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Localized surface plasmon resonance-mediated fluorescence signals in plasmonic nanoparticle-quantum dot hybrids for ultrasensitive Zika virus RNA detection via hairpin hybridization assays

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ABSTRACT

The current epidemic caused by the Zika virus (ZIKV) and the devastating effects of this virus on fetal development, which result in an increased incidence of congenital microcephaly symptoms, have prompted the World Health Organization (WHO) to declare the ZIKV a public health issue of global concern. Efficient probes that offer high detection sensitivity and specificity are urgently required to aid in the point-of-care treatment of the virus. In this study, we show that localized surface plasmon resonance (LSPR) signals from plasmonic nanoparticles (NPs) can be used to mediate the fluorescence signal from semiconductor quantum dot (Qdot) nanocrystals in a molecular beacon (MB) biosensor probe for ZIKV RNA detection. Four different plasmonic NPs functionalized with 3-mercaptopropionic acid (MPA), namely MPA-AgNPs, MPA-AuNPs, core/shell (CS) Au/AgNPs, and alloyed AuAgNPs, were synthesized and conjugated to L-glutathione-capped CdSeS alloyed Qdots to form the respective LSPR-mediated fluorescence nanohybrid. The concept of the plasmonic NP-Qdot-MB biosensor involves using LSPR from the plasmonic NPs to mediate a fluorescence signal to the Qdots, triggered by the hybridization of the target ZIKV RNA with the DNA loop sequence of the MB. The extent of the fluorescence enhancement based on ZIKV RNA detection was proportional to the LSPR-mediated fluorescence signal. The limits of detection (LODs) of the nanohybrids were as follows: alloyed AuAgNP-Qdot646-MB (1.7 copies/mL)) > CS Au/AgNP-Qdot646-MB (LOD =2.4 copies/mL) > AuNP-Qdot646-MB (LOD =2.9 copies/mL) > AgNP-Qdot646-MB (LOD =7.6 copies/mL). The LSPR-mediated fluorescence signal was stronger for the bimetallic plasmonic NP-Qdots than the single metallic plasmonic NP-Qdots. The plasmonic NP-Qdot-MB biosensor probes exhibited excellent selectivity toward ZIKV RNA and could serve as potential diagnostic probes for the point-of care detection of the virus.

1. Introduction

The history of Zika virus (ZIKV) dates back to 1947 when it was first isolated from a rhesus monkey in the East African country of Uganda and was named after the Zika forest, the geographical location where the virus was isolated (Abushouk et al., 2016; Gebre et al., 2016; WHO, http://www.who.int/emergencies/zika-virus/timeline/en/). The first reported occurrence of ZIKV infection in humans was reported in 1952 (Macnamara, 1954), and within 60 years after the virus was first isolated, 13 cases of sporadic infection have been reported (Fagbami, 1979; Moore et al., 1975; Olson et al., 1981). The World Health Organization (WHO) declared on the 1st of February 2016 that Guillain-Barré syndrome and an increased incidence of congenital microcephaly were symptoms associated with ZIKV infection, thus raising an international public health concern (WHO, http://www.who.int/mediacentre/news/statements/2016/emergency-committee-zika-microcephaly/en/). In Brazil alone, approximately 1.5 million people have been estimated to be infected by the virus, and more than 20 countries have reported cases of ZIKV transmission (Kindhauser et al. (2016); Lazear and Diamond (2016)). The ZIKV is a single-stranded positive-sense RNA virus, which belongs to the *Flavivirus* genus of the *Flaviviridae* family (Enfissi et al., 2016). The virus can be transmitted

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sexually, from an infected mother to the fetus (Calvet et al., 2016), through day-time female *Aedes albopictus* and *Aedes aegypti* mosquito bites (Sikka et al., 2016) and through blood transfusion (Marano et al., 2016).

Current laboratory diagnostics for the ZIKV are primarily based on a serological analysis and a molecular diagnosis (reverse-transcriptase polymerase chain reaction (RT-PCR)). The serological analysis, which involves utilizing an enzyme-linked immunosorbent assay (ELISA) to detect IgM antibodies in the serum sample, suffers from cross reactivity of antibodies among flaviviruses, thereby decreasing the specificity of the technique (Gourinat et al., 2015; Lanciotti et al., 2008). RT-PCR can detect ZIKV RNA at 530 aM (320 copies/μL) within a total assay time of 90 min (Faye et al., 2013a, 2013b; Musso et al., 2014). However, due to the decrease in viremia over time, RT-PCR analysis is effective only during the onset of symptoms, which occurs in the first 7 days when the ZIKV load is at a level of ~1 fM in human fluid samples (Barzon et al., 2016; Haug et al., 2016). Beyond this time period, i.e., ≥4 days after symptom onset, reliable data can be obtained only via labor-intensive diagnoses (Petersen et al., 2016). With respect to the sensitivity of Zika RNA detection using RT-PCR, detection limits of 10^3 to 10^{11} copies/ mL (Waggoner et al., 2016), 140 copies viral RNA/ reaction (Balm et al., 2012) and 0.05 pfu/mL (Faye et al., 2013a, 2013b) have been reported. In addition, a paper-based synthetic gene detection platform for ZIKV RNA was reported to exhibit a detection limit of 1.7×10^6 copies/mL (Pardee et al., 2014). The summarized detection limits suggest that improved sensitivity of ZIKV detection is still needed. Generally, there is an urgent need for the development of more efficient and reliable diagnostic probes for ZIKV that can alleviate the challenges of point-of-care testing for pregnant women, blood donors and travelers from regions with endemic transmission.

Biosensor probes that utilize the phenomenon of plasmonic signal transduction have emerged as useful alternatives for enhancing the sensitivity of detection platforms (Adegoke and Park, 2016; Hall et al., 2011). The exploitation of plasmonic metallic nanoparticles (NPs) in biosensing applications has been inspired by the interaction of these particles with electromagnetic waves, which induce oscillations of the plasmons (free electrons) at the particle surface (Law et al., 2011). This phenomenon has led to the development of localized surface plasmon resonance (LSPR)-based biosensors for various analytes (Anker et al., 2008; Hutter et al. (2003)). Exploiting LSPR signals to trigger the transduction of optical changes in fluorescent semiconductor quantum dot (Qdot) nanocrystals within a molecular beacon (MB) biosensor probe for targeting an oligonucleotide sequence can be a unique strategy to construct ultrasensitive detection platforms for viral nucleic acids.

The phenomenon of hybridizing the loop sequence of an MB probe with the target nucleic acid sequence is reflected in the optical transduction changes in the reporter fluorophore. The ultrasensitive detection of the target nucleic acid sequence is dependent on the choice of reporter fluorophore. In this work, we developed four ultrasensitive LSPR-induced Qdot-MB biosensors for ZIKV RNA. ZIKV RNA was extracted, and the loop of the MB was designed to hybridize with the RNA of the virus genome. Four different plasmonic NPs, namely gold NPs (AuNPs), silver NPs (AgNPs), bimetallic core/shell (CS) Au/AgNPs and alloyed AuAgNPs, were synthesized and functionalized with 3-mercaptopropionic acid (MPA). Each of the MPA-functionalized plasmonic NPs was bonded to $_L$-glutathione (GSH)-capped CdSeS alloyed Qdots to form novel fluorescent nanohybrid systems, which were subsequently conjugated to an MB and utilized as ultrasensitive LSPR-fluorescence signal transducers for ZIKV RNA detection. Optical engineering of the alloyed CdSeS Qdots ensured the appropriate selection of the Qdot size based on the photoluminescence (PL) quantum yield (QY) value. Our MB biosensor platform shows that each of the plasmonic NPs can induce LSPR signals to trigger ultrasensitive fluorescence transduction changes in the alloyed Qdots for the detection of extremely low concentrations of ZIKV RNA. Notably, the extent of the LSPR-fluorescence signal enhancement was dependent on the plasmonic nanostructure, with the bimetallic NPs triggering a higher LSPR-fluorescence signal intensity than the single metallic plasmonic NPs. Our work is the first to construct an ultrasensitive LSPR-mediated fluorescence Qdot-MB biosensor for ZIKV RNA.

2. Materials and methods

2.1. Materials

Cadmium oxide (CdO), trioctylphosphine (TOP), octadecene (ODE), hexadecylamine (HDA), trioctylphosphine oxide (TOPO), selenium (Se), $HAuCl_4\cdot3H_2O$, sulfur (S), rhodamine 6G, MPA, GSH, tannic acid, silver nitrate ($AgNO_3$), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma Aldrich Co. LLC (Saint Louis, MO, USA). Oleic acid (OA) was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Potassium hydroxide (KOH), methanol, tri-sodium citrate, acetone and chloroform were purchased from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). Norovirus RNA of genogroup II (G11) was purchased from Vircell Microbiologists (Granada, Spain). Clinically isolated influenza virus A/Yokohama/110/2009 (H3N2) was supplied by Dr. C. Kawakami of the Yokohama City Institute of Health, Japan. ZIKV strain PRVABC-59 was kindly provided by Professor Kouichi Morita of Institute of tropical medicine Nagasaki University. The ZIKV sample was obtained from culture supernatant that was collected from African green monkey kidney-derived Vero cells infected with ZIKV. Due to the absence of ZIKV outbreak in Japan and because only a few reported ZIKV-positive cases introduced from people from other countries to date, it is however quite difficult to obtain virus samples from infected patients. However, we can expect no possible drawback in the case of clinical samples, such as patients' sera because total RNAs are only extracted from human sera as efficiently as those from cell-culture supernatants according to the RNA isolation protocol used in this study.

An ultrapure Milli-Q water system was used for sample preparation. The MB with a loop sequence specific for the target ZIKV RNA was synthesized and purified via HPLC by FASMAC (Kanagawa, Japan). The loop of the MB was composed of a 23-bp single-stranded DNA with the 5' terminus labeled with an amino group (NH_2) and the 3' terminus conjugated to the fluorescence quencher 4-((4-(dimethylamino)phenyl) azo)benzoic acid (dabcyl). The MB oligonucleotide sequence is as follows:

5'-/NH_2-/**<u>GCGAC</u>**CACGGGCTACTCCGCGTTTTAGC**<u>GTCGC</u>**-dabcyl/-3'.

The stem domain is indicated by the underlined sequence.

The sequence of the ZIKV RNA targeted is: GCTAAAACGCGGAGTAGCCCGTG. This target sequence is conserved in ZIKV strains and doesn't match with the closely related *Flavivirus* species.

2.2. Equipment

Fluorescence emission measurements and UV/vis absorption were performed using a filter-based multimode microplate reader (Infinite® F500, TECAN, Ltd., Männedorf, Switzerland). Powder X-ray diffraction (PXRD) measurements were obtained using a RINT Ultima XRD instrument (Rigaku Co., Tokyo, Japan) with a Ni filter and a Cu-Kα source. The data were collected from 2theta =5–90° at a scan rate of 0.01°/step and 10 s/point. Transmission electron microscopy (TEM) images were captured using a TEM (JEM-2100F, JEOL, Ltd., Tokyo, Japan) operated at 100 kV. Dynamic light scattering (DLS) and zeta potential (ZP) measurements were performed using a Zetasizer Nano series. Data analysis was performed using the Malvern Instrument Dispersion Technology software (version 7.1).