pH-Regulated Synthesis of Trypsin-Templated Copper Nanoclusters with Blue and Yellow Fluorescent Emission

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ABSTRACT: In this article, a simple protocol to prepare water-soluble fluorescent copper nanoclusters (CuNCs) using trypsin as a stabilizer and hydrazine hydrate as a reducing agent was reported. It was found that the pH of the reaction solution was critical in determining the fluorescence of CuNCs. CuNCs with blue and yellow fluorescent emission were obtained under basic and acidic conditions, respectively. Although the detailed formation mechanisms of these CuNCs required further analysis, the synthetic route was promising for preparing different fluorescent metal NCs for applications. With good water solubility and excellent photostability, the yellow-emitting CuNCs could serve as a fluorescence probe for detection of Hg²⁺ based on the aggregation-induced quenching mechanism. The fluorescence quenching efficiency had fantastic linearity to Hg²⁺ concentrations in the range of 0.1–100 μM, with a limit of detection of 30 nM. Additionally, the yellow-emitting CuNCs exhibited negligible cytotoxicity and were successfully applied to bioimaging of HeLa cells.

INTRODUCTION

Metal nanoclusters (MNCs), consisted of several to hundreds of metal atoms, have drawn considerable attention due to their unique physical, chemical, and optical properties resulting from their discrete energy levels and band-gap energy structures. In particular, compared with conventional organic fluorophores and semiconductor quantum dots, fluorescent MNCs exhibit several advantages such as strong photoluminescence, good biocompatibility, excellent photostability, and sub-nanometer size. Thus, they have been developed to be used in a wide range of applications in sensing, imaging, and bioanalysis.

Among the studied MNCs, gold NCs (AuNCs) and silver NCs (AgNCs) have received extensive research attention by size-controlled synthesis, structural characterization, and property investigations. In fact, compared with gold and silver, copper was more popular in industry because of its high conductivity and much lower cost. Nevertheless, over the past decades, studies on the synthesis, properties, and applications of copper NCs (CuNCs) were scarce primarily because of their susceptibility to oxidation and the difficulty in preparing extremely tiny particles. In recent years, considerable efforts have been devoted to exploring the synthesis of fluorescent CuNCs and great progress has been achieved. By employing a series of scaffolds or capping agents, such as small molecules, polymers, oligonucleotides, peptides, and proteins, stable CuNCs have been successfully prepared. Among these methods, protein-templated synthesis is particularly attractive as proteins could serve as environmentally benign reducing and stabilizing molecules. However, there were few reports on the discussion of the mechanism for the formation of CuNCs and it remained unclear how the protein template affected the CuNC fluorescence behaviors under various reaction conditions. In a previous report, the pH-dependent synthesis of pepsin–AuNCs with different fluorescent emission was developed. The different charges on pepsin under different pH conditions affected the structure of pepsin chains, which led to the formation of AuNCs with different fluorescent emission. Therefore, it enlightened us whether multicolored CuNCs could be prepared by regulating the reaction pH.

Hg²⁺ is one of the most toxic heavy-metal ion pollutants that exists in water, soil, and food. Mercury can accumulate in organisms and has long-term adverse effects on liver, kidney, central nervous system, and so on. Therefore, developing effective methods for the sensitive and selective detection of Hg²⁺ was especially important for environmental monitoring and clinical research. Traditional methods of Hg²⁺ sensing, including atomic absorption/emission spectroscopy, inductively coupled plasma mass spectrometry, stripping voltammetry, etc., were limited by the disadvantages of requiring expensive instruments, the complex procedures in sample preparation, a specific worker, etc. Electrochemical, colorimetric, and fluorescent sensors for Hg²⁺ have also been...
reported over the past decade. Among these methods, fluorescent Hg\(^{2+}\) sensors based on various nanoparticles have been developed due to their unique advantages such as high sensitivity, simple operation, and fast response.

On the basis of the above conditions, we reported for the first time a pH-dependent synthesis of CuNCs with blue and yellow fluorescent emission using trypsin as a template and hydrazine hydrate as a reducing agent (Scheme 1). Trypsin is an important digestive enzyme produced by pancreatic acinar cells. It is also a good candidate for synthesis of trypsin-stabilized CuNCs as trypsin is rich in amino acid residues, with 7 cysteine (Cys) and 10 tyrosine (Tyr) residues. The different conformational states of the trypsin molecule under different pH conditions could affect the interaction between trypsin and copper ion surface, leading to the formation of CuNCs with different sizes at different pH conditions. Then, the prepared yellow-emitting CuNCs were successfully employed as an effective fluorescent probe for Hg\(^{2+}\) sensing. Because of the low toxicity and good biocompatibility of the yellow-emitting CuNCs, they were also used in cell labeling of HeLa cells.

■ RESULTS AND DISCUSSION

Trypsin contained rich Cys, His, and Tyr that could act as chelating groups for sequestering copper ions and polynuclear ligands for passivating the surface of metallic materials. Next, the reducing agent \(\text{N}_2\text{H}_4\) was applied to quickly reduce Cu\(^{2+}\) cations to CuNCs. It has been reported that proteins exhibit different conformational states at different pH levels, which could affect the size and fluorescence properties of MNCs. Therefore, it was of interest to investigate the synthesis of trypsin-template CuNCs at different pH values. In a typical synthesis, trypsin and CuSO\(_4\) solution was mixed thoroughly.

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Scheme 1. Illustration of the Synthesis of CuNCs with Blue and Yellow Emission

Figure 1. Fluorescence excitation and emission spectra of the yellow- (A) and blue- (B) emitting CuNCs; insets show photographs of the CuNC solution under visible (a, c) and UV (b, d) light irradiation.

Figure 2. TEM images of the blue-emitting (A) and yellow-emitting (B) CuNCs.
For the synthesis of yellow- and blue-emitting CuNCs, the pH of the mixture was adjusted to 3.4 and 12.0, respectively, before addition of N\textsubscript{2}H\textsubscript{4}. It was worth noting that after addition of N\textsubscript{2}H\textsubscript{4} to the mixture, the solution pH changed from 3.4 to 5.1 for the yellow-emitting CuNCs and remained unchanged for the blue-emitting CuNCs. After incubating at 70 °C for 2 h, CuNCs with different fluorescent emission were obtained. Figure 1 shows the maximum fluorescence excitation and emission peaks of the prepared CuNCs. The diluted yellow- and blue-emitting CuNC solutions were nearly colorless (or very pale yellow) and transparent under visible light, whereas they exhibited yellow and blue fluorescence under UV light irradiation (365 nm), respectively (inset of Figure 1). As shown in Figure S1A, the emission wavelength of blue-emitting CuNCs was red-shifted from 415 to 475 nm with the excitation wavelength ranging from 310 to 400 nm, whereas the emission wavelength was almost independent of the excitation wavelength for the yellow-emitting CuNCs (Figure S1B). The difference of the fluorescence behaviors may be caused by the different surface states of the CuNCs with blue and yellow emission. The absolute quantum yields (QYs) for the CuNCs in aqueous solutions were measured as 3.1 and 0.1% for yellow and blue emission, respectively. The morphology and size of CuNCs were clearly revealed by transmission electron microscopy (TEM) images. Figure 2 shows that CuNCs were highly uniform and monodisperse. The average diameters of CuNCs for blue and yellow emission were about 1.8 and 2.5 nm, respectively. These results were highly in accord with the phenomenon of fluorescence wavelength dependence on the size of CuNCs. That is, the larger size of CuNCs corresponded to the red-shifted fluorescence emission wavelength, similar to that for other fluorescent nanostructures such as AuNCs. Figure S2 shows the UV−vis absorption spectra of the as-prepared CuNCs and trypsin. The absorption spectrum of trypsin had a peak centered at 276 nm, and it was changed prepared CuNCs and trypsin. The absorption spectrum of denaturation of trypsin under acidic pH conditions. More conformational change for trypsin occurred because of the band at around 198 nm from the random coil became more Figure 3, compared to that for the trypsin at pH 12.0, a negative band at around 198 nm from the random coil became more predominant for the trypsin at pH 3.4. It indicated that a large conformational change for trypsin occurred because of the denaturation of trypsin under acidic pH conditions. More

Figure 3. CD spectra of the aqueous solution of trypsin at different pHs.

In the present study, the yellow-emitting CuNCs possessed good water solubility and strong fluorescence intensity, they could be explored as a fluorescent probe for practical sensing. To improve the sensitivity of the fluorescent probe, several experimental conditions including the concentration, temperature, and reaction time were optimized to obtain yellow-emitting CuNCs with high fluorescence intensity. In this method, we found that N\textsubscript{2}H\textsubscript{4} was necessary in the preparation of CuNCs. As shown in Figure S4, in the preparation of yellow-emitting CuNCs, the product synthesized in the absence of N\textsubscript{2}H\textsubscript{4} exhibited no fluorescence signal. From this phenomenon, it could be concluded that trypsin alone was not enough to reduce Cu ions. N\textsubscript{2}H\textsubscript{4} was deemed as a reducing agent. In addition to N\textsubscript{2}H\textsubscript{4}, several other reducing agents such as ascorbate and NaBH\textsubscript{4} were also applied for the synthesis of yellow-emitting CuNCs. As shown in Figure S5, the CuNCs with N\textsubscript{2}H\textsubscript{4} as the reducing agent exhibited relatively strong fluorescence intensity. The fluorescence spectra of the CuNCs prepared with different molar ratios of CuSO\textsubscript{4} and N\textsubscript{2}H\textsubscript{4} (keeping the concentration of trypsin constant) are shown in Figure S6A. It could be seen that the product with a molar ratio of 1:1 exhibited the maximum fluorescence intensity at 567 nm. The reaction temperature was also investigated in the synthesis of fluorescent CuNCs. As shown in Figure S6B, external heat
could significantly accelerate the generation of CuNCs; thus, 70 °C was chosen as the reaction temperature. Under these reaction conditions, the fluorescence intensity reached maximum with the reaction time up to 2.0 h, and after that, it decreased (Figure S6C). This result might be attributed to the redistribution or interprotein transfer of copper ions after 2.0 h.46 Therefore, an optimum reaction time of 2.0 h was used in the whole study. Therefore, the yellow-emitting CuNCs prepared under optimal synthetic conditions were used for conducting the following research.

It was well known that Cu was easily oxidized because of its low reduction potential. Therefore, it was important to confirm the oxidation state of Cu in the CuNC sample. An X-ray photoelectron spectroscopy (XPS) survey spectrum showed that the sample was composed of all of the expected elements C, N, O, S, and Cu (Figure 4A). The high-resolution XPS spectrum of the Cu 2p peak of CuNCs is displayed in Figure 4B. Two intense peaks at 951.0 and 931.2 eV were assigned to the binding energies of Cu 2p$^{1/2}$ and 2p$^{3/2}$ from Cu(0), and the result was consistent with the previous report.9,41 In addition, no characteristic satellite peak at around 942 eV implied the absence of Cu$^{2+}$ in CuNCs. This thus precluded any significant oxidation of CuNCs.47 Nevertheless, it was known that the typical 2p$^{3/2}$ binding energy of Cu(0) was only ~0.1 eV away from that of the Cu(I) species.4 Therefore, the valence state of Cu in our samples likely lied between 0 and +1. The powder X-ray diffraction (XRD) pattern of CuNCs showed a broad peak at around 20° (Figure 4C). The result supported the absence of a significant population of crystalline Cu nanoparticles in the sample.48 Next, the surface bonds of the synthesized CuNCs
Table 1. Comparison of the Sensing Performance of Different Fluorescent Probes for Hg²⁺ Detection

| sensing material                        | linear range (μM) | LOD (nM) | response time (min) | reference |
|-----------------------------------------|-------------------|----------|---------------------|-----------|
| nitrogen-doped carbon quantum dots      | 0−25              | 230      |                     | 49        |
| trypsin-stabilized AuNCs                | 0.05−0.6          | 50       |                     | 50        |
| β-lactoglobulin-stabilized AuNCs        | 0.05−500          | 30       | 2                   | 51        |
| BSA–AuAg BNCs                           | 0.05−6.3          | 13       | 5                   | 52        |
| DNA duplex-templated AgNCs              | 0.01−0.3          | 10       | >60                 | 53        |
| oligonucleotide-stabilized AgNCs        | 0.005−1.5         | 5        | 3                   | 54        |
| DNA-templated AgNCs                     | 0.0025−0.05       | 0.9      | 30                  | 55        |
| CuNCs                                   | 0.1−100           | 30       | 2                   | this work |

were analyzed by FT-IR. As shown in Figure 4D, the peaks at 3400−3300 cm⁻¹ due to −NH and −OH stretching vibrations were also prominent in the spectra, indicating the existence of free −NH₂/−COOH groups in CuNCs.

To test the feasibility of using the as-prepared CuNCs in practical sensing applications, the stability of the CuNC probe was investigated. As shown in Figure S7, both the blue- and yellow-emitting CuNCs were observed to be very stable that the fluorescence intensity had no change under continuous light irradiation for 60 min. In addition, CuNCs displayed relatively stable fluorescence intensities even under extreme pH conditions (Figure S8). From Figure S9, it could be noted that the fluorescence intensity remained nearly constant when the concentration of NaCl was as high as 50 mM. These results indicated that the as-prepared CuNCs had excellent photo-stability and salt tolerance.

In this work, it was observed that the fluorescence intensity of the yellow-emitting CuNCs was sensitively quenched in the presence of Hg²⁺. As demonstrated in Figure 5A, with the addition of different concentrations of Hg²⁺, the fluorescence intensity of the CuNC solution decreased proportionately. The fluorescence response was rapid, and the reaction completely achieved a balance within 1 min (Figure S10). To achieve maximum quenching efficiency, the type of buffer solution and detection pH value have been optimized. As shown in Figure S11A, CuNCs exhibited strongest fluorescence intensity in the pH 4.0 phosphate-buffered saline (PBS) buffer solution compared to that in other buffer solutions. In addition, with the addition of Hg²⁺ to the CuNC solution, the fluorescence quenching efficiency reached maximum in the pH 4.0 PBS buffer solution (Figure S11B). Therefore, the pH 4.0 PBS buffer solution was selected for detection of Hg²⁺. Under optimum conditions, the quenching efficiency ($F_0/F$) displayed a good linear relationship ($R^2 = 0.993$) with the concentration of Hg²⁺ ranging from 0.1 to 100 μM, where $F_0$ and $F$ are the fluorescence intensities of the CuNC solution in the absence and presence of Hg²⁺, respectively. The limit of detection (LOD) ($3σ/k$, in which $σ$ is the standard deviation for the control and $k$ is the slope of the calibration curve) was estimated to be 30 nM, which was lower or comparable to that obtained by other fluorescent probes for Hg²⁺ sensing (Table 1). 49−52 It should be noted that the sensitivity of the CuNC sensor for Hg²⁺ was lower than that of DNA-templated fluorescence nanoclusters. 53−55 Nevertheless, the proposed method in this work was much easy-going and time-saving, which made it more convenient for practical applications.

Besides those of Hg²⁺, the effects of some other metal ions and several amino acids on the assay system were further investigated under the same test conditions. As shown in Figure 6, the fluorescence intensity of CuNCs decreased significantly by adding Hg²⁺ to the solution, whereas other metal ions (K⁺, Ca²⁺, Mg²⁺, Mn²⁺, Co²⁺, Ba²⁺, Cu²⁺, Ni²⁺, Fe²⁺, Fe³⁺, Al³⁺, Cd²⁺, and Pb²⁺) and several amino acids (Cys, Trp, Pro, Tyr, His, Thr, Phe) had only a slight or negligible effect on the fluorescence intensity, even when the concentration of the potential interferences was 5-fold higher than that of Hg²⁺. It is worth mentioning that other than Hg²⁺, Ag⁺ ions also led to great decreases in the fluorescence intensity. To eliminate the interference, a chelating ligand, sodium chloride (2.0 mM), which showed effective masking ability for Ag⁺, was added to the solution. As a result, even in the presence of Ag⁺ at a concentration 5 times greater than that of Hg²⁺, no obvious fluorescence quenching was observed, thus exhibiting improved selectivity of the CuNC probe toward Hg²⁺. The results demonstrated that the fluorescent CuNC probe exhibited excellent selectivity toward Hg²⁺.

To date, several Hg²⁺-induced fluorescence quenching mechanisms have been proposed. Morishita et al. noticed a significant quenching of AgNCs by Hg²⁺, and they attributed it to the redox reaction mechanism. 56 In our present work, the...
oxidation state of Cu in CuNCs was investigated by the XPS spectra in the absence and presence of 100 μM Hg²⁺, respectively. As shown in Figure S12, the addition of Hg²⁺ to the CuNC solution had little effect on the oxidation state of Cu, which ruled out the redox-reaction-induced CuNC fluorescence quenching. Other fluorescence quenching mechanisms could be taken into consideration. To study the Hg²⁺-induced fluorescence quenching mechanism, the TEM image of CuNCs after addition of Hg²⁺ was investigated (Figure 7A). It was clear that CuNCs obviously aggregated after Hg²⁺ was added. As it was reported, Hg²⁺ has a strong affinity toward amino and carboxylic groups on the surface of CuNCs. The interaction between Hg²⁺ and CuNCs made the CuNCs close to each other. Thus, fluorescence quenching of CuNCs was ascribed to the aggregation of CuNCs induced by Hg²⁺, thus facilitating the efficient energy transfer. The phenomenon was consistent with the previous report by Huang. In addition, the fact that quenching by Hg²⁺ did not affect markedly either the fluorescence emission spectrum or the absorption spectrum of CuNCs (Figure 7B) further indicated the quenching mechanism of energy transfer between CuNCs and Hg²⁺.

The practical application of this fluorescence method was evaluated through the detection of Hg²⁺ in human urine and serum samples. Three concentrations of Hg²⁺ were spiked into the samples. The recovery values were in the range of 89.0–105.0 and 95.0–108.8% in urine and serum samples, respectively (Table 2). These results demonstrated that the current strategy for Hg²⁺ sensing in practical samples was reliable and feasible.

To apply the yellow-emitting CuNCs in the field of biological imaging, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were carried out to assess the cytotoxicity of the CuNC probes to HeLa cells. HeLa cells were incubated with various concentrations of CuNCs in standard cell culture conditions. After incubation for 24 h, the viability of the cells was determined. As shown in Figure 8, the cell viability was found to be greater than 82% even when the concentration of CuNCs was up to 500 μg/mL. High cell viability demonstrated the low toxicity and excellent biocompatibility of the as-prepared CuNCs, which made them suitable for cell imaging.

As shown in Figure 9, by incubating Hela cells with CuNCs (500 μg/mL) for 1 h at 37 °C, a significant yellow emission from the intracellular region could be observed. All of these results showed that the yellow-emitting CuNCs could be applied in the field of biological imaging and cell labeling.

**CONCLUSIONS**

In summary, CuNCs with yellow and blue fluorescent emission were synthesized with a facile approach in the presence of trypsin and N₂H₄. The pH of the reaction solution was critical in determining whether CuNCs showed yellow or blue fluorescent emission. As the yellow-emitting CuNCs exhibited excellent stability, low toxicity, and good biocompatibility, the fluorescent CuNCs were successfully used in not only the detection of Hg²⁺ but also cell imaging in HeLa cells. Therefore, this facile preparation of multicolored CuNCs offered access to promising candidates for biological labeling and sensing applications.

**EXPERIMENTAL SECTION**

**Materials.** Trypsin from bovine pancreas was obtained from Aladdin Co., Ltd (Shanghai, China). CuSO₄·5H₂O was purchased from Shanghai Bodi Chemical Co., Ltd (Shanghai, China). HgCl₂, KCl, CaCl₂, MgCl₂, MnCl₂, CoCl₂, BaCl₂,
CuCl₂, NiCl₂, FeCl₂, FeCl₃, and AlCl₃ were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). L-Tryptophan (L-Trp), L-proline (L-Pro), L-tyrosine (L-Tyr), L-histidine (L-His), L-threonine (L-Thr), and L-phenylalanine (L-Phe) were purchased from Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China).

**pH-Dependent Synthesis of Copper Nanoclusters (CuNCs).** All glassware was cleaned in a bath of freshly prepared 3:1 HCl/HNO₃ and rinsed thoroughly in water before use. Yellow-emitting CuNCs were prepared as follows. Typically, 1 mL of CuSO₄ solution (10 mM) was added to 1 mL of trypsin (40 mg/mL) under vigorous stirring at room temperature. Five minutes later, the pH of the obtained solution was about 3.4. Then, 100 μL of N₂H₄ solution (100 mM) was added dropwise under vigorous stirring. The reaction mixture was incubated at 70 °C in a water bath for 2 h, and the color changed gradually from light blue to pale yellow. After the reaction, CuNCs were purified by centrifuging at 12 000 rpm to remove large particles. The resultant yellow-emitting CuNCs were stored at 4 °C for further use.

To obtain the blue-emitting CuNCs, similar synthesis was conducted except that the pH of the solution before addition of N₂H₄ was adjusted to 12 by 1 M NaOH. A final dark brown solution of CuNCs exhibited a blue-emitting fluorescence under UV lamp irradiation.

**Fluorescence Detection of Hg²⁺.** For the typical assay of Hg²⁺, 300 μL of the prepared yellow-emitting CuNCs solution was added into 2.2 mL of the PBS buffer solution (pH 4.0, 10.0 mM) to prepare the probe solution. The solution (10.0 μL) with different concentrations of Hg²⁺ was added into the probe solution. Fluorescence emission spectra were collected with excitation at 360 nm after 60 s. In the selectivity experiment, a series of potential metal ions and amino acids were mixed with the probe solution. The concentrations of the these interferences were 500 μM.

**Characterization.** All of the instruments used for characterization were the same as those used in the previous work. Transmission electron microscopy (TEM) images of CuNCs with different fluorescent emission were obtained using a Tecnai G2F30 instrument. Fourier transform infrared (FT-IR) spectra were recorded on a Nicolet Nexus 670 spectrometer using KBr pellets. Powder X-ray diffraction (XRD) patterns were recorded on a D/max 82400 X-ray powder diffractometer (Rigaku, Japan) with Cu Kα radiation (λ = 0.154056 Å). X-ray photoelectron spectroscopy (XPS) measurement was performed using a PerkinElmer PHI-5702 multifunctional photoelectron spectrometer equipped with an Al Kα exciting source. Far-UV circular dichroism (CD) spectra of trypsin under different pH conditions were recorded at 25 °C on an Olis DSM 1000 double-beam spectrophotometer. UV–visible absorption spectra were recorded by a TU-1901 double-beam UV–vis spectrophotometer. Fluorescence measurements were carried out using a RF-5301 spectrofluorophotometer with both excitation and emission slits set at 10.0 nm. The excitation wavelength was set at 360 nm. Samples for absorption and emission measurements were taken in 1 cm × 1 cm quartz cuvette. The absolute photoluminescence quantum yield (QY) of CuNCs was measured and calculated using an “Edinburgh Instruments” FLS 920 spectrometer, which has been reported by our previous work (see Supporting Information). MTT Assay. The human cervical carcinoma HeLa cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum using a 96-well culture plate and kept in an incubator at 37 °C with a humidified atmosphere of 5% CO₂. Prior to test, 1 × 10⁴ cells were incubated in 96-well plates for 24 h at 37 °C in a final volume of 100 μL. Then, 10 μL of CuNCs with different concentrations (0, 50, 100, 200, 300, and 500 μg/mL, respectively) was added and incubated for another 24 h. Afterward, cells were rinsed twice with PBS (10 mM, pH 7.4) followed by addition of 100 μL of fresh medium and 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/mL) to each well. The cells were incubated for additional 4 h at 37 °C. After removing all medium from the wells, 100 μL of dimethyl sulfoxide was added to each well and mixed thoroughly for 5 min. The optical density (OD) of the mixture was measured at 570 nm using a microplate reader. The cell viability was estimated as (OD treated/OD control) × 100%, where OD control and OD treated were obtained in the absence and presence of CuNCs, respectively.

**In Vivo Fluorescence Imaging.** The HeLa cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37 °C with 5% CO₂ overnight. Then, CuNCs (500 μg/mL) were added to the cell culture, and the cells were incubated for another 1 h at 37 °C. After the cells were washed with PBS three times, the fluorescence images were acquired by a fluorescent microscope.

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**Figure 9.** Fluorescence microscopy image (A) and its corresponding bright-field transmission image (B) of HeLa cells incubated with 500 μg/mL CuNCs for 1 h at 37 °C.
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