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Original article

Terminal protection of small molecule-linked ssDNA-SWNT nanoassembly for sensitive detection of small molecule and protein interaction

Yu Wang, Dian-Ming Zhou, Zhan Wu, Li-Juan Tang *, Jian-Hui Jiang *

State Key Laboratory of Chemo/Bio-Sensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha 410082, China

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ABSTRACT

The interactions between small molecules and proteins constitute a critical regulatory mechanism in many fundamental biological processes. A novel biosensing strategy has been developed for sensitive and selective detection of small molecule and protein interaction on the basis of terminal protection of small molecule-linked ssDNA-SWNT nanoassembly. The developed strategy is demonstrated using folate and its binding protein folate receptor (FR) as a model case. The results reveal the developed technique displays superb resistance to non-specific binding, very low detection limit as low as subnanomolar, and a wide dynamic range from 100 pmol/L to 500 nmol/L of FR. Thus, it may offer a simple, cost-effective, highly selective and sensitive platform for homogeneous fluorescence detection of small molecule–protein interaction and related biochemical studies.

1. Introduction

The detection of the interactions between small molecules and proteins is of crucial importance for chemical genetics, molecular diagnostics and drug developments [1]. Conventional methods for the detection of small molecules and target proteins interactions include affinity chromatography [2], kinetic capillary electrophoresis [3], surface plasmon resonance (SPR) [4], fluorescence resonant energy transfer [5], and fluorescence anisotropy [6]. Despite the importance and the success of these techniques, the need of sophisticate instruments, costly labeled reagents, laborious and time-consuming washing steps, or the susceptibility to the non-specific adsorption may limit their general applications. In the context, the development of highly specific, cost-efficient, convenient and rapid assay strategies for the small molecule and protein interaction is still of fundamental importance.

Recently, we have reported a series of studies on terminal protection based on the finding that the binding of the target protein to small molecule-linked single strand DNA (ssDNA) could protect the conjugated ssDNA from degradation by 3′ single-strand specific exonuclease I (Exo I) [7]. Employing this technique, several biosensing strategies with high sensitivity and specificity have been developed for remarkable biological applications [8]. Here, we report the proof-of-principle of a novel homogenous fluorescence assay strategy for the detection of small molecule and protein interaction based on terminal protection technique. To realize the specific, sensitive and rapid detection of protein binding event, a nanoassembly was constructed combining small molecule-linked ssDNA with single-walled carbon nanotubes (SWNTs). SWNTs are well-known as intrinsically fluorescence quenchers for most of fluorophores in close proximity to their surfaces [9]. By assembling fluorescein labeled-DNA on the surfaces of SWNT, fluorescence of these fluorescein labels can be dramatically quenched *via* photo-induced energy or electron transfer when the hydrophobic aromatic fluorescein group binds to the sidewalls of SWNT [10]. The terminal protection assay, when coupled with the unique properties of carbon nanotubes, allows the development of a novel fluorescence-based assay strategy for the detection of small molecule–protein interaction. The biosensing strategy has the advantages in its ability to perform homogeneous single-phase reaction, circumvent specific labeling or immobilizing reagents, and exhibit high resistance to non-specific interactions. Additionally, fluorescence-based readouts permit a wide dynamic range and the use of simple instrumentation. To investigate the characteristics of the developed strategy in responding the small molecule-protein binding event, we chose folate and its binding protein folate receptor (FR), a highly selective molecular biomarker associated with various tumors, as the model target. The results revealed the developed strategy may create a robust, cost-efficient and rapid platform for screening the small molecule–protein interaction with high sensitivity and selectivity.

2. Experimental

2.1. Reagents and materials

Folate receptor (FR), folic acid, 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxysulfosuccinimide (sulfo-NHS) were purchased from Sigma–Aldrich Chemical

* Corresponding authors.
E-mail addresses: tanglijuan@hnu.edu.cn (L.-J. Tang), jianhuijiang@hnu.edu.cn (J.-H. Jiang).

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Co., Single-strand selective exonuclease I (Exo I) was from New England Biolabs (Ipswich, MA, USA). Purified single-wall carbon nanotube (SWNT) with an average diameter of ~5 nm was obtained from Beijing Nachen Co., Ltd. (Beijing, China). Bovine serum albumin (BSA), human serum albumin (HSA), human immunoglobulin G (IgG), carcinoembryonic antigen (CEA), α-fetoprotein (AFP), transferrin (TF), and carbohydrate antigen (CA) 19-9 were provided by National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All other chemicals were of analytical grade and obtained from Sinopharm Chemical Reagent Co., Ltd. All solutions were prepared using ultrapure water, which was obtained through a Millipore Milli-Q water purification system (Billerica, MA, USA) and had an electric resistance >18.3 MΩ. Single strand DNA oligonucleotides used in this study were synthesized from Takara Biotechnology Co., Ltd. (Dalian, China), and the sequence is 5′-GTG TGT GTG TGT GTG TGT(-*X*) GTG TGT GTG TGT TTT TT-NH_2-3′, where *X* denoted the fluorescent label. In the present study, *X* was fluorescein isothiocyanate (FITC), a modifier of T nucleotide.

2.2. Labeling of folate to NH_2-modified oligonucleotides

Folate was conjugated to the 3′-NH_2 moiety of the oligonucleotides using the succinimide coupling (EDC-NHS) method [7]. Briefly, 0.5 mL of 20 μmol/L ssDNA was mixed with 0.5 mL of 10 mmol/L phosphate buffer (PB, pH 7.4) containing 10 mmol/L folate, 1 mmol/L EDC, as well as 5 mmol/L sulfo-NHS, followed by incubation at 37 °C for 2 h in the dark. The solution was then dialyzed against PB using a membrane with molecular weight cutoff of 1000 Dalton to remove excessive folate. The dialysis was performed for 3 days with shield from light and changes with fresh buffer every 4 h.

2.3. Preparation of folate-linked ssDNA-SWNT nanoassembly

Purified SWNTs (1 mg) was added in a 1.6 mL aliquot of reagent solution containing 20 μmol/L folate-linked ssDNA in PBS buffer (10 mmol/L PB, 0.1 mol/L NaCl, pH 7.4). The mixture was sonicated in ice bath for 2 h in the dark under a power of ~10 W using a probe-type sonicator [11]. The resulting suspension was centrifuged at 15,000 *g* for 30 min to remove possible SWNTs aggregates and the supernate containing stable suspension of folate-linked ssDNA-SWNT nanoassembly was collected. Then the suspension was centrifuged at 85,000 *g* for 30 min to remove excessive oligonucleotides followed by re-suspending the sediment in 1 mL ultrapure water. This step was repeated four times to sufficiently remove all excessive oligonucleotides. Subsequently, the folate-linked ssDNA-SWNT nanoassembly was re-suspended in 500 μL ultrapure water. The nanoassembly suspension, which was stored at 4 °C, was found to be stable for weeks with no appreciable aggregates and precipitates.

2.4. Terminal protection-based detection of small molecule–protein interaction

A 3 μL aliquot of folate-linked ssDNA-SWNT nanoassembly suspension was added into 20 μL reaction buffer containing 67 mmol/L Glycine-KOH (pH 9.5), 6.7 mmol/L $MgCl_2$, and folate receptor of a given concentration. The mixture was incubated at 37 °C for 30 min to allow complete interaction between FR and the folate-linked ssDNA-SWNT nanoassembly. Then, 50 U Exo I was added to the reaction buffer and incubated at 37 °C for 30 min followed by the addition of 20 mmol/L EDTA to terminate the reaction. The resulting mixture was diluted to a final volume of 100 μL with ultrapure water and subjected to fluorescence measurements. The fluorescence spectra were measured at room temperature in a 100 μL quartz cuvette on a Fluorolog-Tau-3 spectrofluorometer (Jobin Yvon Inc., NJ). The excitation wavelength was 485 nm and the emission wavelength was collected in the range from 500 nm to 620 nm.

3. Results and discussion

The fluorescence-based small molecule–protein interaction assay strategy relies on it that the binding of a target protein to small molecule-linked ssDNA-SWNT nanoassembly could efficiently protect the nanoassembly from the degradation by Exo I, as illustrated in Fig. 1. A folate is covalently linked to the 3′ end of the ssDNA with repeated $(GT)_{15}$ which is labeled with FITC. The folate-linked ssDNA can easily wrap around the sidewalls of SWNT to form a nanoassembly due to the aromatic interactions between nucleotide bases and SWNT sidewalls [11]. In this nanoassembly, the aromatic groups in FITC labels are located in proximity to the hydrophobic regions of SWNT sidewalls, so their fluorescence is quenched nearly completely due to efficient electron or energy transfer from the fluorophores to SWNTs [10]. In the absence of FR, the folate-linked ssDNA can be digested from the 3′ end and hydrolyzed successively into mononucleotides by Exo I, which releases the FITC labels apart from the SWNT surfaces, thus activating their fluorescence signal. On the other hand, when FR is bound to the folate-linked ssDNA-SWNT nanoassembly, the binding of FR can preclude Exo I from freely accessing the 3′-terminus and protect the ssDNA from the Exo-catalyzed degradation, so the fluorescence activation process will not occur. Since the degradation of small molecule-linked ssDNA-SWNT nanoassembly and the release of FITC labels by exonuclease are highly selective to the binding of target protein, the resulting fluorescence response could allow immediate detection of small molecule–protein interaction.

Fig. 2 depicts typical fluorescence spectral responses of the biosensor in the assays of folate and folate receptor interaction. The folate-linked ssDNA-SWNT nanoassembly was observed to only exhibit a very weak fluorescent signal at 518 nm, which suggested that the fluorescence of the FITC labels was efficiently quenched due to energy transfer from the fluorophores to SWNTs. After incubating the nanoassembly with 2.5 U/μL Exo I for 30 min, the reaction mixture showed a very strong fluorescence signal, evidencing that the fluorophores were dissociated from the surfaces of SWNTs during the hydrolysis reaction catalyzed by Exo I. In contrast, when the nanoassembly incubated with 500 nmol/L FR followed by the Exo I-catalyzed degradation, no substantial fluorescence increase was observed. This revealed that the hydrolysis reaction of the nanoassembly was prohibited by the binding of FR to the folate-linked ssDNA-SWNT nanoassembly. In other words, the fluorescence restoration process could not be achieve when the binding of a target protein to its ligand-linked ssDNA-SWNT nanoassembly. Additionally, we performed the

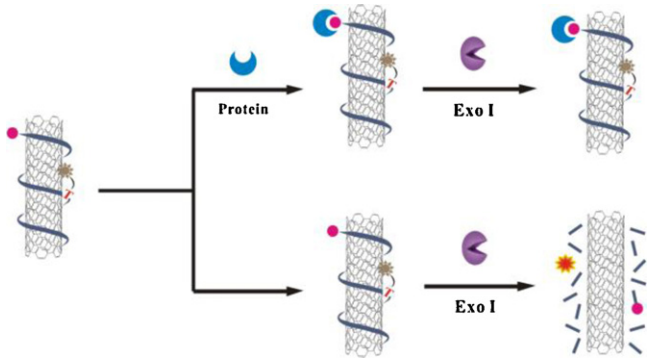

Fig. 1. Illustration of the small molecule–protein interaction assay based on terminal protection of small molecule-linked ssDNA-SWNT nanoassembly.