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PII: S0039-9140(18)31221-9
DOI: https://doi.org/10.1016/j.talanta.2018.11.067
Reference: TAL19300

To appear in: *Talanta*

Received date: 14 September 2018
Revised date: 18 November 2018
Accepted date: 21 November 2018

Cite this article as: Xinge Li, Xiaoman Wu, Fei Zhang, Bing Zhao and Yan Li, Label-free detection of folic acid using a sensitive fluorescent probe based on ovalbumin stabilized copper nanoclusters, *Talanta,* https://doi.org/10.1016/j.talanta.2018.11.067

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# Label-free detection of folic acid using a sensitive fluorescent probe based on ovalbumin stabilized copper nanoclusters

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## Abstract

In this work, ovalbumin (OVA) stabilized copper nanoclusters (OVA-Cu NCs) were prepared through a simple protein-stabilized synthetic method and applied for the sensitive determination of

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FA. $Cu^{2+}$ ions were directly reduced to Cu (0) by a mild reducing agent, $N_2H_4 \cdot H_2O$, at room temperature without any other complicated procedure such as adjusting pH and controlling the temperature of the reaction mixture. The as-prepared OVA-Cu NCs showed good chemical stability, wonderful water solubility and excellent biocompatibility. The OVA-Cu NCs were obtained with an average particle size of 2.0 nm and good dispersibility in aqueous solution. They also showed strong red emitting at 625 nm with a quantum yield of 3.95%. The OVA-Cu NCs were then applied for label-free detection of FA based on a static quenching mechanism. The linear calibration curve of detecting FA served from 0.5 μM -200 μM with the detection limit of 0.18 μM. In addition, the developed method was also successfully applied to determine the content of FA in tablet, spinach, orange juice and biological samples with quantitative spike recoveries from 96.9% to 100.9%. For these reasons, the developed OVA-Cu NCs-based fluorescent strategy can thus offer a convenient label-free biosensor platform for the detection of FA in biomedical applications.

**Keywords:** copper nanoclusters; red emitting; label-free detection; folic acid

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## 1. Introduction

Folic acid (FA) or pteroyl-L-glutamic acid, known as (N-[p-{[(2-amino-4-hydroxy-6-pteridinyl) methyl]-amino} benzoyl]-L-glutamic acid, and it is one of the water-soluble B group vitamins and exists widely in most animals, microorganisms and plant foods [1-3]. FA plays a remarkably significant physiological role in numerous biological processes including the bio-synthesis of purines, pyrimidines and methionine [4]. Simultaneously, FA is intimate for constructing healthy cells which relates to the synthesis, repair and methylation of DNA [5, 6]. In addition, FA also is considered as a blood tonic, anti-anemia and growth factor, due to it can promote the formation of red blood cells [7]. Deficiency of FA is a common cause of cause serious complications during pregnancy, which can result in malformations of the spine, skull and brain malformations of developing foetuses [8]. The lack of FA also gives rise to gigantocytic anemia, associated with leukopenia, devolution of mentality and psychosis. To avoid these risk issues, FA supplementation is particularly significant in the group of the population. However, even though FA is not toxic, it is concerned that FA in excess affect the absorption of vitamin $B_{12}$ and zinc in human body which may lead to other health problems [9]. Therefore, it is necessary and has attracted considerable attention for establishing sensitive, selective and rapid detection methods for the accurate determination of FA in biosamples.

To date, various strategies for detection of FA have been reported, including high-performance liquid chromatography (HPLC) [10-12], cyclic voltammetry (CV) [13, 14], capillary electrophoresis (CE) [15, 16], enzyme-linked immunosorbent assay (ELISA) [17, 18], colorimetric assays [19], UV spectrophotometric method (UV) [20], surface-enhanced Raman scattering (SERS) [21, 22] as well as fluorescence method [6, 23, 24]. Among these reported methods, HPLC can provide an excellent selectivity for detecting FA, it is still faced with the problems in the assay process such as requiring

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expensive equipments and the large time consuming. CE has the advantages that small amounts of samples and high separation efficiency, but it also has disadvantages of poor reproducibility and toxic organic solvent. Identically, ELISA and colorimetric assays have low sensitivity for detecting FA and their detecting speeds are much faster than those of CE and HPLC methods but they also require a preseparation step to remove matrix interferences. Compared with the above pathways, fluorescence methods are rapid, highly sensitive, cost-effective, and exhibit predominant potential for detection of FA in different samples.

Fluorescent-transducer-based probes have been designed for FA detection, including functional gold nanoparticles (Au NPs) [21], Cu nanoparticles [25], quantum dots (QDs) [6, 26] and metal nanoclusters (MNCs) [27] due to their distinct advantages in terms of sensitivity, selectivity and response time. As an emerging new class of fluorescent nanomaterials, MNCs exhibit unique physical and chemical properties, as well as have promising applications in catalysis, chemical sensing, electronic devices, and biological imaging [28]. With discrete energy levels, MNCs could bridge “missing link” between atomic and nanoparticles behavior, and show molecule-like electronic transitions within the conduction band, which leads to a strong fluorescence emitting [29]. Similar to Cd-based QDs, MNCs exhibit tunable fluorescent emissive wavelength and high photoluminescence quantum yield (QY). Compared with semiconductor QDs, MNCs have many advantages including easy preparation, low toxicity, good biocompatibility and multifunctional surface chemistry [30]. In the meanwhile as a new type non-noble MNCs, copper nanoclusters (Cu NCs) display several intrinsic advantages, such as abundant source, low cost and availability. Compared with Cu NPs, Cu NCs have smaller size (< 5 nm) and better biocompatibility because the size of Cu NPs often exceeds 50 nm [25]. Due to these outstanding properties, Cu NCs have attracted a great deal of attention and

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been applied for the detection of metal ions, acid groups and proteins in complex biosamples. Therefore, it is necessary and high-performance to design and establish a sensitive method for determination of FA in biosamples based on Cu NCs as the fluorescent probe.

Herein, a facile one-pot strategy was designed for the preparation of fluorescent ovalbumin (OVA) protected Cu NCs (OVA-Cu NCs) without any turning pH and controlling the temperature of the aqueous solution. In this strategy, OVA was used as a protective agent and a scaffold to prevent nanoclusters from growing large nanoparticles because the cost using OVA as the stabilizer for the preparation of OVA-Cu NCs was obviously lower than that of the other Cu NCs prepared with other templates such as bovine serum albumin (BSA) [31, 32], transferrin (TRF) [33], deoxyribonucleic acid (DNA) [34, 35]. The as-prepared OVA-Cu NCs showed red fluorescence with peaking center at 625 nm. Furthermore, OVA-Cu NCs have some other advantages including low toxicity, good biocompatibility and optical properties. Importantly, the fluorescence of the as-prepared OVA-Cu NCs can be quenched efficiently by FA, which made them excellent candidates as fluorescent probes for the label-free detection of FA. The as-prepared OVA-Cu NCs showed a potential application as probe for monitoring FA owing to their unique electronic structure and subsequently molecule-like optical properties. In this study, we developed a facile, sensitive and selective assay for the sensing of FA, based on fluorescent inner filter effect. This method exhibited a linear response to FA in the range of 0.5-200 μM with the limit of detection of 0.18 μM. The detection system was also successfully applied to determine FA in tablet, spinach, orange juice and biological samples with quantitative spike recoveries from 96.9%-100.9%.

## 2. Experimental section

### 2.1. Materials and chemicals

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All chemicals involved in this work were at least of analytical grade. Ovalbumin (OVA, MW = 45000) and amino acids were purchased from Yunni Technology Ltd. (Tianjin, China). Dopamine hydrochloride (DA) was purchased from Sigma-Aldrich (St. Louis, MO). Uric acid (UA) was purchased from Sangon Biotech Co., Ltd.(Shanghai, China). Hydrazine hydrate ($N_2H_4 \cdot H_2O$, 80%) and ascorbic acid (AA) were purchased from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). Folic acid (FA, 99%+) was purchased from Yinuokai Technology Ltd. (Beijing, China).Ultrapure water was obtained from the Wahaha Co., Ltd. (Hangzhou, China). PBS buffer solution (20×) was purchased from J&K Scientific Ltd. (Beijing, China). Fructose, xylose, glucose, maltose, citric acid, oxalic acid $CuCl_2 \cdot 2H_2O$ (99%), NaCl (98%), KCl (99.8%), $MgCl_2$ (99.8%) and urea (99%) were purchased from Tianjin Kewei Co., Ltd. (Tianjin, China).All of the chemicals employed were used without further purification.

## 2.2. Apparatus and characterization

Fluorescent spectra of all samples were measured on a Cary Eclipse Fluorescence spectrophotometer (Agilent Technologies, USA) equipped with a plotter unit and a quartz cell (1 cm × 1 cm) in the fluorescence mode. The fluorescence emission spectra were recorded in the wavelength range of 450-900 nm upon excitation at 340 nm. The slit widths for excitation and emission were both 10 nm. The photomultiplier tube (PMT) voltage was set at 700 V. UV-Vis spectra (200-400 nm) were recorded on a UV-2450 spectrophotometer (Shimadzu, Japan). Fourier transform infrared (FT-IR) spectra (4000-500 $cm^{-1}$) were recorded using a NICOLET 6700 FT-IR spectroscope with KBr pellets (NICOLET, USA). Circular dichroism (CD) assays were measured on a Jasco J-715 spectropolarimeter (Jasco, Japan) at ambient temperature (cell length: 1 cm; data pitch: 0.1 nm; bandwidth: 1nm; scanning speed: 500 $nm \cdot min^{-1}$; accumulation: 3). Transmission electron microscopy

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(TEM) images were carried out using a Tecnai G2 F20 (FEI, USA), which was operated at an accelerating voltage of 200 kV. X-ray photoelectron spectroscopy (XPS) measurements were carried out on a Kratos Axis Ultra DLD spectrometer (Kratos, UK) employing a monochromated Al-Ka X-ray source (hν = 1486.6 eV), hybrid (magnetic/electrostatic) optics and a multi-channel plate and delay line detector (DLD). All XPS spectra were recorded using an aperture slot of 300 × 700 microns, survey spectra were recorded with a pass energy of 160 eV, and high resolution spectra with a pass energy of 40 eV. The dynamic light scattering (DLS) and the Zeta potential were measured by Malvern Zetasizer Nano ZS (red badge, Malvern, UK) with 633 nm He-Ne laser. The fluorescence decay curve of the OVA-Cu NCs was measured on an FLS920 spectrofluorimeter (Edinburgh, UK). The fluorescent quantum yield (QY) of the OVA-Cu NCs was measured by a reference method using quinine sulphate as reference fluorescent dye (QY = 55%). Powder X-ray diffraction (XRD) measurements were performed ona Bruker D8 diffractometer at a scanning rate of 1º/min in the 2θ ranged from 5º to 80º. Sample's composition quantitative analysis was analyzed on the X7 Series Inductively coupled plasma mass spectrometry (ICP-MS) (Thermo Electron Corporation, USA). Thermo gravimetric analysis (TGA) was performed using a PTC-10A thermal gravimetric analyzer (Rigaku, Japan) from room temperature to 800ºC.

## 2.3. Synthesis of OVA stabilized Cu NCs

The OVA-stabilized Cu NCs were prepared by a one-pot route. In a typical preparation process, 100 μL of $CuCl_2$ aqueous solution (0.1 M) was added into 13.90 mL of OVA solution (7.14 $mg \cdot mL^{-1}$). The above mixture was stirred at room temperature for 10 min and a white hydrogel was formed. After being slowly injected of $N_2H_4 \cdot H_2O$ (0.5 mL) to the reactive mixture and stirred at room temperature for 3 h, the solution changed to light yellow which suggested the formation of OVA-Cu

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NCs. In this study, we also adjusted the dosages of reducing agent and $Cu^{2+}$ to change the fluorescence intensity of the OVA-Cu NCs. And the solution after synthesis was precipitated with absolute ethanol, separated by centrifuging at 12000 rpm for 10 min and then re-dissolved in ultrapure water. The above purification process was repeated for three times, and finally the resultant OVA-Cu NCs were kept at 4 °C.

## 2.4. Quantum yield measurement of OVA-Cu NCs

The quantum yield (QY) of OVA-Cu NCs was obtained by comparing the in tegrated luminescence intensities and the absorbance values of the OVA-Cu NCs with the reference quinine sulfate. The absorption spectra of the samples were obtained from a UV-2450 UV-Vis spectrophotometer (Shimadzu, Japan). Quantum yield was calculated by the following equation[36-39]:

$$Qs = \frac{Fs}{As} \times \frac{Ar}{Fr} \times \frac{{n_s}^2}{{n_r}^2} \times Qr \quad (1)$$

where Qs was the quantum yield of OVA-Cu NCs, or was the quantum yield of quinine sulphate, Fs was the integrated fluorescence intensity of OVA-Cu NCs, Fr was the integrated fluorescence intensity of quinine sulphate, As was the absorbance of OVA-Cu NCs, Ar was the absorbance of the reference quinine sulphate, $n_s$ and $n_r$ were the refractive indices of the sample and reference, respectively, in distilled water, which were assumed to be equal to that of water (1.33). The emission spectra for the sample were recorded at the excitation wavelength of 320 nm, keeping the slit width at 2 nm.

## 2.5. Fluorescence detection of FA

To investigate the fluorescence quenching effect of FA to OVA-Cu NCs, 20 μL of FA with different concentrations (0.5-200 μM) was added to 0.80 mL OVA-Cu NCs (8.50 $mg{\cdot}L^{-1}$) and bring the mixture to 3.18 mL with PBS buffer (10 nM, pH 7.4). The solution was equilibrated for 10 min to



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measure the fluorescence signal intensity. The fluorescent emission spectra (the detecting emission range was 450-900 nm) were measured at an excitation wavelength of 348 nm. The fluorescent spectra of OVA-Cu NCs was measured on a Cary Eclipse Fluorescence spectrophotometer (Agilent Technologies, USA) equipped with a plotter unit and a quartz cell (1 cm ×1cm) in the phosphorescence mode. The excitation and emission slit widths were 10 nm.

## 2.6. Selectivity and anti-interference of detecting FA

To investigate the selectivity of OVA-Cu NCs toward FA, some substances including DA, UA, AA, fructose, xylose, glucose, maltose, citric acid, oxalic acid, and amino acids {threonine (Thr), histidine (His), lysine (Lys), methionine (Met), glycine (Gly), cysteine (Cys), glutamic acid (Glu), phenylalanine (Phe), valine (Val), leucine (Leu)} were also detected. In addition, these environmentally and biologically friendly OVA-Cu NCs probes tested satisfactorily against interference.

## 2.7. Detection of FA in real samples

Tablet sample: folic acid tablet was bought in a local drugstore, each piece (120 mg) contained 0.4 mg of folic acid. The average tablet weight was calculated from 20 tablets and then finely powdered in a porcelain mortar. And then, a portion of this powder, equivalent to 4.00 mg of folic acid was accurately weighed and dissolved in 2 mL of NaOH (1.0 M). The solution was centrifuged for 10 min at 12000 rpm, and filtered with 0.22 μm microporous membrane. The solution was diluted into with 10 mL PBS buffer solution (pH=7.4) [6,27]. Urine sample: the morning urine samples of healthy volunteers were collected. To avoid the fluorescence background of the pure urine sample, the collected sample was centrifuged for 10 min at 12000 rpm and filtered with 0.22 μm microporous membrane to remove particulate matter. The supernatants were collected and diluted 500 times with

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PBS buffer solution (pH=7.4) [40]. Spinach samples: the spinach was purchased from a local supermarket. The spinach samples were prepared as previously described [41]. 100 g of real sample (spinach) were cut into small pieces using a razor blade, and were boiled with water under re flux for 50 min. The mixture was cooled and filtered through a membrane filter. A suitable aliquot of the filtrate was used to determine FA. The orange juice samples: the orange juice was obtained by squeezing fresh orange by manual orange juice extractor. The orange juice was first filtered with 0.22 μm microporous membrane and the filtrate was stored in refrigerator for the use in further analysis [42].

Standard solution of FA was prepared by dissolving FA in biological fluid. To detect FA in the biological fluid, the fluid containing OVA-Cu NCs (0.80 mL, 8.50 $mg \cdot L^{-1}$) and FA (20 μL) was equilibrated at room temperature for 3 min. After different concentrations of FA were added in the fluid for 10 min, the fluorescent spectra were recorded.

## 2.8. HPLC test of the real samples

HPLC analysis was performed on a high performance liquid chromatograph (LC-1260, Agilent) equipped with an Diode-array Detector (DAD). A Baseline C18 column (250 mm long × 4.6 mm i.d. × 5 μm particle diameter) was used for the chromatographic separation. The HPLC conditions were set as follows: the flow rate was 1.0 $mL \cdot min^{-1}$, mobile phase was a mixture of methanol and water (90:10), the column temperature was 25 °C and the injection volume of the sample solution was 20 μL. The mobile phase was filtered through the 0.45 μm nylon membrane filter prior to use. The photodiode array detector was operated between 190 and 400 nm. Multiple wavelengths were used for the quantification of the FA at the 280 nm.

## 3. Results and Discussion



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## 3.1. Synthesis and design of OVA-Cu NCs for detection of FA

OVA-Cu NCs with red emitting were synthesized via a facile and single step approach at room temperature. As shown in Scheme 1, OVA was employed as the stabilizer for the formation of OVA-Cu NCs due to the numerous carboxyl on its surface, since OVA is an glycoprotein derived from chicken egg white that is made up of 385 amino acids and it contains rich amino acid residues with 6 Cys and 10 Tyr. So the numerous carboxyl on the surface of OVA can interact with Cu atoms. In a typical experiment, a reactive mixture was produced by adding $CuCl_2$ (0.1 M, 100 μL) into OVA solution under vigorous stirring, then it was introduced with $N_2H_4 \cdot H_2O$ solution (wt 80%, 1.0 mL) for reducing $Cu^{2+}$ ions to Cu (0). After a strong stirring for 3 h at room temperature, OVA-Cu NCs were obtained in the reactive mixture, which were demonstrated that the color of the reactive mixture solution change from colorless to deep yellow. It is worthy to note that adjusting pH and $N_2$ protection were unnecessary in this work. In the as-developed synthetic route $N_2H_4 \cdot H_2O$ was not only afforded an indispensable alkaline condition for the preparation of OVA-Cu NCs, but also protected the OVA-Cu NCs away oxidation and rejected quenching of their fluorescence. Therefore, the process of the preparation of OVA-Cu NCs is remarkably simple, rapid and low cost.

The OVA-Cu NCs could be used to detect FA straightforwardly without any other functionalizations. The obtained OVA-Cu NCs could specifically identify FA owing to static quenching mechanism. As shown in Scheme 1, the absorbance wavelength of FA was 360 nm (curve a) and the fluorescent excitation wavelength of OVA-Cu NCs was 348 nm (curve b). Because of the spectral overlap between the excitation spectrum of OVA-Cu NCs and the absorbance spectrum of FA, thereby FA could quantitatively quench fluorescent intensity of OVA-Cu NCs. This method could be used to detect FA based on fluorescent inner filter effect.

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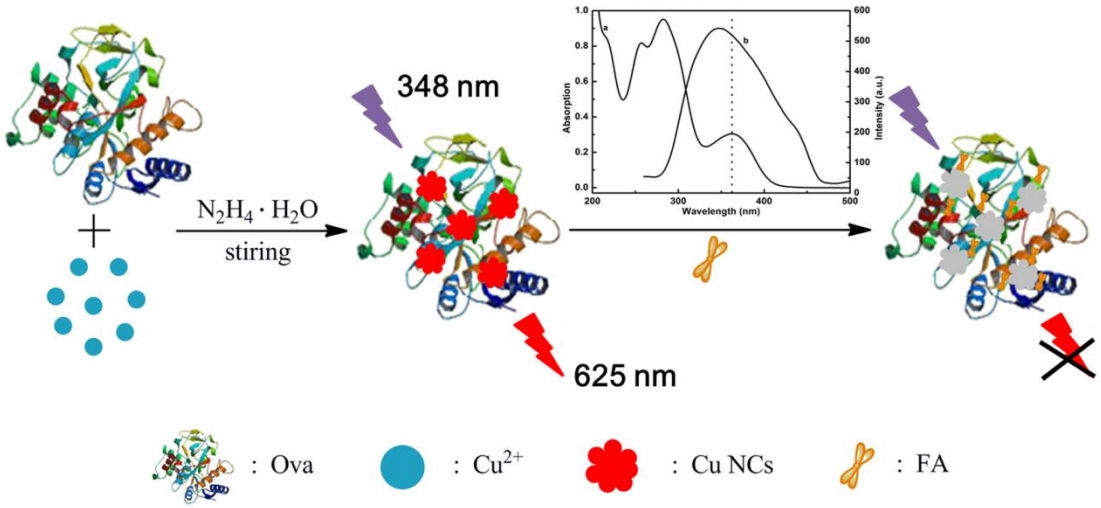


**Scheme 1.** Schematic illustration of the formation process and application process in the detecting FA of red-emitting OVA-Cu NCs. In the illustration, curve a was the absorbance spectrum of FA and curve b was the fluorescent excitation spectrum of OVA-Cu NCs.

## 3.2. Characterization of the as-prepared OVA-Cu NCs.

The HRTEM image and the distribution of OVA-Cu NCs based on the statistics of the HRTEM image illustrated that the average size of the as-prepared OVA-Cu NCs was approximately 1.80 nm without aggregation emerged, depicting their satisfactory dispersion (Fig. 1A and 1B). Fig. 1C showed that the excitation wavelength and emission wavelength of the as-prepared OVA-Cu NCs the as-were 348 nm and 625 nm, respectively. The inset photograph of Fig. 1C showed that the OVA-Cu NCs solution was yellowish and transparent under ambient light and exhibited strong red fluorescence under the radiation of UV light (365 nm). In addition, in Fig. 1D, the XPS spectra of the OVA-Cu NCs showed typical Cu $2p_{1/2}$ and Cu $2p_{3/2}$ peaks of Cu (0) with the measured binding energy of 952.3 and 932.4 eV, respectively. It is notable that the $2p_{3/2}$ binding energy of Cu (0) was very close to that of Cu (I) species, and thus the valence state of the as–prepared OVA-Cu NCs might lie between 0 and +1. XPS survey spectrum of OVA-Cu NCs and XPS spectra of the binding



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energies of C 1s, N 1s, O 1s and S 2p were shown in Fig. S1 and Fig. S2, respectively. The XPS wide spectrum of the OVA-Cu NCs illustrated that the OVA-Cu NCs were composed of all the expected elements including C, O, N, S, and Cu (Fig. S1). Besides, on the total XPS spectrum of OVA-Cu NCs, five peaks appeared at 160.7, 282.2, 397.3 and 529.1 were assigned to S 2p, C 1s, N 1s and O 1s state, respectively (Fig. S2).

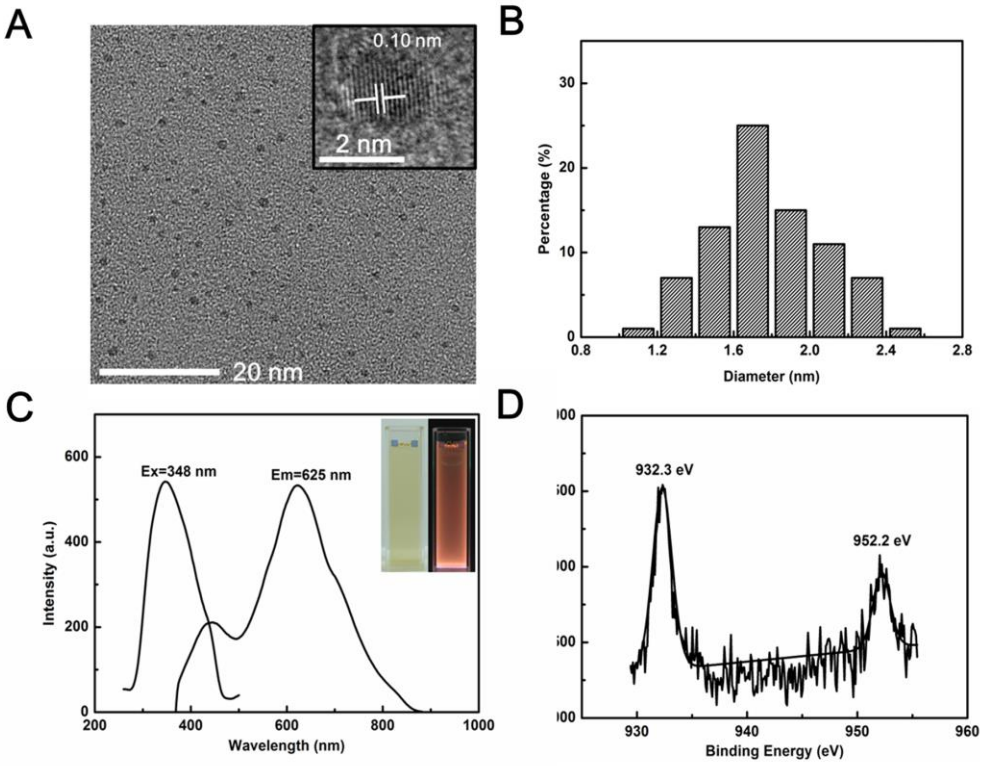


**Fig. 1.** (A) HRTEM image of the as-prepared OVA-Cu NCs; (B) The distribution of OVA-Cu NCs based on the statistics of the HRTEM image; (C) Fluorescent excitation and emission spectra of the as-prepared OVA-Cu NCs. Inset: photographs of the as-prepared OVA-Cu NCs under the irradiation of the daylight (left) and UV light (right); (D) XPS spectrum in the Cu2p region of OVA-Cu NCs.

To affirm the synthesis of OVA-Cu NCs, a series of characterization were taken for comparison, such as FT-IR, UV-vis spectra, CD spectrometry and Zeta potential. As shown in Fig. S3 A, there is

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no obvious change in FTIR patterns of the as-prepared OVA-Cu NCs and the original OVA. Compared with the UV-vis spectrum of OVA-Cu NCs, UV-vis spectrum of the original OVA displayed an obvious absorption peak at 279 nm and high absorbance from 250 to 300 nm (Fig. S3 B). Fig. S3 C demonstrated the conformational behavior of OVA before and after reaction as measured by CD spectrometry. The 208 nm peak was blue-shift to 204 nm with the formation of OVA-Cu NCs, indicating the loss of the α-helix and the increase of β-sheet and random coil structures. The OVA-Cu NCs were synthesized based on protein, which also resulted in the reduction of Zeta potential (Fig. S3 D).All these results demonstrated the successful synthesis of OVA-Cu NCs.

The fluorescent QY of the OVA-Cu NCs was about 3.95%, smaller than that of the trypsin-captured Cu NCs [33] and the glutathione-captured Cu NCs [43], but larger than that of the Cu NCs captured by the other reagents [44, 45]. TGA assay was employed to estimate the thermal stability of OVA-Cu NCs, and the result was conducted in Fig. S4 .In the lower temperature range (up to 200°C), a slight weight loss was observed. The obvious weight loss of OVA-Cu NCs was occurred in the temperature range from 201 to 600°C. The residual weight should be the weight of Cu. Therefore, the OVA-Cu NCs had good thermal stability at 0-100°C

## 3.3. Optimization of the experimental conditions

In order to choose the optimum conditions, further experiments were investigated with different dosages of $Cu^{2+}$ and $N_2H_4{\cdot}H_2O$ about the influence of the fluorescent intensity (FI) of the synthetic OVA-Cu NCs, volume of the reaction mixture and the reaction time. As shown in Fig. S5 A, the FI of the OVA-Cu NCs increased with the increase of $Cu^{2+}$ upon to 100 μL. It reveals a similar influence of $N_2H_4{\cdot}H_2O$ on the FI of the OVA-Cu NCs as that the dosages of OVA on FI (Fig. S5 B).

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Furthermore, we had done many other optimal experiments, such as the volume and time of synthesis system as shown in Fig. S5 C. In Fig. S5 D, time-course measurements of the synthesis system revealed that the synthesis of OVA-Cu NCs was complete within 180 min. The volume of the OVA-Cu NCs solution was 15.0 mL when they were synthesized with the optimal time that 180 min. Therefore, 100 μL $Cu^{2+}$, 1.0 mL $N_2H_4 \cdot H_2O$, 180 min time-course and 15 mL volume were selected as the optimum condition for preparation experiments of OVA-Cu NCs.

To ensure the proposed system for detection of FA, a series of experiment parameters were investigated, including the dosage of OVA-Cu NCs (Fig. S6 A), the species of buffer solution (Fig. S6 B), incubation time (Fig. S6 C) and detection time (Fig. S6 D). The method for detecting FA based on red-emitting OVA-Cu NCs is a simple, rapid and sensitive method. The quantity of FA could be measured via investigating the fluorescent variation of the OVA-Cu NCs probe. As shown in Fig. S6, FA (20 μM) could rapidly quench the fluorescent intensity of the OVA-Cu NCs (0.80 mL, 8.50 $mg \cdot L^{-1}$) in PBS buffer solution. It was revealed that the OVA-Cu NCs probe had the potentiality for the rapid and sensitive detection of FA.

## 3.4. Analytical performance based on the OVA-Cu NCs for detection of FA.

To explore the feasibility of this approach that the OVA-Cu NCs probe for determination of FA, fluorescent spectroscopy was used to investigate the response of the OVA-Cu NCs toward FA. The biosensor system was spiked with varied concentrations of FA and brought the volume to 4 mL with PBS. The solution was equilibrated for 10 min to measure the FI. It was found that in Fig. 2A and Fig. 2B that the fluorescent intensity of the OVA-Cu NCs gradually decreased with addition of FA. Because of a more efficient fluorescence quenching occurred at 625 nm compared with the quenching at around 420 nm, the fluorescence peak at 625 nm was chosen for the analysis of FA.

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The calibration curves were covered by a linear range from 0.50 to 200 μM. The detection limit for FA was 0.18 μM, and the standard deviation for nine replicate measurements of 200 μM FA was 1.1%. The correlation equation could be defined as $F_0/F = 1+Ksv [FA]$, where F and $F_0$ were the maximum emission intensities in the presence and absence of the FA, respectively. The calibration curve formula was established according to the relative fluorescent intensity, which was $F_0/F$. Data were presented as mean ± SE (standard error) of three independent experiments. The estimated detection limit of FA was about 0.18 μ M, which was comparable or lower than some previous results.

A comparison of the linear range and the detection limit for detecting FA obtained by the present method and other techniques was made in Table 1. It was demonstrated that the present method could provide the outstanding selectivity and sensitivity for the detection of FA in the real samples. Clearly, the OVA-Cu NCs probe for detecting FA in this work had a LOD higher than that of ECS, but it was one or four orders of magnitude lower than those of the other methods. The linear rang of the OVA-Cu NCs probe for detecting FA developed in this work was a little narrower than that of the voltammetric immunosensor method, but it was one or two orders of magnitude wider than those of ELISA, MS, other ESC and fluorescent methods.

**Table 1.** Comparison of the linear range and the detection limit of FA by different method

| Method | Linear range (μM) | LOD (μM) | Reference |
|---|---|---|---|
| Synchronous fluorescence | 0.23-0.57 | $4.5\times10^{-3}$ | 22 |
| Fluorescence | $8.0\times10^{-2}$-60.0 | $1.2\times10^{-3}$ | 23 |
| Fluorescence | 0.2–100 | 0.08 | 7 |
| Cyclic voltammetry | $1.0\times10^{-3}$-$44.0\times10^{-3}$ | $0.12\times10^{-3}$ | 12 |

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| | | | |
|---|---|---|---|
| Cyclic voltammetry | $8.79\times10^{-6}$-$1.93\times10^{-3}$ | $1.24\times10^{-6}$ | 13 |
| Capillary electrophoresis | 12.0-48.0 | 0.612 | 15 |
| Capillary electrophoresis | $5.0\times10^{-2}$-10.0 | $2.0\times10^{-2}$ | 14 |
| Enzyme-linked immunosorbent assay | $0.36\times10^{3}$-$2.90\times10^{3}$ | 40.78 | 17 |
| Colorimetric assays | 26.51-132.99 | -- | 18 |
| Surface-enhanced Raman scattering | $9\times10^{-3}$-$180\times10^{-3}$ | -- | 20 |
| Surface-enhanced Raman scattering | 0.5-25.0 | 0.3 | 21 |
| UV | 2.27-39.65 | 0.02 | 19 |
| Fluorescence | 0.20-200.0 | 0.18 | This work |

## 3.5. The mechanism for detection of FA.

It was worthy to notice that the mechanism of OVA-Cu NCs fluorescent properties and quenching mechanism is still lacking, which was mainly caused by the intrinsic complexity of the protein templates and the lack of accurate structural information of the OVA-Cu NCs. Basically, the fluorescent quenching process can be divided into two kinds of different quenching mechanism including static quenching mechanism and dynamic quenching mechanism [46, 47]. The above two mechanisms can be distinguished by their different dependence on the temperature and fluorescent lifetime. In order to understand the detection mechanism, the fluorescent decay curves of OVA-Cu NCs in the absence and presence of FA were both investigated (Fig. 2C and 2D). The average fluorescence lifetime estimated for OVA-Cu NCs was approximately 5.76 ns. In the presence of FA, the average lifetime was 5.51 ns with negligible decrease. The fluorescence lifetime of two different analysts indicated that it could be static quenching mechanism [27]. In addition, the absorption spectra of FA and fluorescence spectra of OVA-Cu NCs were studied to further understand the mechanism of the fluorescence quenching of OVA-Cu NCs by FA. As shown in Scheme 1, the

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absorbance wavelength of FA was 360 nm (curve a) and the fluorescent excitation wavelength of OVA-Cu NCs was 348 nm (curve b). Because of the spectral overlap between the excitation spectrum of OVA-Cu NCs and the absorbance spectrum of FA, thereby FA could quantitatively quench fluorescent intensity of OVA-Cu NCs. This method could be used to detect FA based on fluorescent inner filter effect. Furthermore, to further confirm this consideration, the Stern-Volmer equation $F_0/F = 1+Ksv \times [FA]$, where $F_0$ and F were the fluorescent intensities of the mixture in the presence and absence of FA, respectively, [FA] is the molarity of quencher and Ksv was the Stern-Volmer constant. According to the previous reports, $Ksv = kq \times \tau$, where kq was the quenching rate constant and $\tau$ was the fluorescence lifetime without an added quencher. The quenching constant of this work was $9.43 \times 10^3$ L $mol^{-1}$. Given the SV relationship in the above equation, the quenching rate constant was $kq=1.71 \times 10^{12}$ $M^{-1}$ $s^{-1}$ ($\tau$=5.51 ns, Fig. 2D). Based on quenching rate constant, it should be noted that the observed quenching might be attributed to the static quenching, not the dynamic quenching. To sum up, the possible mechanism using OVA-Cu NCs as fluorescent probes to effectively detect FA should be fluorescent inner filter effect [48].

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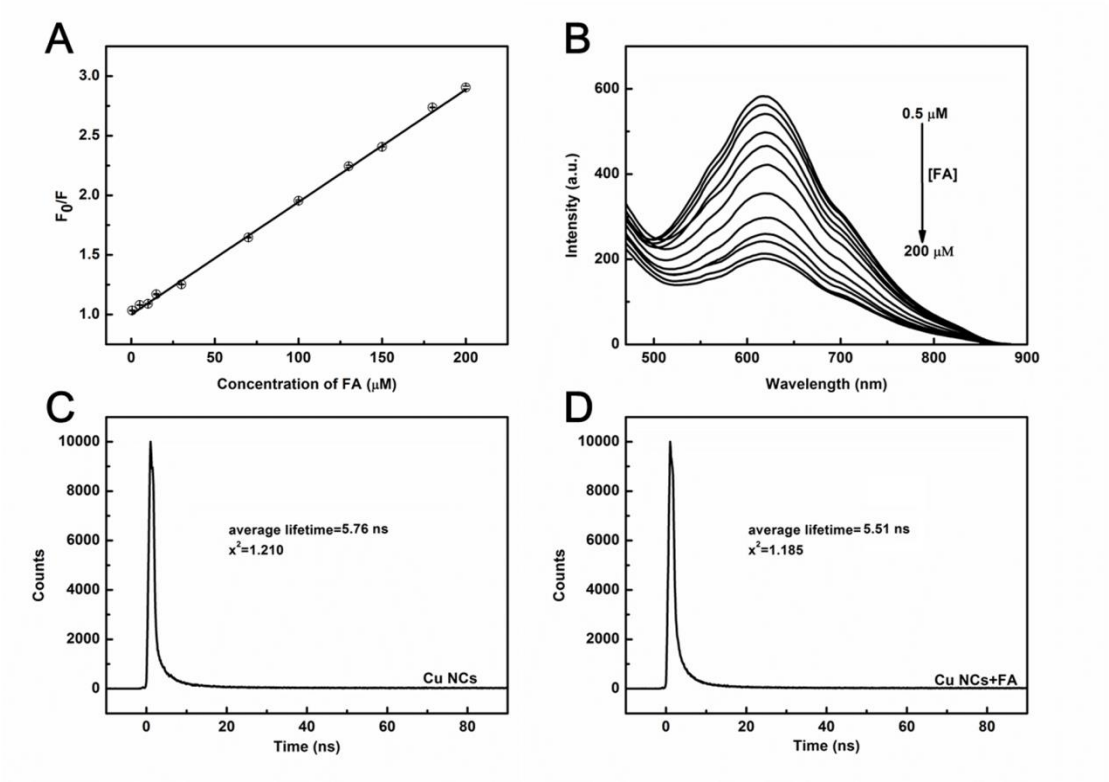


**Fig. 2.** (A) The calibration curve of the logarithm of FA concentration versus the fluorescent intensity of the OVA-Cu NCs at 625 nm.(B) Fluorescent emission spectrum of OVA-Cu NCs in PBS buffer (pH = 7.4) with declining concentrations of FA: 0.5μM -200 μM. (C)The fitted fluorescent decay curves of OVA-Cu NCs with the excitation wavelength of 348 nm and the fluorescent intensity was collected at 625 nm. (D)The fitted fluorescent decay curves of OVA-Cu NCs (with 25.0μM) with the excitation wavelength of 348 nm and the fluorescent intensity was collected at 625 nm.

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## 3.6. Selectivity of the OVA-Cu NCs for detection of FA

To investigate the selectivity of the OVA-Cu NCs toward FA, the effect of some probable ions as well as other kinds of common biomolecules (DA, UA, AA, fructose, xylose, glucose, maltose, citric acid, oxalic acid, Thr, His, Lys, Met, Gly, Cys, Glu, Phe, Val, Leu ) was investigated under identical conditions. Fig. 3A. showed that other kinds of biomolecules could not lead to any significant fluorescence decrease of OVA-Cu NCs. The results showed that only FA gave significant quenching

of the fluorescent intensity of OVA-Cu NCs, indicating the high selectivity of the hybrid system for detection of FA.As shown in Fig. 3B, although the concentration of coexisting substances was 10-fold higher than that of FA, the obtained signals for detecting FA had not changed much, indicating that there is no contribution to detect FA from the common coexisting substances in this system.

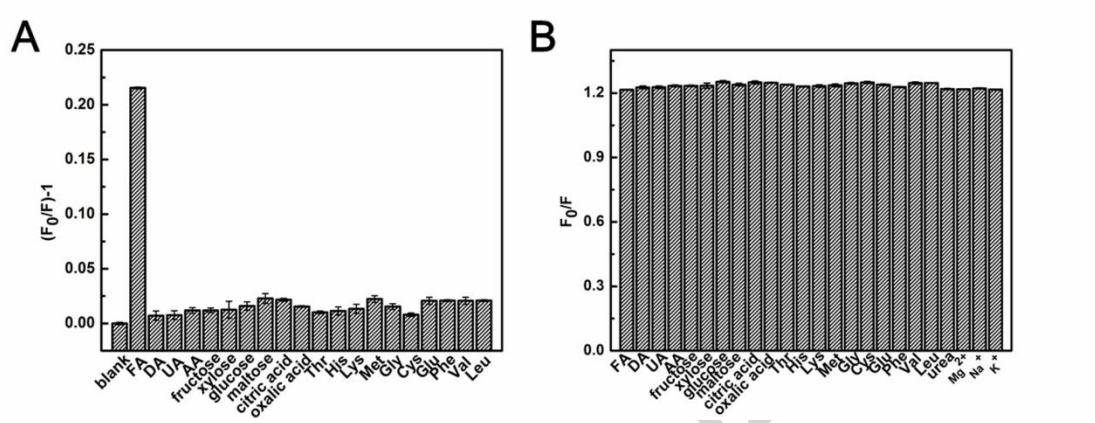


**Fig. 3.** (A) The fluorescent responses of FA and other interfering substance to OVA-Cu NCs at 625 nm. (B) The relative fluorescent intensity of the OVA-Cu NCs system in the presence of other control molecules and ions in coexistence with FA in PBS buffer (pH=7.4) at 625 nm.

## 3.7. Analytical applications of OVA-Cu NCs for determination of FA in real samples

FA plays a significant physiological role in numerous biological processes. It is essential that the high selectivity and sensitivity for detecting FA in tablet, spinach, orange juice and biological samples. Four kinds of samples were collected and validated the application of the method for the detection of FA. The real samples (tablet, spinach and orange juice) were purchased in local drugstore, market and department store, respectively.

To evaluate the validity and reliability of the proposed sensor in practical applications, the

analytical determination of FA in two human urine samples were performed using the standard addition method. The human urine samples were diluted 100 times using 0.1 M PBS buffer solution (pH 7.4). In order to ascertain the correctness of the results, certain amounts of FA were added into the above mentioned diluted sample and the reactive mixture were then detected. The analytical results were summarized in Table 2. The recovery of the spiked samples ranged from 96.6% to 100.9%, indicating the successful applications of the OVA-Cu NCs for the determination of FA in tablet, spinach, orange juice and biological samples.

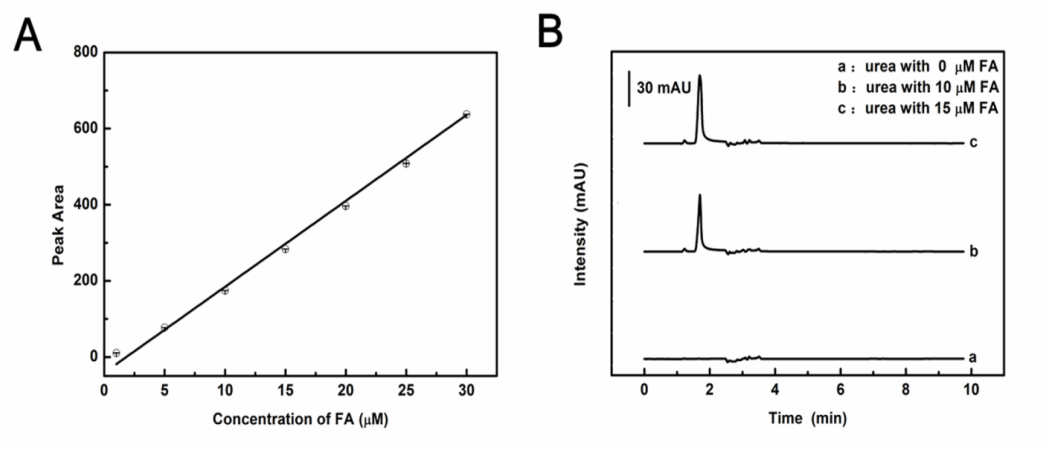


**Fig. 4** (A) The calibration curve of the logarithm of FA concentration versus the liquid chromatography intensity of the OVA-Cu NCs at 1.7 min. (B) Liquid chromatography of FA: 0 μM, 1.0 μM, 30.0 μM with conditions that the flow rate was 1.0 $mL \cdot min^{-1}$, the column temperature was 25 °C, the photodiode array detector was operated between 190 and 400 nm and multiple wavelengths were used for the quantification of the FA at the 280 nm.

Furthermore, we conducted the recovery test by HPLC to test and verify the accuracy of the strategy for real samples. The average recoveries of FA in biological samples were found to be in the ranges from 98.5% to 100.8% in Table 2. The calibration curve of the logarithm of FA concentration versus the liquid chromatography intensity of the OVA-Cu NCs at 1.7 min and liquid

chromatography of FA: 0 μM, 1.0 μM, 30.0 μM were shown in Fig. 4A and Fig. 4B, respectively. Therefore, the above results demonstrated the accuracy and reliability of the OVA-Cu NCs hybrid system for selective detection of FA in biological samples.

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**Table 2.** Determination of FA in real samples by fluorescence spectrum and HPLC

| Samples | Found by this method | | | | Found by official HPLC method | | | |
|---|---|---|---|---|---|---|---|---|
| | Spiked (μM) | Measured (μM) | Recovery (%) | R.S.D. | Spiked (μM) | Measured (μM) | Recovery (%) | R.S.D. |
| Urea-1 | 0 | nd | | | 0 | nd | | |
| | 10 | 9.89±0.13 | 99.9±5.3 | 4.63 | 10 | 9.98±0.25 | 99.9±2.4 | 3.76 |
| | 15 | 14.79±0.22 | 98.6±1.5 | 1.25 | 15 | 14.81±0.36 | 99.7±2.0 | 2.17 |
| Urea-2 | 0 | nd | | | 0 | nd | | |
| | 10 | 10.09±0.50 | 100.9±5.0 | 1.06 | 10 | 9.99±0.45 | 98.5±4.6 | 2.07 |
| | 15 | 14.49±0.55 | 96.6±3.7 | 3.54 | 15 | 14.63±0.37 | 100.8±3.9 | 1.95 |
| Tablet | 0 | 4.36±0.10 | | | | | | |
| | 10 | 14.13±0.17 | 97.2±1.2 | 0.97 | | | | |
| Spinach | 0 | 0.80±0.03 | | | | | | |
| | 10 | 10.85±0.05 | 96.2±2.2 | 1.94 | | | | |
| Orange juice | 0 | 0.50±0.06 | | | | | | |
| | 10 | 10.45±0.05 | 95.6±3.4 | 1.37 | | | | |

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## 4. Conclusions

In summary, a simple protein-directed synthesis method was applied to prepare stable and water-soluble of fluorescent copper nanoclusters (OVA-Cu NCs). The surface of the as-prepared OVA-Cu NCs was protected by a layer of OVA and they showed stability in various common anions. With the help of a mild reducing agent, $N_2H_4 \cdot H_2O$, the OVA-Cu NCs could be easily prepared at room temperature. The as-prepared OVA-Cu NCs showed red fluorescence with a peaking center at 625 nm (quantum yield 3.95%). It was verified that the resultant OVA-Cu NCs could be used as the probe for detection of FA due to an arresting set of features, including water-dispersibility, red fluorescence, good biocompatibility, surface bioactivity and small size. In this study, our developed method with using the probes OVA-Cu NCs was particularly suitable for detecting of FA by the quenching the fluorescent signals of the OVA-Cu NCs based on static quenching mechanism. In addition, the hybrid system of OVA-Cu NCs presented a simple and feasible strategy to solve the discrimination and detection of different species. All in all, the proposed method is a promising approach to selectively detect FA in biological samples.

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## Acknowledgements

We are grateful for the financial support from the National Natural Science Foundation of China (21375095, 20975054), Program for “131” InnOVAtive Talented Personnel Training Project in Tianjin (ZX110185, 135305QS15), the Tianjin Natural Science Foundation (No. 17JCQNJC05800), and Doctor Foundation of Tianjin Normal University (52XB1510).

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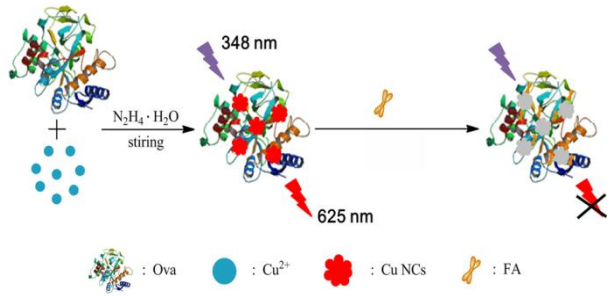
## Graphical Abstract




The ovalbumin (OVA) stabled Cu NCs (OVA-Cu NCs) was prepared in a facile route with red emission and applied for label-free detection of folic acid (FA) with high sensitivity and selectivity.

## Research highlights

1. The facile synthesis of Ova-Cu NCs without adjusting the pH value and controlling the temperature

2. The as-prepared Ova-Cu NCs with red emitting fluorescence, uniform shape and narrow size-distribution

3. Application for detection of FA base on static quenching mechanism

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