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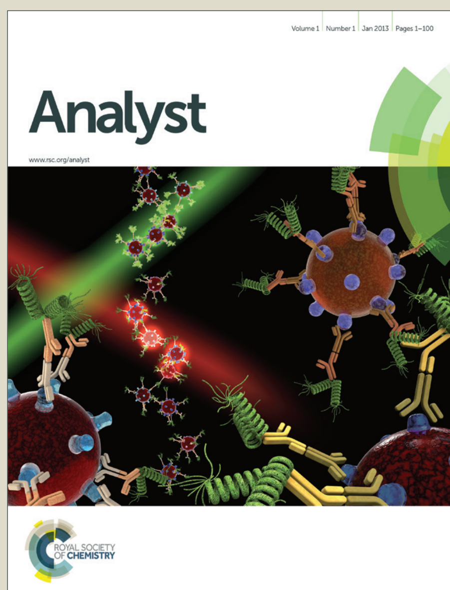

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PAPER

Sensitive Assay of Trypsin Using Poly(thymine)-Templated Copper Nanoparticles as Fluorescent Probes

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A new, simple and sensitive fluorescent strategy was developed for trypsin assay based on copper nanoparticles (CuNPs) and its different fluorescence response toward trypsin-catalyzed hydrolysis of cytochrome c (Cyt c). Polythymine (poly T)-templated CuNPs were served as effective fluorescent probes. Cyt c is well-known to act as a quencher. However, herein, a low concentration of Cyt c was designed specially acting as the substrate of trypsin to avoid the quenching effects by electron transfer from Cyt c to CuNPs. In the presence of trypsin, Cyt c would be hydrolyzed to small peptides, releasing free cysteine residues. A nonfluorescent coordination complexes were formed upon exposure to free cysteine residues by metal-ligand bond between Cu atoms and sulfur atoms, leading to a decreased fluorescence response to CuNPs. This novel method for quantitative determination of trypsin has a linear detection range from 0.25 μg/mL to 1000 μg/mL and a relatively low detection limit of 42 ng/mL. To the best of our knowledge, this is the first application of the trypsin-catalyzed hydrolysis reaction of Cyt c to produce quenching effect in bioanalysis, which provided a novel approach for biochemical sensing strategy.

Introduction

Trypsin, which can catalyze the hydrolysis of peptide bonds on the C-terminal side of arginine or lysine residues, is an important digestive enzyme produced in the pancreas.[1] Trypsin promotes other pancreatic proenzymes into the active forms and controls pancreatic exocrine function.[2] The level of trypsin is connected to several diseases, such as pancreatitis, meconium ileus, and apoptosis.[3-5] Therefore, the analysis of trypsin activity plays important roles in therapeutics of both pancreatic diseases and epidemic diseases.

Many traditional methods for trypsin assays have been developed, for example gel electrophoresis,[6] high-performance liquid chromatography,[7] radioimmunoassay[8] and gelatin-based film techniques,[9] which suffer from time-consuming, cost-expensive, laborious and radioactive labeling. Recently, peptide has been extensively utilized to construct various sensitive, label-free biosensors for trypsin assay by fluorimetry,[10-18] colorimetry,[19,20] electrochemistry[21] or SERS.[22] However, most of these reported methods are based on conjugated polyelectrolytes,[10,11] quantum dots (QDs),[12-14] gold/silver nanoparticles,[15,20] which require sophisticated synthesis processes, many types of reagents and the toxicity of QDs. Moreover, these approaches need specific synthesis of peptides or complex instrumentations, which are of high cost. In addition, some nonspecific signals could be generated by charged interfering proteins in these reported conjugated polyelectrolyte-based fluorescent techniques. Hence, it is still highly desirable to develop rapid, low-cost, efficient and precise strategies to assess trypsin activity.

More recently, using CuNPs as the fluorescent probes to detect biomolecules have gained much interests due to their facile synthesis, excellent fluorescence emission, good biocompatibility and water solubility.[23-30] Here, based on poly T-templated CuNPs, we developed a novel fluorescent label-free strategy to detect trypsin using Cyt c as its natural substrate. Cyt c is a well-known electron transfer protein, which is able to quench the fluorescence of CuNPs through electron transfer between the CuNPs and the heme cofactor in Cyt c.[31] However, the cleaved Cyt c leading the fluorescence quenching instead of electron transfer quenching from Cyt c has not yet been reported. In this work, a low concentration of Cyt c is designed particularly not only to serve as a substrate for enzyme hydrolysis but also to work as a medium for fluorescence quenching. At low concentration of Cyt c, almost no fluorescence signal changes for CuNPs were obtained. After addition of trypsin to the substrate solution, Cyt c was digested into small peptides and cysteine residues was released. Fluorescence quenching of CuNPs was observed due to the formed coordination complexes between biothiols and CuNPs. This strategy can lead to a label-free fluorescence detection technique, which is sensitive and selective to trypsin.

Experimental

Reagents and materials

The Oligonucleotides poly-T (T30) (5'-TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT-3') was synthesized by Sangon Biotechnology (Shanghai) Co. Ltd. and purified by HPLC. The stock solutions of poly-T DNA were prepared by dissolving them in sterile deionized water. Cytochrome c (Cyt c), trypsin, NaCl, KH_2PO_4 and Na_2HPO_4 were of analytical purity and obtained from the Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Supplies of 3-(N-morpholino)-propane sulfonic acid (MOPS), $CuSO_4·5H_2O$, lysozyme and thrombin were commercially obtained from Dingguo Biotechnology Co. Ltd. (Beijing, China). We purchased (+)-sodium ascorbate, human serum albumin (HSA), bovine serum albumin (BSA) and hemoglobin (Hb) from Sigma-Aldrich (Germany). The MOPS

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buffer (10 mM MOPS, 150 mM NaCl, pH 7.6) was used for the formation of fluorescent CuNPs. All reagents were of analytical grade and solutions were prepared using ultrapure water (electric resistance >18.3 MΩ).

Fluorescence measurements were carried out on F-7000 fluorescence spectrometer (Hitachi Ltd., Japan). The optical path length of the quartz cuvette was 1.0 cm. The excitation wavelength was 340 nm and the emission spectra were recorded from 360 to 660 nm with both excitation and emission slits of 5 nm.

Synthesis of fluorescent copper nanoparticles

DNA-templated CuNPs were synthesized according to previous reports.[23] Briefly, 2 mM sodium ascorbate was added to a 10 μM DNA solution (diluted in 10 mM MOPS buffer, 150 mM NaCl, pH 7.6). After blending completely, 0.5 mM $CuSO_4$ was added to this mixture and incubated under a gentle stirring at room temperature in the dark to form fluorescent CuNPs.

Fluorescence measurements of CuNPs in the presence of different amounts of Cyt c

Cyt c stock solutions (1 mM) were mixed with 10 μL buffer (200 mM PBS pH=8.86) in sterilized tube, respectively. Then 50 μL CuNPs were mixed with the samples. Subsequently, water was added to sterilized tube to ensure the total volume of the reaction mixture was 100 μL and the emission spectra were recorded at room temperature.

Trypsin detection

Trypsin of different concentrations was added to 20 mM PBS buffer solution (pH 8.86) containing 25 μM Cyt c (the substrate) and 5 μM CuNPs. The final concentrations of trypsin were 0, 0.25, 0.5, 1, 5, 10, 40, 60, 100, 200, 300, 600 and 1000 μg/mL, respectively. Assay solution was then incubated at 37°C for 30 min. The fluorescence spectra of the mixture were measured under excitation at 340nm. The emission spectra were recorded at room temperature.

Results and discussion

Sensor design

The design of the trypsin label-free fluorescence assay is illustrated in Fig 1. Highly fluorescent CuNPs were first prepared by using poly T ssDNA as a template according to previous reports.[23] Meanwhile, a low concentration of Cyt c was designed specially to prevent the quenching of CuNPs induced by Cyt c through electron transfer between the CuNPs and the heme cofactor. However, in the presence of the trypsin, the fluorescence of the CuNPs was greatly decreased. The mechanism is still not very clear at present. Presumably, such a quenching effect might be attributed to the free biothiols products produced during the trypsin-catalyzed hydrolysis of Cyt c. It is reported that two cysteine residues of the protein covalently link to the heme cofactor in Cyt c through a C–S bond.[32] The cysteine residues are closely surrounded by the protein matrix, which make the bound cysteine residues have difficulty accessing the CuNPs surfaces. Once trypsin was introduced, Cyt c was hydrolyzed to small peptides,[33] and free cysteine residues were released, resulting in a formation of a nonfluorescent coordination complex by the Cu-S metal-ligand bond and fluorescence quenching.[34] A possible

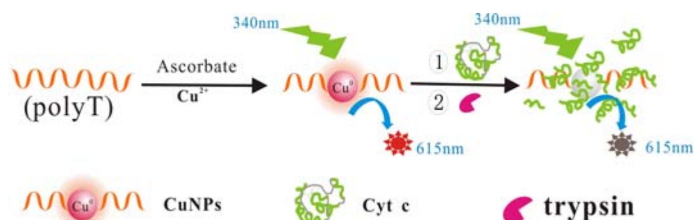

Fig. 1 Schematic illustration of the trypsin sensing strategy based on poly T-templated CuNPs.

quenching mechanism for the coordination complex is that the strong Cu-S may weaken the interaction between the DNA template and the CuNPs which induced fluorescence quenching.

Proof-of-principle of sensor

CuNPs were synthesized according to our previous reports at the optimal conditions of 0.5 mM Cu^{2+}, 2 mM sodium ascorbate, 5 μM T30, with excitation and emission wavelengths of 340 nm and 615 nm, respectively (Fig 2). The fluorescence intensity at the maximum emission wavelength was used to evaluate the effect of trypsin on the fluorescence of the CuNPs.

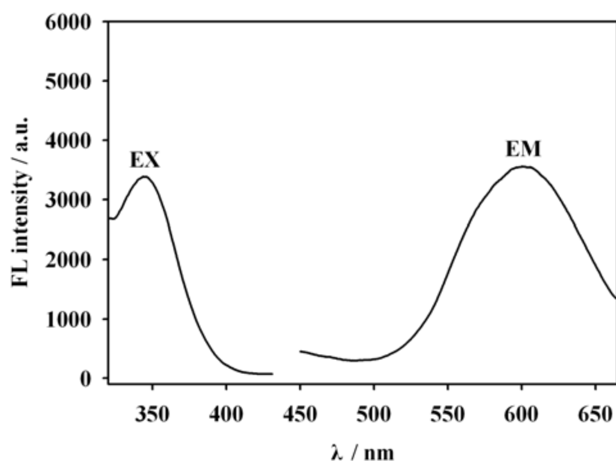

Fig. 2 The maximum excitation and emission spectra of the highly fluorescent poly T-templated CuNPs (ex: 340 nm, em: 615 nm).

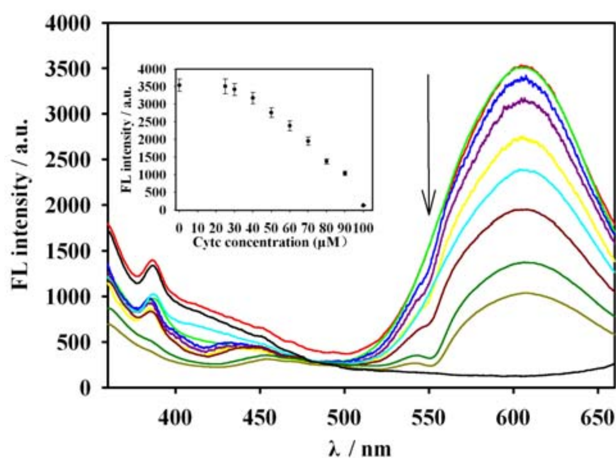

Fig. 3 Fluorescence emission spectrum of CuNPs upon the addition of varying concentrations of Cyt c (from top: 0 μM, 25 μM, 30 μM, 40 μM, 50 μM, 60 μM, 70 μM, 80 μM, 90 μM, 100 μM). Inset: plot of the fluorescence intensity at 615 nm versus the Cyt c concentration.

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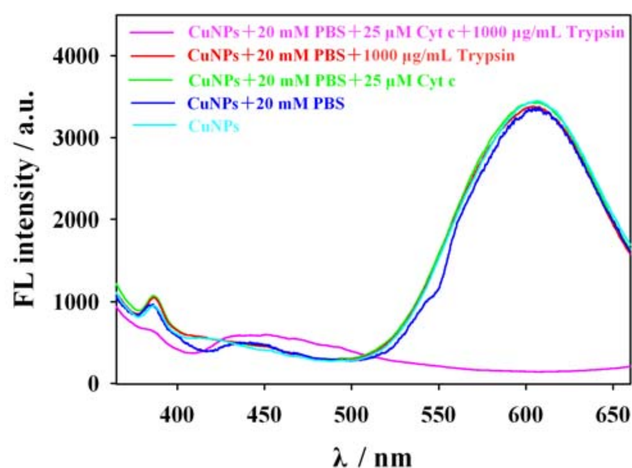

Fig. 4 Fluorescence emission spectra of CuNPs under different conditions: CuNPs (cyan line); CuNPs + PBS (blue line); CuNPs + PBS + Cyt c (green line); CuNPs + PBS + trypsin (red line); CuNPs + PBS + Cyt c + trypsin (pink line). [CuNPs] = 5 μM, [PBS] = 20 mM, [Cyt c]=25 μM, [trypsin] = 1000 μg/mL.

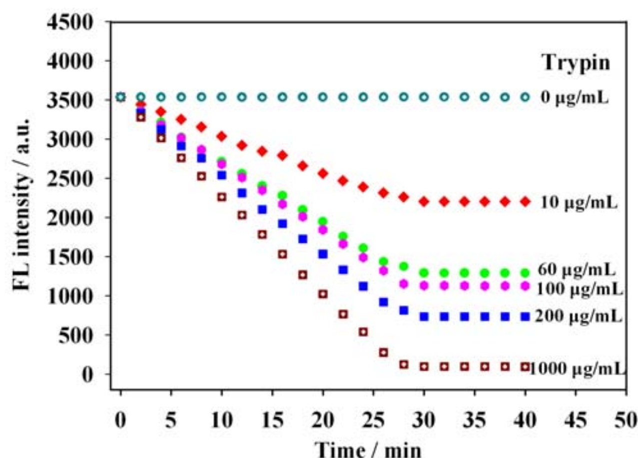

Fig. 5 The time-dependent CuNPs fluorescence intensity as a function of hydrolysis time with varying trypsin concentration.

According to previous reports, Cyt c is an electron transfer protein, which is able to quench the fluorescence of CuNPs effectively through electron transfer from the CuNPs to the metal-containing heme in Cyt c. Therefore, before implementing the label-free fluorescent detection of trypsin, the quenching effects of the Cyt c at a series of concentration on the fluorescence of CuNPs are investigated. As shown in Fig 3, the fluorescence signals decreased with the increasing concentrations of Cyt c from 0 μM to 100 μM. And when 100 μM Cyt c was added, a quenching efficiency of 96.4 % was obtained. However, when the concentration of Cyt c was as low as 25 μM, no obvious decrease of fluorescence intensity for CuNPs could be observed. To avoid possible fluorescence quenching of CuNPs from Cyt c, 25 μM Cyt c was selected for the subsequent trypsin assay.

To further verify the feasibility of the method for the detection of trypsin, a series of control experiments was performed under the same conditions and the results are shown in Fig 4. It was clear that, strong fluorescence emission signals were observed at 615 nm from the separate CuNPs (cyan line), CuNPs in PBS buffer (blue line), CuNPs with Cyt c (green line) or trypsin (red line) in PBS buffer in the absence of trypsin. However, after adding trypsin to the system, no noticeable fluorescent signal could be observed at 615 nm (pink line). These observations demonstrated that the hydrolysis effect of trypsin to Cyt c could quench the fluorescence of CuNPs effectively. These control experiments suggested that the quenching effects to CuNPs were derived from the hydrolysis reaction mediated by trypsin rather than the PBS buffer, the individual Cyt c or the separate trypsin.

Sensor optimization

To get the best conditions for the performance of the developed fluorescent method for detecting trypsin, various variables of the measuring system were optimized in our studies, such as concentrations of poly T DNA, Cu^{2+}, sodium ascorbate for the synthesis of CuNPs and the reaction time of trypsin hydrolysis.

According to previous studies,[23] highly fluorescent CuNPs were synthesized when the solution contained 5 μM T30, 0.5 mM Cu^{2+} and 2 mM sodium ascorbate. Under the optimal condition, the time-dependent changes in CuNPs emission upon incubation of trypsin from 0 to 1000 μg/mL are shown in Fig. 5. The fluorescence intensity decreased with increasing incubation time at each trypsin concentration. After a 30 min reaction, the fluorescence intensity reached a plateau. In addition, the fluorescence intensity decreased more rapidly for the cleavage

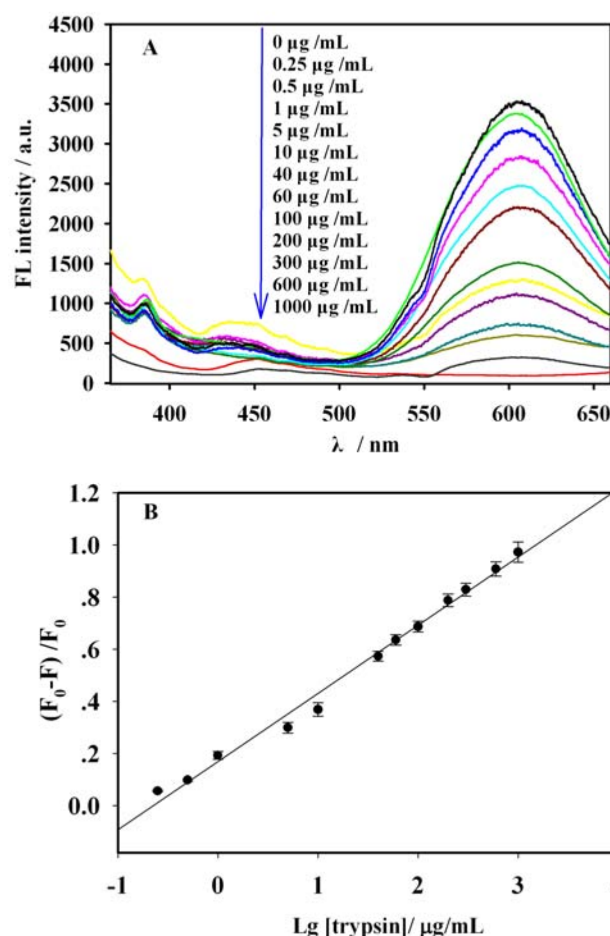

Fig. 6 (A) Fluorescence spectra of the sensor in response to trypsin of various concentrations. (B) Plot of the values of $(F_0-F)/F_0$ for CuNPs versus the logarithm of trypsin concentrations rang from 0.25 μg/mL to 1000 μg/mL (F_0 and F are the fluorescence peak intensities at 615 nm in the absence and presence of trypsin, respectively). Error bars are standard deviation across three repetitive experiments. Conditions: [CuNPs]= 5 μM; [Cyt c]= 25 μM; [PBS]= 20 mM (pH 8.86).

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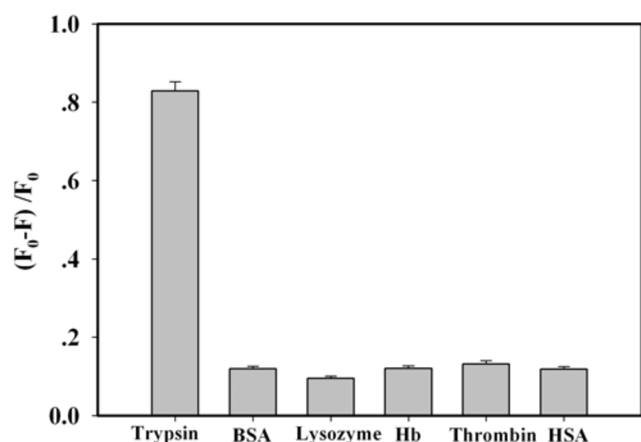

Fig. 7 Selectivity of trypsin analysis. Different proteins were used (trypsin, BSA, lysozyme, Hb, thrombin and HSA). Conditions: [CuNPs]= 5 μM; [Cyt c]= 25 μM; [trypsin], [BSA], [lysozyme], [Hb], [thrombin], [HSA]= 300 μg/mL each; [PBS]= 20 mM (pH 8.86).

reaction at a higher concentration of trypsin with an increased reaction rate.

Performance of the sensor

The ability of the CuNPs-based biosensor for quantitative analysis of trypsin was further evaluated. The fluorescent response of the sensor to trypsin at varing concentrations are shown in Fig. 6A. The peak fluorescent intensity was dynamically decreased in the presence of increasing trypsin concentration in the range of 0.25 μg/mL to 1000 μg/mL. A linear relationship (R^2=0.995) (Fig. 6B) was observed from 0.25 μg/mL to1000 μg/mL between the values of $(F_0\text{-}F)/F_0$ and the logarithm of trypsin concentrations with a robust detection limit of 42 ng/mL. The detection limit of the present strategy is comparable to or higher than that of the previous reported approaches,[10,14,35] but operation convenience, label-free property, low cost and easy preparation of CuNPs can offer additional advantages for this analytical strategy.

To investigate the specificity of the biosensor for trypsin, other interfering proteins, including BSA, lysozyme, Hb, thrombin and HAS, were used instead of trypsin under the same conditions. As shown in Fig. 7, none of the interfering proteins can significantly quench the fluorescent signal except trypsin. It demonstrated that the designed sensing strategy exhibited attractive selectivity to trypsin.

Conclusion

In conclusion, we have developed a novel strategy for the selective detection of trypsin using CuNPs as fluorescent probes. The protocol relies on the fact that the fluorescence quenching of CuNPs induces by the hydrolysis of Cyt c triggered by trypsin rather than the electron transfer effect of Cyt c towards CuNPs. This proposed method provides desirable sensitivity, with a detection limit of 42 ng/mL, and also excellent selectivity, with no interference from other proteins. Additionally, compared with other reported approaches for trypsin assay, this biosensor also has potential advantages, such as easy operation without tedious synthesis processes for conjugated polymers or quantum dots; low-cost without any labels or costly peptide. Therefore, this sensing technique may hold great promise for protease activity related biochemical applications.

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