Biosensors and Bioelectronics 65 (2015) 327–332

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Quantum dots and duplex-specific nuclease enabled ultrasensitive detection and serotyping of Dengue viruses in one step in a single tube

Wei Shen, Zhiqiang Gao *

Department of Chemistry, National University of Singapore, Singapore 117543, Singapore

ARTICLE INFO

Article history:
Received 2 September 2014
Received in revised form
21 October 2014
Accepted 27 October 2014
Available online 30 October 2014

Keywords:
Quantum dot
Duplex-specific nuclease
Dengue virus
Fluorescence
Biosensor.

ABSTRACT

Leveraging on the enzymatic processing of Dengue virus (DV) RNA hybridized quantum dot-capped DNA capture probes (QD–CPs), an ultrasensitive assay for the detection and serotyping of DVs is described in the report. Briefly, DV-specific DNA CPs are first capped by QDs and then conjugated to magnetic beads. In a sample solution, strands of DV RNA form heteroduplexes with the QD–CPs on the magnetic beads. The CPs together with the QDs in the heteroduplexes are subsequently cleaved off the magnetic beads by a duplex-specific nuclease (DSN), releasing the QDs to the solution, freeing the target RNA strands, and availing them for another around of hybridization with the remaining QD–CPs. After removing the magnetic beads along with unreacted (uncleaved) QD–CPs by using a permanent magnet, ultrasensitive fluorescent detection of DV is realized through the cleaved QDs. Serotyping of DV is accomplished by a judicious design of the QD–CPs. The assay combines excellent signal generation by the highly fluorescent QDs and the effortlessness of utilizing magnetic beads in the removal of the unreacted QD–CPs. The highly efficient DSN cleavage in conjunction with its excellent mismatch discrimination ability permits serotyping of DVs in one tube with excellent sensitivity and selectivity.

1. Introduction

Dengue virus (DV) poses significant global health risks for people living in the tropical and subtropical regions. The global prevalence of Dengue has grown dramatically in the past century with around 100 million cases of acute febrile disease each year (Scott, 2007). Although many infections are mild or asymptomatic, 5–10% of patients may experience severe and fatal Dengue hemorrhagic fever and hemorrhagic shock syndrome that claim more than 12,000 lives every year (Gubler, 1998). In spite of extensive research efforts, no vaccines for Dengue are yet available (Whitehead et al., 2007). The most effective prevention is to arrest the multiplication of mosquito vectors. On the other hand, the first clinical syndromes are not reliable means for diagnosing DV infection since similar syndromes are often observed in many other virus infections. Dengue diagnosis based on clinical syndromes must be verified by laboratory tests because more than half of infected individuals either are asymptomatic or have a mild undifferentiated fever (Endy et al., 2002). Early and rapid detection of DV infection during the febrile period is crucial for proper patient management and prevention of disease spread. Apart from vector control, the burden of Dengue on society can also be reduced through appropriate and timely clinical interventions to prevent severe morbidity and mortality. The clinical diagnosis of Dengue is mainly based on the physiological symptoms and physical examination; this is especially adopted in Dengue endemic areas (Whitehorn and Farrar, 2010). However, the symptoms of Dengue infection in the first few days are difficult to differentiate from other viral infections.

There is a great demand for the rapid detection and differentiation of DV infection in order to provide timely clinical treatment and etiologic investigation and disease control. A simple, highly sensitive and selective, and easy to use assay is needed for routine DV screening especially in the resource-limited rural healthcare settings where Dengue is endemic. Based on the nature of the target (analyte), laboratory diagnosis of DV infection can be classified to the detection of (1) specific virus, (2) viral antigen, (3) genomic sequence, and (4) antibody (World Health Organization, 1997). Considered as the “gold standard” for virus identification, the diagnosis of DV infection may be unambiguously confirmed by microbiological laboratory testing (Wiwanitkit, 2010).

With the backdrop of frequent outbreaks of Dengue all over tropical and subtropical countries, the cell microbiological laboratory tests are increasingly being replaced by viral antigen detection, specific antibodies (serology), and nucleic acid detection by PCR (Guzman et al., 2010). Among them, PCR-based nucleic acid assays and quantitative PCR (qPCR) in particular, are more sensitive than the antigen assays. Despite its excellent sensitivity and

* Corresponding author. Fax: +65 6779 1691.
E-mail address: chmgaoz@nus.edu.sg (Z. Gao).

http://dx.doi.org/10.1016/j.bios.2014.10.060
0956-5663/

acceptable specificity, widespread applications of qPCR at point-of-care are severely hindered by the stringent demands on sample preparation and the requirement for the complex instruments in signal readout (World Health Organization, 2014; Pritchard et al., 2012). More seriously, qPCR is subject to false-negative results and all tests may be negative in the early stage of DV infection (Gijavanekar, 2011; Ranjit and Kissoon, 2011).

The above-mentioned assays are only of diagnostic value during the acute phase of the illness. Therefore, there is an ever-increasing need for rapid and accurate diagnostic assays to detect DVs at point-of-care. In this work, we developed a simple and ultrasensitive assay for the detection and serotyping of all four DVs in one step in a single tube through engaging an isothermal target cycling amplification process. The amplification was facilitated by a duplex-specific nuclease (DSN) and the uses of quantum dots (QDs) as signal generators. Comparing to organic dyes, QDs are 20 times brighter and much more photostable (Chan and Nie, 1998). Moreover, the exceptional amplification power of the DSN, the cumulative nature of the signal generation process, and the high fluorescence intensity of QDs permitted the detection of DVs down to 10^2 viral RNA strands/μL. The excellent mismatch discrimination ability of the DSN will allow DVs to be detected with high confidence. The isothermal amplification scheme coupled to the simple assay protocol will make direct, rapid, and ultrasensitive detection of DVs in pristine clinical samples with little sample preparation possible.

2. Experimental section

2.1. Materials and apparatus

The DSN (EC 3.1.30.-, extracted from *Paralithodes camtschaticus*) was purchased from Evrogen (Moscow, Russia). Exonuclease I (Exo I) was purchased from New England Biolabs (Ipswich, MA, USA). Four types of carboxylic acid-coated CdSeS/ZnS alloyed QDs with λ_{em} at 450, 520, 580, and 660 nm were purchased from Sigma-Aldrich (St. Louis, MO). Dual-labeled (terminal amine and biotin) DNA CPs of the following sequences: 5′-A_9 TGA TAT GTC TCT GAG TAT GTA TCC AAG TT A_9 (for DV-1), 5′-A_9 GGA AAT GTC CCT TAA TAT GTA GCC TAG CT A_9 (for DV-2), 5′-A_9 GCT CAC ATC TCT TAA AAT GTA GCC TAG CC A_9 (for DV-3), 5′-A_9 GTC TAT CTC CTC CAG GAT ATA TCC CAA TC A_9 (for DV-4), synthetic RNAs, and all other oligonucleotides of PCR purity were obtained from Integrated DNA Technologies (Coralville, IA, USA). Ethyl (dimethylaminopropyl) carbodiimide (EDC) was purchased from Pierce (Rockford, IL, USA). Streptavidin-coated magnetic beads were acquired from Life Technologies (Carlsbad, CA, USA). The hybridization/DSN incubation medium was made of pH 8.0 50 mM Tris buffer containing 20 mM $MgCl_2$ and 1.0 mM DTT (TMD buffer). All centrifuge tubes were autoclaved and all surfaces were treated with RNaseZap to eliminate any possible RNase contaminations. Nuclease-free ultrapure water was used in all solutions preparations. Fluorescence spectra were acquired on a Fluorolog®-3 spectrofluorometer (Jobin Yvon Inc., Edison, NJ, USA) using a microcuvette.

2.2. Preparation of the QD–CP tethered magnetic beads

The four types of CPs were first conjugated to their respective QDs through carbiimide coupling chemistry. Briefly, the carboxylic acid-coated QDs were suspended in 200 μL of pH 5.5 10 mM phosphate buffer. Then, the amine-terminated CPs were added into above solution with a final concentration of 1.0 μM. Subsequently, 100 μL of 0.020% freshly-prepared EDC solution was added and the reaction mixture was incubated for 24 h at room temperature with gentle shaking. After centrifugation, the supernatant was carefully removed. Purification of the QD–capped CPs (QD–CPs) form excess oligonucleotides was performed by three cycles of repetitive centrifugation and dispersion of the QD–CPs after conjugation. The purified QD–CPs were then tethered to the magnetic beads by incubating them with the streptavidin-coated magnetic beads in pH 8.0 10 mM Tris buffer containing 10 mM EDTA and 0.20 M NaCl for 30 min. Thereafter, the excess of CPs on the QDs was hydrolyzed into mononucleotides by a period of 60 min incubation with Exo I, whereas the CPs linked to the magnetic beads remained intact because of significant steric hindrance and terminal protection. It was found that $\sim 4.5 \times 10^5$ QD–CPs are attached to each magnetic bead, which is ~20% of the binding capacity of the magnetic beads (Invitrogen, 2011). After three thorough washes with ultrapure water, the QD–CP-tethered magnetic beads were kept in the dark at 4 °C in a refrigerator and ready for use. The QD–CPs tethered to the magnetic beads were stable for at least six months. However, the fluorescence intensity of the QDs was found to decrease over a much longer storage time due to the deterioration of the QDs.

2.3. DV detection and serotyping

Total RNA in DV was extracted using a viral RNA extraction kit from Qiagen (Valencia, CA, USA) according to the manufacturer′s recommendation. Briefly, 0.50 mL of DV sample was added to 5 mL of Qiazol solution. The mixture was vortexed and incubated for 15 min at room temperature. After adding 1.0 mL of chloroform the mixture was thoroughly mixed and centrifuged at $1.3–1.5 \times 10^4$ g for 10 min at 4 °C. The total RNA in the aqueous phase was further purified according the procedure recommended by the manufacturer. DV detection and serotyping were performed in microplates or microcentrifuge tubes. Each reaction mixture contains 1 × TMD buffer, 0.5 U/μL of RNase inhibitor, 500 nM QD–CPs, and 0.50 U the DSN. The total volume of the reaction mixture was ≤ 20 μL. The obtained mixture was incubated at room temperature for 60 min to allow target RNA hybridization and the QD–CP cleavage. After removing the magnetic beads together with the unreacted QD–CPs, the fluorescence intensities of the cleaved QDs were used to quantify DVs and their emission profiles (emission wavelengths) were used to serotype DVs. In addition, a control was run in parallel for each assay. The control solution contained all components of the assay and a totally noncomplementary synthetic RNA. The fluorescence readings were always subtracted from that of the control.

3. Results and discussion

3.1. Assay strategy

The working principle of the assay is schematically described in Fig. 1. As shown in Fig. 1, the principal steps of the assay are: (a) preparation of the QD–CP-magnetic bead conjugates, (b) hybridization of DV RNA strands to the DNA CPs to form RNA–DNA heteroduplexes, (c) cleavage of the CPs in the heteroduplexes by the DSN and freeing the target RNA stands, (d) hybridization of the freed target RNA strands to the remaining QD–CPs on the magnetic beads – the establishment of an isothermal target cycling amplification process, and (e) magnetic separation of the magnetic beads along with unreacted QD–CPs. As a result, a large amount of the highly fluorescent QDs are released in solution, resulting in the appearance of a fluorescence signal. The target-recycling amplification and the cumulative nature of the DSN cleavage process eventually lead to significant signal enhancement when a sufficient amount of the DSN is used and a long enough hybridization/DSN incubation time is employed. DV serotyping is accomplished