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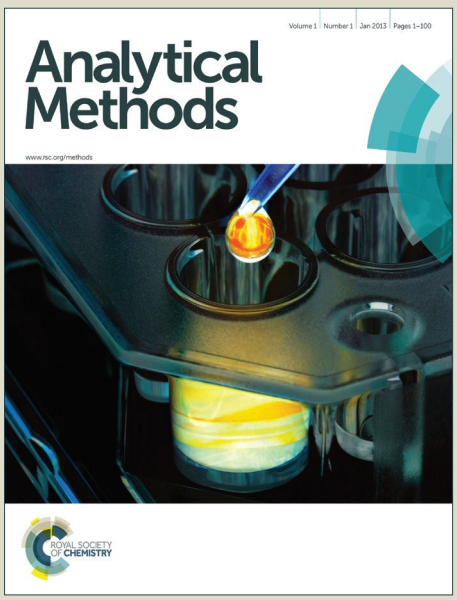

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ARTICLE

Graphene oxide-based chemiluminescent sensing platform for label-free detection of trypsin and its inhibitors

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Huanhuan Zhang, Luyan Yao, Xiaoqian Yu, Yanjun Zhao and Aiping Fan [a,b*]

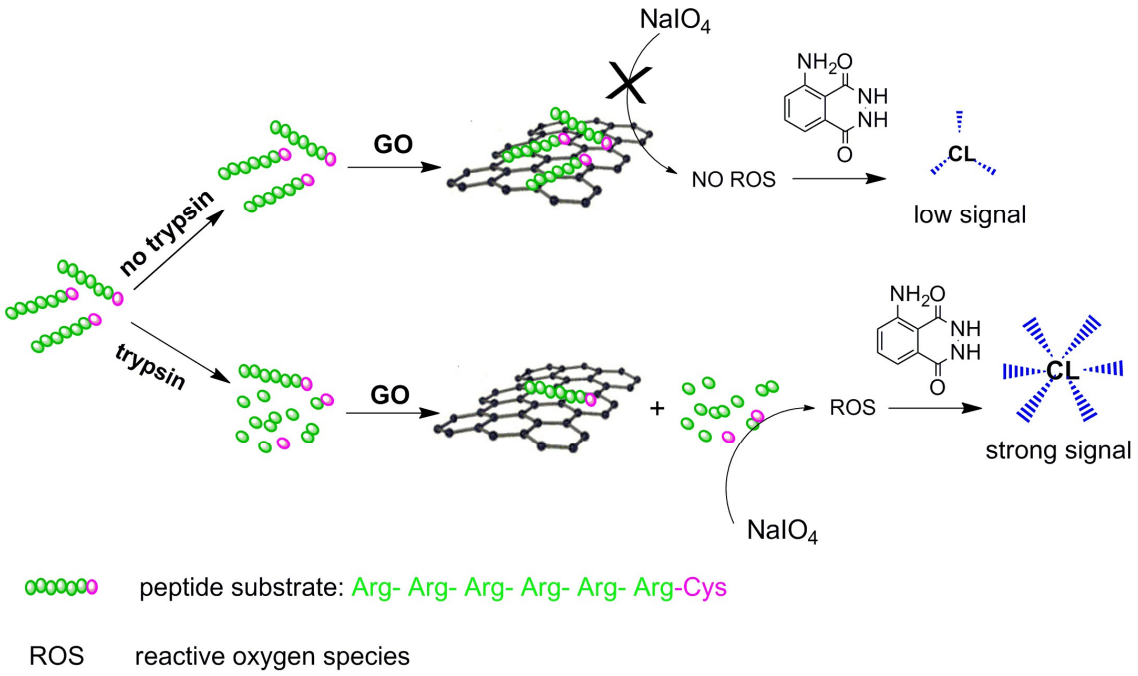
In this study, a novel label-free chemiluminescent (CL) sensing platform for trypsin and its inhibitors screening was developed by taking advantage of luminol-$NaIO_4$-(Cysteine (Cys)-containing peptide) system and graphene oxide (GO). The assay was based on different catalytic activity of Cys-containing peptide and Cys-containing peptide-GO complex in luminol-$NaIO_4$ CL reaction. A strong CL emission was obtained when Cys-containing peptide mixed with $NaIO_4$ and basic luminol, while a much lower CL signal was observed when Cys-containing peptide-GO complex instead of Cys-containing peptide was injected into the mixture of $NaIO_4$ and basic luminol. Based on the room-temperature electron spin-resonance spectroscopy (ESR) study, we speculated that the catalytic activity of Cys-containing peptide in luminol-$NaIO_4$ CL system may be inhibited when the peptides were adsorbed on GO surface. Based on the above observation, a label-free CL sensing platform for trypsin was developed. In the absence of trypsin, the Cys-containing peptide was adsorbed on GO surface *via* electrostatic interactions resulting in a low CL signal. In contrast, in the presence of trypsin, the peptide can be catalytically hydrolyzed at the C-terminus of Arginine (Arg), resulting in the release of Cys-containing residues from the surface of GO and subsequent CL recovery with the addition of luminol and $NaIO_4$. The proposed method enabled the determination of trypsin with a detection limit of 7.3 pM, which can also be employed for screening of trypsin inhibitors. The method can be easily generalized for monitoring the hydrolysis activity of other proteases by simply changing the peptide substrate sequence.

1. Introduction

Trypsin which is a major digestive enzyme produced in the pancreas hydrolyzes peptide on the bonds forming by the C-terminal Arg or Lys.[1,2] It participates in various physiological processes such as playing an irreplaceable role in protein degradation and converting other pancreatic proenzymes into their active forms in intestine.[3,4] The unbalanced quantity and quality of trypsin leads to some pancreatic diseases, such as acute pancreatitis, cystic fibrosis, and so on.[5,6] So it is of great significance to measure trypsin activity in clinical diagnosis. Various techniques have been established for measurement of trypsin activity, including electrochemical methods,[7] colorimetry,[8] surface imprinting strategies,[9] gel electrophoresis,[10] capillary isoelectric focusing,[11] surface-enhanced Raman scattering techniques[12] and numerous fluorescent methods.[13,14] Grant's group reported a high surface area nanoporous organosilicate and dual fluorescent dye labeled peptide sequences based method for fluorescent detection of trypsin.[15] Demaille's group established an electrochemical method by using ferrocene-labeled peptides.[16] However, many of these methods are often laborious and time-consuming, or require the use of labels or sophisticated instruments. The development of label-free, simple and rapid methods for trypsin detection has become increasingly desirable.

In recent years, several label-free methods for the detection of trypsin using fluorescent technology,[17] quartz crystal microbalance[18] and colorimetry[19] have been reported. CL analysis, as a well-established trace analysis, has been widely applied in constructing biosensors owing to its low cost apparatus, wide calibration ranges, high sensitivity and selectivity.[20,21] However, few label-free CL methods for the detection of trypsin have been reported. Yu's group developed a label-free CL assay for trypsin detection using a natural substrate, which is very simple and sensitive.[22] In recently, our group found that biotinylated Cys-containing peptide could catalyze luminol-$NaIO_4$ reaction to emit strong light, and then developed a streptavidin-modified magnetic beads-based CL sensing platform for trypsin detection.[23] This assay is rapid, versatile and sensitive. However, biotinylated peptide and streptavidin-modified magnetic beads were employed which increased the cost of the determination. GO, as water-soluble derivative of graphene, is a single-atom-thick and two-dimensional carbon nanomaterial that has recently attracted

[a] *Collaborative Innovation Center of Chemical Science and Engineering (Tianjin), Tianjin 300072, PR China.*
[b] *School of Pharmaceutical Science and Technology, Tianjin University, Tianjin 300072, PR China.*
*E-mail: aipingfan1980@163.com
Electronic Supplementary Information (ESI) available: the sequences of the peptide used in this study, results of CL response of P_3 in luminol-$NaIO_4$ solution, ESR spectra of aqueous solution of DMPO under different conditions and MS spectra of P_2 and P_3 under different conditions.



Scheme 1 The schematic illustration of the GO-based CL sensing platform for monitoring trypsin activity.

intense interest owing to its remarkable electronic, mechanical, and thermal properties.[24,25] GO exhibits good water solubility and unique capacity of adsorbing biomolecules, such as nucleic acids and peptides, which creates a robust platform for the development of biosensors. So far, GO has been used for many biomolecules analysis such as DNA,[26,27] thrombin,[28] ATP,[29] and glucose.[30] Most of them relied on the preferential binding of GO to single-stranded DNA (ssDNA) over rigid double-stranded DNA (dsDNA). Ye's group reported that GO exhibited strong binding ability to the amino acid Arg via electrostatic interactions as a driving force.[31] Inspired by the above work, a label-free CL sensing platform for trypsin detection based on the interaction between GO and peptides was developed.

In the present study, a series of peptides which contained several Arg and a terminal Cys (Table S1, Supporting Information) were designed. The designed peptides were incubated with GO together resulting in the adsorption of Cys-containing peptides on the surface of GO, the resulting reaction mixture without any washing steps was then injected into basic solution of luminol and $NaIO_4$. We found that the CL signal decreased to a much lower level. Based on this phenomenon, a label-free CL sensing platform for trypsin was developed (Scheme 1). In the absence of trypsin, the Cys-containing peptide was adsorbed onto GO surface, resulting in a low CL signal of luminol-$NaIO_4$-(Cys-containing peptide) system. On the contrary, in the presence of trypsin, the peptide can be catalytically hydrolyzed at the C-terminus of Arg, resulting in the release of Cys-containing residues from the surface of GO and subsequent CL recovery with the addition of luminol and $NaIO_4$. The CL intensity is directly proportional to the concentration of trypsin added in the assay solution. In addition, the hydrolysis of the peptide by trypsin will be retarded in the presence of its inhibitors. Thus, the CL of the luminol-$NaIO_4$-(Cys-containing peptide) system should reduce accordingly. Therefore, the GO-based CL sensing platform can also be employed for the screening of trypsin inhibitors.

2. Experimental

2.1 Materials and chemicals

All chemicals were of analytical reagent grade and were used as received. Ultrapure water (18.2 MΩ cm^{-1}) was used throughout the current work. Trypsin was obtained from Beijing Solarbio Science & Technology Co., Ltd. (China). Cytochrome c and lysozyme were purchased from Puboxin Biotechnology Co., Ltd. (Beijing, China). Bovine serum albumin (BSA), hemoglobin, casein and calponin 1 were purchased from Sigma-Aldrich (Beijing, China). The peptide substrates of trypsin were obtained from Zhejiang Ontores Biotechnologies Co., Ltd. (Zhejiang, China). The details of the peptides were shown in Table S1 (Supporting Information). Soybean trypsin inhibitor (SBTI) was obtained from Yuanye Bio-Technology Co., Ltd. (Shanghai, China). 5, 5-Dimethyl-1-pyrroline N-oxide (DMPO) was purchased from J&K Scientific Ltd. (Beijing, China). Luminol was purchased from Alfa Aesar (Tianjin, China). EDTA, sodium periodate ($NaIO_4$), and other chemical reagents were obtained from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). A mother solution of trypsin was prepared daily at a concentration of 10^{-4} M in 10 mM PBS solution (pH 8.0). A stock solution of peptides (100 μM) in 10 mM PBS (pH 8.0) was divided into 50 μL aliquots and stored at

-20 °C. Stock solution of 10^{-2} M luminol was made in 0.1 M NaOH solution and the working solution of luminol was made by gradually diluting stock solution with 0.1 M Na_2CO_3-0.01M NaOH buffer solution (pH 13.0).

2.2 Apparatus

CL measurements were performed with a BPCL chemiluminescence analyzer (Beijing, China). The mass spectra (MS) were recorded on a Bruker Daltonics micrOTOF-QII (United States) operating with electrospray ionization (ESI) in positive ion mode.

2.3 Preparation of graphene oxide

GO was synthesized from natural graphite powder by a modied Hummers method,[32] and characterized using transmission electron microscopy, X-ray diffraction, Fourier transform-infrared spectroscopy and energy dispersive X-ray spectroscopy as reported in our previous work.[33]

2.4 CL detection of P_3 and P_3-GO complex

P_3 was a peptide substrate with six Arg and a termianl Cys. For CL detection of *P_3*, a 25 μL of designed concentration of *P_3* (0, 0.1, 0.2, 1.0, 2.0, 4.0, and 6.0 μM) in 10 mM PBS (pH 8.0) was mixed with 25 μL of 10 mM PBS (pH 8.0). EDTA (5 μL, 4 mM) and HCl (5 μL, 0.1 M) was then added into the above solution, respectively. Afterwards the result mixture was transferred into 14×40 mm glass tube containing 100 μL of CL reaction solution that consisted of 10 μM $NaIO_4$ working solution and 2.5 μM luminol working solution, and the CL signal was displayed in the BPCL analyzer. The measurements were repeated three times.

For CL detection of *P_3*-GO complex, a 25 μL of 2 μM *P_3* in 10 mM PBS (pH 8.0) was mixed with 5 μL of 4 mM EDTA, and then mixed with 25 μL of designed concentration of GO (4, 8, 20, and 30 μg mL^{-1}) or polylysine blocked GO [Polylysine blocked GO was prepared by mixing designed concentration of GO (4, 8, 20, and 30 μg mL^{-1}) and 10 mM polylysine in PBS buffer (pH 8.0) at 25 °C for 10 min and subsequent centrifugation (10,000 *rpm*, 3 min) for the removal of unreacted polylysine]. The mixture was incubated at room temperature for 3 min for the formation of *P_3*-GO complex. Then, 5 μL of 0.1 M HCl was pipetted into the mixture. The result mixture was transferred into 14×40 mm glass tube and the CL signal was measured as described above. The measurements were repeated three times.

2.5 CL detection of trypsin

A 25 μL of reaction solution consisting of 10 mM PBS (pH 8.0), 6 μM *P_3*, and designed concentration of trypsin was incubated at 25 °C for 20 min with gentle shaking, after which EDTA (5 μL, 4 mM) and GO (25 μL, 60 μg mL^{-1}) were added into the reaction solution and incubated at room temperature for 5 min. HCl (5 μL, 0.1 M) was then pipetted into the mixture. Finally, the result mixture was transferred into 14×40 mm glass tube and the CL signal was measured as described above. The measurements were repeated three times.

2.6 Screening of trypsin inhibitors

25 μL of reaction mixture consisting of 10 mM PBS buffer, 2 nM trypsin, 6 μM *P_3*, and designed concentration of trypsin inhibitor was incubated at 25 °C for 20 min with gentle shaking, after which EDTA (5 μL, 4 mM) and GO (25 μL, 60 μg mL^{-1}) were added into the reaction solution and incubated at room temperature for 5 min. HCl (5 μL, 0.1 M) was then pipetted into the mixture. Finally, CL signal of result mixture was measured as described above. The measurements were repeated three times.

3. Results and discussion

3.1 CL detection of P_3 and P_3-GO complex

Firstly, the CL response of *P_3* which has six Arg and a terminal Cys in the mixture solution of $NaIO_4$ and basic luminol was investigated. A good linear relationship between the CL intensity and the *P_3* concentration 0.1-6 μM was produced with a correlation coefficient of 0.991 (Fig. S1, Supporting Information). As low as 0.1 μM *P_3* could be sensitively detected under the optimum CL reaction conditions. Then, the effect of GO on the signal of luminol-$NaIO_4$-(Cys containing peptide) CL system was investigated by adding different concentrations of GO to a solution containing a fixed concentration of *P_3* (2 μM). According to previous report,[31] *P_3* with six Arg would be strongly adsorbed on the surface of GO through electrostatic interaction resulting in the formation of *P_3*-GO complex. In the experiment, with the increasing concentration of GO, the more *P_3*-GO complex should be generated, and consequently the less *P_3* stayed in the solution. The resulted reaction mixture without any washing steps was then mixed with basic luminol and $NaIO_4$. As shown in Fig. 1, referring to the CL intensity of 2 μM *P_3* as 100%, the CL decreased to 49.8%, 18.9%, 6.3%, and 3.8% in the presence of 4, 8, 20, and 30 μg mL^{-1} of GO, respectively. Control experiments were carried out using polylysine blocked GO instead of GO. The polylysine blocked GO was prepared by incubating GO with 10 mM polylysine

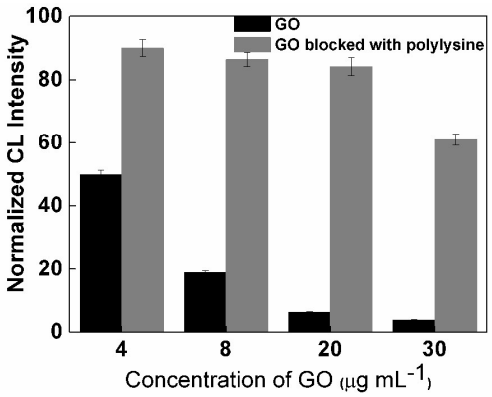


Fig. 1 Normalized CL intensity of luminol-$NaIO_4$-*P_3* system in the presence of GO and polylysine blocked GO with concentration ranging from 4 to 30 μg mL^{-1}. Experimental conditions: *P_3*, 2 μM; luminol, 2.5 μM; $NaIO_4$, 10 μM; GO (or polylysine blocked GO), 4, 8, 20, 30 μg mL^{-1}; *T_{GO}*, 3 min.

resulting in masking of the negative charge of GO and subsequent unable adsorption of P_3. The results were shown in Fig. 1. After an identical incubation procedure, the CL only decreased to 90%, 86.4%, 84%, and 61% in the presence of 4, 8, 20, and 30 μg mL^{-1} of polylysine blocked GO. The above results indicated that the adsorbing of Cys-containing peptide to the surface of GO is critical for the decrease of the CL intensity of the luminol-$NaIO_4$-(Cys containing peptide) system.

We consider that there are two possible mechanisms for the decrease of CL intensity when Cys-containing peptides are adsorbed on GO surface. One is that the chemiluminescence resonance energy transfer (CRET) between GO and Cys-containing peptide catalyzed luminol CL reaction. The other is that Cys-containing peptide is inactive or semiactive when the peptides were adsorbed on GO surface. To conform the mechanism, reactive oxygen species (ROS) such as OH• generated during the oxidation process in the absence and presence of GO were determined by using a room-temperature ESR spectroscopy. DMPO, a specific target molecule of OH•, was used for capturing OH• which was generated during the oxidation process. ESR spectra of aqueous DMPO solutions (100 mM) containing (I) $NaIO_4$ and P_3, (II) $NaIO_4$ and P_3-GO complex, (III) $NaIO_4$ was investigated, respectively. As shown in Fig. S2 (Supporting Information), a typical ESR spectrum for DMPO-OH adducts and a triplet peak for DMPO-ring broken adduct[34] was observed in the presence of $NaIO_4$ and P_3 (situation I). The results indicated the existence of OH• during the catalytic oxidation process, which is consistent with Lau's report.[35] In contrast, when P_3-GO complex instead of P_3 to react with $NaIO_4$ and DMPO (situation II), only a triplet peak for DMPO-ring broken adduct was observed, and no DMPO-OH signal was observed. The results were similar to that of control experiment (situation III). Based on above observation, we speculated that the catalytic activity of Cys-containing peptide may be inhibited when the peptides were adsorbed on GO surface. The low catalytic activity of P_3-GO complex may lead to little or no oxygen related radical produce during the oxidation process. Therefore, a low CL intensity would be obtained when P_3-GO complex instead of P_3 was added into luminol-$NaIO_4$ system.

3.2 Feasibility of the CL biosensor for trypsin detection

On the basis of the above results, a GO-based CL sensing system for label-free CL detection of trypsin was developed. In the absence of trypsin, the peptides with several Arg and a terminal Cys were adsorbed on the surface of GO leading to a relative low CL signal. Conversely, the peptides can be hydrolyzed in the presence of trypsin, leading to the release of Cys-containing residues into reaction solution and subsequent CL recovery in the presence of basic luminol and $NaIO_4$.

We then conducted a proof-of-concept experiment for the label-free and turn-on CL detection of trypsin using P_3 as substrate peptide. Fig. 2 displayed the CL kinetic curves under different conditions. Referring to the CL intensity of 2 μM P_3 as 100%, the CL intensity decreased to 3.3% upon addition of 20

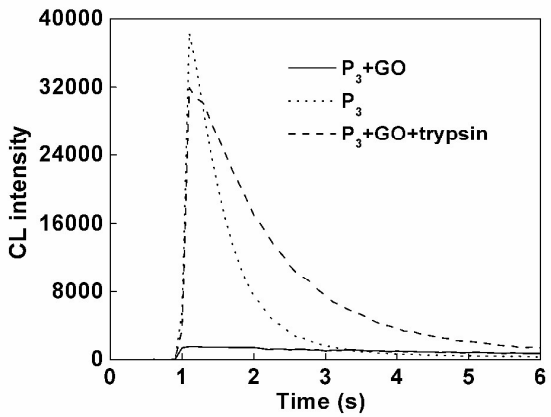


Fig. 2 The CL kinetic curves of luminol under different conditions. Experimental conditions: P_3, 2 μM; GO, 20 μg mL^{-1}, trypsin, 2 nM; luminol, 2.5 μM; $NaIO_4$, 10 μM; T_{GO}, 3 min.

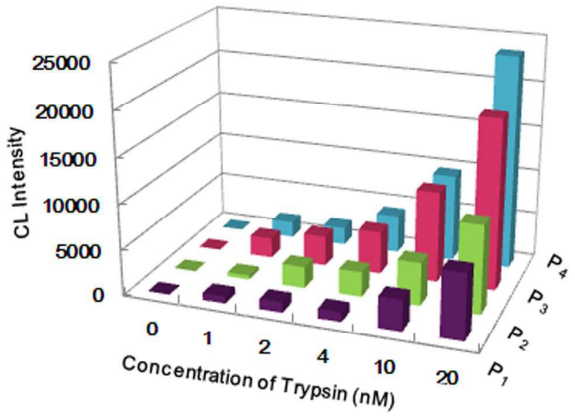


Fig. 3 Effect of peptide length on assay performance. Experimental conditions: peptide (P_1-P_4), 4 μM; trypsin, 1, 2, 4, 10 and 20 nM; GO, 100 μg mL^{-1}; T_E, 20 min; T_{GO}, 3 min; luminol, 2.5 μM; $NaIO_4$, 10 μM.

μg mL^{-1} GO. And in the presence of 2 nM trypsin, the CL intensity increased to about 83.3% indicating that Cys-containing residues are released from the surface of GO. To further confirm the above observation, MS characterizations were performed to monitor the change of the digestion products in liquid supernate before and after incubation with GO (Fig. S3, Supporting Information). Fig. S3B illustrated that the main digestion products of enzymatic digestion were Arg_3, Arg_2-Cys, Arg_2, Arg-Cys, and Arg. After incubation with GO, an obvious change of the peak height ratio of the above peptide residues was observed. For example, the peak height ratio of Arg_2 to Arg-Cys changed from 2.0:1 to 1.3:1 after incubation with GO, while the peak height ratio of Arg_3 to Arg_2-Cys changed from 3.2:1 to 1.1:1. Similar MS results were obtained using P_2 as peptide substrate (Fig. S4, Supporting Information). From the above observation, we speculated that both Arg_n and Arg_n-Cys residues were released after the enzymatic digestion. However, the Cys-containing residues showed much slower adsorption rate onto GO surface than that of Arg_n indicating that more Cys-containing residues stayed in the liquid phase. The MS observations are consistent with the CL results

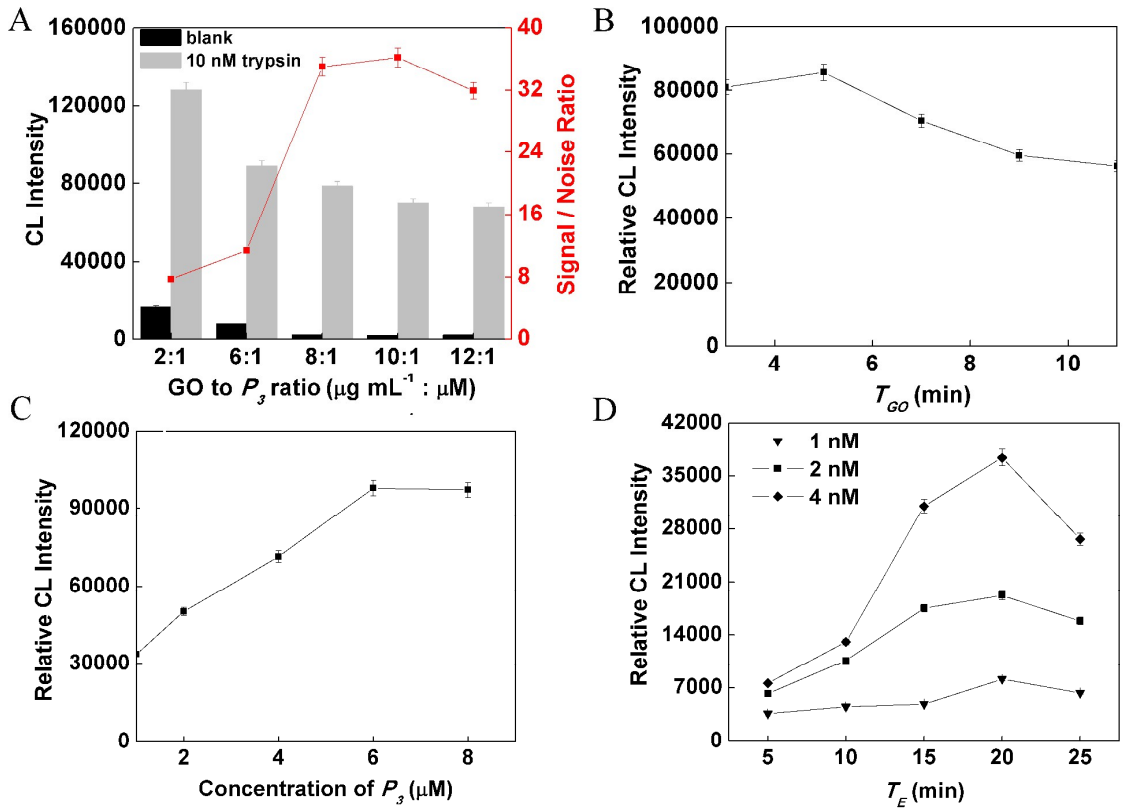


Fig. 4 Optimization of experimental conditions for label-free detection of trypsin. **(A)** Effect of GO to P_3 ratio (μg mL^{-1}: μM) on assay performance. Experimental conditions: P_3, 4 μM; trypsin, 10 nM; GO to P_3 ratio (μg mL^{-1}: μM), 2: 1, 6: 1, 8: 1, 10: 1 and 12: 1; other experimental conditions were the same as Fig. 3; **(B)** Effect of T_{GO} on relative CL intensity. Experimental conditions: P_3, 4 μM; GO to P_3 ratio (μg mL^{-1}: μM), 10: 1; T_{GO}, 3, 5, 7, 9 and 11 min; other experimental conditions were the same as Fig. 4A; **(C)** Effect of P_3 concentration on relative CL intensity. Experimental conditions: P_3, 1, 2, 4, 6 and 8 μM; T_{GO}, 5 min; other experimental conditions were the same as Fig. 4B; **(D)** Effect of T_E on relative CL intensity. Experimental conditions: P_3, 6 μM; trypsin, 1, 2 and 4 nM; T_E, 5, 10, 15, 20 and 25 min; other experimental conditions were the same as Fig. 4C. Error bars were calculated based on three replicates determination.

3.3. Optimization of assay conditions for trypsin detection

In order to achieve the best sensing performance, some important experimental parameters influencing the detection of trypsin, including the length of peptide substrate, ratio of GO to P_3, incubation time between GO and P_3 (T_{GO}), concentration of P_3, and enzymatic digestion time (T_E) were optimized.

The length of peptide was investigated firstly. According to Fan's report,[36] the cleavage rate was slow when the length of peptide substrate was less than Arg_4. Hence, a series of peptide substrates with four or more Arg residues and a terminal Cys were designed. Shorter peptide substrates with less Arg were not studied in the present experiment. The All of the peptides were employed as peptide substrates for the CL recovery studies of the CL platform. As depicted in Fig. 3, all of the peptides exhibited a gradual enhanced CL signal in the presence of increasing concentration of trypsin. P_3 (with six Arg and a terminal Cys) and P_4 (with seven Arg and a terminal Cys) exhibited similar CL responses which are better than that of P_1 (with four Arg and a terminal Cys) and P_2 (with five Arg and a terminal Cys). Considering better CL response of P_3 at low concentration of trypsin, P_3 was selected for the further studies.

The ratio of GO to P_3 has direct influence on the CL detection of trypsin. The ratio of GO and P_3 was investigated by using a fixed concentration of P_3 (4 μM). As shown in Fig. 4A, both signal (CL signal in the presence of trypsin) and blank (CL signal in the absence of trypsin) decreased with the increase of GO concentration. And the signal to noise ratio was highest at 40 μg mL^{-1} GO. The optimal ratio of GO and P_3 was calculated to be 10:1 (μg mL^{-1}: μM). The effect of T_{GO} on the CL signal of trypsin detection was shown in Fig. 4B. It was found that both signal and blank decreased with the increase of T_{GO}. A maximum relative CL intensity (refers to signal minus blank) was obtained at 5 min of T_{GO}. Thus, T_{GO} used in the further experiments was set at 5 min. By using the optimal ratio of GO to P_3, the concentration of P_3 used in this work was optimized (Fig. 4C). The relative CL intensity increased with the increase of P_3 concentration indicating more Cys residues were release in the solution, and then leveled off beyond 6 μM P_3. Hence, 6 μM P_3 was selected for the following study. Since the enzyme reaction is dependent on the digestion time under a certain enzyme concentration, the effect of T_E on the assay performance was investigated at 1, 2, and 4 nM trypsin,

respectively. As shown in Fig. 4D, higher concentration of trypsin leads to the faster increase rate and thus a higher level of CL recovery at the same time point. However, the relative CL intensity of the three concentration levels of trypsin all reached to maximum at 20 min of T_E. Hence, the optimal T_E was selected as 20 min for further study.

3.4. Assay performance of the GO-based CL platform for trypsin detection

To demonstrate the sensitivity of the GO-based CL platform for trypsin analysis, different concentrations of trypsin (0, 0.02, 0.1, 0.2, 0.4, 1, 2, 4 and 10 nM) from one stock solution were tested. The relative CL intensity gradually increased with the increasing concentration of trypsin in the GO based CL system, indicating that more Cys-containing residues were released in the presence of higher concentration of trypsin. A linear relationship between relative CL intensity and concentration of trypsin was observed in the range from 0.02 nM to 10 nM with a correlation coefficient of 0.997 (Fig. 5A). The limit of detection (taken to be three times the standard deviation corresponding to the blank solution) was 7.3 pM, which is about 1165-, 58- and 12-fold lower than that of previous reported fluorescent,[37] electrochemical,[5] and colorimetric[38] detection of trypsin, respectively. In addition, the proposed assay did not need any labeling procedure, tedious operation, and expensive equipment. Thus, the present study is highlighted by its sensitivity, simplicity, and rapidity. A series of seven repetitive measurements of 2 nM trypsin yielded reproducible signals with a relative standard deviation of 6.2%, indicating the good reproducibility of the proposed method.

Recently published results for trypsin detection including the label, detection limit, linear range, and assay time were summarized in Table S2 (Supporting Information). As can be seen from Table S1, the proposed method exhibits much lower detection limit than that of other methods (fluorescent, electrochemical, or colorimetric method) reported previously. The assay time (30 min) is superior to most of the reported method, and a wider linear range is obtained using the proposed CL method compared to other methods. In addition, the proposed method does not need any labelling process and washing step.

To investigate the selectivity of the proposed method, we firstly investigated the effect of several potential interferents including lysozyme, casein, calponin 1, BSA, cytochrome c, and hemoglobin on the CL detection of trypsin with the same procedure as for trypsin. 2 nM potential interferents led to little changes of the CL signals, while a strong CL signal was obtained by adding 2 nM trypsin indicating selective cleavage of P_3 by trypsin, and subsequent release of Cys-containing residues and catalytic oxidation of luminol. Then the effect of higher concentration of interferents on the CL detection of trypsin was further investigated. As displayed in Fig. 5B, little changes of the CL signals were observed in the presence of 20 nM potential interferents. CL reaction is prone to trace metal ions. Hence, the influence of common metal ions was studied.

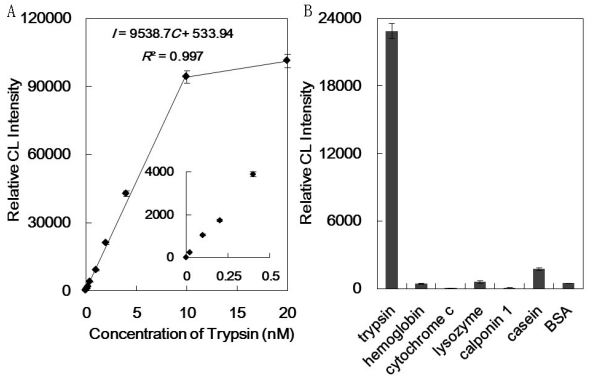


Fig. 5. (**A**) The linear relationship between relative CL intensity and trypsin concentration in the range 0.02-10 nM. Experimental conditions: P_3, 6 μM; trypsin, 0, 0.02, 0.1, 0.2, 0.4, 1, 2, 4, 10 and 20 nM; T_E, 20 min; other experimental conditions were the same as Fig. 4D. (**B**) Specificity of the proposed method. Experimental conditions: trypsin, 2 nM; hemoglobin, cytochrome c, lysozyme, calponin 1, casein and BSA, 20 nM; other experimental conditions were the same as Fig. 5A. Error bars were calculated based on three replicates determination.

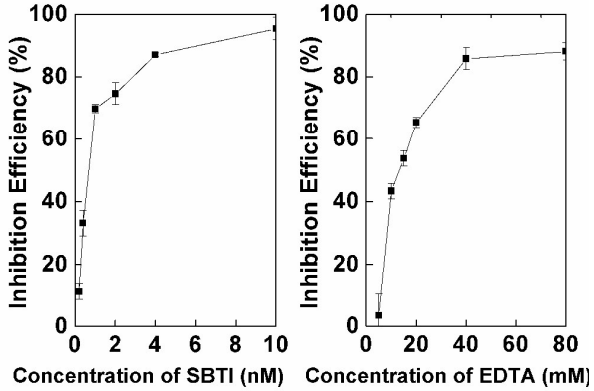


Fig. 6. The plots of inhibition efficiency *vs* the concentration of SBTI and EDTA. Experimental conditions: SBTI, 0.2, 0.4, 1, 2, 4, and 10 nM; EDTA, 5, 10, 15, 20, 40 and 80 mM; trypsin, 2 nM; other experimental conditions were the same as Fig. 4A. Error bars were calculated based on three replicates determination.

The tolerance of each metal ion was taken as the largest concentration yielding an error of less than ±5% in the CL signal of 2 nM trypsin. No interference was found when up to 100 μM Na^+, K^+, Ag^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, and Al^{3+} was contained in sample solution. These results demonstrated that the proposed strategy had good selectivity for trypsin detection.

3.5 Screening of trypsin inhibitors

Since the screening and analysis of protease inhibitors is of great importance in pharmaceutical industry. The proposed CL sensing platform was also applied for monitoring the inhibition of trypsin activity in the presence of its inhibitors, such as soybean trypsin inhibitor (SBTI) and ethylene diamine tetraacetic acid (EDTA), two general inhibitors for the serine protease family, as examples. Fig. 6 displayed the plot of the inhibition efficiency I (%) = $(CL_0\text{-}CL_1)/CL_0\times$ 100% (where CL_0 and CL_1 were the CL intensity of the sensing platform without and with the addition of inhibitor, respectively) of inhibitors

toward trypsin versus the concentration of its inhibitors. With an increasing concentration of trypsin inhibitors, the inhibition efficiency increased rapidly. In this way, the IC_{50} value (the concentration at 50% inhibiting) of SBTI and EDTA was estimated to be 0.7 nM and 12 mM, respectively. The results demonstrated that the proposed GO-based label-free CL platform could be employed for trypsin inhibitors screening.

4. Conclusion

In summary, a label-free, sensitive and versatile GO-based CL sensing system for trypsin and its inhibitors screening has been developed. The established method is designed by taking advantage of inactivity of Cys-containing peptide-GO complex in luminol-$NaIO_4$ CL system, and the hydrolysis of the peptide and subsequent CL recovery in the presence of trypsin. The proposed method enabled the determination of trypsin with a detection limit of 7.3 pM, which is much lower than that of previously reported detection of trypsin. The whole process was very simple, and did not need any washing steps. What's more, it took no more than 30 min to find out the concentration of trypsin. The proposed assay did not need any labeling procedure, and expensive equipment. The assay was also validated with known inhibitors of trypsin. From the analytical chemistry point of view, the concept can be easily generalized for other proteases by simply changing the peptide substrate sequence for monitoring the hydrolysis activity of other proteases. We believe the proposed GO-based CL sensing platform has great potential in high-throughput screening for protease-targeted drug discovery.

Acknowledgment

This study was supported by the National Basic Research Program (973 program) of China "The fundamental and frontier studies of the new topology of molecular functional carbon materials" (nos. 2015CB856500), National Natural Science Foundation of China (21475094), Tianjin Research Program of Application Foundation and Advanced Technology (15JCQNJC11200) and Collaborative Innovation Center of Chemical Science and Engineering (Tianjin).

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Graphical Abstract

A simple and versatile GO-based chemiluminescent sensing platform for trypsin and its inhibitor screening has been developed.