Analytical Methods

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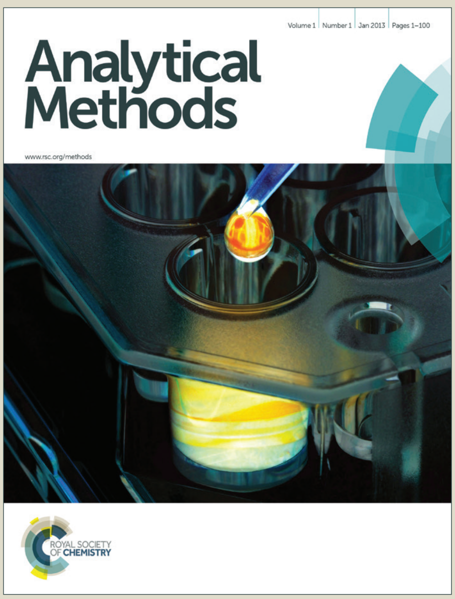

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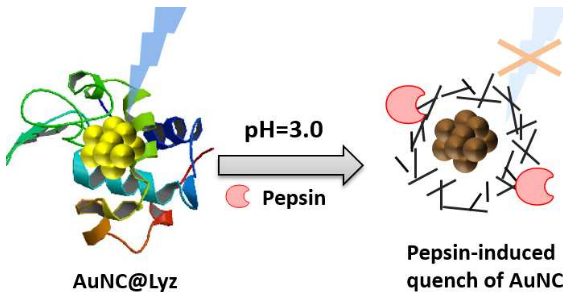
A highly selective and sensitive detection of pepsin was developed through peptic hydrolysis of lysozyme-stabilized gold nanoclusters at pH 3.



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ARTICLE

Scissor-based fluorescent detection of pepsin using lysozyme-stabilized Au nanoclusters †

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Pepsin is an active digestive enzyme present in the acidic environment of animal stomachs, and has been widely used to prepare bioactive peptides in the food industry. In this work, a simple fluorescence sensor for scissor-based detection of pepsin activity was developed by using lysozyme-stabilized gold nanoclusters (AuNCs@Lyz) in aqueous media. Under acidic conditions (pH 3.0), enzymatic digestion of AuNCs@Lyz with pepsin results in a significant decrease of fluorescence intensity. Notably, its acidic environment not only helps maintain the maximum fluorescence of gold nanoclusters, but also ensures the highest pepsin enzymatic activity. In addition to offering high selectivity because of the unique proteolytic action of pepsin under acidic conditions, this facile method provides high sensitivity. With the sensing system, the linear range for pepsin detection is found to be 1 μg/mL to 100 μg/mL, with a detection limit of 0.256 μg/mL at a signal-to-noise ratio of 3. Furthermore, the AuNCs@Lyz based fluorescent sensing system could find applications in highly sensitive and selective detection of pepsin in food and biological samples.

Introduction

Pepsin, a member of the aspartic proteinase family, is one of three principal proteolytic enzymes in the digestive system.[1] Pepsin is widely used in the food industry, such as in soy and beer production, because it is the most efficient enzyme in cleaving peptide bonds between hydrophobic and preferably aromatic amino acids. As one of the most important proteinases in human gastric juices, pepsin has a significant function in gastro-esophageal reflux disease because it could cause injury to the esophagus,[2, 3] hypopharyngeal,[4] oropharynx, and upper airway.[5] Therefore, sensitive detection methods for pepsin are required in food industry and disease detection. To date, several analytical techniques have been reported for pepsin assay, such as colorimetric[6] and chromatographic[7-9] techniques. Among these methods, the colorimetric method involves the use of phenol reagent and suffers from poor applicability for mixture or real samples, as well as poor selectivity toward other proteases,[10] whereas chromatographic analysis often requires hours of work, skilled labor, and complicated procedures. In addition, these methods are limited by their sensitivity, in which only milligram levels of pepsin are detectable. For these reasons, substantial efforts are still needed for the development of simple and efficient methods for direct pepsin assay with high sensitivity and selectivity.

Recently, fluorescent metal nanoclusters have drawn substantial attention due to their advantageous features, such as ultra-small size, good biocompatibility and excellent photostability, which make them ideal fluorescent labels for biological applications.[11-14] As a promising fluorescent probe, metal nanoclusters have exhibited great potential for detecting various biologically important analytes (e.g. metal ions, small biomolecules, and biomacromolecules).[15, 16] Recent advances have made possible the facile preparation of water-soluble fluorescent metal nanoclusters with different ligands and tunable emission colors in various biocompatible scaffolds.[17, 18] Among these nanoclusters, protein-mediated metal nanoclusters have been studied extensively, and found to play an important role in biological detection.[19-22] For instance, highly fluorescent Au nanoclusters have been successfully prepared by Ying et al. by using BSA as a template.[11, 23] And BSA-stabilized gold nanoclusters had been applied for fluorescence sensing of trypsin,[21] papain and its inhibitor,[22] respectively. But those nanoclusters, which were synthesized under alkaline conditions, were not stable enough for pepsin detection in acidic environment. The acquired nanoclusters showed great promise for biological labeling and sensing (e.g., ascorbic acid, dopamine, hydrogen peroxide, glutaraldehyde, mercury ion, silver ion, copper ions and cyanide).[24-28] Lysozyme-stabilized Au_8 clusters have been applied to detect glutathione through core-etching.[29] Red emissive Ag nanoclusters have been obtained by Irudayaraj using denatured BSA as the stabilizing agent; the nanoclusters showed high selectivity toward mercury ion.[30] Goswami used Cu quantum clusters capped with BSA to construct a fluorescence sensor for lead ion sensing.[31] Moreover, pepsin-stabilized blue, green, and red fluorescent

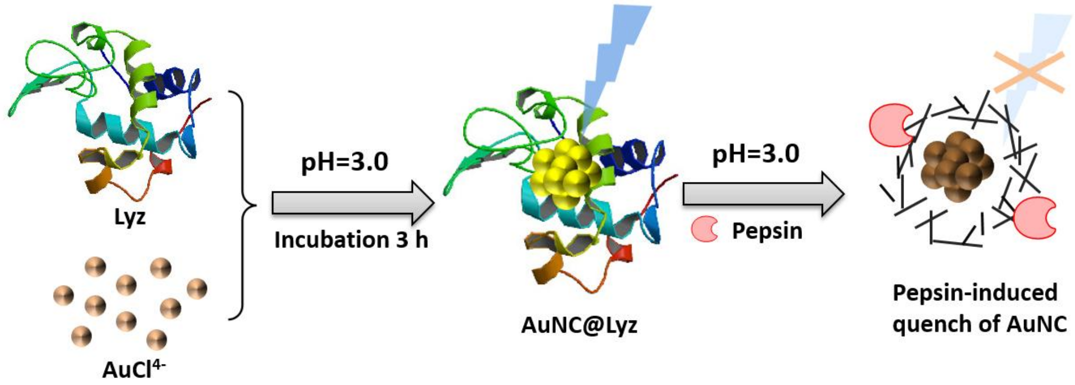

Fig. 1 A schematic illustration of pepsin detection by using scissor-based assay with lysozyme stabilized AuNCs.

gold nanoclusters have been obtained by adjusting pH.[32] Trypsin-stabilized AuNCs have also been used for mercury ion detection.[33] Most of the above sensing systems are based on the direct interactions between metal core and target molecules. Notably, the fluorescence intensity from nanoclusters has been reported to depend on both the metal core and the surface ligand shell.[34, 35] Combining the characteristics of the ligand shell and the target molecule contributes to the rational design of an effective sensing platform that can endow the sensing system with better specificity and sensitivity.

In this study, we achieved a specific interaction, namely enzymatic reaction, between the luminescent nanoclusters (AuNCs@Lyz) and the analyte (pepsin) for recognition and signal generation. As shown in Fig. 1, highly fluorescent gold nanoclusters were synthesized in aqueous solution under acidic conditions with lysozyme as stabilizing and reducing agent. Under acidic conditions, the hydrolysis of pepsin toward a template protein, which in this case was lysozyme, may cause the decomposition of the template protein followed by the decrease in fluorescence intensity of the nanocluster. A sensitive and selective pepsin sensing platform could be achieved. Several factors that may interfere with the detection of pepsin were investigated. We further evaluated the sensitivity and specificity of this sensing platform.

Materials and methods

Materials

Unless otherwise noted, all reagents and materials were purchased from commercial sources and used as received. Lysozyme, trypsin, and alkaline protease were purchased from Beijing Probe Bioscience Corporation (Beijin, China). Pepsin, glucose oxidase, thrombin, papain, and lipase were obtained from Sigma-Aldrich. $HAuCl_4 \cdot 4H_2O$, sodium hydroxide, and glycine were purchased from Aladdin Reagent Company (Shanghai, China). All other reagents and chemicals were of analytical or HPLC grade. Ultrapure deionized water was obtained from a Millipore purification system (18.2 MΩ cm resistivity).

Apparatus

The fluorescence spectra were measured by Cary Eclipse Fluorescence spectrophotometer (Agilent Technologies, USA). The UV–VIS absorption spectra were recorded using a TU-1810 spectrophotometer (Pgeneral, China). The AuNCs@Lyz were characterized using a JEM-2100F transmission electron microscope (JEOL, Japan) with an accelerating voltage of 200 KV. Dynamic light scattering measurements were performed using a Zetasizer Nano ZS (Malvern Instruments Ltd., UK). The pH values of all the solutions were measured by SevenGo™ pH – SG2 (Mettler Toledo, Switzerland). AuNC synthesis and enzymatic reaction of pepsin were both performed in a shaking water bath (Julabo GmbH, Germany).

Preparation of lysozyme-stabilized AuNCs

All glass vessels used in the experiment were steeped in aqua regia (HNO_3/HCl: 1/3, v/v) overnight, washed by double-distilled water, and then dried in an oven before use. The lysozyme-stabilized AuNCs were synthesized according to a method reported previously.[36] Typically, 5 mL $HAuCl_4$ (10 mM) was added into 5 mL of totally dissolved lysozyme solution under vigorous stirring. After 2 min, 1 M NaOH was introduced to adjust the pH to 3. The mixture was maintained at room temperature for another 2 min before it was incubated at 37 °C for 3 h. The color of the solution changed from brown to light yellow. The final solution was stored at 4 °C in a refrigerator for further use.

Fluorescence detection of pepsin activity

A stock solution of pepsin (1 mg/mL) was prepared in a 50 mM glycine-HCl buffer at a pH of 3.0. Pepsin solution with various concentrations was obtained by serial dilution of the stock solution. For pepsin detection, 3600 μL of the pepsin solution with different concentrations was added into 400 μL AuNC solution in the glycine-HCl buffer at pH 3.0. Then, the mixture solution was equilibrated at 37 °C for 3 h in shaking water bath (200 rpm). The samples were maintained at room temperature prior to spectral measurements. Fluorescence spectra of all samples were recorded using a Cary Eclipse Fluorescence spectrophotometer.

Selectivity and interference measurements for pepsin activity

The selectivity of this sensing system for pepsin was evaluated by testing the fluorescence response of this system to other substances using the following enzymes: trypsin, alkaline protease, lysozyme, glucose oxidase, thrombin, papain and lipase. The solution of the above substances were mixed with AuNC solution and reacted for 3 h before the spectral measurements.

Results and discussion

Characterization of lysozyme-stabilized AuNCs

Lysozyme-stabilized AuNCs were prepared based on the method described previously. As shown in Fig. 2A, the aqueous solution of AuNCs@Lyz was light yellow in color under visible light and exhibited a strongly blue fluorescence under the UV lamp (Fig. 2A, inset), which implied the preparation of fluorescent AuNCs. The fluorescence properties of the as-prepared AuNCs were further examined. The fluorescence measurements showed that AuNCs had the excitation and emission peaks at 348 and 420 nm, respectively.

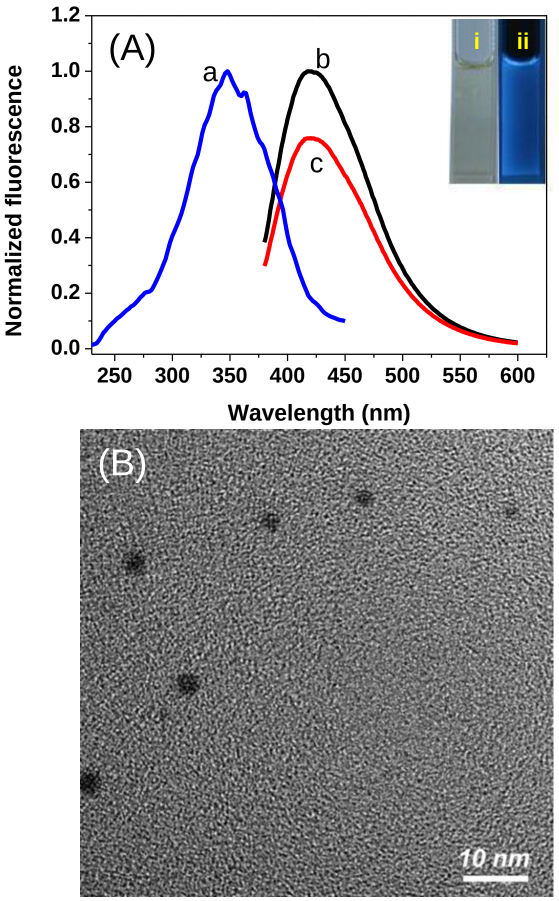

Fig. 2 (A) Fluorescence excitation (blue, curve a) and emission spectra of AuNCs@Lyz in the absence (black, curve b) and presence (red, curve c) of 100 μg/ml pepsin. The inset contains the photographs of the AuNCs@Lyz under visible light (i) and UV light (ii). (B) TEM image of AuNCs@Lyz. (The AuNCs@Lyz was synthesized with a reaction time of 180 min)

To further confirm the formation of the AuNCs@Lyz, transmission electron microscopic images were obtained. The TEM image in Fig. 2B shows that the as-prepared gold nanoclusters were spherical in shape and about 2 nm in diameter. The characteristics of the gold nanoclusters were consistent with those reported in the literature. According to Tseng et al.,[29] the emission at 420 nm of lysozyme stabilized AuNCs was attributed to the presence of Au_8 clusters.

Scissor-based fluorescent quenching of Lyz-AuNCs

Upon the addition of 100 μL AuNC solution into 900 μL pepsin solution at a concentration of 100 μg/mL, the corresponding emission of the system decreased significantly (Fig. 2A). We hypothesize that, under acidic conditions, enzymatic digestion of the AuNCs@Lyz with pepsin results in a significant decrease in the fluorescence intensity. We next performed a series of control experiments to investigate the proposed fluorescence quenching mechanism. The results of dynamic light scattering measurements (Fig. 3) showed that the hydrodynamic diameter of the AuNCs@Lyz decreased from around 80 nm to around 60 nm upon addition of pepsin, which is consistent with the size change of lysozyme in the presence and absence of pepsin. However, if denatured pepsin was added into the AuNCs, no fluorescent change or size change could be observed. These results suggested that the drop in fluorescence intensity was caused by the degradation of the protein shells by pepsin. Thus, the AuNCs@Lyz could be used as a fluorescent probe for pepsin in aqueous solution. The working principle of the scissor-based fluorescent sensor for pepsin detection is schematically represented in Fig. 1.

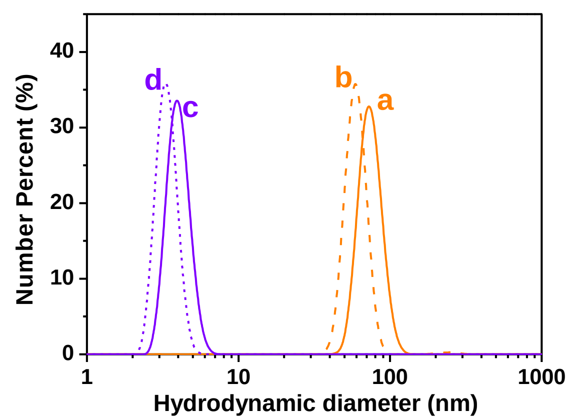

Fig. 3 DLS analysis of lysozyme stabilized AuNCs with (a) and without (b) treatment by pepsin, and lysozyme with (c) and without (d) treatment by pepsin.([pepsin]=100 μg/ml).

Optimization of sensing conditions

Prior to the application of the fluorescent sensor for pepsin detection, several influencing factors were investigated. First, we should confirm the synthesis time to obtain suitable AuNCs. As shown in Fig. 4, the intensity growth is very rapid during the first 60 min, but gradually reduces in the next few hours, indicating the short time it took for the reaction to be completed. As a result, the intensity increase slows down after

3 h, so 180 min is determined to be the synthesis time. And we also found that the fluorescence intensity of ten times diluted AuNCs@Lys was almost unchanged after 1.5h incubation (Fig. S1, ESI†).

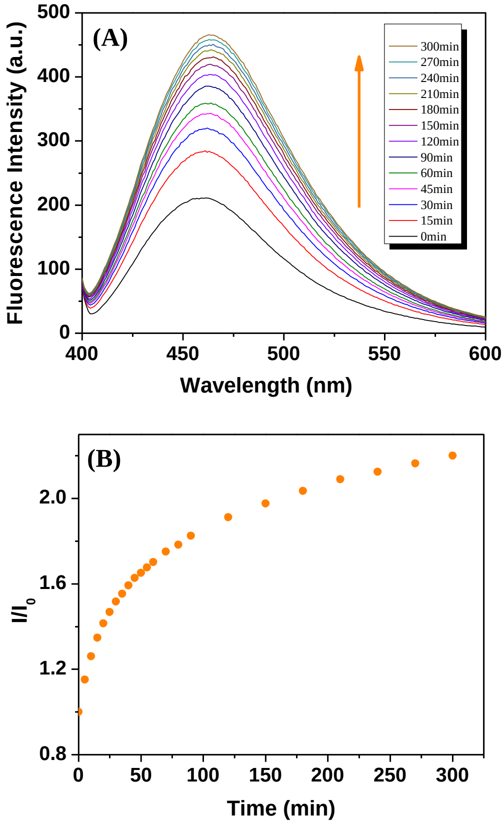

Fig. 4 (A) Time evolution of the fluorescence emission spectrum for the reaction solution of $HAuCl_4$ with lysozyme at 37 °C. (B) The plot of the relative fluorescent intensity (I/I_0) versus time. I_0 is the fluorescence intensity at the beginning of the reaction and I is the fluorescence intensity at the following time.

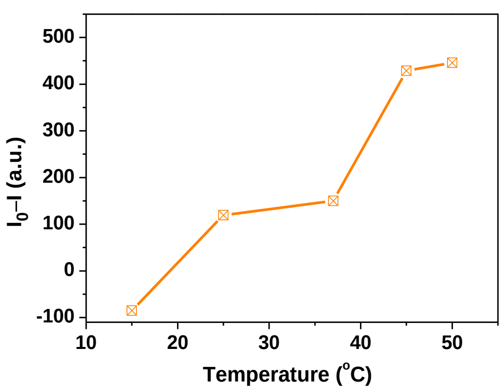

Fig. 5 Plot of the fluorescence decrease (I_0-I) versus the reaction temperature. I_0 and I are the fluorescence intensities of AuNCs in the absence and presence of pepsin, respectively. The reaction time was set as 180 min.

Another key factor is the pH value of the sensing system. Pepsin reportedly retains its significant activity at pH 1.5 to 4.0. At pH value higher than 4.0, pepsin activity begins to decline as the pepsin becomes denatured. Besides, the AuNCs themselves were found to undergo a decrease in fluoresce intensity with increasing pH (Fig. S1, ESI†). Meanwhile, the most appropriate pH for pepsin is 2-4. Therefore, the acidic environment not only helps maintain the maximum fluorescence of gold nanoclusters, but also ensures the highest pepsin enzymatic activity. To keep pepsin in their native state and to maintain the maximum fluorescence of gold nanoclusters, we selected pH 3.0 (glycine-HCl buffer, 50 mM) to optimize the detection of pepsin.

To achieve sensitive pepsin detection, the effect of reaction temperature on the fluorescence of AuNCs@Lyz was investigated. After incubation with pepsin at different temperatures for 180 min, fluorescence spectra of all samples were tested. As shown in Fig. 5, the fluorescence quenching signal is more apparent with increasing reaction temperature. For good sensitivity, the higher temperature may be selected. However, a moderate condition should be selected to obtain a large detection range and avoid the decrease in fluorescence intensity caused by high temperature. Combined with the fact that pepsin is most active in acidic environments between 37 and 42 °C, 37 °C is selected as the reaction temperature according to these observations.

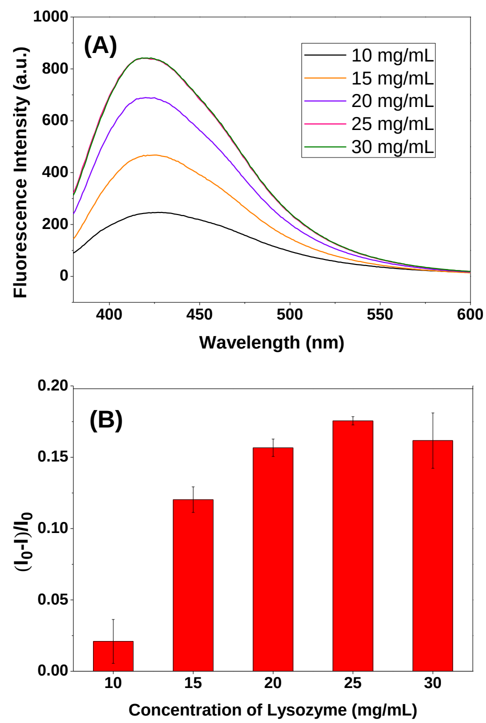

Fig. 6 Fluorescence emission spectra of AuNCs prepared with different lysozyme concentrations (A) and the fluorescence signal [$(I_0-I)/I_0$] they can create (B). The reaction time was set as 180 min.

The effect of lysozyme concentration in the initial reaction solution was investigated as the amount of lysozyme that could affect the formation of AuNCs. Reaction conditions were optimized to obtain the best detection signal. The ratio of lysozyme to $HAuCl_4$ was first adjusted. Lysozyme solutions with the concentration of 10, 15, 20, 25, and 30 mg/mL were used to synthesize AuNCs through the method previously mentioned. As demonstrated in Fig. 6A, fluorescence intensity increased as the concentration was elevated from 10 mg/mL to 25 mg/mL. However, after that trend, the sequential increase in concentration did not help obtaining an apparent fluorescence intensification. As shown in Fig. 6B, at a lysozyme concentration of 10mg/mL, the relative intensity difference was remarkably small, indicating that the AuNCs synthesized by low-density lysozyme were too weak to provide an apparent detection signal. This explanation can be proven by the fact that the AuNCs synthesized by higher lysozyme concentration could provide better signal.

Another information we can derive from Fig. 6B is that 25 mg/mL is the most suitable lysozyme concentration for the reaction system, not only because its signal is the highest, but also because a lower concentration could not guarantee a large detection range and a higher concentration could not guarantee good sensitivity. Thus, 25 mg/mL was selected.

The kinetic behavior of the interaction between AuNCs@Lyz and pepsin was monitored to further test the effect of reaction time on the fluorescence emission of AuNCs@Lyz. Fig. 7 shows the time-dependent fluorescence responses of AuNCs@Lyz after addition of 100 μg/mL pepsin. The fluorescence intensity at 420 nm decreased remarkably during the early stages of the reaction and gradually reached a plateau after 180 min. Considering the total time consumption and the detection sensitivity, an incubation time of 180 min was selected.

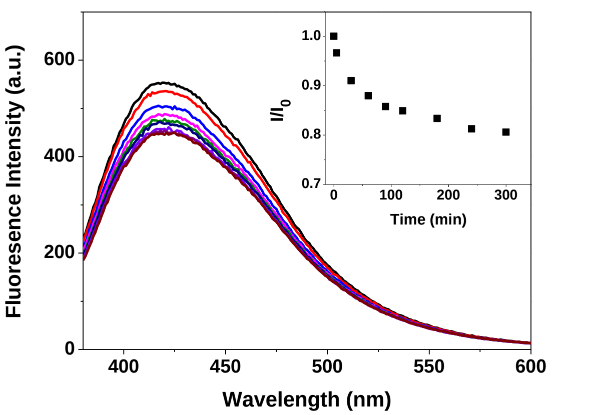

Fig. 7 Fluorescence emission spectra of AuNCs in the presence of 100 μg/mL pepsin for different times. The inset: plot of relative fluorescence intensity at 420 nm versus reaction time.

AuNCs@Lyz as a fluorescent probe for pepsin

To investigate the sensitivity of this sensing system under the optimized conditions discussed above, the fluorescence changes were monitored upon adding increasing concentrations of pepsin for a fixed time of 180 min.

As shown in Fig. 8A, the fluorescent intensity of AuNCs@Lyz at 420 nm decreased gradually with increasing concentrations of pepsin, indicating that the fluorescence intensity of AuNCs@Lyz was highly dependent on the concentration of pepsin. Fig. 8B shows that fluorescence decrease (I_0-I) versus pepsin concentration on a logarithmic scale was linear in the range from 1 μg/mL to 100 μg/mL (R^2=0.99262). The detection limit for pepsin was 0.256 μg/mL at a signal-to-noise ratio of 3. The fluorescence response proved to be very sensitive and the method was much more sensitive than that in previous studies.[1, 6, 7, 10, 11] Therefore, the results demonstrated that our sensing platform could be a simple and sensitive approach for pepsin detection.

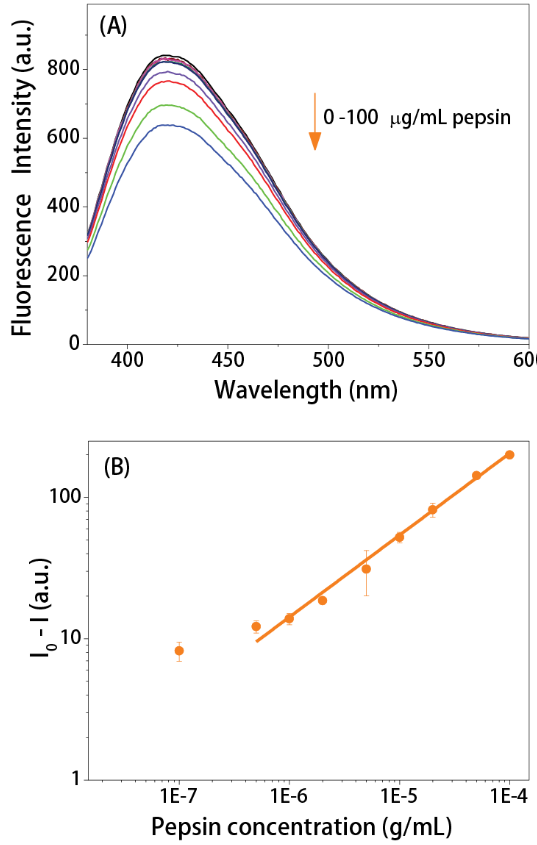

Fig. 8 (A) Fluorescence emission spectra of AuNCs treated with different concentrations of pepsin (from bottom: 100, 20, 10, 5, 2, 1, 0.5, 0.1, 0 μg/mL). (B) Plot of the florescence decrease at 420 nm as a function of pepsin concentration. The error bars represent the standard deviation of three measurements.

Selectivity of the sensing system

The selectivity of AuNCs@Lyz for pepsin determination was studied by comparing the relative fluorescence decrease caused by pepsin and other enzymes, including trypsin, lysozyme, glucose oxidase, thrombin, papain, alkaline protease and lipase.

As shown in Fig. 9, only 50 μg/mL pepsin induced 20% decrease in the fluorescence intensity, whereas no obvious fluorescence changes were observed in the presence of other interfering substances. The high sensitivity of the proposed method could be attributed to the unique proteolytic action of pepsin under acidic conditions. Specifically, the rationale behind the sensing platform was based on the scissor-based interaction between enzyme and its substrate. In our case, the fluorescence decrease was caused by peptic hydrolysis of lysozyme on the surface of AuNCs, and hence only protease could achieve the hydrolysis of the peptide bonds. What's more, under acidic condition (pH 3.0), only pepsin maintains high proteolysis activity toward peptide bonds compared with other common proteases. As a result, this sensing platform has the potential application in the analysis of biological and food samples.

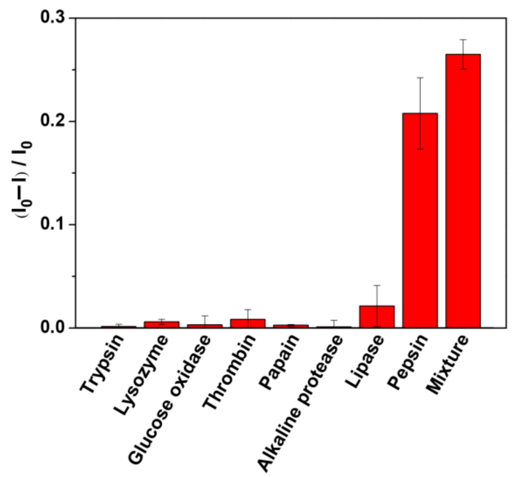

Fig. 9 The fluorescence responses of pepsin and other interfering substances to AuNCs. The concentration of pepsin and other substances is 50 μg/mL.

Conclusions

In summary, we have established a new fluorescent sensing platform based on lysozyme-stabilized AuNCs for pepsin assay with high sensitivity and selectivity. The detection mechanism is based on the change in fluorescence intensity for AuNCs@Lyz when the protein shell is degraded by pepsin under acidic conditions. In addition to offering excellent selectivity, this facile method provides high sensitivity with a detection limit of 0.256 μg/mL, which is currently high competitive in this field. Furthermore, the AuNCs@Lyz based fluorescent sensing system could be used in applications requiring highly sensitive and selective detection of pepsin in food and biological samples.

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Notes and references

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