An intracellular temperature nanoprobe based on biosynthesized fluorescent copper nanoclusters†

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Temperature variation is related to a series of biological reactions and abnormal medical processes of living cells. Fluorescence-based temperature nanoprobes have great potential for cellular imaging and temperature measurement. In this study, we have established a facile, efficient and green strategy for the preparation of an intracellular temperature nanoprobe specifically in situ biosynthesized fluorescent copper nanoclusters (CuNCs). Our observations demonstrate that the fluorescent CuNCs could specifically be biosynthesized spontaneously in MDA-MB-231 cancer cells through a particular molecular process, but not in normal cells (i.e., L02 cells). The resultant CuNCs, with an average diameter of 2.4 ± 0.4 nm, were found to exhibit red fluorescence emission ($\lambda_{em} = 610$ nm) and could further efficiently accumulate for biomaging in target cancer cells. More importantly, the fluorescence signal of the biosynthesized CuNCs is sensitively thermo-responsive over the physiological temperature range in MDA-MB-231 cells (relative sensitivity: $-3.18\%$ per Celsius). This provides an efficient nanothermometer based on the in situ biosynthesized CuNCs for cellular fluorescence imaging and other biomedical applications.

Introduction

Monitoring temperature is essential in biochemistry and other scientific research. Almost all of the cell processes including corresponding dynamics and reactivity of biomolecules are strongly dependent on temperature.¹,² The real-time monitoring of temperature in vivo may provide detailed information about target cells for biomedical diagnosis and therapy, since a variety of abnormal physiological phenomena are associated with inflammation or tumor growth, which is usually accompanied by a temperature change. Nevertheless, the accurate, fast and real-time measurement of intracellular temperature is still facing a big challenge.³,⁴

So far, there have been two typical methods to measure temperature. One is the contact method that includes thermocouples or thermistors, which needs electrical wiring and has strong electromagnetic noise simultaneously, and thus is difficult to use in living systems. The other is the non-contact method that monitors the signal from emitted light,⁵ which requires stable and constant emission from related materials. As the non-contact method is based on the emission signal of fluorescent materials, the relevant temperature measurement could exploit an accurate measurement of the related temperature according to the changes in the fluorescence signal.⁶–⁸ Presently, a series of fluorescent materials, such as quantum dots,⁹ lanthanoid-based nanomaterials,¹⁰ upconverting nanoparticles¹¹ and GFP-based thermosensors,¹² have been reported to act as nanothermometers in living systems. Nanoclusters (NCs) including Au NCs have also been explored as sensitive nanoprobes to detect the physiological temperature range in living cells.¹³,¹⁴ Compared to the extensively investigated Au NCs and Ag NCs, only a few research studies have focussed on Cu based materials. Since small-dimensional Cu based materials like Cu nanoclusters (NCs) were found to have weak stability and slight toxicity,¹⁵ various kinds of synthesis strategies have been exploited to reduce the possible cytototoxicity¹⁶ and improve the stability of CuNCs during the relevant process.¹⁷ Therefore, to overcome these problems, there is an urgent need to establish a simple method for the synthesis of CuNCs with excellent performances.

Based on these considerations, in this study we have explored a green and efficient strategy to biosynthesize highly fluorescent and biocompatible CuNCs in target cancer cells upon incubation with a special copper precursor (i.e., the complex solution of glutathione (GSH) and copper(II)). Our observations demonstrate that in situ biosynthesized fluorescent nanoclusters have ultratine size below the renal clearance barrier (namely, 5.5 nm), i.e., with an average diameter of 2.4 ± 0.4 nm, so that they can efficiently induce their accumulation in target living systems, thus preventing their potential toxicity over a long term.¹⁸ The biosynthesized CuNCs exhibit near red fluorescence emission ($\lambda_{em} = 610$ nm), which can efficiently avoid interference from the auto-fluorescence of cells and could...
be especially useful for sensitive measurement. More importantly, the relevant fluorescence intensity of the biosynthesized CuNCs is highly sensitive to physiological temperatures in cancer cells like MDA-MB-231 cells. Taking these advantages together, we have devised a new accurate, biocompatible and real-time method to measure intracellular temperature change. This provides an efficient intracellular thermometer based on in situ biosynthesized CuNCs for in vivo bioimaging and related biomedical applications.

**Experimental**

**Materials**

Reduced l-glutathione (GSH) (CAS: 10031-43-3), copper nitrate trihydrate (\(\text{Cu(NO}_3\text{)}_2\cdot 3\text{H}_2\text{O}\)), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), fetal calf serum (FBS), trypsin, penicillin and dimethyl sulfoxide (DMSO) were purchased from Sigma. DMEM (high glucose) culture solution was purchased from Hyclone. MDA-MB-231 cells (cervical carcinoma) were purchased from the Shanghai Institute of Cells, Chinese Academy of Sciences. Other reagents were all of analytical grade and purchased from Sinopharm Chemical Reagent Co. (China). All the reagents were used as received without further purification. Deionized water (\(\geq 18 \text{ M}\Omega \text{ cm}^{-1}\)) obtained from a Milli-Q water purification system was used for all the experiments.

**Characterization**

UV-Vis absorption spectra and fluorescence were recorded using a UV 3600 spectrophotometer and an RF-5301 PC spectrometer (Shimadzu), respectively. X-ray photoelectron spectroscopy (XPS) analysis was performed on a thoremoelectric instrument (Thermo VG Scientific ESCALAB 250) using Mg K\(\alpha\) excitation (1253.6 eV). Infrared spectroscopy (IR) was carried out on a Thermo Fisher Nicolet iS10 in the wavelength range from 4000 to 500 cm\(^{-1}\). To verify the morphology and the particle size distribution of the in situ biosynthesized CuNCs, transmission electron microscopy (TEM) was carried out on a JEM-2100 under the accelerating voltage of 200 kV. Energy dispersive X-ray spectroscopy (EDS) was measured using a field-emission scanning electron microscope (Zeiss, Ultra Plus). The confocal microscopy images were observed under a confocal fluorescence microscope (PerkinElmer, LS-55).

**Cell culture**

MDA-MB-231 cells (human breast carcinomas) were purchased from the Shanghai Institute of Cells, Chinese Academy of Sciences. L02 cells (human embryo liver cells) were provided by Third Military Medical University (Chongqing, China). MDA-MB-231 cells and L02 cells were grown in DMEM medium supplemented with 10% FBS, 100 U mL\(^{-1}\) penicillin, and 100 mg mL\(^{-1}\) streptomycin at 37 °C with 5% CO\(_2\) in a 95% humidified atmosphere.

**MTT assay**

MDA-MB-231 cells were cultured in DMEM medium on a 96-well plate at 37 °C with 5% CO\(_2\). It was important to prepare the copper precursor solution. In a typical procedure, an aqueous solution of copper nitrate trihydrate (\(\text{Cu(NO}_3\text{)}_2\cdot 3\text{H}_2\text{O}\)) was freshly prepared, and a specific amount of GSH was added dropwise to it and stirred for 10 min at ambient temperature; the pH value of the resultant solution was regulated, and this was used as the precursor solution for the following experiments. For MTT cell viability assay, various concentrations of the precursor solution (i.e., 5, 10, 20, 30 and 40 μg mL\(^{-1}\)) were incubated with MDA-MB-231 cells. After culturing for 24 h, 20 μL MTT solutions were added to each well for 4 h followed by the removal of the culture medium with MTT and subsequent addition of 150 μL of DMSO. The same dose of copper precursor was added to normal embryo liver cells (L02 cells) using the same protocol. The resulting mixture was shaken for approximately 10 min at room temperature. At last, by detecting the absorbance of the suspensions, cell viability could be measured. And the relevant equation was expressed as follows: cell viability (%) = [\(A\)\(_{\text{test}}\)/\(A\)\(_{\text{control}}\) \times 100%], where \(A\) represents the absorbance value at 492 nm.

**Biosynthesis of CuNCs**

The well-monodispersed and thermoresponsive CuNCs were spontaneously biosynthesized in situ in MDA-MB-231 cells through treatment with a special precursor. Typically, reduced l-glutathione and copper nitrate trihydrate were dissolved in pure water to form a slightly yellow solution. Then, NaOH of 0.1 mol L\(^{-1}\) was added dropwise to adjust the solution pH to 5, where the solutions changed from the suspension solution to a clearly visible solution. The resultant solution was used as a precursor to be injected into MDA-MB-231 cells. After incubating MDA-MB-231 cells with 20 μg mL\(^{-1}\) of copper precursor solution for 24 hours, bright fluorescent Cu NCs could be easily biosynthesized in cancer cells. Then, the biosynthesized CuNCs were isolated from MDA-MB-231 cells through a classical repetitive freeze–thaw method (ESI† S1). The as-obtained CuNCs were characterized by UV-Vis absorption spectroscopy, IR spectroscopy, fluorescence spectroscopy, EDS and TEM. The same protocol was used for the normal cells (L02 cells) as the control group. The quantum yield (QY) of the biosynthesized CuNCs was calculated by using eqn (S1), ESI†.

**Confocal fluorescence imaging of cells**

Confocal fluorescence microscopy imaging of living cells incubated with the copper precursor solution was performed on a laser confocal fluorescence microscope. For fluorescence microscopy imaging, a continuous wave laser at 405 nm provided excitation, and fluorescence emission at 500–650 nm was collected as the output signal. A 60 \(\times\) oil-immersion objective lens was used. The temperature was adjusted using a heater. To avoid cell death, the above-mentioned processes must be very fast and directly monitored under a confocal microscope. All temperature measurements were recorded using a thermometer.

**Results and discussion**

**Characterization of the CuNCs biosynthesized in cancer cells**

The special copper precursor, which was prepared using copper nitrate trihydrate and GSH in a certain proportion, was initially
transported into the cells via a classical endocytosis pathway. After incubation for 24 h, the intracellular fluorescence signal appeared, indicating the fluorescent CuNCs biosynthesized in target cancer cells. As shown in Scheme 1, it is evident that the fluorescent CuNCs could be spontaneously biosynthesized in cancer cells for intracellular fluorescence imaging and temperature measurement, which could not be biosynthesized in normal cells. Remarkably, the relevant fluorescence intensity of the as-formed CuNCs was reversibly and sensitively responsive to temperature changes in MDA-MB-231 cancer cells. The rationale behind it could be attributed to the special microenvironment of cancer cells and the relevant CuNCs may be formed by reduction and etching with excess GSH in cancer cells, which was much more abundant in cancer cells than in normal cells.

Although copper ions are essential microelements of the human body, which play a pivotal role in many fundamental physiological processes, they usually show slight toxicity. Thus, in order to avoid potential toxicity, we have used GSH, a natural peptide, to coordinate with copper ions through a relevant thiol group, forming a chelate which is used as a precursor. This can not only efficiently enhance the biocompatibility of copper ions but also facilitate the process efficiency of the biosynthesized CuNCs. Meanwhile, our observations demonstrate that the relevant molar ratio and the pH value also play a key role in the biosynthesis of CuNCs in cancer cells. An optimal composition of GSH contents (i.e., Cu/GSH = 1/4) was found to be desirable for obtaining highly fluorescent biosynthesized CuNCs in situ in cancer cells. Meanwhile, considering that cancer cells had a special microenvironment including a relatively low pH value and specifically physiological metabolism and the pK_a values of both the relevant carboxyl groups are around 4.25, we have chosen pH 5 for copper solution as the special copper precursor for the biosynthesis of CuNCs in cancer cells. Fig. 1 shows the typical UV-Vis absorption spectra and bright fluorescence of the intracellular self-biosynthesized CuNCs, extracted from targeted cancer cells. It is evident that under the excitation wavelength of 400 nm, the biosynthesized CuNCs in aqueous solution exhibited fluorescence emission with a peak at 610 nm with a normal symmetry (Fig. 1a). The QY of the biosynthesized CuNCs in aqueous solution was found to be around 4.5% using rhodamine 6G (QY = 95% in ethanol). When the biosynthesized CuNCs were dispersed in 70 vol% ethanol solution, the fluorescence intensity was significantly enhanced compared with that in aqueous solution (Fig. S1, ESI†), suggesting that the biosynthesized CuNCs may exhibit the aggregation-induced emission enhancement (AIEE) effect, which agrees with previous reports in the literature. The UV-Vis absorption spectroscopy (Fig. 1b) demonstrates a spectral band centered near 300 nm, which is due to relevant ligand-to-metal-charge-transfer transition (i.e. LMCT), indicating the formation of CuNCs. It is worth mentioning that fluorescent CuNCs could not be formed in normal cells even after incubation for longer times, suggesting that fluorescent CuNCs can be in situ biosynthesized only in cancer cells.

The TEM study clearly characterizes the biosynthesized CuNCs in cancer cells. As observed from the TEM images (Fig. 1c), CuNCs show good uniformity and mono-dispersion, with an average diameter of 2.4 ± 0.4 nm (Fig. 1d), and larger size metal nanoparticles are not formed. It is noted that the interplanar crystal spacing is 2.05 Å, indicating the (111) diffraction planes of face-centered Cu. The relevant ultrafine size of CuNCs is below the renal clearance barrier (about 5.5 nm), which can efficiently reduce their accumulation in living systems, thus preventing the potential toxicity over a long term. Thus, the biosynthesized CuNCs could be biocompatible and valuable for the in vivo imaging of cancer cells. Furthermore, we have measured EDS of the biosynthesized fluorescent CuNCs extracted from MDA-MB-231 cells to confirm their elemental composition. As shown in Fig. 2a, the EDS study indicates the presence of the Cu element in the biosynthesized nanoclusters, and the absence of other metallic chemical elements.

Meanwhile, we have further utilized XPS analysis to verify the oxidation state of copper in the biosynthesis of fluorescent CuNCs. As shown in Fig. 2b, no characteristic peak around 942.0 eV demonstrated the absence of Cu(II). While two obvious
peaks appeared at 932.1 eV and 953 eV, which can be respectively assigned to the binding energies of the 2p_{3/2} and 2p_{1/2} electrons of Cu(0), consistent with the previous report in the literature. In addition, it is worth noting that the difference in the binding energy of Cu(0) and that of Cu(I) species is nearly 0.1 eV only. Thus, the formation of Cu(i) cannot be readily excluded. Some reports in the literature indicate that there are two steps involved in the formation of metal NCs. At first, Cu(II) was reduced to Cu(I) or Cu(0) by GSH. This process occurred very fast. Then, the resultant product coordinates with GSH through a thiol group and an amino group. According to the relevant study, a similar phenomenon has been found for CuNC aggregation induced emission enhancement. One possible reason is that charge transfer occurs in Cu–S bonds, so the valence state most likely lies between 0 and +1.

For purified dry samples extracted from MDA-MB-231 cells, we use FT-IR spectra to acquire further information for the relevant structural or surface properties of the biosynthesized fluorescent CuNCs. As shown in Fig. 2c, a typical absorption band of the carboxyl (−COO−) group at ca. 1713 cm\(^{-1}\) corresponds to the successful coordination of the glycine residue of GSH on the surface of CuNCs. Compared with pure GSH alone, the characteristic absorption peak at ca. 2525 cm\(^{-1}\), which closely corresponds to the stretching band (ν\(_{\text{SH}}\)), disappeared in the biosynthesized CuNCs. This provides evidence for the binding of GSH via deprotonation and coordination of a thiol group with CuNCs. A similar observation at ca. 390–370 cm\(^{-1}\) assigned to the stretching band of Cu–S in GS–CuNCs further confirms the binding of copper ions with a sulphur (S) group. Additionally, it is evident that at ca. 3500–3400 cm\(^{-1}\) the band assigned to ν\(_{\text{OH}}\) appears obviously in the biosynthesized CuNCs, which can be attributed to GSH residues.

**Confocal fluorescence imaging of in situ biosynthesized CuNCs**

To evaluate the possible cytotoxic effects of the biosynthesized fluorescent CuNCs in MDA-MB-231 cells, the MTT assay was conducted on the MDA-MB-231 cells loaded with the copper precursor solution. From the results of the MTT assay (Fig. S2, ESI†), it is evident that the cell viability still remained above 85% after incubation with the copper precursor solution even at the concentration of 40 μg mL\(^{-1}\) for 24 h. When the same dose of copper precursor solution was incubated with the normal cells (L02 cells), similar results were obtained (Fig. S3, ESI†), which indicates that the biosynthesized fluorescent CuNCs had low cell-cytotoxicity and was safe for in vivo studies.

The bioimaging and intracellular distribution of spontaneously biosynthesized fluorescent CuNCs in cancer cells were evaluated by laser confocal fluorescence microscopy (Fig. 3). From the bright-field images, it is evident that MDA-MB-231 cells maintained their morphology after incubation with 20 μg mL\(^{-1}\) of the copper precursor solution for 24 h at 37 °C, indicating good biocompatibility for the biosynthesis of CuNCs (Fig. 3a). Meanwhile, the bright red fluorescence signal detected in MDA-MB-231 cells was not only in the cytoplasm, but also in the cellular nucleus (Fig. 3a). This observation agrees well with that reported in the literature. Besides, the fluorescence signal in the cytoplasm of MDA-MB-231 cells is most significant, and the rationale behind it could be attributed to the fact that the as-formed CuNCs exhibit the aggregation-induced emission enhancement (AIEE) effect, where most of the fluorescent CuNCs were spontaneously biosynthesized in the cytoplasm. Furthermore, in control experiments, DMEM and copper precursor solution were incubated in MDA-MB-231 cells, and no fluorescence signal was observed (Fig. 3d). In addition, when the same protocol was used in normal cells (L02 cells), no obvious fluorescence signals could be obtained (Fig. S4, ESI†). It is observed that the fluorescent CuNCs can be biosynthesized only in cancer cells after incubation with the copper precursor solution, but not in normal cells. Recent studies revealed that AuNCs and AgNCs could also be biosynthesized in cancer cells, and not in normal cells, which could be due to the special microenvironment of cancer cells. Our observations for the biosynthesized CuNCs were consistent with those reported in the literature, which can efficiently avoid interference from the auto-fluorescence of the cells and could...
be especially useful for the sensitive and specific fluorescence imaging of living cancer cells. The luminescence mechanism of biosynthesized CuNCs in cancer cells alone is not yet completely clear. However, our recent studies indicate that the molecular process of the biosynthesized fluorescent complexes in cancer cells may be involved in the important NAD(P)H-oxidase and metabolic pathway.29

Thermo-responsive images based on the biosynthesized CuNCs in cancer cells

Intriguingly, in this study we have found the temperature sensitivity of the biosynthesized CuNCs in target cancer cells, which was closely dependent on the fluorescence emission intensity. As shown in Fig. 4, after incubation with copper precursor solution for 24 h, the biosynthesized CuNCs were extracted from MDA-MB-231 cells. The fluorescence spectra were recorded for the biosynthesized CuNCs at a physiological temperature ranging from 20 to 45 °C. In Fig. 4a, by increasing the temperature from 20 to 45 °C, the emission intensity of the biosynthesized CuNCs shows a significant decrease, whereas no obvious shifts in the emission peak wavelength of CuNCs was observed within the temperature range. On the other side, when we decreased the temperature from 45 to 20 °C, the fluorescent intensity was almost recovered (Fig. S5, ESI†). This phenomenon is attribute to a typical behaviour of temperature based fluorescent materials. When the temperature increases, the molecule collision frequency increases, which leads to an increase in the non-radiative transition rate. So this can reduce the fluorescent intensity.30 The reproducibility of the biosynthesized CuNCs is an essential property of temperature-sensitive fluorescent materials. Fig. 4b further indicates that there is a linear relationship between fluorescence intensity and temperature. In addition, the fluorescent intensity was quite stable (Fig. 4b inset). So the temperature change operation was repeated for five cycles between 20 and 40 °C (Fig. S6, ESI†). The fluorescence intensity trend shows no obvious change, which is similar to other conventional fluorophores.31 According to the relative sensitivity calculated using eqn (S2), ESI†, the biosynthesized CuNCs decreased at a rate of −3.18% per °C, while the temperature varied from 20 to 45 °C. This means that the biosynthesized CuNCs can meet the requirement for cellular temperature measurement.

![Fig. 4](image-url)  
**Fig. 4** Temperature dependent changes in the fluorescence emission of biosynthesized CuNCs extracted from MDA-MB-231 cells. (a) The fluorescence emission spectra from 20 to 45 °C, with a temperature interval of 5 °C. Excitation wavelength: 400 nm. (b) The relationship between the fluorescence intensity and temperature. (Inset: the relationship between the fluorescence intensity and time at 20 °C.)

By taking advantage of this characteristic of the relevant CuNCs, the biosynthesized CuNCs in cancer cells had enormous potentiality to act as temperature nanoprobes in living systems in a physiological temperature range. Since the temperature could change in living biological systems including the cancer microenvironment, which often occurs in a small range, the sensitivity and stability of the relevant detection are very important.

Based on these observations, we have further explored the effective thermo-responsive images based on the biosynthesized CuNCs in cancer cells. After incubating MDA-MB-231 cells with 20 μg mL⁻¹ of copper precursor solution for 24 h, the fluorescence images of the MDA-MB-231 cells at different temperatures were obtained by laser confocal fluorescence microscopy. As shown in Fig. 5, our observations demonstrate that the in situ biosynthesized CuNCs could be readily used to monitor the intracellular temperature change in MDA-MB-231 cancer cells. It is evident that the fluorescence intensity decreased markedly with the increase of relevant temperature (i.e., from 20 to 40 °C); when the temperature is above 45 °C, the fluorescence intensity decreased sharply. The average gray scale values per unit area were calculated for different temperatures (Fig. S7, ESI†). As expected, the fluorescence intensity decreased with an increase of relevant temperature, which was consistent with that observed in Fig. 5. This has raised the possibility of using the in situ biosynthesized CuNCs as bioprobes in cancer cell fluorescence imaging and temperature sensing at the cellular level.

**Fig. 5** Typical confocal fluorescence images of MDA-MB-231 cells. The fluorescent CuNCs in situ biosynthesized after incubation with 20 μg mL⁻¹ of copper precursor solution for 24 h, and then tested at different temperatures.

Conclusions

In summary, in this study we have explored a facile and green strategy to in situ biosynthesize fluorescent CuNCs in cancer cells, which can accurately act as nanothermometers in the
in vivo cancer cell fluorescence imaging and temperature measurement at the cellular level. Our observations demonstrate that the fluorescent CuNCs could be spontaneously biosynthesized in MDA-MB-231 cancer cells through a particular molecular process. The resultant CuNCs exhibit good biocompatibility and red fluorescence, with an average diameter of 2.4 ± 0.4 nm, which could further efficiently accumulate for bioimaging in target cancer cells. The resultant CuNCs were found to exhibit red fluorescence emission ($\lambda_{em} = 610$ nm), which can efficiently avoid auto-fluorescence from cells and could be especially useful for sensitive measurement. Moreover, the in situ degrading them into small molecular complexes, this new target cancer cells over the physiological temperature range. Since the kidneys could efficiently clear GSH–Cu nanoparticles by degrading them into small molecular complexes, this new nanoprobe shows promising potential in cellular temperature bioimaging and clinic diagnosis related biomedical applications with the assistance of super resolution imaging technology.

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Notes and references

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