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Salicylicylphenylacetylene fluorophore mixed with graphene oxide for selective lysozyme detection

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ABSTRACT

A highly selective method of lysozyme detection based on salicylicylphenylacetylene (**1**) fluorophores and graphene oxide (GO) was successfully developed. The fluorescent emission of **1** was decreased fivefold in the addition of GO (0.6 Abs) due to π-π and hydrophobic interaction between **1** and GO graphitic plane. In the presence of lysozyme (Lys), the non-emissive **1**-GO became highly fluorescent as a result of strong interaction of **1** with the cationic Lys preventing the complexation between **1** and GO. The linear dynamic response of fluorescence intensity was observed in the Lys concentration range of 0–80 μg/mL with the limit of detection of 65 ng/mL. This label free technique was developed for quantitative determination of Lys in egg white sample with an analytical accuracy of less than 10% error.

1. Introduction

The development of protein detection and quantitation is extremely important for chemical and biological fields [1–4]. Presently, polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) are the prime techniques available for construction of biosensors. The major advantage of these techniques is their specificity [5–11] while some of their drawbacks include the use of unstable biological reagents, sophisticated fabrication, multisteps and long detection time [12–15]. The faster and simpler techniques for protein detection and quantification are ever needed [16–19].

Lysozyme is a small globular protein which contains 129 amino acid residues and has molecular weight of 14.4 kD [20]. Lysozyme is one of the defensing enzymes preventing bacterial infection by catalyzing hydrolysis of bacterial cell walls. It is worth noticing that fluid samples of egg white, milk, serum and urine include fairly high amounts of lysozyme which has been used as an important biomarker. Its abnormal concentration in serum and urine is an indication to many diseases such as leukemia, renal diseases and cancers [21–23]. Thus, novel lysozyme detection and quantification is of considerable importance.

Graphene oxide (GO) [19] has recently been applied in development of novel sensors for gases [24,25], DNA [26] and proteins [27–29] based on its fluorescent quenching property, mostly via π–π interactions with aromatic fluorophores [19,30]. Fluorometry is indispensable techniques for protein detection and measurement due to its high sensitivity and selectivity [31,32]. Currently, lysozyme assay has been reported by using high affinity lysozyme aptamer labeling fluorescein derivative [33–36]. However, a few label-free aptamer based methods for lysozyme detection have recently been reported using triazolylcoumarin-AuNPs [37], carboxymethyl chitosan QDs [38] and $CuInS_2$ QDs [39] as the fluorescent signal transducers.

Number of research on practical applications of using organic fluorophores as label free biosensors for detection and quantification of protein is limited. Nevertheless, our recent works demonstrated that electrostatic and hydrophobic interaction between protein and phenylacetylene fluorophores having different electric charges could produce distinct photophysical effects on the fluorophores that can be used for identification of proteins. [40–42]. The approach is fast and simple that it is suitable for high throughput screening and authentication of a large numbers of protein samples.

In this study, we utilized GO to quench the fluorescence of an anionic salicylicylphenylacetylene (**1**). Lysozyme could competitively

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bind to **1** thus perturb the interaction between **1** and GO that lead to the recovery of the fluorescence signal. Therefore, we designed a novel label-free fluorescence strategy for lysozyme assay using **1** and GO with good precision and selectivity.

2. Experimental

2.1. Materials and methods

Chemicals and materials: concanavalin A (Con a, from Jack bean), cytochrome c (CytC, from equine heart), histone (His, from calf thymus, type III-S), human serum albumin (HSA), lysozyme (Lys, from hen egg white) and myoglobin (Myo, from equine heart) were purchased from Sigma. *N*,*N*-dimethylaniline, trimethylsilylacetylene, $PdCl_2(PPh_3)_2$, sodium thiosulfate, benzyltrimethylammonium chloride, potassium hydroxide, potassium carbonate, and calcium carbonate and bovine serum albumin (BSA) as reagent grade were purchased from Fluka. Triphenylamine, iodine monochloride, copper(I) iodide, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), quinine sulfate and 4-iodomethylsalicylate as reagent grade were purchased from Aldrich and used as received without further purification. Solvents were purified and dried according to standard methods prior to use. All reactions were carried out under nitrogen atmosphere unless specified otherwise. Column chromatography was performed on Merck silica gel 60 (70–230 mesh).

Analytical Instruments: ^{1}H spectra were recorded on Bruker 400 MHz NMR spectrometer using the residual solvent proton resonance of $CHCl_3$ at 7.26 ppm as the reference. ^{13}C NMR spectra were recorded on the same instruments but using the solvent carbon resonance of $CDCl_3$ at 77.1 ppm. Mass spectra were recorded on a Microflex MALDI-TOF mass spectrometer (Bruker Daltomics) using doubly recrystallized α-cyano-4-hydroxy cinnamic acid (CCA) as the matrix. Absorption spectra were measured by a Varian Cary 50 UV–vis spectrophotometer or Perkin-Elmer 35/FIAS300. Fluorescence spectra were performed on Hitachi F-2500 and EnSpire multimode plate reader on Perkin-Elmer.

2.2. Synthesis of 1

2.2.1. Compound 1

A mixture of triiodotriphenylamine (1.9852 g, 3.1 mmol), $PdCl_2(PPh_3)_2$ (0.10 g, 0.15 mmol), CuI (0.0267 g, 0.12 mmol), methyl 4-ethynyl-2-hydroxybenzoate (1.3014 g, 7.20 mmol) in toluene (20 mL) was added to DBU (1.0 mL) and then stirred at room temperature (RT) for 3 h. After the combined filtrate was evaporated, the residue was eluted through a silica gel column by using gradient solvents as an eluent ranging from pure hexane to CH_2Cl_2–hexane (2/1 v/v). The solvents were then removed resulting in yellow solid residue. The triester product (0.60 g, 0.48 mmol) was dissolved in THF (15 mL) and methanol (15 mL). The saturate KOH aqueous solution (1.0 mL) was added to the solution and then heated to 70 °C for 24 h. The solution was evaporated and the residue was dissolved in water (20 mL). Approximately 50 g of ice was added to the solution and the resulting solution was acidified with 1 M (M) HCl. The acidic aqueous solution was filtered to afford compound 1 in a yellow solid form (0.40 g, 89% yield), melting point (mp.) > 203 °C (decompose). ^{1}H NMR (CD_3OD, 400 MHz): δ (ppm) 7.70 (d, J = 8.0 Hz, 3H), 7.33 (d, J = 8.0 Hz, 6H), 6.82–6.95 (m, 12H) [42].

2.2.2. Compound 2

A mixture of triiodotriphenylamine (0.35 g, 0.50 mmol), $PdCl_2(PPh_3)_2$ (100 mg, 0.16 mmol), CuI (20 mg, 0.10 mmol), 4-ethynyl-*N*,*N*-dimethylaniline (0.27 g, 1.8 mmol) in toluene (10 mL) was added to DBU (1 mL). Then, the mixture was stirred at room temperature for 3 h. After evaporating the mixture, the residue was eluted through a silica gel column by using gradient eluent starting from pure hexane to methylene chloride/hexane (2/3) giving the triamine product a yellow solid. The triamine (0.20 g, 0.70 mmol) was dissolved in CH_3CN (15 mL) and was then added with CH_3I (0.5 mL). The mixture was stirred at 70 °C in sealed tube for 24 h. The solution was then evaporated to afford compound 2 as a yellow solid (0.35 g, 75% yield). mp: > 348 °C (decompose); ^{1}H NMR (CD_3CN, 400 MHz) δ (ppm) 7.88 (d, J = 8.0 Hz, 6H), 7.76 (d, J = 8.4 Hz, 6H), 7.53 (d, J = 8.4 Hz, 6H), 7.15 (d, J = 8.0 Hz, 6H) [43].

2.3. Fluorescence quenching study

GO was synthesized from graphite according to a modified Hummers method [44]. Aqueous dispersion with the concentration of 0.60 mg/mL was obtained by adding 10 mL pure water to 300 mg of GO, followed by sonication for 1 h. GO stock solution (Abs_{280} = 2) was prepared by diluting with Deionized water (DI water). The stock solutions of compound **1** and **2** (200 μM) in PBS buffer pH 7.4 (10 mM) were transferred to a cuvette (1.5 mL) to yield a solution of **1** or **2** = 20 μM. GO (Abs_{280} = 2) stock solution was subsequently added to the cuvette. After 10 min, the PL spectra were collected in the range of 400–600 nm upon the excitation at 375 nm. Then, the optimized condition for Lys bioassay was investigated.

2.4. Lysozyme detection

Different concentrations of lysozyme were added to the **1** (20 μM) mixed with GO (0.6 Abs) solution in 10 mM PBS buffer pH 6.0, which was afterward shaken for 6 min. Next, the abovementioned was measured by fluorescent spectrophotometer. The increase of fluorescence emission was defined by ΔI, where I_0 and I were embodied sensing system in the absence and presence of lysozyme, respectively. The interference study of the method was prepared by using the solution **1** (20 μM) and GO (0.6 Abs) mixed with other proteins (BSA, Cyt C, His, Hem, Lip, Myo (50 μg/mL)). All measurements were repeated three times and the standard deviation was calculated as the error bars in the plot.

2.5. Zeta potential and dynamic light scattering (DLS)

Zeta potential and DLS method with irradiation from a standard 633 nm laser was used to comprehend the interaction among **1**, GO and lysozyme. The zeta potentials and hydrodynamic diameters of **1** (20 μM) mixed with GO (0.6 Abs), lysozyme (20 μg/mL) were recorded by using DLS under the condition at pH 6 and 150-fold dilution.

2.6. Detection of lysozyme in hen egg white

Hen egg whites was used for lysozyme detection and carried out by the following method. First, the hen egg whites and egg yolk were separated. Only egg white was used and diluted with 10 mM PBS buffer pH 6.0 at the ratio of 1–50. Second, various amounts of the prepared samples were added to the **1** (20 μM) and GO (0.6 Abs) solution. The percent recoveries of the lysozyme detection were investigated by using the standard addition method. All measurements were repeated three times

3. Results and discussion

3.1. Synthesis and fluorescence quenching study

Compound **1** was successfully synthesized by using the Sonogashira coupling between triiodotriphenylamine core with methyl-4-ethylsalicylate ester branches followed by a base catalyzed hydrolysis of the ester to get a compound **1** [36]. Likewise, the Sonogashira coupling between triiodotriphenylamine and *N*,*N*-dimethyl-4-ethynylaniline