, USA). Transmission electron microscopy (TEM) images were obtained on a JEM-2200FS instrument (Jeol Ltd., Tokyo, Japan) operating at an accelerating voltage of 200 kV. X-ray photoelectron spectroscopy (XPS) analysis was performed on a Thermo Scientific ESCALAB 250Xi spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) using a monochromatic Al $K\alpha$ X-ray source. Time-resolved fluorescence decay profiles were collected using an FLS920 spectrometer (Edinburgh Instruments Ltd., Livingston, UK). Absolute fluorescence quantum yields were determined with a steady-state/transient fluorescence spectrometer (Edinburgh Instruments Ltd., Livingston, UK).

2.2. Synthesis of Au-AgNCs

Thiolactic acid-protected gold–silver bimetallic nanoclusters (Au-AgNCs) were synthesized via a modified hydrothermal method based on a previous report [20]. Briefly,

HAuCl₄ solution (0.70 mL, 10 mM) was mixed with NaOH solution (0.55 mL, 200 mM) and stirred at room temperature for 4 min. Subsequently, thiolactic acid (0.65 mL, 100 mM) was added, followed by the addition of AgNO₃ solution (0.3 mL, 20 mM). The mixture was then diluted to a final volume of 10.0 mL with ultrapure water (7.8 mL) under vigorous stirring. The resulting solution was transferred into a stainless-steel autoclave and heated at 110 °C for 2 h. After cooling naturally to room temperature, the crude product was purified by precipitation with acetone (added at a 1:2 *v/v* ratio of product solution to acetone). The mixture was centrifuged at 16,000× *g* for 20 min. The supernatant was discarded, and the precipitate was collected and stored at 4 °C for further use.

2.3. Fluorescence Sensing Procedure

A stock solution of CTC (0.50 mM) was prepared in ultrapure water. The sensing assembly solution was prepared by mixing appropriate volumes of Au-AgNCs stock solution (100 µg·mL⁻¹) and bovine serum albumin (BSA, 100 µM) stock solution, followed by dilution with Tris-HCl buffer (20 mM, pH 7.0) to obtain final concentrations of 5.0 µg·mL⁻¹ for Au-AgNCs and 4.0 µM for BSA. For the detection assay, different aliquots of the CTC stock solution were titrated into 1.0 mL of the assembly solution. After incubation at room temperature for 2 h, the fluorescence spectra were recorded using a fluorescence spectrophotometer with an excitation wavelength of 360 nm. All measurements were performed in triplicate to ensure reproducibility.

2.4. Selectivity and Interference Studies

The selectivity of the Au-AgNCs@BSA assembly towards CTC was evaluated against various potential interferents. These included: amino acids (Arg, Met, Trp, Thr, His, Ser, Glu, Gly, Leu, Asn, Lys, Phe, and Asp), common inorganic ions (Na⁺, K⁺, Ca²⁺, Mg²⁺, Al³⁺, Zn²⁺, Fe²⁺, CO²⁺, Ni²⁺, Ag⁺, Fe³⁺, NH₄⁺, F⁻, Cl⁻, Br⁻, I⁻, SO₄²⁻, CO₃²⁻, NO₃⁻, PO₄³⁻, and S²⁻), and other tetracycline antibiotics (TC, OTC, DC). For selectivity tests, the assembly solution (prepared as described in Section 2.3) was treated with each individual interferent at a final concentration of 5.0 µM. For anti-interference tests, the assembly solution was treated with a mixture containing both CTC (5.0 µM) and one of the interferents (5.0 µM). In all cases, the samples were incubated under the same conditions as in Section 2.3, and their fluorescence spectra were recorded accordingly. In a typical interference test, an appropriate volume of the interferent stock solution was added to a fixed volume of the Au-AgNCs@BSA sensing solution. For instance, to test Na⁺ at a final concentration of 5 µM, 5 µL of a 1.0 mM Na⁺ stock solution was added to 995 µL of the sensing solution (final composition: Au-AgNCs at 5.0 µg·mL⁻¹ and BSA at 4.0 µM).

3. Results and Discussion

3.1. Preparation, Optimization and Characterization of Au-AgNCs

Gold–silver bimetallic nanoclusters (Au-AgNCs) were synthesized via a one-pot hydrothermal method using thiolactic acid as the ligand, with HAuCl₄ and AgNO₃ as metal precursors (Figure 1A). The as-prepared product exhibited a broad excitation range (360–560 nm), strong red emission centered at 687 nm, and characteristic absorption in the 280–500 nm region (Figure 1B), confirming the successful formation of fluorescent Au-AgNCs. This broad, featureless absorption profile is characteristic of the molecular-like electronic transitions of ultras-small metal nanoclusters. Given their sub-2 nm size, the contribution from light scattering to the measured extinction is negligible, and the signal is therefore attributed to absorption. To achieve optimal optical performance, key synthesis parameters, including reaction time, temperature, NaOH and thiolactic acid concentrations, and the Au/Ag molar ratio, were systematically optimized. The growth process of Au-

AgNCs was first monitored by fluorescence spectroscopy at 15 min intervals. As shown in Figure S1A, the precursor mixture exhibited negligible fluorescence prior to heating. Upon hydrothermal treatment, a weak emission band emerged at 687 nm after 1.5 h and intensified rapidly until 2 h, followed by a gradual quenching thereafter. The initial increase in fluorescence intensity is attributed to the nucleation and maturation of Au-AgNCs, while the subsequent decrease likely results from further particle growth or aggregation [23,24]. A concomitant red-shift in emission wavelength was observed over time, consistent with the proposed growth mechanism. UV-Vis absorption spectroscopy further corroborated this process (Figure S1B). While no distinct absorption was detected in the 350–700 nm range at $t = 0$ h, a broad absorption band (300–550 nm) developed progressively during the reaction, indicative of nanoclusters formation. It is worth noting that the absorption around 300 nm decreased while that around 550 nm increased slightly with prolonged reaction time, indicating the progressive formation and growth of Au-AgNCs into nanoparticles, which correspondingly modulated the fluorescence emission intensity.

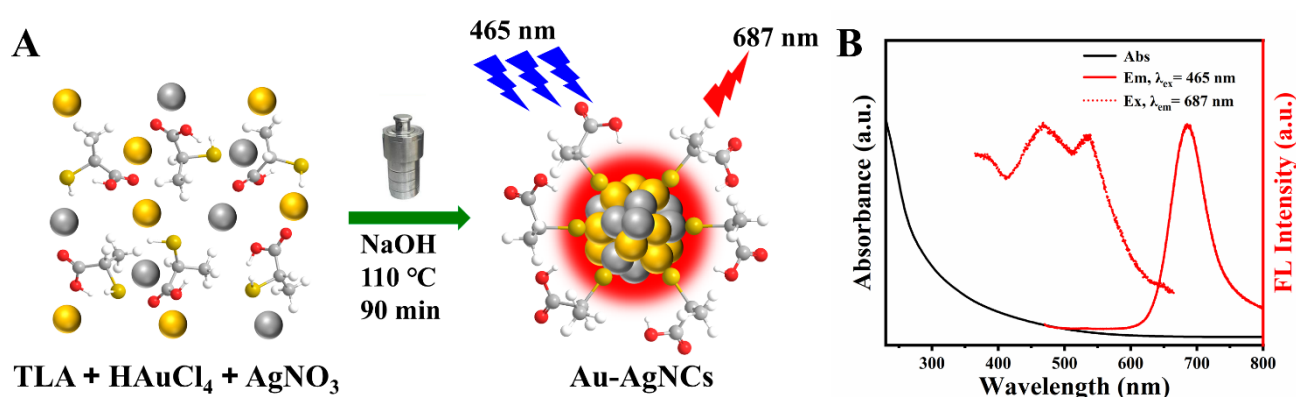


Figure 1. (A) Schematic illustration of the controlled synthesis of Au-AgNCs. (B) Normalized UV-vis absorption (black solid line), fluorescence excitation (red dashed line), and emission (red solid line) spectra of Au-AgNCs.

Thiolactic acid serves dual roles as both a reducing agent and a stabilizing ligand, necessitating its use in excess relative to metal ions. As illustrated in Figure S2, the fluorescence intensity of Au-AgNCs increased with thiolactic acid concentration, reaching a maximum at 6.5 mM. This optimal concentration was therefore selected for subsequent syntheses, as it provides sufficient surface protection and ligand distribution to maximize fluorescence. Given that alkaline conditions facilitate the reduction of Au(III) [25], the concentration of NaOH and its pre-incubation time were also optimized. The fluorescence intensity of the resulting NCs showed a volcano-shaped dependence on NaOH concentration, with a maximum at 4.0 mM (Figure S3). A pre-incubation time of 4 min was found to be optimal (Figure S4). The Au/Ag molar ratio was another critical factor; a ratio of 8:2 (HAuCl₄:AgNO₃) yielded the highest fluorescence intensity (Figure S5). Finally, the reaction temperature was varied between 90 and 130 °C. The product synthesized at 110 °C exhibited the strongest emission (Figure S6) and was therefore selected for all further experiments. In summary, the optimal synthesis conditions were determined as follows: 110 °C, 120 min reaction time, Au/Ag molar ratio of 8:2, 4.0 mM NaOH, and 6.5 mM thiolactic acid.

The absolute fluorescence quantum yield (PLQY) of the optimized Au-AgNCs was measured to be 2.50% using an integrating sphere. This value falls within the typical range (often 1–10%) reported for many thiolate-protected gold or gold–silver nanoclusters emitting in the red to near-infrared region, where fluorescence originates from the ligand-to-metal charge transfer (LMCT) or intra-core transitions. For instance, similar bimetallic Au-AgNCs protected by glutathione have reported QYs between 2.47% and 7.2% [26,27].

Transmission electron microscopy (TEM) revealed their size and morphology. As shown in Figure 2A, the NCs are well-dispersed with an average diameter of 1.85 ± 0.20 nm (based on statistical analysis of over 200 particles), primarily distributed between 1.50 and 2.30 nm. This size dispersion is likely due to inherent structural inhomogeneity and non-uniform ligand distribution. High-resolution TEM (inset, Figure 2A) displayed a lattice fringe spacing of 0.23 nm, consistent with the (111) plane of face-centered cubic gold [28].

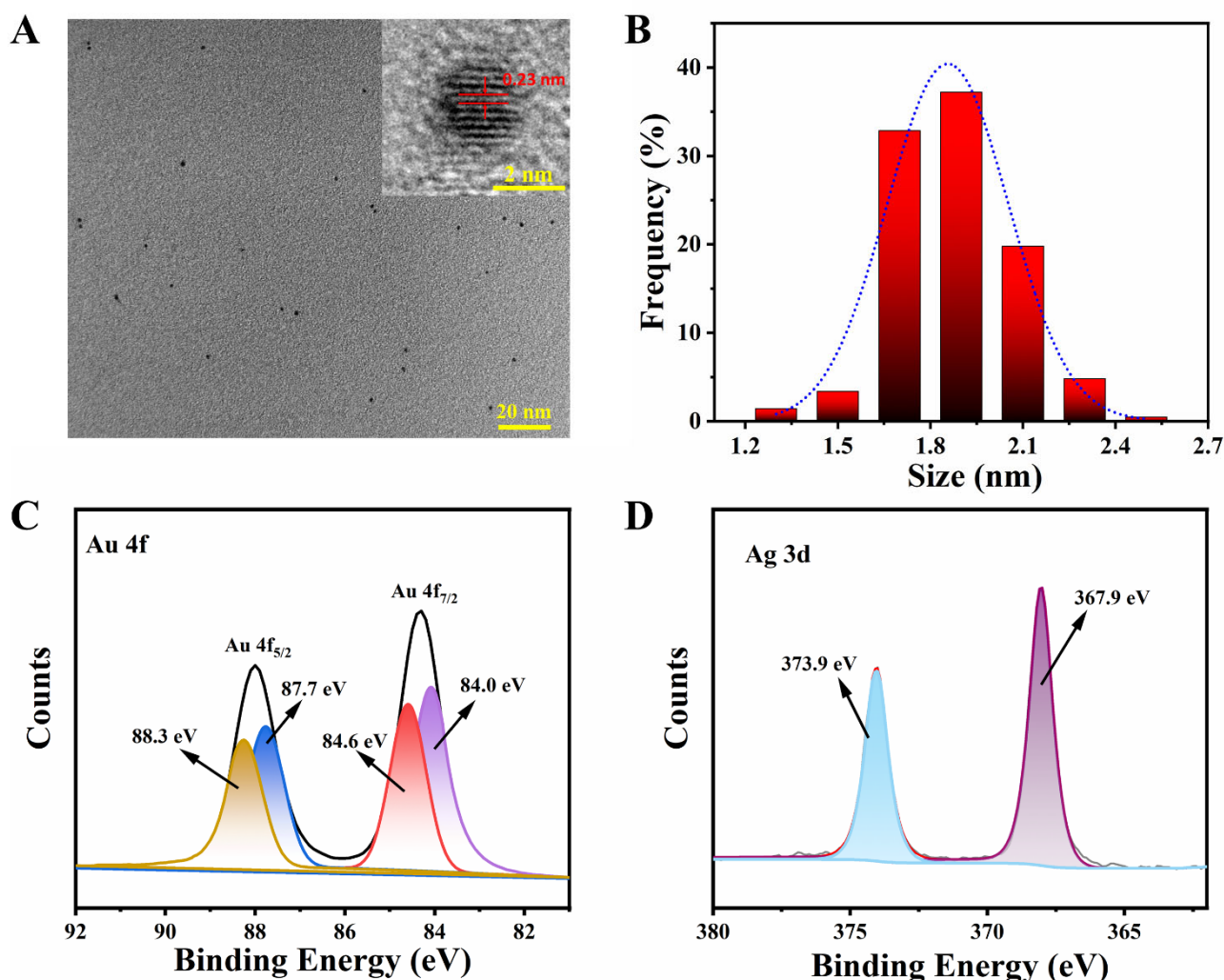


Figure 2. (A) Representative TEM image (inset: enlarged view) and (B) particle size distribution (statistical analysis of over 200 particles) of Au-AgNCs. (C) Au 4f and (D) Ag 3d XPS spectra of the Au-AgNCs.

X-ray photoelectron spectroscopy (XPS) was employed to elucidate the elemental composition and oxidation states (Figure S7A). In the Au 4f region (Figure 2C), two peaks at binding energies (BEs) of 84.3 eV (Au 4f_{7/2}) and 88.0 eV (Au 4f_{5/2}) were observed. Deconvolution of the Au 4f_{7/2} peak revealed two components at 84.0 eV and 84.6 eV, assigned to Au(0) and Au(I), respectively [29,30]. The presence of both components confirms the reduction of HAuCl₄ to a mixture of metallic and oxidative states within the clusters, with the Au(I) state likely forming a stable Au(I)-thiolate complex on the surface. In the Ag 3d spectrum (Figure 2D), peaks at 367.9 eV (Ag 3d_{5/2}) and 373.9 eV (Ag 3d_{3/2}) are characteristic of Ag(0) [31,32], suggesting the co-existence of metallic Ag(0) and Au(0) in the core. Based on XPS atomic percentages, the bimetallic NCs consist of approximately 77.81% Au

and 22.19% Ag, which is consistent well with the proportion of the raw materials (Au:Ag molar ratio of 8:2).

Further elemental composition and oxidation states analysis was conducted. The S 2p peak at 162.9 eV (Figure S7B) corresponds to sulfur atoms bound to the metal core (e.g., Au–S), with no detectable signal near 168.2 eV for oxidized sulfur species [33,34]. This indicates the complete formation of a stable Au(I)-thiolate complex on the NC surface. The O 1s peak at 531.2 eV and 532.3 eV (Figure S7C) is attributed to C–O and C=O groups from thiolactic acid [35], while C 1s spectrum showed two components at 284.8 eV (C–C) and 288.2 eV (C=O), consistent with the organic ligand (Figure S7D) [36]. Collectively, the TEM and XPS results confirm the successful synthesis of red-emitting Au-AgNCs with a core-shell-like nanostructure and an average size of ~1.85 nm.

3.2. Formation and Properties of the Au-AgNCs@BSA Assembly

Building on our previous observation that thiolactic acid-protected gold nanoclusters exhibit assembly-induced emission enhancement (AIEE) upon binding to bovine serum albumin (BSA) [20], we explored the interaction between BSA and the synthesized Au-AgNCs. As depicted in Figure 3A, the fluorescence intensity of Au-AgNCs increased progressively with the addition of BSA, reaching a maximum (a 3.2-fold enhancement) at a BSA concentration of 4.0 μ M. Further increase in BSA concentration led to a gradual quenching of fluorescence accompanied by a slight red-shift. This phenomenon may be attributed to competitive interactions between the nanoclusters and multiple binding sites within the BSA molecules at higher protein concentrations. The assembly process did not significantly alter the optical absorption characteristics of the Au-AgNCs, as evidenced by the nearly unchanged UV-Vis absorption spectrum (Figure S8), indicating good stability of the nanoclusters during complex formation. The corresponding changes observed in the enlarged UV-vis absorption spectra align well with the fluorescence trends, which further corroborates the dynamic assembly process between BSA and the nanoclusters. In addition, the fluorescence lifetime of the assembly was measured to gain further mechanistic insights (Figure S9). The results showed that the lifetime of Au-AgNCs increased from 1.42 μ s to 1.89 μ s upon assembly with BSA, which is consistent with the observed fluorescence enhancement, further supporting the occurrence of AIEE and the formation of a rigidified nanostructure.

Transmission electron microscopy (TEM) further confirmed that the core structure and dispersion state of the NCs were preserved after assembly with BSA. Statistical analysis (Figure 3B) revealed an average particle size of approximately 1.90 nm, which is consistent with that of the isolated NCs, confirming that the assembly did not induce notable aggregation or changes in the metal core. Based on prior work with analogous AuNCs@TLA and the unchanged dispersion state observed by TEM [20], the term ‘assembly’ here describes the formation of discrete BSA-nanocluster complexes, primarily driven by electrostatic interactions, rather than the aggregation of nanoclusters. The observed fluorescence enhancement along with the increased absolute fluorescence quantum yield (from 2.50% for Au-AgNCs to 7.78% for the Au-AgNCs@BSA assembly), strongly suggests a specific and effective interaction between the nanoclusters and BSA. It also represents a key advancement, highlighting the effectiveness of the protein-induced rigidification strategy in boosting the emission efficiency of these nanoclusters. Given that the photophysical properties of metal nanoclusters are primarily governed by their ligand shell and core–ligand interface [32–35], we propose that the interaction with BSA modulates the local environment of the ligands and/or the ligand-core coupling, thereby activating the AIEE effect and leading to the significant fluorescence enhancement.

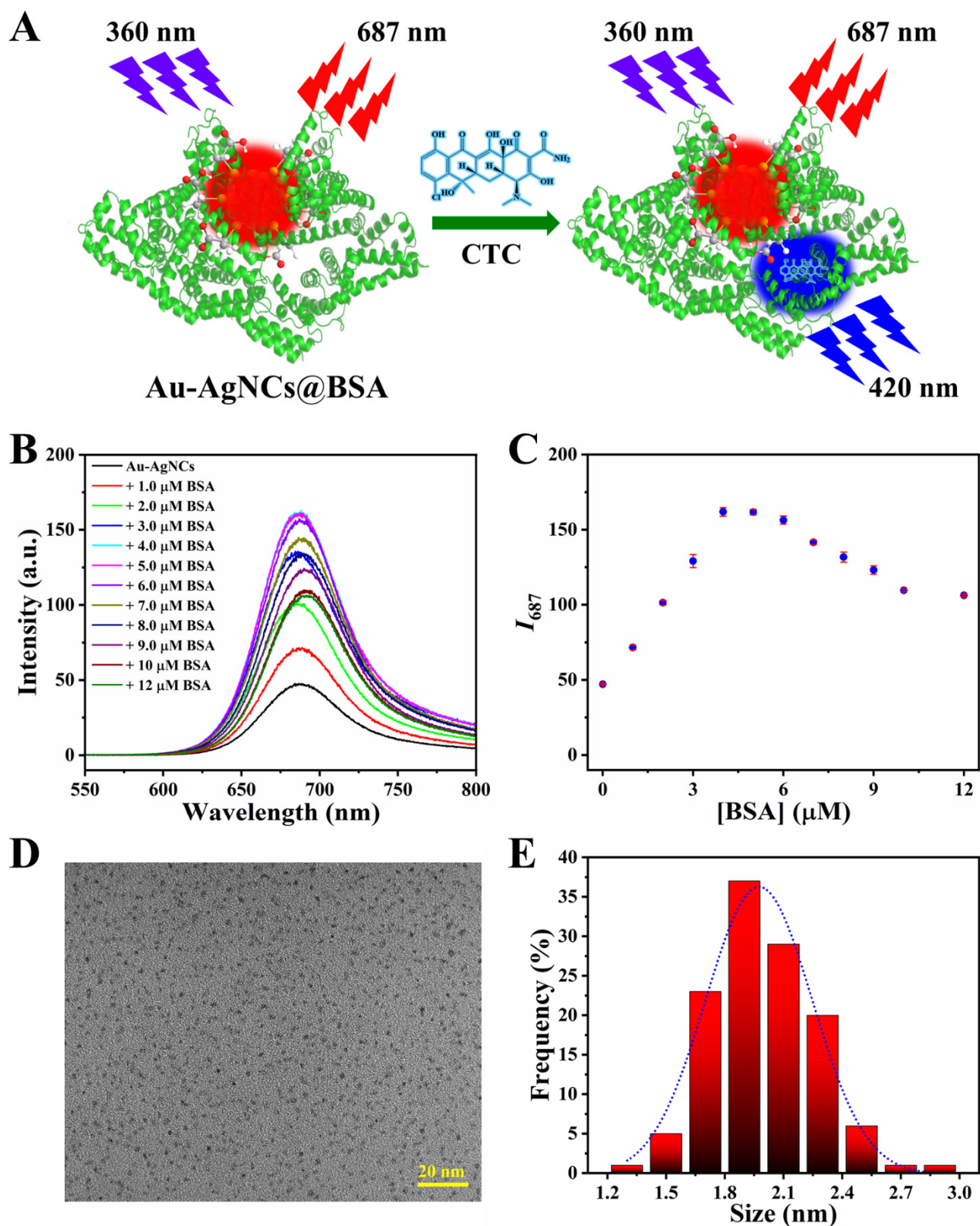


Figure 3. (A) Schematic illustration of the AIEE-based fluorescence enhancement of Au-AgNCs upon BSA assembly. (B) Fluorescence spectra of Au-AgNCs ($5.0 \mu\text{g}\cdot\text{mL}^{-1}$) titrated with increasing BSA concentrations (0–12 μM) in Tris-HCl buffer (pH 7.0; $\lambda_{\text{ex}} = 465 \text{ nm}$). (C) Corresponding emission intensity changes at 687 nm (Error bars represent the standard deviation from three independent measurements ($n = 3$)). (D) Representative TEM image and (E) particle size distribution (statistical analysis of over 200 particles) of the Au-AgNCs@BSA assembly.

3.3. Fluorescence Detection of CTC by Using the Au-AgNCs@BSA Assembly

Upon addition of CTC to the Au-AgNCs@BSA assembly solution, as shown in Figure 4A, the intensity of the new emission peak at 420 nm (I_{420}) increased progressively with CTC concentration. However, the original emission of the Au-AgNCs at 687 nm (I_{687}) remained largely unaffected. The intensity of this new peak (I_{420}) increased progressively with higher CTC concentrations (Figure 4), enabling the system to function as a ratiometric fluorescence sensor for CTC. To achieve optimal sensing performance, the incubation time was first optimized. The fluorescence response required approximately 2.0 h to reach equilibrium (Figure S10), which was consequently adopted as the incubation time for all detection experiments. This timescale is attributed to the kinetics of the specific biomolecular interaction between BSA and CTC, which involves the diffusion and encapsulation of CTC into the hydrophobic binding site of the protein, a process characterized by a moderate association rate [37,38]. Consequently, a 2 h incubation period was adopted for all subsequent detection experiments.

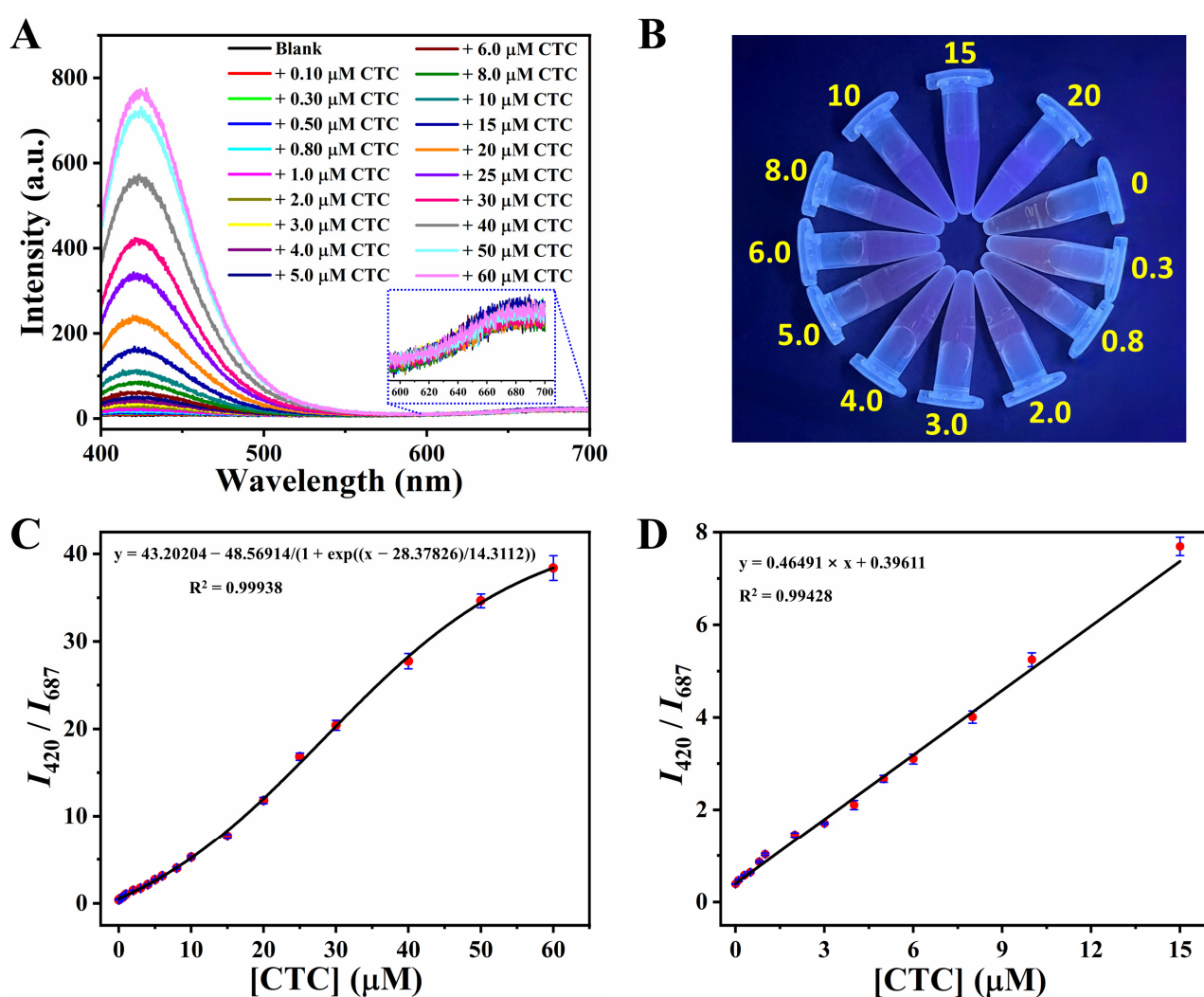


Figure 4. (A) Fluorescence spectra of the Au-AgNCs@BSA assembly ($5.0 \mu\text{g}\cdot\text{mL}^{-1}$ and $4.0 \mu\text{M}$) upon titration with CTC ($0\text{--}60 \mu\text{M}$; $\lambda_{\text{ex}} = 360 \text{ nm}$). (B) Photographs of the corresponding solutions of Au-AgNCs@BSA in the presence of CTC ($0\text{--}20 \mu\text{M}$) under 365 nm UV light. (C,D) Linear calibration plot of I_{420}/I_{687} versus CTC concentration (Error bars represent the standard deviation from three independent measurements ($n = 3$)).

Under the optimized conditions, the sensing performance was evaluated by titrating increasing concentrations of CTC into the Au-AgNCs@BSA solution. As depicted in Figure 4A, the intensity of the emission at 420 nm (I_{420}) intensified with increasing CTC concentration, ultimately showing an enhancement of approximately 100-fold at saturation. Notably, the original emission at 687 nm (I_{687}) remained stable throughout the titration. The ratiometric response also translates into a distinct visual color change under UV light (365 nm). As shown in Figure 4B, the solution fluorescence transitions from faint red to bright blue with increasing CTC concentration, demonstrating the potential of this system for visual or semi-quantitative analysis. The ratio of the two emission intensities (I_{420}/I_{687}) exhibited an excellent linear correlation with CTC concentration over the range of 0.10 to 15 μ M (Figure 4C,D). The corresponding linear regression equation was $I_{420}/I_{687} = 0.46491 \times [\text{CTC}] + 0.39611$ ($R^2 = 0.99428$). The limit of detection (LOD) was calculated to be 20 nM based on the formula $3\sigma/k$, where σ is the standard deviation of the blank signal and k is the slope of the calibration curve. As summarized in Table 1, the LOD and linear range of the proposed ratiometric method are comparable to, and in some cases superior to, those of previously reported turn-on or turn-off fluorescence sensors for CTC. More importantly, as demonstrated in subsequent sections, this sensor exhibits exceptional selectivity for CTC against other tetracycline antibiotics and common interferents, highlighting its significant practical application potential. Furthermore, we compared this assembled probe with the previously reported NiNCs probe based on BSA [39]. While both systems utilize BSA-nanoclusters complexes, the NiNCs probe operates via a single-signal, aggregation-induced turn-on mechanism. In contrast, our Au-AgNCs@BSA platform employs a self-calibrating ratiometric design that not only offers higher reliability against environmental fluctuations but also provides a distinct visual color transition (red-to-blue), enabling both quantitative analysis and intuitive semi-quantitative readout.

Table 1. Comparison between Au-AgNCs@BSA and reported fluorescent sensors for CTC detection.

Sensor	Detection Pattern	Working Range (μ M)	Assay Time	LOD (nM)	Real Samples	Ref.
BSA-NiNCs	Turn on	0.01–75	30 min	4.2	honey and milk	[39]
[MQDA-Eu ³⁺]	Turn on	5.0–95.0	30 s	0.93	pip water, milk, and river water	[40]
CuNC-Al ³⁺	Ratiometric	0.1–3.0	15 min	25.3	environmental water, milk	[41]
YCDs-mSiO ₂ @PVA	Turn on	5.0–300	3 min	24	tap water and lake water	[42]
CdTe QDs@ZIF-8	Ratiometric	1.0–30	10 min	37	milk, honey and urine	[43]
CuNCs@TA	Turn on	0.5–200	120 min	84	lake water, and urine	[44]
Au-AgNCs@BSA	Ratiometric	0.10–15	120 min	20	tap water and lake water	This work

3.4. Selectivity and Anti-Interference Performance of Au-AgNCs@BSA for CTC Detection

To assess the practical applicability of the Au-AgNCs@BSA assembly for monitoring antibiotic residues in natural water, its selectivity against common potential interferents was systematically evaluated. These interferents included various amino acids (Arg, Met, Trp, Thr, His, Ser, Glu, Gly, Leu, Asn, Lys, Phe, and Asp), metal ions (Na⁺, K⁺, Ca²⁺, Mg²⁺, Al³⁺, Zn²⁺, Fe²⁺, CO²⁺, Ni²⁺, Ag⁺, Fe³⁺, and NH₄⁺), and anions (F[−], Cl[−], Br[−], I[−], SO₄^{2−}, CO₃^{2−}, NO₃[−], PO₄^{3−}, and S^{2−}). As shown in Figure 5A, the fluorescence response (I_{420}/I_{687}) of the assembly toward these substances was negligible. Subsequently, selectivity among tetracycline antibiotics was also investigated. While the assembly exhibited a pronounced ratiometric response to CTC, other TCA, including tetracycline (TC), oxytetracycline (OTC), and doxycycline (DC), induced only minimal signal changes (Figure 5B). The presence of these analogous antibiotics did not substantially alter the specific response to CTC. The

ratiometric response (I_{420}/I_{687}) to each individual interferent is summarized in Table S1, quantitatively confirming the exceptional selectivity for CTC.

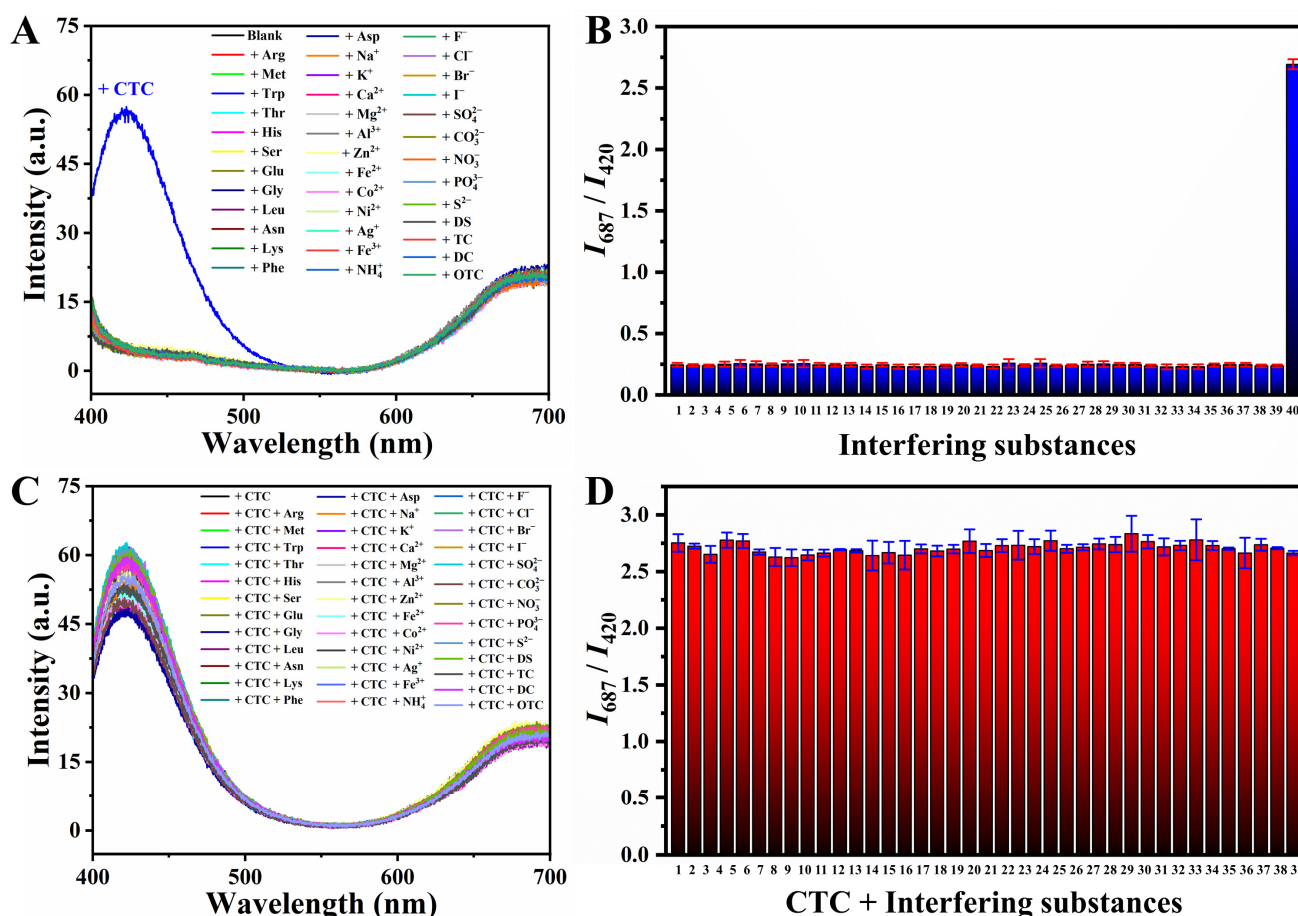


Figure 5. (A) Selectivity test: Fluorescence spectra of Au-AgNCs@BSA ($5.0 \mu\text{g}\cdot\text{mL}^{-1}$ and $4.0 \mu\text{M}$) upon addition of various potential interferents ($5.0 \mu\text{M}$). (B) Corresponding ratiometric response (I_{420}/I_{687}) (Error bars represent the standard deviation from three independent measurements ($n = 3$)). (C) Anti-interference test: Fluorescence spectra of the assembly in the presence of $5.0 \mu\text{M}$ CTC and each interferent ($5.0 \mu\text{M}$). (D) Corresponding ratiometric response (I_{420}/I_{687}) (Error bars represent the standard deviation from three independent measurements ($n = 3$)). Interferent list (1–40): Blank, Arg, Met, Trp, Thr, His, Ser, Glu, Gly, Leu, Asn, Lys, Phe, Asp, Na^+ , K^+ , Ca^{2+} , Mg^{2+} , Al^{3+} , Zn^{2+} , Fe^{2+} , CO_3^{2+} , Ni^{2+} , Ag^+ , Fe^{3+} , NH_4^+ , F^- , Cl^- , Br^- , I^- , SO_4^{2-} , CO_3^{2-} , NO_3^- , PO_4^{3-} , S^{2-} , DS, TC, DC, OTC, and CTC, respectively.

To further simulate complex matrices, anti-interference tests were conducted by introducing potential interferents into a solution containing a fixed concentration of CTC. As illustrated in Figure 5C, the presence of common amino acids, metal ions, or anions did not significantly alter the sensor's specific response to CTC. While a minor signal variation was observed in the presence of the other tetracycline antibiotics, the core ratiometric response to CTC remained strong and clearly distinguishable (Figure 5D), demonstrating the method's practical utility for selective CTC detection even in the presence of structurally related analogs. In addition, diclofenac sodium (DS), a common non-steroidal anti-inflammatory drug, was also introduced as a reference. As shown in Figure 5, DS induced no significant ratiometric response, and its presence did not interfere with the detection of CTC. This result provides additional evidence for the sensor's specificity in complex matrices that may contain diverse drug residues. Considering that inorganic ion concentrations in natural waters can be substantially higher than the target

analyte, the interference from key ions (Na^+ , K^+ , Ca^{2+} , Mg^{2+} , Cl^- , CO_3^{2-} , and NO_3^-) was re-evaluated at a concentration of 100 μM . As shown in Figure S11, no significant interference was observed, further confirming the robustness of the sensor in complex ionic matrices. The results demonstrate the method's practical utility for selective CTC detection even in the presence of structurally related analogs and large amounts of other interfering substances.

3.5. Investigation of the Sensing Mechanism

To clarify the origin of the emission at 420 nm, the intrinsic fluorescence of free CTC was examined for comparison. As shown in Figure S12, free CTC displayed only a weak emission band centered around 570 nm. The exclusive emergence of an intense, blue-shifted peak at 420 nm in the presence of the Au-AgNCs@BSA assembly provides direct evidence that this strong signal results from CTC molecules whose fluorescence is dramatically enhanced upon binding to the hydrophobic cavity of BSA within the composite.

The specific recognition of CTC by the Au-AgNCs@BSA assembly is driven by the strong interaction between BSA and CTC. Previous studies have quantitatively characterized this interaction. For instance, fluorescence spectroscopy gave a binding constant of $5.74 \times 10^4 \text{ M}^{-1}$ [37], confirming stable complex formation. Molecular docking further revealed that CTC binds inside the hydrophobic pocket of BSA via multiple hydrogen bonds and hydrophobic contacts [38]. Additionally, Ni et al. established a 1:1 binding stoichiometry and highlighted the role of BSA's site I (involving Trp residues) in enhancing tetracycline fluorescence [45]. These findings are fully consistent with the 2 h equilibration time observed in our system. The pronounced blue-shift from 570 nm (free CTC) to 420 nm (assembly-bound CTC) confirms that CTC resides in a unique, rigidified microenvironment within the Au-AgNCs@BSA composite. This encapsulation restricts molecular vibration and rotation, shielding CTC from water-mediated quenching and thereby greatly enhancing its intrinsic fluorescence in a hydrophobic environment [46]. This mechanism aligns perfectly with our experimental observation that introducing CTC to the assembly triggers a new, intense emission at 420 nm (Figure 4), which can be attributed to the BSA-enhanced fluorescence of CTC itself.

Crucially, the original fluorescence of the Au-AgNCs at 687 nm remained virtually unchanged upon CTC addition (Figure 2). Furthermore, the UV-Vis absorption profile of the Au-AgNCs was also unaffected by CTC (Figure 6). These two key observations indicate the following: (1) CTC binds to BSA at a site distinct from where the Au-AgNCs are anchored, resulting in no competitive binding; and (2) there is no fluorescence resonance energy transfer (FRET) occurring between the Au-AgNCs (acceptor) and the BSA-bound CTC (donor).

Based on these findings, we propose a dual-role mechanism for the assembly, as illustrated in Scheme 1. First, BSA enhances the fluorescence emission of the Au-AgNCs via an assembly-induced emission enhancement (AIEE) effect, providing a stable internal reference signal at 687 nm. Second, BSA simultaneously and specifically binds to CTC, dramatically enhancing its intrinsic fluorescence at 420 nm. The constant reference signal (I_{687}) and the analyte-dependent signal (I_{420}) together constitute a robust ratiometric sensing platform. This work thus presents an effective design strategy for achieving highly selective fluorescence detection of CTC by leveraging the multifunctional role of BSA within a nanoclusters–protein composite system.

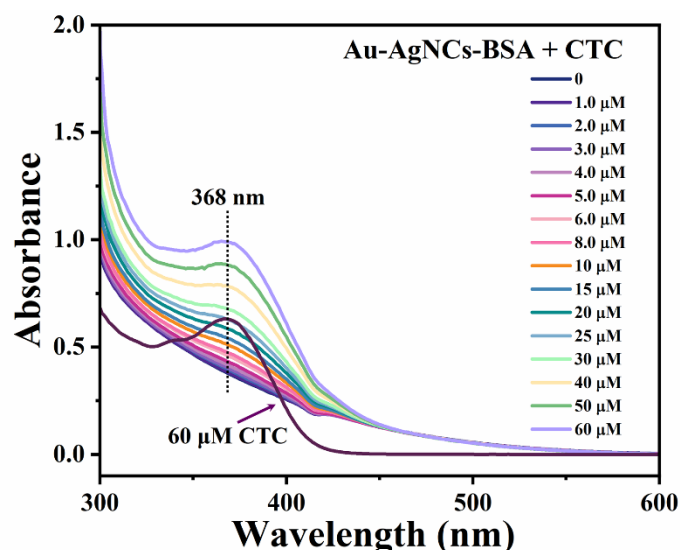


Figure 6. UV-vis absorption spectra of Au-AgNCs@BSA ($5.0 \mu\text{g}\cdot\text{mL}^{-1}$ and $4.0 \mu\text{M}$) upon addition of varying CTC concentrations (0–60 μM).

3.6. Practical Application: Detection of CTC in Real Water Samples

To evaluate the practical applicability of the developed ratiometric sensor, it was employed to detect CTC in spiked tap water and lake water samples. The lake water was collected from Qing Lake on the Qianwei Campus of Jilin University. As summarized in Table 2, the sensor demonstrated good analytical performance, with relative standard deviations (RSDs) below 5% and satisfactory recovery rates ranging from 98.6% to 102.5% for both sample matrices. These results confirm the reliability and accuracy of the Au-AgNCs@BSA-based sensor for quantifying CTC in complex real-water environments. The minimal matrix interference and high recovery rates underscore the sensor's strong potential for practical application in monitoring antibiotic residues in natural water systems.

Table 2. Analytical parameters for the determination of CTC in tap water and lake water samples ($n = 3$).

Sample	Spiked (μM)	Found (μM)	RSD (%)	Recovery (%)
Tap water	0	N.d.	0	0
	5.0	4.95 ± 0.102	3.3	99.0
	10	10.14 ± 0.203	2.7	101.4
	15	15.38 ± 0.262	3.6	102.5
Lake water	0	N.d.	0	0
	5.0	5.12 ± 0.114	3.8	102.4
	10	9.86 ± 0.223	3.2	98.6
	15	15.08 ± 0.308	4.3	100.5

4. Conclusions

In summary, this work presents a novel ratiometric fluorescence sensing platform for chlortetracycline (CTC) detection, based on the strategic design of an Au-Ag bimetallic nanoclusters and bovine serum albumin (BSA) assembly (Au-AgNCs@BSA). Its core innovation lies in the dual-functional role of BSA, which simultaneously enhances the red emission of the nanoclusters as a stable internal reference (via AIEE, QY increased from 2.50% to 7.78%) and acts as a specific host to dramatically enhance the intrinsic blue fluorescence of CTC, enabling robust ratiometric sensing. The assay demonstrates key competitive advantages, including exceptional selectivity, high sensitivity, and strong prac-

tical applicability. Significantly, it successfully distinguishes CTC from other tetracycline antibiotics (TC, OTC, DC), overcoming a major challenge in this field. The method exhibits a wide linear detection range (0.10–15 μM) and a low detection limit of 20 nM. Notably, it has been successfully applied for the accurate determination of CTC in real environmental samples, such as tap water and lake water. A recognized limitation is the relatively long assay time (2 h), attributed to the kinetics of the BSA-CTC biomolecular interaction. This highlights a clear avenue for future development through protein engineering or composite structure optimization to accelerate response. This study not only provides a promising tool for environmental CTC monitoring but also establishes a versatile design principle. The strategy of employing a single protein for both signal enhancement and selective recognition offers a promising blueprint for developing future ratiometric sensors targeting other biologically relevant molecules.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/chemosensors14030056/s1>. Figure S1: (A) Fluorescence and (B) UV-vis absorption spectra of Au-AgNCs synthesized with different reaction times (inset: enlarged absorption spectra). Figure S2: (A) Fluorescence and (B) UV-vis absorption spectra of Au-AgNCs synthesized with different amounts of TLA (4.5–8.5 mM, $\lambda_{\text{ex}} = 465 \text{ nm}$). Figure S3: (A) Fluorescence and (B) UV-vis absorption spectra of Au-AgNCs synthesized with varying NaOH concentrations (2.0–10.0 mM, $\lambda_{\text{ex}} = 465 \text{ nm}$). Figure S4: (A) Fluorescence and (B) UV-vis absorption spectra of Au-AgNCs synthesized with different NaOH incubation times (2–6 min, $\lambda_{\text{ex}} = 465 \text{ nm}$). Figure S5: (A) Fluorescence and (B) UV-vis absorption spectra of Au-AgNCs synthesized with different ratio of Au to Ag (5:5–9:1, $\lambda_{\text{ex}} = 465 \text{ nm}$). Figure S6: (A) Fluorescence and (B) UV-vis absorption spectra of Au-AgNCs synthesized with different temperature (90–130 $^{\circ}\text{C}$, $\lambda_{\text{ex}} = 465 \text{ nm}$). Figure S7: (A) XPS full-survey spectrum of the Au-AgNCs. High-resolution spectra of (B) S 2p, (C) O 1s, and (D) C 1s regions. Figure S8: (A) UV-vis absorption spectra of Au-AgNCs in the presence of different amounts of BSA (0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 9.0, 10, and 12 μM) in Tris-HCl buffer. (B,C) The amplified UV-vis absorption spectra of Au-AgNCs in the presence of different amounts of BSA. Figure S9: Fluorescence decay curves of Au-AgNCs at 687 nm in the absence and presence of BSA. Figure S10: Fluorescence spectra of Au-AgNCs@BSA in the presence of CTC (15 μM) with different response times (0–4.0 h, $\lambda_{\text{ex}} = 360 \text{ nm}$). Figure S11: (A) Fluorescence spectra of Au-AgNCs@BSA (5.0 $\mu\text{g}\cdot\text{mL}^{-1}$ and 4.0 μM) upon addition of various potential interferents (100 μM), respectively. (B) Fluorescence spectra of the assembly in the presence of 5.0 μM CTC and each interferent (100 μM). Figure S12: (A) Fluorescence spectra of CTC with varying concentrations (0.10–60 μM , $\lambda_{\text{ex}} = 360 \text{ nm}$). (B) Fluorescence spectra of CTC (50 μM) and the mixture of Au-AgNCs@BSA and CTC (50 μM) under the same equipment parameters. Table S1: Summarizes the ratiometric fluorescence response (I_{420}/I_{687}) for all interferents tested individually.

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