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Fluorescence energy transfer inhibition bioassay for cholera toxin based on galactose-stabilized gold nanoparticles and amine-terminated quantum dots

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ABSTRACT

A simple and rapid bioassay for cholera toxin (CT) has been developed by using fluorescence resonance energy transfer (FRET) between galactose-stabilized gold nanoparticles (AuNPs) and amine-terminated CdTe quantum dots (QDs). The target CT from the bacterium *Vibrio cholerae* has five carbohydrate binding sites which selectively bind to the synthesized β-galactose derivative immobilized on AuNPs. The H-bonding between amino groups of QDs and hydroxyl groups of galactose on AuNPs leads to the formation of QDs-AuNPs assembly, thus causing an effective FRET from the donor QDs to the acceptor AuNPs. However, the selective binding of CT to the galactose derivative immobilized on AuNPs inhibits the H-bonding formation between QDs and AuNPs, thereby leading to the fluorescence recovery. The degree of FRET recovery is strongly dependent upon the amount of added CT, which enables highly sensitive quantitation of CT. The present bioassay gave a linear response ($r^2 = 0.999$) for CT from 23.6 nM to 2.36 μM with a theoretical detection limit (S/N = 3) of 280 pM without the use of time-consuming, expensive, and complex signal amplification steps generally employed in typical sandwich-type immunoassays. Therefore, the present bioassay is simple and rapid (less than 10 min). Moreover, the galactose-stabilized gold nanoparticles are very stable over long-term period of storage (over 6 months).

1. Introduction

Cholera is an acute infectious disease leading to severe dehydration through intestinal fluid loss and ultimately even death. Cholera toxin (CT), secreted by the bacterium *Vibrio cholerae*, is a hexameric protein (AB_5) composed of an active single A subunit and five identical B subunits [1]. The A subunit surrounded by five B subunits is the pathogenic agent responsible for the symptoms of cholera [2]. The B subunits are nontoxic and bind specifically to pentasaccharide ganglioside (GM_1) presented on the surface of mucous cells in small intestine [3]. CT is easily transmitted to humans by water and food contaminated with CT-producing bacterium *Vibrio cholerae*. Cholera is a serious danger to human health especially in underdeveloped countries and can be fatal if rapid medical treatment is not provided [4].

In recent years, the detection of CT has drawn considerable research interest in order to prevent mass outbreak of cholera and also the possibility of bioterrorism utilizing biological toxins. Until now, several methods have been developed for the detection of CT. The majority of these detection techniques are based on the use of either CT-specific antibody or pentasaccharide GM_1 ganglioside natural receptor as recognition element for CT. For example, antibody-based multiplexed fluoroimmunoassays for CT were developed in a format of either microarray using organic fluorophores [5,6] or microtiter plate using semiconductor quantum dots [7]. Antibody-based CT immunosensors were also developed using several transduction methods such as impedance measurement coupled with surface plasmon resonance (SPR) on an ion selective field effect transistor device [8], SPR [9], electroptode based on luminol chemiluminescence [10], and capacitance measurement [11]. However, the GM_1 ganglioside receptor has been most widely utilized for the detection of CT because the interaction of the GM_1 ganglioside with CT is very specific and strong as studied by SPR [12,13]. For example, fluorescence CT assays based on the GM_1 ganglioside was developed through either distance dependant FL self-quenching or fluorescence resonance energy transfer (FRET) [14–16]. Microarray GM_1 ganglioside biosensors based on optical waveguide were also developed through either direct assay using fluorescently labeled CT or sandwich immunoassays using fluorescently labeled tracer antibodies [17]. Since the GM_1 ganglioside is lipophilic, it can be easily incorporated into either self-assembled lipid bilayers or liposomes. The liposome incorporated with the lipophilic GM_1 ganglioside can be used as a signal transducer in colorimetry or a signal amplifier in sandwich immunoassay. For example, colorimetric CT assay based on poly(diacetylene) liposomes incorporated with the lipophilic GM_1 ganglioside was developed [18]. The binding of CT at the interface of the liposome resulted in a

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colour change induced by conformational changes in the conjugated ene-yne polymer backbone. In sandwich immunoassays, target CT was first captured by CT-specific antibody and then exposed the GM_1-incorporated liposome incorporating signalling agents such as fluorophore, colouring agent, enzyme, or electroactive redox probe. For example, CT immunoassay coupled with the liposome containing over 22,000 fluorophores was benefited from significant signal amplification for each binding event [19]. CT immunoassays were also developed using GM_1-sensitized, dye-entrapped liposomes in a format of nitrocellulose test strip assay [20] and a flow injection analysis [21]. Luminol chemiluminescence CT immunosensor was also reported using supported lipid layer incorporated with ganglioside GM_1, where the captured CT was chemiluminescently detected with liposome probe functionalized with horseradish peroxidase as luminol CL catalyst [22]. The GM_1-functionalized liposomes containing either HRP or potassium ferrocyanide were also used in electrochemical sandwich immunosensors to enhance electrochemical signals in electrochemical impedance spectroscopy [23] and in adsorptive stripping voltammetry [24]. Although CT assays based on antibody or GM_1 ganglioside have shown good sensitivity and selectivity toward CT, they still raise a stability issue. In order to solve this limitation, monosaccharides composed of either sialic acid or *N*-acetylgalactosamine were used in a microarray to detect fluorescently tagged cholera toxin [25]. In addition, simple and rapid colorimetric assay based on disaccharide lactose-stabilized gold nanoparticles was developed [26]. The CTB binding to the lactose derivative on gold nanoparticles resulted in the aggregation of the gold nanoparticles and thus colour change.

In the present study, a simple and rapid bioassay for CT has been developed by using FRET between monosaccharide galactose-stabilized gold nanoparticles (AuNPs) and amine-terminated CdTe quantum dots. We have previously reported that specially synthesized monosaccharide α-mannose derivative self-assembled on AuNPs has strong affinity toward mannose binding protein (lectin) such as concanavalin A (Con A) and these mannose-stabilized AuNPs can be used for the determination of Con A as signal amplifiers in quartz crystal microbalance (QCM) [27] and also in stripping analysis [28]. In addition, it is reported that FRET can be obtained by combining the mannose-stabilized AuNPs with quantum dots [29]. On addition of the target Con A leads to the recognition-induced inhibition of FRET and thus fluorescence recovery. In the present work, it is shown that the specially synthesized monosaccharide β-galactose derivative self-assembled on AuNPs has strong affinity toward CT and thus can be used as a basis for a selective bioassay for CT. The association constant between the synthesized galactose and CT has been determined by using a quartz crystal microbalance study. The present bioassay provides a quantitative information on CT concentration over two orders of magnitude with a theoretical detection limit ($S/N = 3$) of 280 pM without the use of an extra step of signal amplification generally employed in typical sandwich-type immunoassays. Moreover, the present method is quite simple and rapid (within 10 min) because the fluorescent tagging of either analytes or secondary enzymes is not required.

2. Material and methods

2.1. Reagents

CT and Tris (hydroxymethyl) aminomethane hydrochloride were obtained from Sigma. Tris (hydroxymethyl) aminomethane was purchased from Sigma-Aldrich Chemical Co. Amine terminated PEG QDs (CdTe) were purchased from Invitrogen (ca. 4 nm diameter, Qdot® ITK™ amino (PEG), 8.0 μM). The gold nanoparticle (diameter: 10 nm) colloid was purchased from British Biocell International (Cat. No. EM.GC10, 5.7×10^{12} particles/mL). A 50 mM Tris buffer at pH 7.5 was prepared. Cholera toxin solutions were prepared in 50 mM Tris buffer at pH 7.5. Water for all solutions was purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Instrumentation

Absorption and fluorescence spectra were obtained using a UV-Vis absorption spectrophotometer (V-660, JASCO, Japan) and a spectrofluorometer (FP-6300, JASCO, Japan), respectively. Centrifugation of galactose-stabilized Au NPs was carried out with HA-1000-3 centrifuge (Hanil Science Industrial, Seoul, Korea). QCM study was carried out by QCA922 (Seiko EG&G Co., Ltd.) as a frequency analyzer. A gold QCM electrodes from Seiko EG&G Co., Ltd. (AT-cut, 9 MHz, area: 0.196 cm^2) was mounted in a custom-made QCM cell. 1 Hz of frequency change is equivalent to 5.45 ng/cm^{-2} of mass change in this experimental condition. Frequency shift during the experiments was recorded by WinEchem software (Seiko EG&G Co., Ltd.).

2.3. Preparation of galactose-stabilized AuNPs

The β-galactoside containing a thiol functional group at the terminal position (Fig. 1) has been prepared in a multistep sequence from galactosyl trichoroacetimidate and alcohol by K. S. Kim's laboratory according to our previous report [27] and the prepared thiolated galactose derivative was characterized by high-resolution mass spectrometer. The derivative showed peak at m/z 1017.55 corresponding to $C_{46}H_{90}O_{18}S_2$ $[M + Na]^+$. This derivative was present in disulfide form.

The poly-ethyleneglycol (PEG) spacer in the thiolated galactose was introduced to reduce nonspecific adsorption of proteins to AuNPs. The galactose-stabilized AuNPs were prepared by the displacement self-assembly in which commercially available citrate-capped AuNPs (ca. 10 nm diameter, British Biocell International, 5.7×10^{12} particles/mL, 1.0 mL) were reacted with 80 μM thiolated galactose solution (1.0 mL) for 12 hours. The mixture was centrifuged and filtered with Amicon Ultra-4 (Millipore, USA) in order to obtain the galactose-stabilized AuNPs. The filtered galactose-stabilized AuNPs were resuspended in 50 mM Tris buffer (pH 7.5). The final concentration of the AuNPs was determined to be 5.0×10^{12} particles/mL by UV-visible spectrophotometry.

2.4. Quartz crystal microbalance experiments

For the QCM experiments, a fresh QCM electrode was cleaned with ethanol, acetone and water to establish the optimal condition for the formation galactose self-assembled monolayer (SAM) on the electrode surface. The electrode was soaked overnight in 0.5 mM thiolated galactose solution. Sonication was carried out for 2 min after washing repeatedly with water, followed by drying in the air at room temperature. All QCM experiments have been performed at room temperature. A prepared galactose SAM-modified Au QCM electrode was mounted in a batch cell that allows only single side of QCM electrode to contact with solution. After incubating in 125 μL of phosphate buffer (pH 7.0) until a stable frequency obtained, 20 μL of CT solution with various concentrations were injected into the cell and the resulting frequency changes were recorded.

2.5. Bioassay for CT

FRET-based inhibition assay has been carried out for the detection of CT. Different concentrations of CT were added to the galactose-stabilized AuNPs (8.31 nM) and were incubated for 10 min. Then, the

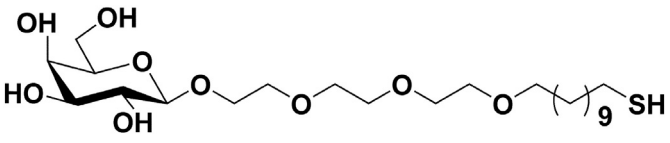

Fig. 1. Structure of thiol-modified β-galactose derivative.