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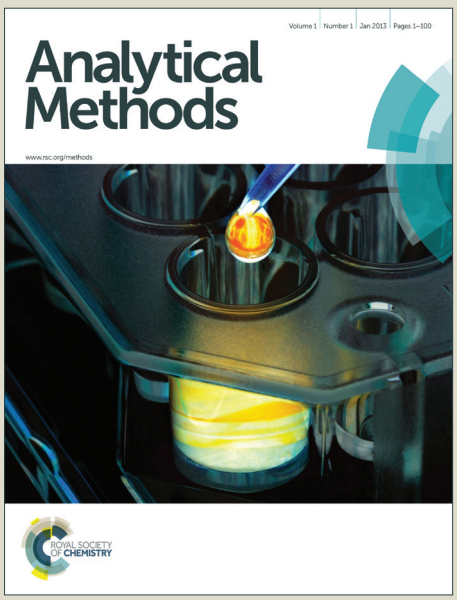

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Successive detection of glucose and bio-copper in human serum based on multiplex biosensor of gold nanorods

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Abstract

In this paper, a promising combined assay for successive detection of blood glucose and sera copper levels based on etching of gold nanorods (GNRs) was developed. A hydroxyl radical-enhanced GNR oxidation under ultraviolet irradiation facilitates to establish a plasmonic biosensor that may quickly detect blood glucose. A linear relationship between the change of plasmonic wavelength and glucose concentration was found ($\Delta\lambda$=4.2284+132.0c) at range of 0.23 to 0.928 mM and the LOD was 0.45 μM. Determination of blood glucose by this proposed method was satisfactory and closely comparable with the results given by the local hospital. On the other hand, a blue-shift of longitudinal plasmon wavelength induced by various forms of copper in the presence of $Na_2S_2O_3$ provides a sensitive approach to detect total copper level in biological sample. The copper levels of human sera were measured and corroborated by flame atomic absorption spectrometry, which confirms that this approach might be applicable to bio-copper analysis with high accuracy. A combined assay for successive detection of blood glucose level and serum copper was subsequently developed. Compared with other relative biosensors requiring modified design, bio-molecular modification or/and sophisticated instruments, the dual glucose and copper sensor is very simple, cost-effective and easy detection, suggesting great potential applications for successively monitoring blood glucose and copper concentrations and their changes during the progression of diabetes.

Introduction

Gold nanorods (GNRs) have attracted much attention in the past decade due to their physical and chemical properties.[1-4] Their unique properties make them suitable for photothermal therapy,[5,6] imaging,[7,8] gene delivery,[9] data storage.[10] Numerous biosensors for detection of biological targets such as antibody (or antigen), DNA were developed due to GNRs sensitive to the dielectric constant of the surrounding medium.[11-16] As chemical sensor, determination of dissociated metal ions in the aqueous solution could be achieved by modulating the plasmonic properties of GNRs.[17,18] These works greatly expanded the biosensing application of GNRs, which provoke further efforts to develop their bio-analytical applications.

Diabetes mellitus is one of the leading causes of death and disability which is a worldwide public health problem. The main feature of diabetes is chronic hyperglycemia, which leads to the disturbance of carbohydrate, fat and protein metabolism.[19,20] Moreover, various secondary complications may occur such as renal dysfunction and failure,[21] cardiac abnormalities,[22] diabetic retinopathy,[23] neuropathy,[24] atherosclerosi.[25] The blood glucose level is used as a clinical indicator of diabetes mellitus. Therefore, efforts to explore biosensors for fast and reliable glucose monitoring in the diagnosis of diabetes have received continuous interest. Meanwhile, there is accumulating evidence that the metabolism of several trace elements such as copper and zinc are altered in diabetes mellitus and these nutrients might have specific roles in the pathogenesis and progression of this disease.[26-28] Plasma copper levels have been found to be elevated in type-1 diabetes mellitus

patients and the urinary excretion of copper has been found to be affected by diabetes mellitus.[29] Recently, novel approaches for detection of copper have attracted increasing attention.[30-32] Specific glucose and copper detection of diabetes mellitus by using simple and low-cost assays is important in clinical diagnostics. Most current methods for the quantification of blood glucose level and serum copper require costly and sophisticated instruments with time-consuming, labor intensive and expensive with complicated procedures. Therefore, it is necessary to develop quick and successive assay for combined detection of glucose and serum copper in order to diagnose and monitor progression of diabetes as well as glucose metabolism of cells.

In this study, we present a simple combined assay for successive detection of blood glucose level and serum copper based on etching of GNRs. Under irradiation of ultraviolet (UV) light, the glucose may be quickly detected. In addition, the serum copper level including copper-containing proteins in human serum can be quickly determined, requiring no pretreatment with high sensitivity and selectivity, which is different from most of the previous approaches that only measured the dissociated copper ion rather than the undissociated copper-containing compounds. This dual glucose and copper sensor is quick response not only to blood glucose level but also serum copper, suggesting great potential applications for successively monitoring glucose and copper concentrations and their changes during the progression of diabetes.

Material and Methods

Materials and Reagents

Cetyltrimethylammonium bromide (CTAB), ascorbic acid, sodium thiosulfate, silver nitrate, sodium borohydride, $HAuCl_4 \cdot 4H_2O$, 30% H_2O_2, glucose, glucose oxidase, zinc nitrate, calcium nitrate, ferric chloride, manganese sulfate, copper nitrate, cobalt sulfate, nickel sulfate, aluminum sulfate, sodium chloride, chromium sulfate, mercuric chloride were purchased from Sinopharm Chmical Reagent Co., Ltd. (Shanghai, China). All of the chemicals, unless mentioned otherwise, were of analytical reagent grade and used as received. The aqueous solutions were prepared in doubly distilled water.

Preparation of GNRs

GNRs were synthesized using a seed-mediated silver-assisted growth method.[33, 34] The main steps include two steps: (1) Preparation of gold seeds. The CTAB solution (1.5mL, 0.1 M) was mixed with 100 μL of 0.02 M $HAuCl_4$. One hundred microliters of ice-cold 0.01 mM $NaBH_4$ was added while the solution was stirred, resulting in the formation of a brownish yellow solution. The particles in this solution were used as seeds within 2-5 h after preparation. (2) Synthesis of GNRs. 1.5 mL of 0.02 M $HAuCl_4$ and 0.8 mL of 0.01M $AgNO_3$ were added to 30 mL of 0.1 M CTAB, followed by the addition of 0.8 mL of 0.08 M ascorbic acid. Finally, 70 μL of the seed solution was added to the solution and the color changed gradually in 30 min.

Detection of hydrogen peroxide and glucose

A series of concentrations of H_2O_2 solution (0.5, 1, 3, 5, 10, 20 mM) were added to

1 mL of GNR dispersion under the irradiation of ultraviolent for 30 min before the vis−NIR absorption measurement. 30μL of various concentrations of glucose solution (0.5, 1, 3, 5, 10, 20 mM) and 30 μL glucose oxidase (0.5mg/mL) was mixed for incubation at 37 °C in a water bath about 30 min, 1 mL GNR dispersion was added to the solution, and then the mixture was irradiated by UV light for 35 min before recording absorption spectra.

Detection of copper

40 μL of 0.01M $Cu(NO_3)_2$, 20 μL of 0.25 M $Na_2S_2O_3$ were added to 2 mL of the synthesized GNRs at room temperature. A longitudinal plasmon wavelength (LPW) of GNRs increased with the increment of concentrations of Cu^{2+}. Various stages were monitored at different reaction time intervals and the final spectrum was recorded.

Blood collection and handling

All human serum samples were obtained from hospital and they were enclosed in centrifuge tube and stored in the refrigerator until the performance of experiment.

Experimental instrumentation

Transmission electron microscopy (TEM) was performed on a Tecnai G2 20 (USA) operating at 100 kV. For TEM sample preparation, 1-2 μL of the nanorods solution was placed on a carbon coated copper grid and allowed to dry at room temperature. Absorption spectra of the GNR dispersion were measured using Lambda 35 (PerkinElmer, USA). To assess the validity of the proposed method, as a comparative experiment, the copper content of RBC were also determined by atomic absorption spectrometry (AAS) using a Perkin Elmer atomic absorbance

spectrophotometer (PEAAnalyst 300).

Results and discussion

Detection of H_2O_2 based on etching of GNRs under UV irradiation

First, detection of glucose based on enzymatic etching of GNRs was explored. A simple and fast method for detection of H_2O_2 under UV irradiation without introduction of any other reactions would be achieved, subsequently, the detection of glucose based on enzymatic etching of GNRs may be accomplished, as shown in Scheme 1. The catalysis of UV light toward H_2O_2 to generate a hydroxyl radical (•OH) may significantly shorten the reaction time,[35,36] resulting from much stronger oxidizing ability of •OH than H_2O_2. GNRs might be oxidized by some oxidized cations such as Fe^{3+} and Cr^{3+}.[37,38] The strong oxidizing species •OH can readily etch GNRs, reducing the aspect ratio of GNRs and leading to a significant blue-shift of longitudinal peak of the localized surface plasmon resonance (LSPR). Under the existence of oxidase (GOx), the enzymatic etching of GNRs might be realized by combining reactions of glucose oxidation to H_2O_2 and subsequently decomposed •OH under UV irradiation. The degree of change of longitudinal plasmon wavelength (LPW) of the GNRs directly depends on the concentration of glucose, which indicates the concentration of glucose may be detected using this GNRs-based plasmonic H_2O_2 monitor.

The effect of UV on GNR etching was studied. Two identical samples consisting of GNRs and H_2O_2 were investigated. Figure 1a shows one was directly oxidized by H_2O_2 with a total blue-shift of 115 nm within 35 min under the irradiation of UV light.

However, no clear blue shift of LPW was observed on another sample without the irradiation of UV light at the same time. Meanwhile, various concentrations of H_2O_2 were added to GNRs under the UV irradiation at the same time, which show the degree of blue-shift of LPW was significantly dependent on the concentration of H_2O_2, as shown in Figure 1b. It is found that the LPW gradually blue shifts and decreases in intensity, while the transverse plasmon wavelength stays at 520 nm. The as-prepared GNRs were characterized by TEM shown in Figure 2a, which indicates that the transverse width and axis length of the GNRs are 11±2 and 50±3 nm, respectively. After GNRs oxidized by H_2O_2 under UV irradiation, a clear decrease in length of the resultant nanorods displayed in Figure 2b was observed, resulting in a decrease of aspect ratio of GNRs. This reveals that the strong oxidizing ability of hydroxyl radical can not only accelerate the oxidation of GNRs but also enable the oxidation of GNR at low concentration of H_2O_2.

The influence of UV intensity and irradiation time on GNRs was further investigated. With the addition of H_2O_2 to GNRs at final concentration of 10 mM, a blue-shift of LPW was negligible in 60 min at room temperature. However, when the mixture solution was irradiated by strong UV light (2.31 mW/cm^2), a remarkable blue-shift of LPW occurred. Meanwhile, a slight blue-shift of LPW of the GNRs was found under the same strong intensity of UV light at the same irradiated time, as shown in Figure 3a. A detailed investigation on light density of UV exhibited that when the UV light density was less than 0.58mW/cm^2, no detectable change of LPW of GNRs would be found, as shown in Figure S1 (supplementary material). On the

other hand, the irradiation time of UV was explored. Figure 3b shows the relationship between degree of blue-shift of LPW and the irradiation time under 0.58 mW/cm^2 UV light, indicating the optimized time is 35 min.

The determination of H_2O_2 was performed under the optimized conditions. As an indicator of the oxidation of GNRs, different concentration of H_2O_2 may be reflected by the degree of blue-shift ($\Delta\lambda$) of LPW. Obviously, different concentration of H_2O_2 might lead to varying degree of blue-shift of LPW. A linear relationship between the corresponding $\Delta\lambda$ of GNRs and the concentration of H_2O_2 was found within the concentration range from 0.015 to 0.16 mM, as shown in Figure 4. The linear equation is $\Delta\lambda$=4.2284+132.0*c* (mM), where the *c* represent the concentration of H_2O_2, the regression coefficient of R^2 =0.9832 is satisfactory and the limit of detection (LOD) is 2.9×10^{-9} M. The degree of blue-shift of LPW could be directly reflected by the H_2O_2 concentration. Thus, the feasibility of this plasmonic sensor for H_2O_2 detection was demonstrated by longitudinal plasmon band measurement.

Detection of glucose based on enzymatic etching of GNRs

This plasmonic biosensor was employed to detect glucose by GOx-catalyzed H_2O_2 generation. The effect of glucose and GOx on GNRs was investigated and the results illustrated that no detectable change of LPW was found from the addition of the glucose and GOx to GNR dispersion within 60 min. However, the addition of glucose and GOx to the GNRs solution under UV irradiation quickly led to a clear blue-shift of LPW of the GNRs. On the other hand, the sole addition of glucose or GOx in the GNRs solution did not result in such changes, suggesting that the glucose

and GOx independently had no effect on the GNRs.

The quantitative analysis of glucose was carried out under the optimized conditions for detection of H_2O_2. Figure 5 shows that the LPW shift of GNRs in 35 min was recorded under the UV irradiation. Similarly, a linear relationship between $\Delta\lambda$ and glucose concentrations at range of 0.23 to 0.928 mM was obtained ($\Delta\lambda$=4.2284+132.0*c*, R^2=0.9914) and the LOD was 0.45 μM. Thus, this GNRs-based sensor presents a potential to measure blood glucose directly by the shift of plasmonic wavelength of GNRs.

This plasmonic glucose sensor was practically applied to analyze human serum samples from ten volunteers. Fresh serum samples were first analyzed in the local hospital with Roche cobas 8000 Modular Analyzer (Mannheim, Germany). The glucose levels of these samples were determined by the proposed UV-induced decomposition of H_2O_2 approach and the detected blood glucose concentration were displayed in Table 1, which were satisfactory and closely consistent with the results given by the hospital. This confirms that the proposed sensing system is applicable to blood glucose analysis with high accuracy.

Copper-induced plasmonic change of GNRs

When $Cu(NO_3)_2$ solution was added to the remainder GNRs after reacting with H_2O_2 under UV irradiation, no appreciable change of LPW was observed. With the addition of $Na_2S_2O_3$ to this mixture, however, a significant blue-shift of longitudinal peak of the LSPR spectra appeared, as shown in Figure 6a. The GNRs were characterized by TEM displayed in Figure 6b and 6c. Following the reaction of GNRs

with $Cu(NO_3)_2$ in the presence of $Na_2S_2O_3$, a clear decrease in length of the resultant nanorods was observed, namely, a significant decrease of aspect ratio of the generated GNRs occurred. A control experiment about the reaction of $Na_2S_2O_3$ and remainder GNRs in the absence of $Cu(NO_3)_2$ was carried out, a remarkable red-shift of LPW occurred companied by the decrease of absorption intensity. The results imply that the plasmonic modulation arises from the GNRs reacting with $Na_2S_2O_3$ and Cu^{2+}, resulting in change of size of nanorods.

In fact, the previous studies found that Au and Ag constitute GNRs prepared by the silver ion-assisted seed-mediated method in the medium of CTAB according to the previous studies.[39,40] The previous works also demonstrated that the GNRs would react with $Na_2S_2O_3$ to produce core-shell nanorods, companied by the red-shift of LPW.[41,42] Surprisingly, the present of Cu^{2+} in the solution changes the reactions, and shortened GNRs take the place of core-shell nanorods while GNRs reacting with $Na_2S_2O_3$. Thus, Cu^{2+} can oxidize gold and silver in the presence of $S_2O_3^{2-}$ to produce $Cu(S_2O_3)_3^{5-}$.[43,44] Owing to the axial surface of GNRs surrounded by CTAB and fewer gathered the ends of GNRs, the corroded GNR reactions are preferentially taken place on the ends of GNRs, resulting in a decrease of aspect ratio of nanorods and a significant blue-shift of LPW. Meanwhile, the concentration of $Na_2S_2O_3$ play an important factor, if the concentration of $Na_2S_2O_3$ was too high, the blue-shift of LPW would swiftly transform to a red-shift due to the remainder $Na_2S_2O_3$ reacting with GNR to form a core-shell nanostructure. On the other hand, too low concentration of $Na_2S_2O_3$ led to greatly slowing down the reaction. The investigation shows that the

proper concentration of $Na_2S_2O_3$ was 0.25M.

Evaluation of the GNRs response to Cu^{2+} ion.

A significant blue-shift of LPW induced by copper may provide a potential opportunity for detection of Cu^{2+}. To evaluate the feasibility, various concentrations of Cu^{2+} in the presence of $Na_2S_2O_3$ were investigated and the corresponding absorption spectra were subsequently recorded. The degree of blue-shift of LPW was clearly increased with the increment of concentration of Cu^{2+} (Figure S2). This method exhibits high sensitivity with the LOD 1×10^{-10} M. Further investigation indicates a linear relationship will be found within a certain concentration range of Cu^{2+} ions.

To examine the selectivity of this approach for Cu^{2+}, other metal ions such as Na^{+}, Zn^{2+}, Ni^{2+}, Co^{2+}, Ca^{2+}, Fe^{3+}, Al^{3+}, Hg^{2+}, Mn^{2+}, Cr^{3+}, and Pb^{2+} was investigated. There was no clear variation of plasmon peak wavelength when individual metal ion reacted with GNRs under the same reaction condition illustrated in Figure 7. From this Figure, it can be seen that no detectable changes of LPW occurred even if the concentration of these metal ions increased up to 1×10^{-4} M, indicating that all competitive metal ions have no obvious interference toward the detection of Cu^{2+} ion. These results imply that this method exhibits excellent selectivity.

Feasibility of detection bio-copper in human serum samples

As discussed aforementioned, the copper ions may be readily detected by this approach. However, the main existed form of copper-containing compounds is present as undissociated copper such as protein in the biological body fluids and organs. Various forms of copper including copper-containing compounds and copper

nanoparticles, such as coordinate compound Cu-EDTA and copper nanocluster were employed to investigate the feasibility for expanding this method to detect various forms of copper. Interestingly, the expected blue-shift of LPW was observed, as shown in Figure 8. Cu-EDTA is very stable water soluble complex with high stability constants (log*K*=18.80). Copper nanoclusters are small nanoparticles comprising several to tens of copper atoms and were prepared according to previous report,[45] showing blue emission (410 nm) under exited 325 nm, as shown in Figure. S3**.** The experimental results indicate that this assay can readily detect copper regardless of the forms of copper, implying that this assay can be applied to determination of bio-copper in the biological samples.

Human serum samples were employed to demonstrate the practical application for measuring the bio-copper level. When the human serum sample was added to GNR dispersion, no noticeable change of LPW was found. However, a significant blue-shift of LPW appeared with the addition of human serum sample to GNR dispersion in the presence of $Na_2S_2O_3$ and quick response of the LSPR signal was obtained within 10 min. Figure 9a shows that the detection of bio-copper level of human serum samples by using this method. The same samples of human serum as the above were measured utilizing this approach. The concentration of copper range is between 13.18 ~ 23.06 μM displayed in Figure 9b. One of copper levels of these samples is 19.22 μM and the validity is demonstrated by AAS, which confirms that the copper-induced plasmonic modulation is applicable to bio-copper analysis with high accuracy.

Combined assay for successive detection of blood glucose and serum copper.

As discussed aforementioned, both glucose and copper levels of human sera may be directly measured without requiring bio-molecular modification and pretreatment. As shown in Scheme 1, only GOx was added in the procedure of analyzing glucose of human serum and, apparently, the addition of this copper free protein will not affect the subsequent measurement of copper level of human serum. This suggests that copper level of human serum may be successively measured after the accomplishment of detection of blood glucose in the human serum sample. Figure 10a shows the LSPR spectra acquired from the addition of human serum to the GNR dispersion, indicating the feasibility for successive detection of glucose and copper level. The above human serum samples were analyzed again using this combined assay and the same results of the glucose and copper were obtained. Other human serum samples from four volunteers were analyzed and Figure 10b shows their concentrations of glucose and copper.

Conclusion

In summary, we demonstrate a simple combined assay for successive detection of blood glucose and serum copper level based on etching of GNRs. Hydroxyl radical-enhanced GNRs oxidation under UV irradiation was introduced, which greatly shorten the etching time of GNRs. A novel plasmonic blood glucose monitor based on glucose oxidase-mediated oxidative etching of GNRs was thereby established. A linear relationship between the change of plasmonic wavelength and glucose concentration was found ($\Delta\lambda=4.2284+132.0c$) at range of 0.23 to 0.928 mM with LOD 0.45 μM. Determination of blood glucose by this proposed method was

satisfactory and closely comparable with the results given by the local hospital. On the other hand, a highly sensitive biosensor with the LOD 1×10^{-10} M for detection of bio-copper was established based on the blue-shift of LPW induced by various forms of copper in the presence of $Na_2S_2O_3$. The copper levels of human sera were measured and corroborated by AAS. Both glucose and copper level of human serum may be directly measured without requiring bio-molecular modification and pretreatment. A combined assay for successive detection of blood glucose level and serum copper was subsequently established. Compared with other relative biosensors requiring modified design, bio-molecular modification or/and sophisticated instruments, the dual glucose and copper sensor is very simple, cost-effective and easy detection, suggesting great potential applications for successively monitoring glucose and copper concentrations and their changes during the progression of diabetes.

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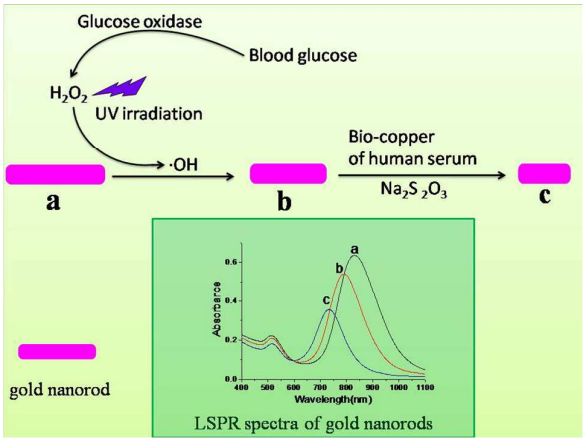
Figures and Captions



Scheme 1. Schematic illustration of successive detecting glucose and bio-copper in human serum based on GNRs.

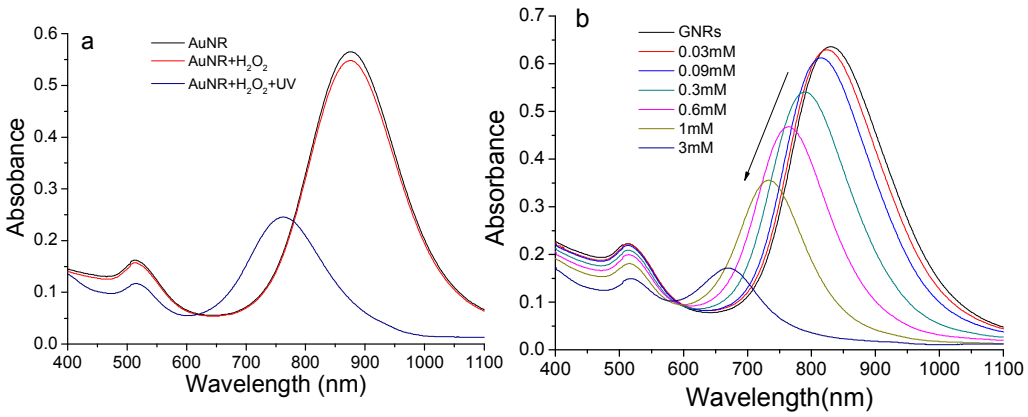


Figure 1. *a*: Absorption spectra acquired from GNRs reacting with H_2O_2 in the presence and absence of UV irradiation at the same time. *b*: Absorption spectra obtained by GNRs reacting with various concentration of H_2O_2 under the irradiation of UV light.

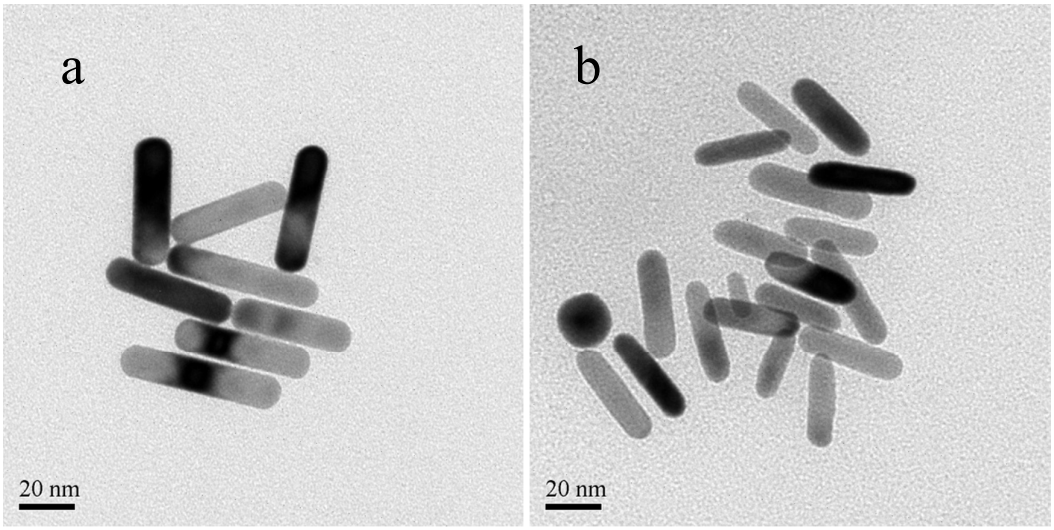


Figure 2. TEM images before (a) and after (b) GNRs oxidized by H_2O_2 under UV irradiation.

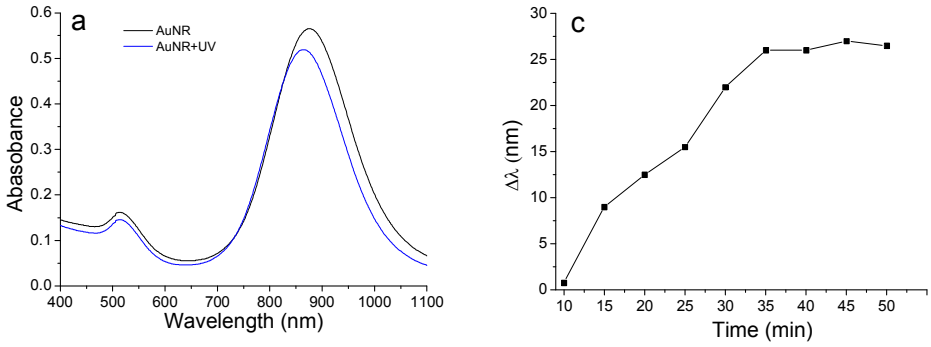


Figure 3. *a* represents a slight blue-shift of LPW of the as-synthesized GNRs under 2.31 mW/cm^2 of UV light in 60 min. *b* shows the relationship between degree of blue-shift of LPW and the irradiation time under irradiation of 0.58mW/cm^2 UV light.

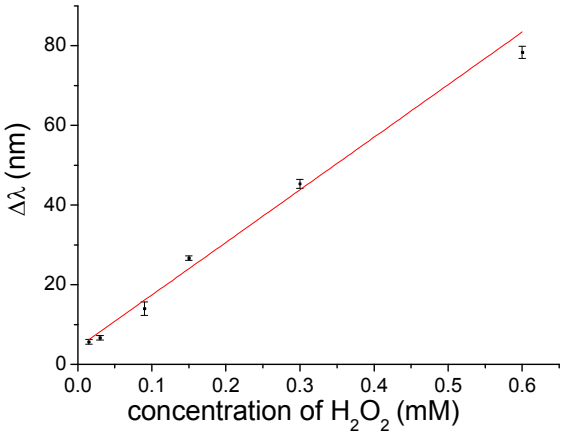


Figure 4. The relationship between the concentration of H_2O_2 and the change of LPW of GNRs.

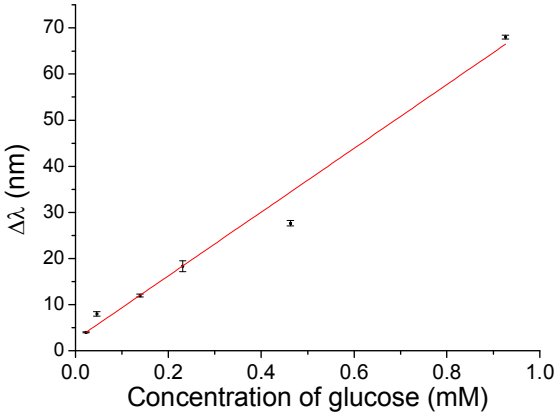


Figure 5. The relationship between the concentration of glucose and the change of LPW of GNRs.

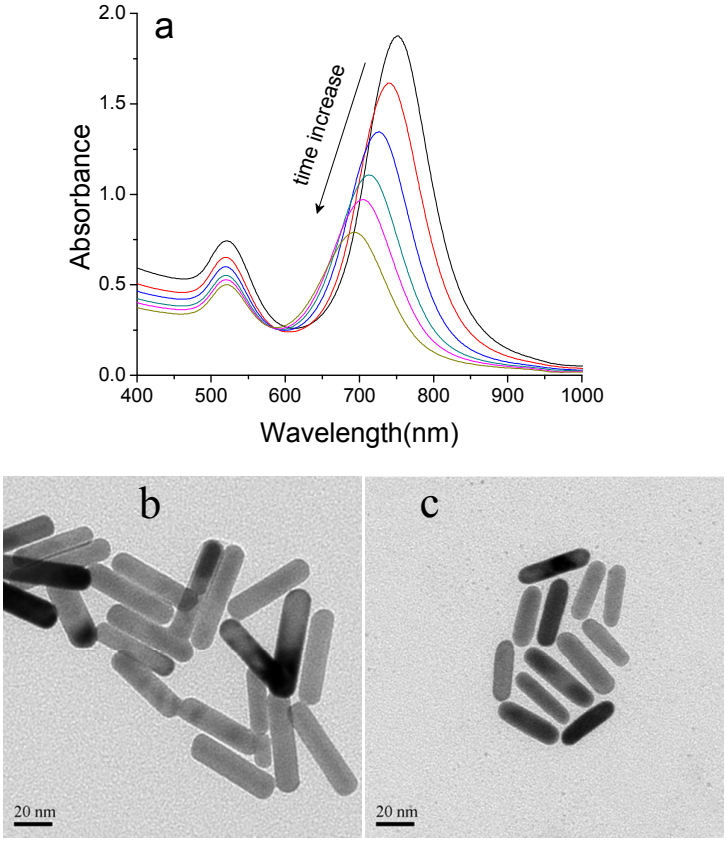


Figure 6. a: Absorption spectra acquired from the GNRs reacting with $Cu(NO_3)_2$ and $Na_2S_2O_3$. TEM images of as-synthesized GNRs (b) react with Cu^{2+} and $S_2O_3^{2-}$ to produce shortened GNRs(c).

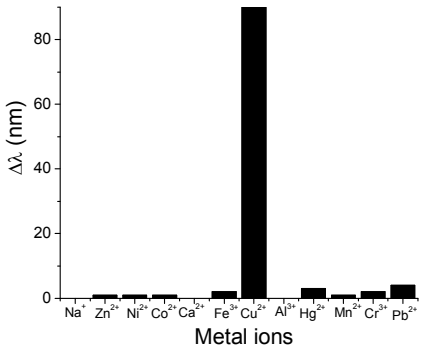


Figure 7. The degree of blue-shift of LPW of GNRs induced by various metal ions in the presence of $Na_2S_2O_3$.

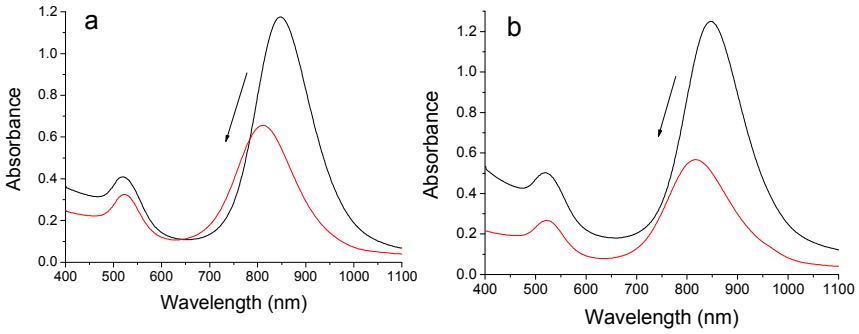


Figure 8 a: Absorption spectra acquired from as-synthesized GNRs reacting with coordinate compound Cu-EDTA in the present of $Na_2S_2O_3$, and the longitudinal plasmon wavelength change from 847 nm to 812 nm. b: Absorption spectra acquired from as-synthesized GNRs reacting with copper nanocluster in the present of $Na_2S_2O_3$, and the LPW change from 846 nm to 816 nm.

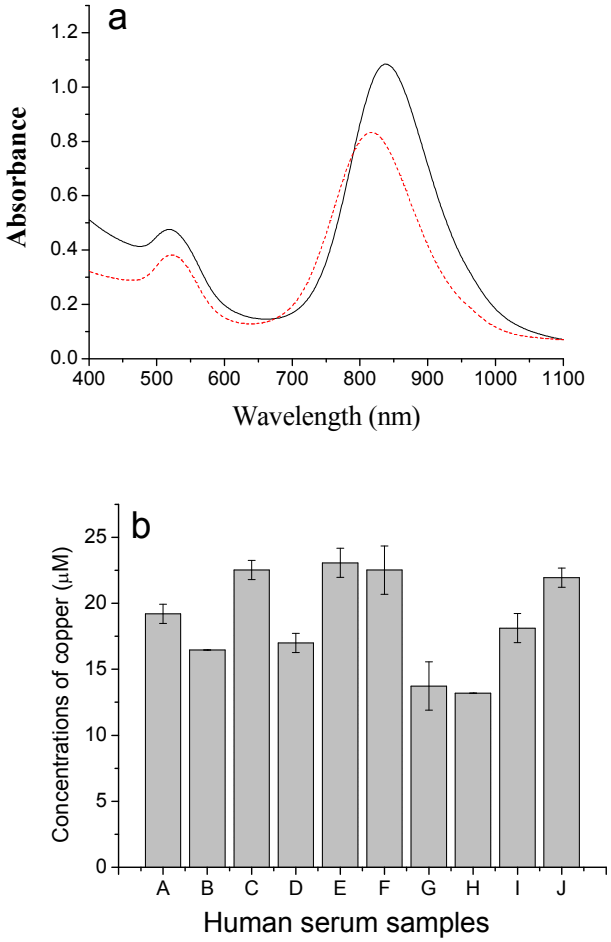


Figure 9. *a*: Absorption spectra acquired from as-synthesized GNRs reacting with human serum in the present of $Na_2S_2O_3$, and the LPW change from 837 nm (solid line) to 816 nm (dash line). *b*: bio-copper levels of ten samples of human serum detected by this assay.

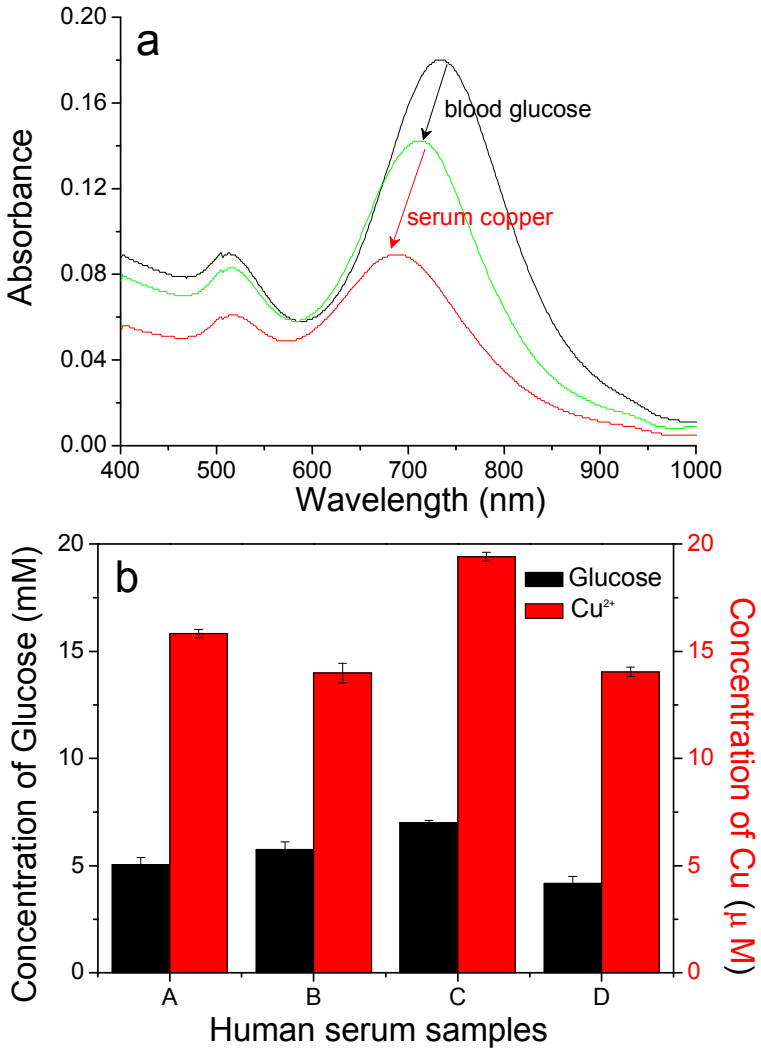


Figure 10. *a*: Absorption spectra acquired from the addition of human serum to the GNR solution for successive detection of glucose and copper level. *b* represents the glucose and copper levels in human serum samples obtained by four volunteers utilized this assay.

Table 1. Determination of blood glucose in human serum samples.

Samples	1	2	3	4	5	6	7	8	9	10
Glucose found by cobas 8000 Modular Analyzer (mM)	7.43	4.89	5.40	6.22	5.20	5.15	11.10	6.02	4.83	5.91
Glucose found by the proposed sensor （mM）	7.55	4.65	5.51	6.39	5.03	5.33	10.65	6.24	5.07	5.98