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Synthesis of gold–cellobiose nanocomposites for colorimetric measurement of cellobiase activity

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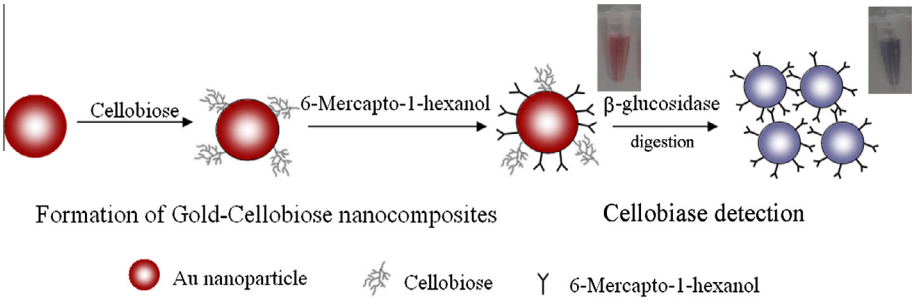
HIGHLIGHTS

- Gold–cellobiose nanocomposites were prepared for cellobiase activity assay.
- The probe displayed advantages of rapid response, high selectivity and sensitivity.
- Cellobiase activity could be detected with a wide linear from 3.0 to 100.0 U L^{-1}.

GRAPHICAL ABSTRACT



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ABSTRACT

Gold–cellobiose nanocomposites (GCNCs) were synthesized by reducing gold salt with a polysaccharide, cellobiose. Here, cellobiose acted as a controller of nucleation or stabilizer in the formation of gold nanoparticles. The obtained GCNCs were characterized with UV–visible spectroscopy; Zetasizer and Fourier transform infrared (FT-IR) spectrophotometer. Moreover, 6-Mercapto-1-hexanol (MCH) was modified on GCNCs, and the MCH–GCNCs were used to determine the cellobiase activity in compost extracts based on the surface plasmon resonance (SPR) property of MCH–GCNCs. The degradation of cellobiose on MCH–GCNCs by cellobiase could induce the aggregation, and the SPR absorption wavelength of MCH–GCNCs correspondingly red shifted. Thus, the absorbance ratio of treated MCH–GCNCs (A650/A520) could be used to estimate the cellobiase activity, and the probe exhibited highly sensitive and selective detection of the cellobiase activity with a wide linear from 3.0 to 100.0 U L^{-1} within 20 min. Meanwhile, a good linear relationship with correlation coefficient of $R^2 = 0.9976$ was obtained. This approach successfully showed the suitability of gold nanocomposites as a colorimetric sensor for the sensitive and specific enzyme activity detection.

Introduction

Lignocellulose is the most abundant renewable resource, and the enzymatic hydrolysis of lignocellulosic biomass has led to an increasing interest since it can be used to produce environment-friendly biofuels and chemicals [1–5]. The hydrolysis of β-1,4-glycosidic linkages of cellulose depends on the degradation capacity

Abbreviations: GCNCs, gold–cellobiose nanocomposites; MCH–AuNPs, 6-Mercapto-1-hexanol modified gold nanoparticles; MCH–GCNCs, 6-Mercapto-1-hexanol modified gold–cellobiose nanocomposites.

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of cellulase, which is produced by cellulolytic microorganisms, and usually contains three components: β-1,4-endoglucanase (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91) and cellobiase (EC 3.2.1.21). Low activity of cellobiase may restrict the conversion of cellobiose to glucose, and the accumulation of cellobiose will cause feedback inhibition to the cellulase reaction. Therefore, the role of cellobiase in the hydrolysis of cellulose is significant, and improving cellobiase activity is the key to raising the saccharification yield [6]. Thus, significant efforts are being made to develop technologies for improving the production of cellobiase [2,7–9]. The activity of cellobiase is an important parameter since it could characterize the performance and efficiency of those methods. As such, development of the specific methods for the detection of cellobiase activity has become crucial.

A widely used method to assay cellobiase activity is the protocol recommended by the International Union of Pure and Applied Chemistry (IUPAC) [10]. One international unit of cellobiase activity is the amount of enzyme that forms 2 μM of glucose per minute from cellobiose, and glucose was determined by using a 3,5-dinitrosalicylic acid (DNS) colorimetric assay method. The enzyme blanks are necessary when glucose is present in the diagnostic process [10,11]. In addition, some methods based on chromophore, fluorescent group release or product measurement are able to perform such task [12]. A simple and novel method for the efficient detection of cellobiase activity is still necessary since most of the current methods require tedious sample preparation, or the chemicals involved in such procedures are expensive or toxic [11,13].

Gold nanoparticles (AuNPs) exhibit attractive optical properties, friendly biocompatibility with biomolecules, and easily controllable size-distribution [14–17], the size and relative distance between nanoparticles could modulate its surface plasmon resonance (SPR). Taking advantages of these characteristics, AuNPs has been widely used as optical labels for biospecific interaction analysis [18–20]. On the other hand, polysaccharides have a series of virtues such as non-toxic, special structure characteristics, and biodegradability. Moreover, the commercial availability makes them to be convenient choices for the synthesis of nanomaterials. It has been reported that the reductive polysaccharides could be used as the reducing or stabilizing agent for producing AuNPs [21,22]. The synthesis of gold-polysaccharides nanocomposites has also attracted much attention due to their specific properties and application in biotechnology [23].

In this research, a colorimetric probe based on the synthesis of gold–cellobiose nanocomposites (GCNCs) is proposed for the detection of cellobiase activity. Cellobiose acted as the controller of nucleation or stabilizer in the formation of AuNPs, and 6-Mercapto-1-hexanol (MCH) was modified on GCNCs to promote the sensitivity. The SPR of MCH–GCNCs correspondingly changed with the activity of cellobiase due to the specific interaction between cellobiase and cellobiose, and the change in SPR of cellobiase treated MCH–GCNCs was detected by UV–visible spectrometer. The absorbance ratio of treated MCH–GCNCs (A650/A520) was used to estimate the cellobiase activity. The linear ranges, sensitivity, and accuracy of the colorimetric probe were also investigated. This detection method has advantages of easy operation and non-toxic. It also showed satisfactory sensitivity and selectivity. It is believed that this research could expand the application field of gold nanocomposites.

Material and methods

Materials

Cellobiose, chloroauric acid ($HAuCl_3 \cdot 3H_2O$), MCH, glucose oxidase from *Aspergillus niger*, laccase from *Trametes versicolor* and superoxide dismutase from *Bacillus stearothermophilus* were purchased from Sigma–Aldrich. Sodium borohydride ($NaBH_4$) was purchased from Sinopharm Chemical Reagent Co., Ltd. All the other chemicals were of analytical grade or the highest purity commercially available. Ultrapure water (18.2 MΩ) was obtained from a Milli-Q purification system and used throughout the experiments.

Synthesis of gold–cellobiose nanocomposites

GCNCs were synthesized by the chemical reduction of cellobiose-$HAuCl_4$ mixtures with sodium borohydride. An aqueous solution of freshly prepared $HAuCl_4$ (0.1 ml, 1%) was mixed with a diluted solution of cellobiose (10 ml, various concentration). After 2 h of continuous stirring, 0.1 ml sodium borohydride (0.5 M) was rapidly added to the solution and the mixture solution was left continuous stirring. The color of solution rapidly changed from pale yellow to deep red. The mixture solution was then centrifuged at 1200 rpm for 10 min. After discarding the pellet, the centrifugation was undertaken at 15,000 rpm, 4 °C for another 20 min to remove excess cellobiose. After two centrifuge/wash cycles, the supernatant solution was discarded and the pellet was resuspended in total 5 ml of pH 5.0 citrate–phosphate buffer. The obtained nanocomposites were analyzed by zetasizer (Zetasizer nano Zs, Malvern, UK) and Fourier transform infrared (FT-IR) spectrophotometer (Nicolet, Nexus-670). A UV–visible absorption spectrophotometer (Shimadzu UV-2550) was used to measure the absorbance of GCNCs.

Modification of gold–cellobiose nanocomposites

The obtained GCNCs were modified with MCH to increase the sensitivity for the detection of cellobiase activity. Firstly, 0.01 ml MCH (1 mM) was added to freshly prepared GCNCs solution (10 ml), and incubated at room temperature for 1 h. Then the mixture solution was centrifuged according to the above-mentioned procedure and the pellet was resuspended in 5 ml of pH 5.0 citrate–phosphate buffer. In addition, bare AuNPs were modified with corresponding concentration of MCH to demonstrate the detectability of GCNCs. The modification process was characterized by UV–visible absorption spectrophotometer (Shimadzu UV-2550).

GCNCs-based probe for cellobiase activity assay

For the cellobiase activity assay, 0.1 ml different concentrations of enzymes were added to 2 ml of modified GCNCs. After quick mixing, the solution was incubated at 30 °C for 20 min. Absorption spectra of the mixture solution was recorded and the concentration of cellobiase activity was calculated based on the correlation curves of the absorbance ratio (A650/A520). Controls were treated with thermo-inactivated enzymes in the same conditions as described above. Moreover, laccase, glucose oxidase and superoxide dismutase were treated in the same conditions to evaluate the specificity property.

Measurement in compost extracts

The determination of cellobiase activity in compost extracts was respectively carried out by 3,5-dinitro-salicilic acid (DNS) colorimetric method and GCNCs based method. The composting material is consisted of wheat straw, sawdust, bran, and soil. The above-mentioned materials were mixed in the ratio of 17:4:5:27 and the mixture was controlled to 65% water content. The compost experiment was performed under the condition of 30 °C temperature and 0.1 $m^3\ h^{-1}$ ventilation, and lasted 50 days [2]. 10 g of compost sample was added in 200 ml water, and the supernatant was centrifuged at 12,000 rpm for 10 min. Then, it was filtered to get

the filtrate, and the filtrate was sterilized as the compost extract [24]. The pH value of extract was adjusted to 5.0, and the resulting compost extract samples were added with certain concentration of cellobiase solution. After that, the GCNCs based measurement was performed. At the same time, the DNS colorimetric assay method was carried out according to Shen et al. for comparison [11].

Results and discussion

Characterization of GCNCs

The UV–visible absorption spectra of the GCNCs solutions are shown in Fig. 1. Different concentrations of cellobiose were used to prepare GCNCs, and all spectra exhibited an absorption band around 520 nm. It is a typical SPR band for AuNPs, suggesting the formation of AuNPs [21,22,25]. The intensity of the absorption band varied with the concentration of added cellobiose. When the concentration of cellobiose increased from 0.005% to 1%, the intensity of absorption band first decreased, and then no obvious changes could be observed until the concentration increased to 0.5%, while the intensity of absorption decreased at 1% of cellobiose. The same phenomenon was observed at the gold nanocomposites prepared with low molecular weight chitosan [22,23]. The results indicated that the size of GCNCs changed with the concentration of cellobiose, which may be attributed to that cellobiose could act as the controller of nucleation or stabilizer in the formation of gold nanoparticles as well as chitosan.

The difference of GCNCs particle size was also confirmed by zetasizer observation. The average diameter, polydispersity index (PDI) and Zeta potential of GCNCs prepared from different concentration cellobiose are shown in Table 1. The Zeta potential and PDI values showed no correlation with the concentration of cellobiose. The relatively low value of PDI of all samples indicated the good monodispersity of formed nanoparticles [26], and the Zeta potential values indicated the stability of nanoparticles solution [27]. The size of GCNCs prepared with 0.005% cellobiose was large. However, when the concentration of cellobiose increased from 0.01% to 0.2%, no obvious changes in particle diameter were observed while the size of GCNCs decreased at 1% of cellobiose. These results further indicated that cellobiose might act as the controller of nucleation or stabilizer by adsorbing at the surface of AuNPs. The interaction sites of cellobiose molecules and $AuCl^-$ are very few at the low concentration of cellobiose (0.005%). Thus, large size of AuNPs was produced when few cellobiose molecules worked as controller or stabilizer [22]. As for the decrement of nanoparticles size in the higher cellobiose concentration (1%), the growth rate of AuNPs could be prevented due to the protective action by cellobiose, which resulted in the formation of small AuNPs [23]. Therefore, the intermediate concentration, i.e. 0.1% of cellobiose, was chosen for further application.

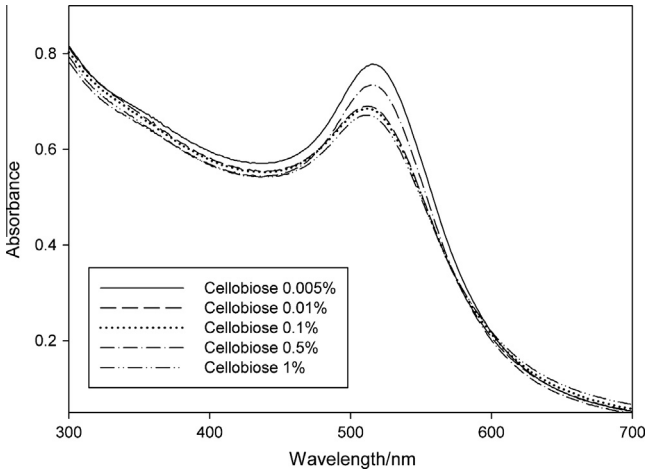

Fig. 1. UV–visible absorption spectra of the gold–cellobiose nanocomposites solutions prepared with different concentrations of cellobiose.

Table 1
The average diameter, polydispersity index (PDI) and Zeta potential of gold–cellobiose nanocomposites in samples at different cellobiose ratios. These results are presented as mean ± standard deviation (n = 3).

Sample	M (%)	Average diameter (nm)	PDI	Zeta potential (mV)
C1	0.005	32.7 ± 0.89	0.087 ± 0.16	−38.3 ± 4.1
C2	0.010	16.0 ± 1.25	0.420 ± 0.08	−40.8 ± 3.5
C3	0.050	14.5 ± 2.01	0.584 ± 0.04	−39.5 ± 6.5
C4	0.100	14.9 ± 2.84	0.172 ± 0.12	−35.5 ± 3.3
C5	0.200	18.5 ± 2.39	0.309 ± 0.11	−34.6 ± 7.4
C6	0.500	21.9 ± 0.79	0.148 ± 0.18	−25.4 ± 6.8
C7	1.000	13.7 ± 3.01	0.489 ± 0.03	−31.4 ± 3.2

[a] M = cellobiose concentration in percentage (high-level 1.000, low-level 0.005).

To further determine the interaction between AuNPs and cellobiose, the prepared GCNCs were separated from the solvent by centrifugation and then analyzed by FT-IR spectrophotometer. As shown in Fig. 2, the characteristics absorption bands among 3230–3600 cm^{-1} of GCNCs are attributed to the OH stretches, the bands at 2860 cm^{-1} and among 1200–1400 cm^{-1} are assigned to CH deviational vibration of saccharides and CH stretching vibration. These are characteristics of saccharides [21,28,29]. In addition, the relative intensities around 890 cm^{-1} (β-D-configuration) and 1080 cm^{-1} (C–O–C ether linkage) are weak. Thus, we speculated that the cellobiose bound to AuNPs, which prevented the pyranose ring and skeletal vibration of cellobiose [30]. This bound was probably caused by the electrostatic interactions between electropositive transition metal cations and the ether, hydroxyl groups of cellobiose [31,32].

The effect of MCH modification was analyzed by UV–visible absorption spectrophotometer. In Fig. 3A, the MCH modified gold–cellobiose nanocomposites (MCH–GCNCs) displayed an intense SPR absorption located at 520 nm, which was similar with AuNPs and GCNCs. However, a slight increase in the intensity of 650 nm occurred, which indicated that the stability of GCNCs was slightly influenced by the modification of MCH [18]. To confirm that whether this influence on the stability of GCNCs would affect the detection precision, the MCH modified bare AuNPs (MCH–AuNPs) were prepared to detect the cellobiase activity for comparison. The results indicated that the modification of MCH also slightly affected the stability of bare AuNPs (Fig. 3B). However, after the treatment of 30 U L^{-1} of cellobiase, no obvious change could be observed at the UV–visible absorption spectrum of MCH–AuNPs. A slight decrease in the intensity at 520 nm was probably caused by the change of MCH–AuNPs concentration in the solution after adding cellobiase. The results revealed that there is no response between MCH–AuNPs and cellobiase. The MCH connected to the AuNPs through the thiol group, and its hydroxyl group exposed on AuNPs, which could enhance the attraction among AuNPs [33,34]. With the consumption of cellobiose, the AuNPs lost stabilizer, and the attraction force of the hydroxyl group of MCH can cause the aggregation of AuNPs [35,36]. Therefore, the modification of MCH can be used to promote the sensitivity of GCNCs. In addition, the time-bound stability of MCH–GCNCs was investigated, and the nanocomposites showed no aggregation under the experimental conditions within 36 h (Fig. S1). In our GCNCs-based colorimetric assays, the absorbance ratio (A650/A520) increased up to a steady value with the increase of

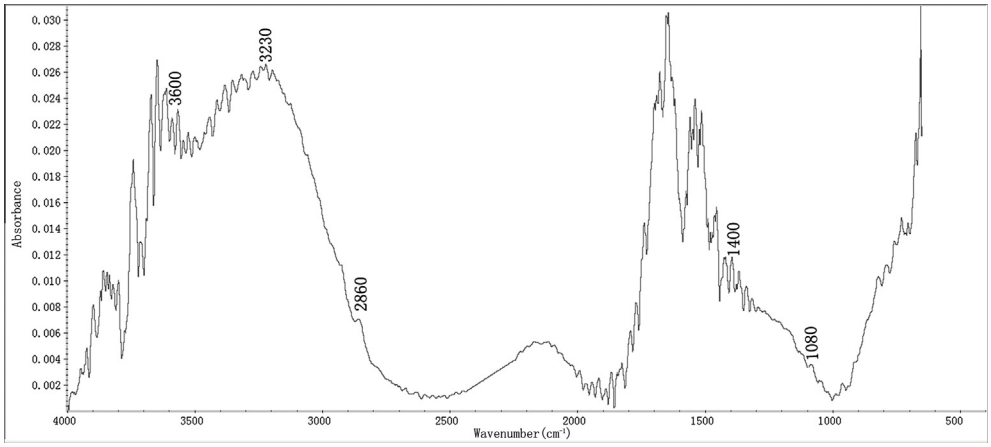

Fig. 2. Fourier transform infrared spectrum of gold–cellobiose nanocomposites.

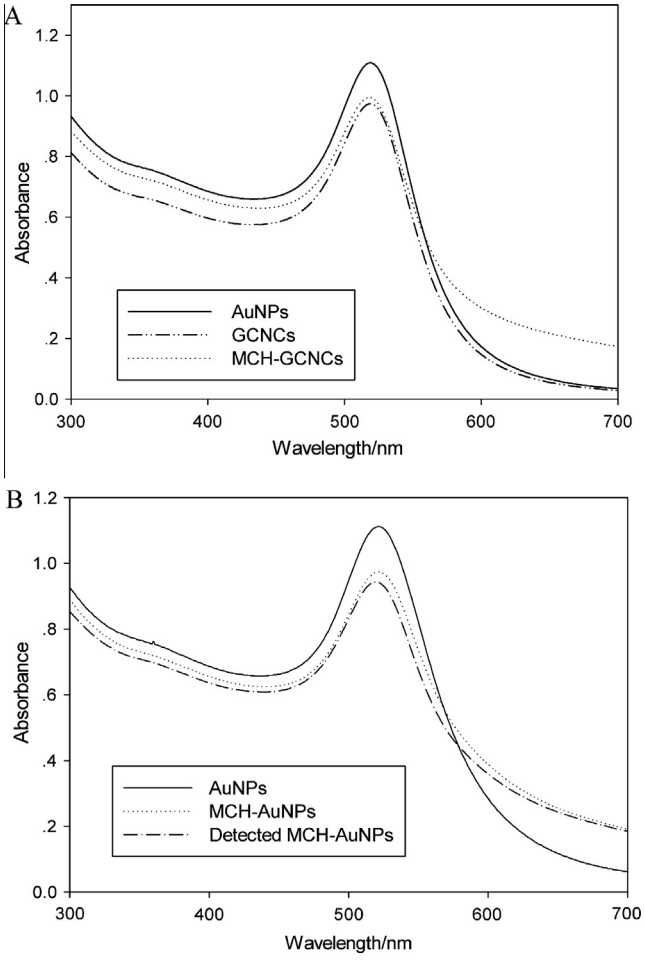

Fig. 3. UV–visible absorption spectra of gold nanoparticles, gold–cellobiose nanocomposites, and 6-Mercapto-1-hexanol (MCH) modified gold–cellobiose nanocomposites (A); UV–visible absorption spectra comparison of gold nanoparticles, MCH modified gold nanoparticles (MCH–AuNPs), and 30 U L^{-1} cellobiase treated MCH–AuNPs (B).

reaction times, and reached a plateau within 15 min. Thus, the reaction time was set to 20 min. The reaction time was lower than the threshold of nanocomposites aggregation. Therefore, the reaction time did not affect the aggregation of GCNCs under the experimental conditions.

Working curve, linear range and sensitivity for cellobiase activity assay

Since the change of reaction conditions may affect the activity of cellobiase, the optimal conditions of reaction were explored, and the results showed that the optimized temperature and pH were located at 30 °C and pH 5.0. Therefore, 0.1 M citrate–phosphate buffer of pH 5.0 was used as reaction buffer to detect cellobiase activity. The colorimetric assay of cellobiase activity was carried out with concentrations over the range of 0.3–250.0 U L^{-1}. The color change of MCH–GCNCs was a sensitive function of cellobiase concentration; it gradually turned to purple along with the increase of cellobiase concentration, which implied the increased aggregation state of MCH–GCNCs (Fig. S2) [37]. Fig. 4A showed the colorimetric response and wavelength change of MCH–GCNCs after treated with different concentrations of cellobiase. The absorbance at 520 nm gradually decreased while the absorbance at 650 nm increased with the increasing of cellobiase activity. The wavelength change suggested the aggregation of MCH–GCNCs [18]. After a 20 min reaction time, the cellobiase activity was estimated by the absorbance ratio (A650/A520) of the cellobiase treated MCH–GCNCs. Fig. 4B depicted the linear relationship between the ratio of spectral absorbance A650/A520 and the log concentration of cellobiase. Under the optimum conditions, the activity of cellobiase was linear with A650/A520 in the range of $10^{0.48}$ to $10^{2.0}$ U L^{-1} (3.0–100.0 U L^{-1}); the linear regression equation was obtained by fitting the experimental data obtained:

$$I = 0.5643n - 0.0586$$

where I was the ratio of spectral absorbance A650/A520, and 10^n was the cellobiase activity (U L^{-1}), and correlation coefficients (R^2) were 0.9976. The detection limit of cellobiase activity by this method was estimated on the basis of 3 times the blank test standard deviation and it is 1.0 U L^{-1}. The results implied that this method is highly sensitive for cellobiase activity quantification. To our knowledge, this nanocomposites-based probe is among the sensitive methods reported for cellobiase activity quantification [13,38,39].

Sample analysis

The cellobiase activity in three compost extract samples were determined by the DNS colorimetric assay method [11] and MCH–GCNCs based method. As shown in Table 2, the cellobiase activity detected using MCH–GCNCs agreed well with those obtained by DNS assay method, showing good accuracy. The

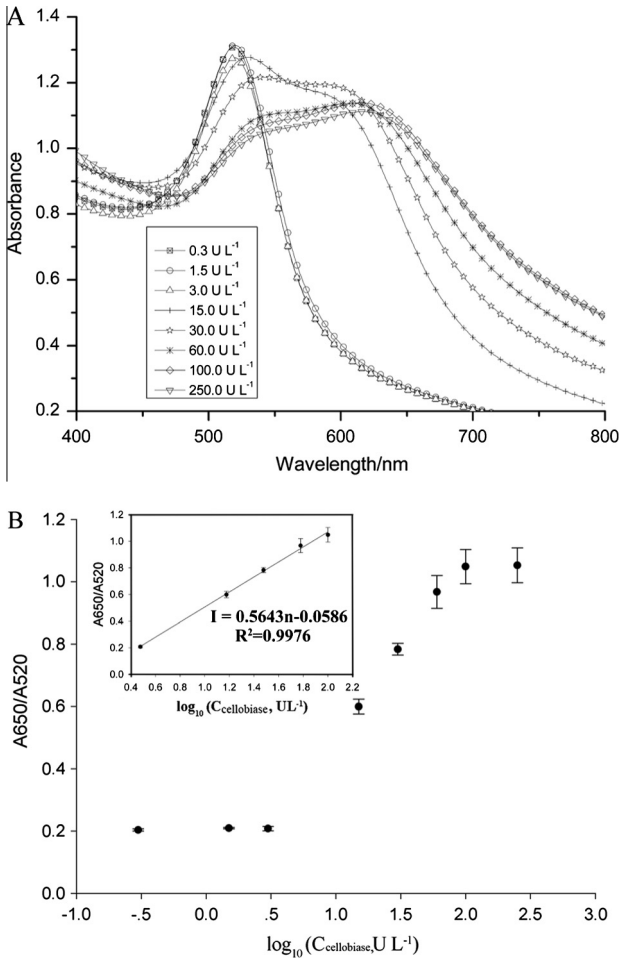

Fig. 4. UV–visible absorption spectrum of 6-Mercapto-1-hexanol modified gold–cellobiose nanocomposites (MCH–GCNCs) after cellobiase treatment. Colorimetric assay of MCH–GCNCs at 20 min after incubation with different concentrations of cellobiase (A). The ratio of A650/A520 calculated from UV–visible absorption spectrum was plotted against the log concentration of cellobiase (B). The inset is standard curve, linear range, and correlation coefficient between the relative A650/A520 values and log concentration of cellobiase. Each value represents mean ± standard deviation derived from three independent detections.

Table 2
The cellobiase activity in compost extracts determined by gold–cellobiose nanocomposites (GCNCs) based method and 3,5-dinitro-salicilic acid (DNS) colorimetric assay method.

Samples	Cellobiase activity (U L^{-1})		RSD (%)
	GCNCs based method[a]	DNS assay method[b]	
1	12.4 ± 0.5	10.5 ± 1.2	4.1
2	53.3 ± 2.4	50.6 ± 4.5	4.5
3	74.8 ± 3.7	71.9 ± 5.4	4.9

[a] An average of three replicate measurement.
[b] An average of two replicate measurement.

results obtained using MCH–GCNCs were measured for 3 times, and the RSD (%) was within 4.1% and 4.9%, showing good precision. Both above-mentioned colorimetric assay methods could precisely detect the cellobiase activity. The MCH–GCNCs based method has the virtue of easy operation, and could be carried out by just measuring the absorbance values of A650 and A520. More importantly, the extension of this method to other substrate polysaccharides to detect enzymes such as sucrase, diastase, maltase, xylosidase seems feasible.

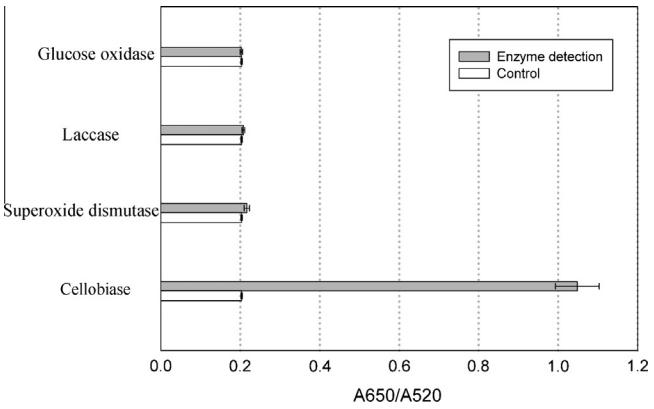

Fig. 5. Specificity of 6-Mercapto-1-hexanol modified gold–cellobiose nanocomposites in the detection of cellobiase activity. Enzymes, including 100 U L^{-1} of glucose oxidase, laccase, superoxide dismutase, and cellobiase were used to test the specificity of the colorimetric probe. Each value represents mean ± standard deviation derived from three independent detections.

Specificity of GCNCs used in cellobiase activity assay

To evaluate the specificity property of this probe, a control experiment was carried out by using several known lignocellulose-degrading enzymes, including glucose oxidase, laccase and superoxide dismutase. As shown in Fig. 5, the value of A650/A520 ratio of the 100 U L^{-1} cellobiase treated sample increased to 1.0483, whereas low signals were obtained in the other enzymes treated samples. The relative A650/A520 ratio was 0.2025 after 100 U L^{-1} glucose oxidase was applied to assess the selectivity of the probe, and it was 0.2078 and 0.2166 for 100 U L^{-1} laccase and superoxide dismutase. It is clear that other lignocellulose-degrading enzymes failed to cause a detectable colorimetric change of MCH–GCNCs compared with corresponding concentration of cellobiase. These results demonstrated the specificity of this probe, which could be attributed to the high substrate specificity of cellobiose and the specific enzymatic activity of cellobiase.

Conclusions

In the present study, the gold–cellobiose nanocomposites were prepared, and a colorimetric probe based on the SPR property of GCNCs was developed for the measurement of cellobiase activity. The cellobiase activity was linearly related to the absorbance ratio A650/A520 of treated GCNCs solution, ranging from 3.0 to 100.0 U L^{-1}. The selectivity of the probe was also illustrated. The authors believe that the proposed method could provide an alternative tool for the cellobiase activity detection due to its low cost, rapidity, high selectivity, and simplicity.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.saa.2014.04.091.

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