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Label-free fluorometric detection of chymotrypsin activity using graphene oxide/nucleic-acid-stabilized silver nanoclusters hybrid materials

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ABSTRACT

Pancreatic function tests are used to determine the presence of chronic pancreatitis, particularly in the early stage of the disease. Chymotrypsin is an indicator of pancreatic function and is thus related to pancreatic diseases. A new fluorescent biosensing method for assay of chymotrypsin activity was developed using DNA (dC_{12})-templated silver nanoclusters and graphene oxide (GO). A peptide probe was also designed using chymotrypsin-cleavable amino acid sequence and a cysteine terminus. The peptide probe formed Ag-S bond to dC_{12}-AgNCs to enhance the fluorescence of dC_{12}-AgNCs. After the addition of GO, the peptide was adsorbed to the negative GO surface and the fluorescence of dC_{12}-AgNCs was quenched by FRET. The peptide was then degraded into amino acid fragments upon addition of chymotrypsin; these fragments were released from the GO surface, and the FRET was terminated. The developed label-free method features lower cost and higher sensitivity to chymotrypsin activity assay compared with conventional fluorescence analysis. The method can be used to analyze chymotrypsin (as low as 3 ng/mL, signal/noise =3) across a dynamic range of 0.0–50.0 ng/mL. The proposed biosensing strategy can also be extended to other proteases by using different peptide substrates.

1. Introduction

Proteases, including chymotrypsin, participate in numerous biological processes and play major roles in various human diseases. Chymotrypsin acts as an indicator of pancreatic function and is related to many secondary diseases in patients suffering from pancreatitis. Therefore, a reliable and sensitive method for measurement of chymotrypsin is important for pancreatic function testing (Lieb 2nd and Draganov, 2008). Methods for detection of chymotrypsin activity have been proposed to assess the functional capacity of the exocrine pancreas. Traditional methods, such as high-performance liquid chromatography (HPLC) (Wainer et al., 1988) and mass spectrometry (MS) (Šalplachta et al., 2005; Yan et al., 2011) are sensitive and accurate. However, these methods are unsuitable for on-site assays because they require specific equipment. Scholars have also developed methods based on fluorescence resonance energy transfer (FRET) (Sun et al., 2007) by using advanced materials, such as quantum dots (Wu et al., 2014), gold or silver nanoparticles (Wang et al., 2010; Huang et al., 2013) and green fluorescent protein (Dantuma et al., 2000; De et al., 2009). These laborious probes labeling involves short synthetic peptide substrates with fluorogenic tags, and high background signals can limit the sensitivity of the system.

GO is a promising nanomaterial with large hydrophobic basal plane and hydrophilic edges and with high efficiency on fluorescence quenching (Zhang et al., 2011; Liu et al., 2013, 2016; Xing et al., 2016; Xu et al., 2016b). The planar structure of GO contains π electron structure and exhibits large specific surface area. Biomolecules can be immobilized onto the GO surface through non-covalent methods. Liu et al reported the hybrid material consisting of nucleic-acid-stabilized AgNCs and GO as a composite functional matrix for the fluorescence detection of DNA or aptamer-substrate complexes (Liu et al., 2013). The detection strategy depended on two points: (1). The conjugation of single-stranded nucleic acid (long chains, the complementary sequences of target genes or aptamer sequences) to the DNA-AgNCs led to the adsorption of the hybrid nanostructures on GO and the fluorescence quenching of the AgNCs; (2). In the presence of the target DNAs or aptamer sequences, desorption of nucleic acid from GO led to the

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generation of fluorescence as readout signal for the sensing event. Long single-stranded DNA can be efficiently absorbed onto GO, short single-stranded DNA not (He et al., 2014). The interaction between GO and amino acids has been examined (Zhang et al., 2011; Russell and Claridge, 2016). Results showed that a strong interaction could occur between GO and amino acids, such as Arg, His, and Lys. Methods for determination of enzyme activity (Zhang et al., 2011; Song et al., 2013; Zhou et al., 2013; Wang et al., 2014; Zhang et al., 2015) have also been developed based on the strong adsorption of fluorescent-labeled polypeptide to GO. However, these methods require laborious fluorescent labeling procedures.

Emerging few-atom DNA-templated silver nanoclusters (DNA-AgNCs) feature high quantum yield, photo stability, and small size (Song et al., 2016; Xu et al., 2016a). Depending on the DNA sequence, the emission spectrum of DNA-AgNCs can be easily modulated; hence, these materials are excellent alternatives for a wide range of chemical and biological detections and cellular imaging applications (Cao et al., 2015; Javani et al., 2016; Teng et al., 2016; Yin et al., 2016; Zhang et al., 2016). In this study, a chymotrypsin-cleavable sequence with a cysteine terminus was designed. dC_{12}-AgNCs can bond to the cysteine of the peptide and the fluorescence of dC_{12}-AgNCs is enhanced because of the formation of Ag-S bond. In the presence of negatively charged GO, the dC_{12}-AgNCs-peptide complex was adsorbed onto the GO surface, resulting in quenching of the enhanced fluorescence of dC_{12}-AgNCs. The peptide was digested when chymotrypsin was added. Consequently, the enhanced fluorescence of dC_{12}-AgNCs was restored because of desorption of dC_{12}-AgNCs from the GO surface. The proposed method utilizes a peptide probe with a cysteine terminus and is label-free, low cost, and highly sensitive.

2. Experimental

2.1. Reagents and materials

Silver nitrate ($AgNO_3$, 99.99%), Chymotrypsin, bovine serum albumin (BSA), Lysozyme and thrombin were purchased from Sigma-Aldrich (Beijing, China). The peptide probes (Peptide 1: RRHFFGC and Peptide 2: RRWHHFFGC) were obtained from Bioxvision (Anhui, China). Ltd. GO (0.5 mg/mL) were purchased from Nanoon (Beijing, China). $NaBH_4$ (98%) was purchased from J & K Chemical Technology (Beijing, China). Oligonucleotides with 12 polycytosine (dC_{12}) were synthesized and purified by Sangon Biotechnology (Shanghai, China). The other reagents are all analytically. All solutions were prepared using Millipore Milli-Q water (18.2 MΩ cm).

2.2. Instruments

The UV–vis and fluorescence spectra of dC_{12}-AgNCs were obtained using LS55 fluorescence spectrometer and Lambda-35 UV–vis spectrometer (Perkin Elmer, England) respectively. Characterization of dC_{12}-AgNCs-peptide conjugate were recorded by X-ray photoelectron spectroscopy (XPS) (Kratos Analytical Ltd.).

2.3. Preparation of dC_{12}-AgNCs

dC_{12}-stabilized AgNCs were synthesized according to the literature reported (Ritchie et al., 2007). Phosphate buffer solution (2 mM, pH 7.4) was added with dC_{12} and silver nitrate ($AgNO_3$) solution and then placed in an ice water bath for 15 min. The solution was added quickly with fresh $NaBH_4$ solution for reduction, shocked for 1 min, and stored in the dark at room temperature for 2 h. Finally, the solution was preserved at 4 °C overnight. The ultimate concentrations of dC_{12}, $AgNO_3$ and $NaBH_4$ were 10, 60, and 60 μM (molar ratio is 1:6:6). The acquired dC_{12}-AgNCs were characterized with UV–vis spectrophotometer and fluorescence spectrophotometer.

2.4. Sequence-dependent responses of dC_{12}-AgNCs to peptides

Two different polypeptide chains were designed, namely, peptide 1 (RRHFFGC) and peptide 2 (RRWHHFFGC). The effects of these peptides on the fluorescence intensity of dC_{12}-AgNCs were analyzed. Different volumes (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 μL) of 5×10^{-5} M peptide 1 and 2 was added to 2.0 μM dC_{12}-AgNCs to obtain a total volume of 500 μL, respectively. The ultimate concentrations of peptide 1 and 2 solution are 0, 50, 100, 150, 200, 250, 300 and 350 nM. Fluorescence intensity was then recorded. The data of fluorescence intensity were normalized by setting the fluorescence intensity of 2 μM dC_{12}-AgNCs of every measurement as 1.

2.5. Optimizing the amount of GO

The response of GO to the fluorescence of the peptide-dC_{12}-AgNCs complex was investigated. Briefly, 2.0 μL of 5.0×10^{-5} M peptide 1, 20 ng/mL chymotrypsin in PBS buffer solution, and 100 μL of dC_{12}-AgNCs (10 μM) were mixed. The mixture was added with 0, 5, 10, 15, 20, 25, 30, and 35 μL of 0.5 mg/mL GO solution to obtain a final volume of 500 μL. The solution was mixed completely and allowed to stand for 10 min. Fluorescence intensity was then determined. When the fluorescence intensity ceased to reduce, the corresponding concentration was selected as the optimal concentration of GO.

2.6. Dynamic detection of chymotrypsin

To maximize chymotrypsin activity, we conducted the following experiment to determine the optimal reaction time. A series of 2.0 mM pH 7.4 phosphate buffer solution were added with 2.0 μL of 5.0×10^{-5} M peptide 1 and 20 ng/mL chymotrypsin. The solution was mixed and incubated at 37 °C in a water bath for 5, 10, 15, 20, 25, 30, 35, and 40 min. The solution was added with 100 μL of 10 μM dC_{12}-AgNCs to obtain a final concentration of 2 μM and a total volume of 500 μL. The solution was thoroughly mixed and let stand for 10 min. Finally, the solution was added with 20 μg/mL GO after stewing for 10 min. Fluorescent intensity at 635 nm with an excitation of 570 nm was recorded.

2.7. Assay of chymotrypsin activity

A series of 2.0 mM pH 7.4 PBS buffer solution were added with 2 μL of 5×10^{-5} M peptide 1 solution and different concentrations of chymotrypsin, completely mixed, and incubated at 37 °C in a water bath. After 20 min, the solution was added with 100 μL of dC_{12}-AgNCs, mixed, and stand for 10 min. The solution was then added with 20 μL of GO (0.5 mg/mL), mixed, and stand for 10 min. Fluorescence intensity was determined at 635 nm with an excitation of 570 nm.

2.8. Selectivity of the method

The solutions of peptide 1 (2.0 μL, 5.0×10^{-5} M) were mixed with a series of phosphate solution (2.0 mM, pH 7.4), then, the individual solution was added with chymotrypsin (50 ng/mL), thrombin (500 ng/mL), BSA (500 ng/mL) and lysozyme (500 ng/mL), respectively. Afterwards, the ensembles were measured as described above under the same conditions. Two artificial mixtures were also tested: Protein mixture contained thrombin, BSA and lysozyme with total concentration of 500 ng/mL, and the

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