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Single-step detection of norovirus tuning localized surface
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Abstract

A new method of label free sensing approach with superior selectivity and sensitivity towards virus detection is presented here, employing the localized surface plasmon resonance (LSPR) behavior of gold nanoparticle (AuNPs) and fluorescent CdSeTeS quantum dots (QDs). Inorganic quaternary alloyed CdSeTeS QDs were capped with L-cysteine via a ligand exchange reaction. Alternatively, citrate stabilized AuNPs were functionalized with 11-mercaptoundecanoic acid to generate carboxylic group on the gold surface. The carboxylic group on the AuNPs is subjected to bind covalently with the amine group of L-cysteine capped CdSeTeS QDs to form CdSeTeS QDs/AuNPs nanocomposites. The fluorescence of CdSeTeS QDs/AuNPs nanocomposite shows quenched spectrum of CdSeTeS QDs at 640 nm due to the close interaction with AuNPs. However, after successive addition of norovirus-like particles (NoV-LPs), steric hindrance-induced LSPR signal from the adjacent AuNPs triggered the fluorescence enhancement of QDs in proportion to the concentration of the target NoV-LPs. A linear range of $10^{-14}$ to $10^{-9}$ g mL$^{-1}$ NoV-LPs with a detection limit of 8.2 fg mL$^{-1}$ was obtained. This method was further applied on clinically-isolated norovirus detection, in the range of $10^{2}$ – $10^{5}$ copies mL$^{-1}$ with a detection limit of 87 copies mL$^{-1}$, which is 100-fold higher than commercial ELSA kit. The superiority of the proposed sensor over other conventional sensors is found in its ultrasensitive detectability at low virus concentration even in clinically-isolated samples. This proposed detection method can pave an avenue for the development of high performance and robust sensing probes for detection of virus in biomedical applications.

Keywords: Biosensor; CdSeTeS; Gold nanoparticle; Localized surface plasmon resonance; Norovirus detection; Quantum dots.
1. Introduction

The current progress in search of optically active nanocomposite has driven the development of variety of applications in diverse fields ranging from biomedical engineering to environmental safety (Dutta Chowdhury and Doong, 2016; Ganganboina et al., 2017; Hsu et al., 2016; Kuila et al., 2011; Lee et al., 2007). Although several developments of optical biosensors have been experienced an exponential growth during the last decade due to the incorporation of nanotechnology for the direct, real-time and label-free detection of many chemical and biological substances (Anh et al., 2017; Chowdhury et al., 2012; Dutta Chowdhury et al., 2017), but there are very few reports which come to appear in real applications. Fluorometric assays are the most often applied methods on optical sensing and comes in a variety of schemes due to its easy technique and reliable outcomes (Al-Ogaidi et al., 2014; Huang et al., 2014). Parameters that are being analyzed in such sensors include fluorescence intensity, decay time, quenching efficiency and regeneration of fluorescence or luminescence energy transfer. Among these, the most innovative and recently evolved optical biosensors are those based on surface plasmon resonance (SPR) properties using different gold nanocomposites (Kawaguchi et al., 2008; Lee et al., 2015; Singh and Strouse, 2010; Yeom et al., 2013). These biosensors have been widely used in the fields for detection of infectious diseases related with cells, bacteria or viruses. Generally, fluorescent quantum dots (QDs) have been widely applied as fluorescence reporters in various LSPR based biosensor whereas the SPR generated AuNPs plays the crucial role to influence the fluorescence signal depending on the size, shape and distance (Lee et al., 2015; Takemura et al., 2017). Triggering with the analyte, it can alter the position as well as distance between these two nanocomponents (QDs and AuNPs), resulting in the variation of signal detection.

Inorganic quantum dots (QDs) are semiconductor nanocrystals whose electrons and holes are quantum-confined from all dimensions. Due to their unique physical and optical properties
with respect to the corresponding bulk materials such as conventional organic dyes or fluorescent proteins, QDs have emerged as a new class of fluorescent agents for biomedical applications (Ahmed et al., 2016; Anderson and Chan, 2008; Lee et al., 2015; Tian et al., 2012). However, poor bio-conjugating ability and high toxicity restrict their potential use for the development of biosensor. To overcome this, lots of efforts have been suggested to improve the performance of QDs in biomedical applications through proper surface modifications. Few chalcogenides such as ZnS, TeS etc. are widely used to shell-coat the QD cores to improve the physiochemical and optical performance of the QDs as well as their biocompatibility in biological system (Adegoke et al., 2015; Liang et al., 2009; Liang et al., 2010).

There are many attempts on optical sensing which are reported on virus detection as the conventional diagnostic systems still have certain limitations. Induced antibody detection on serological analysis can lead to false negative or false positive misguided data interpretation. Most authentic viral culture analysis is time consuming whereas immunofluorescence assays are limited to their sensitivity. Therefore, there is an utmost need for the development of rapid, highly sensitive and selective diagnostic sensor for the virus detection. Pang et al., (2015) reported a fluorescent aptasensor system for the sensitive detection of influenza virus H5N1 in human serum by guanine-enriched anti-rHA aptamers immobilized on the surface of the Ag@SiO$_2$ nanoparticles which performed as a metal-enhanced fluorescence sensing platform. Similarly, Wu et al., (2015) developed an enzyme-induced bi-functional magnetic electrochemical immunosensor to detect Influenza virus A (H7N9) in complex media. In our previous work, detection of influenza virus A (H1N1) has been reported on a combination of LSPR-induced optical transduction from antibody-labeled AuNPs and the fluorescence signal generated from adjacent antibody-conjugated CdSeTeS QDs (Takemura et al., 2017). However, using several antibodies or aptamer-conjugated antibodies is making those systems complicated and expensive. Moreover, the high fluorescence signal of background also
hampers the enhancement of surface plasmon signal, which is disadvantage of this method. To overcome this, here we have developed a new method of LSPR-induced optical transduction between AuNPs and CdSeTeS QDs with a single step process to detect NoV-LPs. The covalent attachment between AuNPs and CdSeTeS QDs forms the rigid sensing probe of CdSeTeS QDs/AuNPs which can sufficiently decreases the nonspecific interaction, resulting the increasing sensitivity. Norovirus (NoV) which is mostly common causes for gastroenteritis disease, generally transmitted through shellfish consumption and food and waterborne routes. As the levels of enteric viruses in bivalve mollusk or in mussels are generally in very low concentration, the high sensitive technique is in demand for its early detection. To establish the detection technique, initially we have taken the NoV-LPs as a target analyte, because there is no robust cell culture system for the NoV propagation to date. The anti-Nov antibody-conjugated CdSeTeS QDs are covalently linked with AuNPs, quenching the fluorescence of CdSeTeS QDs/AuNPs nanocomposites which has been used as the sensing probe for a single step label free NoV-LPs detection. The detection mechanism of the biosensor involves the regeneration of quenched fluorescence of CdSeTeS QDs/AuNPs due to LSPR while the attached NoV-LPs create steric hindrance between two nanomaterials as depicted in Scheme 1.

2. Methods and Materials

2.1. Materials

PBS buffer, sodium citrate, polyoxyethylene (20), sorbitan monolaurate (Tween 20), hydrogen peroxide, sulfuric acid, methanol, potassium hydroxide (KOH), tri-sodium citrate, chloroform and acetone were purchased from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). Tetramethylbenzidine (TMBZ) was purchased from Dojindo (Kumamoto, Japan). HAuCl₄, N-
(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-hydroxy succinimide (NHS), bovine serum albumin (BSA), 11-mercaptoundecanoic acid (MUDA), 1-octadecene, cadmium oxide (CdO), tellurium (Te), L-cysteine, hexadecylamine (HDA), trioctylphosphine oxide (TOPO), trioctylphosphine (TOP), selenium (Se) and sulfur (S) were purchased from Sigma Aldrich Co., LLC (Saint Louis, MO, USA). Oleic acid was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Goat anti-rabbit IgG-horseradish peroxidase (HRP) was purchased from Santa Cruz Biotechnology (CA, USA). Anti-NoV monoclonal antibody against NoV was purchased from Abcam Inc. (Cambridge, UK). Zikavirus and influenza virus A (H3N2) for selectivity test were kindly provided by Professor K. Morita of Institute of Tropical Medicine Nagasaki University and Dr. C. Kawakami of the Yokohama City Institute of Health (Yokohama Japan), respectively.

2.2. Synthesis of CdSeTeS QDs

Organometallic hot-injection synthesis of quaternary-alloyed CdSeTeS QDs was carried out according to our previously reported method using CdO, Se, S as the basic precursors (Adegoke et al., 2015).

2.3. Capping of CdSeTeS QDs

To make the hydrophilic QDs from the synthesized hydrophobic CdSeTeS QDs and to functionalize its surface with amine group, the L-cysteine was conjugated via a ligand exchange reaction. A methanolic-KOH-L-cysteine solution was prepared by dissolving 3 g of KOH in 40 mL of methanol and 2 g of L-cysteine. The hydrophobic QDs in chloroform solution were added to the methanolic-KOH-L-cysteine solution, and an appropriate volume of ultrapure deionized (DI) water was added to precipitate the hydrophilic QDs from solution. The solution was stirred for several mins and was allowed to stand overnight for complete separation of the
organic phase from the water-soluble phase. The QDs were repeatedly purified using acetone and chloroform.

2.4. Synthesis of AuNPs

For the preparation of AuNPs, 35 µL of 2 mM HAuCl₄ and finally 300 µL of 100 mM trisodium citrate were added into 25 mL of pure boiling water under vigorous stirring condition (Zhao et al., 2008). The whole solution was boiled and stirred for 15 min until the color changes to pink.

2.5. Functionalization of AuNPs with 11-mercaptoundecanoic acid

The AuNPs was attached with the MUDA to generate carboxylic acid group in to the surface. The AuNPs solution was stirred for 2 h with 0.1 mM of MUDA at pH 3 where the thiol group has been covalently linked with the AuNPs via soft acid soft base interaction. After successful synthesis of the AuNP-MUDA, the nanoparticle was washed several times with DI water and centrifuged at 6000 × g to obtain excess MUDA free AuNPs.

2.6. Synthesis of sensing probe

Initially, the anti-NoV antibody was conjugated with the free carboxylic group of L-cysteine capped CdSeTeS QDs via EDC/NHS covalent chemistry. Then, the MUDA functionalized AuNPs was covalently linked with the free amine group of L-cysteine capped antibody-linked QDs via EDC/NHS reaction (Valeur and Bradley, 2009). In brief, EDC is mixed with the carboxylic functionalized AuNPs and then further activated with NHS for 30 min before addition of the antibody conjugating QDs (Ab-QDs). The conjugate mixture was stirred overnight at 7 °C to form antibody-conjugating nanocomposites (Ab-CdSeTeS QD/AuNPs) which were purified by centrifugation (3000 × g) for 5 min and subsequently dissolved in 2 mL of ultrapure DI water.
2.7. Enzyme linked immunosorbent assay (ELISA)

Antibody conjugated CdSeTeS QD/AuNPs nanocomposite before and after NoV-LP addition along with its negative controls of bare QDs, AuNPs, BSA and DI water were taken (100 µL) in different well of a nonsterile polystyrene 96-well flat-bottom microtiter plate (Becton Dickinson Labware, NJ, USA) to perform ELISA test. 100 µL of 5 % skim milk solution was added as a blocking agent after washing 3 times with PBS buffer, containing 0.1 % Tween 20. After blocking, the 5% skim milk was removed by washing 3 times with the buffer. After that, anti-rabbit IgG-HRP was diluted to 1:4000 with 2 % BSA, and 100 µL of this solution was added to the well and was incubated for 1 h. 3,3’,5,5’-tetramethylbenzidine or TMB (100 µL) was then added to the each well which was initiated by its characteristic blue coloration. The reaction was then stopped by adding 50 µL of 10 % H₂SO₄, which changed the color of the solution from blue to yellow due to its diimine formation. The absorbance of the solution was measured using a microplate reader at 450 nm with a reference filter of 655 nm.

2.8. Physicochemical analysis

To check the size and surface morphology, transmission electron microscopy (TEM) images were obtained using a TEM (JEM-2100F; JEOL, Ltd., Tokyo, Japan) operated at 100 kV. UV-Vis absorption and fluorescence emission measurements were carried out using a filter-based multimode microplate reader (Infinite® F500; TECAN, Ltd, Männedorf, Switzerland). Powder X-ray diffraction (PXRD) analysis was carried out using a RINT ULTIMA XRD (Rigaku Co., Tokyo, Japan) with a Ni filter and a Cu-Kα source. Dynamic light scattering (DLS) measurements were performed using a Zetasizer Nano series (Malvern Inst. Ltd., Malvern, UK). Conjugation of the antibody to the Ab-QDs and Ab-CdSeTeS QD/AuNPs nanocomposites were confirmed using a plate reader from Bio-Rad (Model 680; Hercules, USA).
2.9. Preparation of NoV-LPs and clinically-isolated NoVs

NoV-LPs were prepared according to the standard method of VLP preparation (Ahmed et al., 2017; Jiang et al., 1992). Clinically isolated NoVs were collected from fecal samples of the patients with infectious gastroenteritis, including foodborne illness, by inspections based on laws and ordinances. This NoV sampling was carried out according to the guideline, after getting the approval by Ethics Committee of Environment and Hygiene Institute in Shizuoka Prefecture (September 14, 2016).

2.10. Fluorometric sensing of NoV-LPs and clinically-isolated NoVs using the CdSeTeS QD/AuNPs sensing probe

CdSeTeS QD/AuNPs nanocomposite was mixed in different concentration of 20 µL volume of the target NoV-LPs as well as clinically-isolated NoVs and incubated for 1 min before fluorescence measurements were acquired. The detection of NoV-LPs in the concentration range of 10 fg mL⁻¹ – 100 ng mL⁻¹ was carried out in DI water. The sample solution was excited at 450 nm, and the fluorescence intensity was measured in a range of 500 – 700 nm. The human serum was diluted 10 times before to spike the NoV-LPs in to it. Clinically isolated NoVs were also detected with the sensor probe in the similar way to measure the fluorometric response.

2.11. Quantification of clinically-isolated NoVs using real-time PCR

NoV RNAs were extracted from 10 % fecal suspension in PBS by using QIAamp Viral RNA Mini Kit (QIAGEN, Tokyo Japan), and after treated with recombinant DNase (RNase-free) (TaKaRa Bio Inc., Shiga, Japan), reverse transcription was performed by using Prime Script RT Reagent Kit (Perfect Real Time) (TaKaRa Bio Inc.). Obtained cDNAs were detected and quantified by real-time PCR technique by using Premix EX Taq (Probe qPCR) (TaKaRa...
Bio Inc.) in accordance with the notice of the Ministry of Health, Labor and Welfare, Japan (2003).

3. Results and discussion

The central theme in this work is to build a new and simple method to detect virus directly without any pretreatment of analytes. Here, we have successfully synthesized an Ab-CdSeTeS QD/AuNPs sensing probe which is able to detect the NoV-LPs by measuring the fluorescence intensity after 1 min of the sensor probe addition (as depicted in Scheme 1). Due to the covalent attachment between CdSeTeS QDs and AuNPs, it causes strong fluorescence quenching of the QDs, initially. After addition of different virus concentration, the Ab-CdSeTeS QD/AuNPs bind with the target due to the presence of monoclonal antibody in between the QDs and AuNPs. This antibody-antigen interaction induces steric hindrance, which causes the optimum distance for LSPR between these two nanoparticles, resulting fluorescence enhancement. The enhancement is proportionated with the concentration of the target NoV-LPs as well as in real NoV analytes, confirming proficient detection ability of the proposed nanobiosensor. Unlike other conventional methods of LSPR detection, here, the sensor nanocomposite is conjugated with a single antibody and capable to detect the virus, without any pre-treatment just after addition in to the sensing sample.

3.1. Synthesis of sensing probe and characterizations

The morphology as well as size distribution of two individually synthesized nanoparticles was examined first. The shape and morphological properties of the CdSeTeS QDs were analyzed using TEM. A monodisperse particle distribution is observed while the particle shape is consistently spherical across the entire TEM image (Fig. 1A). The particle size distribution
has been given in the inset of Fig. 1A where the highly homogenous distribution is found in
the range of 3 – 9 nm with the average particle size of 5.9 ± 0.6 nm. The UV-Vis spectrum of
the synthesized CdSeTeS QDs is given in Fig. S1, showing the signature absorption hump of
CdSeTeS QDs even after the antibody conjugation which confirms the successful synthesis.
Similarly, the citrate stabilized AuNPs are also evenly distributed in the range of 7 – 15 nm
with the average particle size of 11.4 ± 0.5 nm (Fig. 1B). A single TEM image of an isolated
AuNP is given in the inset of Fig. 1B where it is clearly seen the exact spherical nature of the
AuNPs. After incorporation with the MUDA, the agglomeration has been reduced to some
extent due to the coating of the organic layer on the surface. The capping of the organic layer
is verified by the UV-Vis spectra, presented in the supplementary information (Fig. S2). After
successful preparation of the Ab-CdSeTeS QD/AuNPs with anti-Nov antibody conjugation,
the nanocomposite was further characterized by TEM. In Fig. 1C, it is clearly observed the
close conjugation of the QDs with the AuNPs due to their covalent attachment. The antibody
conjugation with the CdSeTeS QD/AuNPs nanocomposites was confirmed by ELISA. The
absorbance peak in ELISA of the bare CdSeTeS QDs is negligible as expected. The antibody
loading is increased to the highest level in case of Ab-CdSeTeS QD/AuNP, confirming the
successful antibody conjugation. However, after the NoV-LPs loading, the ELISA signal is
decreased obviously due to the less availability of the active site of the NoV-LPs covered Ab-
CdSeTeS QD/AuNPs nanocomposites. Overall, the ELISA of different stages of CdSeTeS
QD/AuNPs supports the successful conjugation of antibodies with the nanocomposites.

The CdSeTeS QD/AuNPs nanocomposites were further characterized by XRD spectra to
illustrate the crystal nature of the QD nanocrystals. The diffraction pattern of the QDs indicates
that the QDs are crystalline and cubic in nature (Fig. 2A), exhibiting three characteristic peaks
at 2theta of 24.9°, 42.3° and 50.6° for (111), (220) and (311) crystal planes (Adegoke et al.,
2015; Li et al., 2016; Yang et al., 2013). The position of all these peaks remains unchanged
after functionalization with AuNPs, indicating that the attachment only takes place in the functional groups of the CdSeTeS QD nanocomposites without affecting the crystal structure. In addition, a small but clear peak at 2theta = 37.9° has been introduced due to the incorporation of the (111) plane of AuNPs on the nanocomposites (Krishnamurthy et al., 2014), supporting the successful formation of the CdSeTeS QD/AuNPs nanocomposites.

The hydrodynamic diameter as well as the dispersity of CdSeTeS QD/AuNPs nanocomposites along with its individual components was determined by DLS (Fig. 2B). The distribution of the AuNPs and MUDA-AuNPs are shown particle size of 8.5 ± 1.1 nm and 11.2 ± 1.2 nm, respectively. It proves the monodisperse nature of AuNPs which is not altered even after conjugation with MUDA. Similarly, the cysteine capped CdSeTeS QDs and antibody-conjugated QDs show the hydrodynamic diameter of 29.4 ± 2.3 and 54.2 ± 3.4 nm respectively which differs from the size distribution finding in TEM images. This may be due to the fact that being a small sized and charged particles, the QDs have a strong tendency to agglomerate in aqueous medium, increasing the hydrodynamic radius (Reghuram et al., 2015). However, after the conjugation of these two nanoparticles, the Ab-CdSeTeS QD/AuNPs nanocomposite shows the diameter of 102.1 ± 3.2 nm which is increased up to 107 ± 2.2 nm after the NoV-LPs attachment, confirming the agglomerated distribution, further supported by TEM image, later.

3.2. Optimization of CdSeTeS QD/AuNPs sensor and its mechanism of sensing

After proper characterizations, the Ab-CdSeTeS QD/AuNPs nanocomposite has been used as the fluorometric sensor probe for NoV-LPs detection. Being a strong inorganic QDs with high quantum yield of 0.57 (Takemura et al., 2017), the bare Ab-CdSeTeS QDs show a strong fluorescence signal at 640 nm at the excitation of 450 nm (Fig. 2C). After the covalent attachment with AuNPs, the spectral intensity of Ab-CdSeTeS QDs has been quenched more
than 65% due to the close interaction with AuNPs. The spectral overlap between the surface plasmon spectrum of AuNPs and the emission spectrum of CdSeTeS QDs confirms the quenching interaction, given in Fig. S3. However, after successive addition of NoV-LPs on the nanocomposites, a significant enhancement of the fluorescence signal has been observed due to the enhanced distance between AuNPs and QDs which is the key mechanism of virus detection. It is well known fact that the LSPR properties between any two nanoparticles are highly dependent on their distance and sizes (Guo et al., 2015). Here, the size of QDs and AuNPs are always kept constant at ~6 nm and 12 nm respectively. Therefore, the distance between these two nanoparticles plays the major role of altering fluorescence intensity of QDs. Due to the covalent attachment through a small organic chain of MUDA, the AuNPs and the CdSeTeS QDs are situated in a very closely packed structure (within 6 nm distance), resulting in the initial quenching. However, after the NoV-LPs addition, the NoV-LPs are bound to the Ab-CdSeTeS QD/AuNP due to the antibody-NoV-LPs conjugation. The large size of NoV-LPs compared to those of AuNPs and QDs, induces strong steric repulsion which creates larger distance between two nanoparticles, initiating the LSPR mediated fluorescence enhancement.

In most cases of virus detection methods on LSPR, the nanomaterials are conjugated with different specific bio-markers or antibodies, corresponding to the analyte. After analyte addition, the nanomaterials get close towards each other using analyte as a bridge molecule. In this case, QDs are existed and shows high fluorescence intensity before inducing LSPR in the detection system, which is background fluorescence intensity of detection system. In spite of using more than one costly biomarkers or antibodies, this antibody/aptamer linked analyte bridged system highly suffers from non-specific interaction between two nanomaterials, resulting high background signal hence lowering sensitivity. However, here we have made the system rigid by covalent bonding between two nanomaterials, initially which causes strong fluorescence quenching of the QDs. Then, creating steric repulsion, induced by analyte
conjugation promotes the LSPR interaction. To optimize the exact size for best results, we have varied the sizes of interacting AuNPs from 5 to 100 nm, maintaining the constant size of CdSeTeS QDs for the detection of 1 ng mL$^{-1}$ NoV-LPs. As shown in Fig. 2D, smaller sized AuNPs have better quenching effects on CdSeTeS QDs. However, evaluating the enhancement as well as quenching factor, the 10 – 12 nm AuNPs has shown best performance for this work.

3.3. Fluorometric sensing of NoV-LPs using the Ab-CdSeTeS QD/AuNPs sensing probe

Detection of the NoV-LPs was carried out to demonstrate the performance of the sensor probe. The LSPR-induced immunofluorescence enhancement for the detection of NoV-LPs and its calibration curve is given in Figs. 3A and B, respectively. The fluorescence at 640 nm of CdSeTeS QDs has been monitored as sensing signal whose intensity is quenched and thereafter enhanced by adjacent AuNPs. At increasing concentrations of NoV-LPs, progressive enhancement of the fluorescence has been achieved without any notable peak shift, providing evidence that the QDs were highly stable during the detection period. The response time is around 1 min after the addition of the target NoV-LPs. The corresponding linear calibration curve is shown in Fig. 3B where the limit of detection (LOD) is found of 8.2 fg mL$^{-1}$, based on $3 \times$ standard deviation of lowest concentration divided by the slope of calibration curve (Dutta Chowdhury et al. 2017).

To verify the LSPR behavior from AuNPs influences, the sensitivity of the biosensor was further carried out by a control test using Ab-CdSeTeS QD. Instead of covalently bonded AuNPs, the AuNPs was only physically mixed with the Ab-CdSeTeS QDs for the detection of the targeted NoV-LPs. As shown in Fig. S4, the fluorescence emission of the Ab-CdSeTeS QDs after addition of AuNPs by only physical mixing was almost unaffected, indicating that without LSPR signal, the target virus cannot be detectable. The changes of fluorescence intensity of the Ab-CdSeTeS QD/AuNPs have been also observed in naked eye in the 450 nm
UV light chamber (Fig. 3C). The highly flourished bare Ab-CdSeTeS QDs is significantly quenched after the formation of CdSeTeS QD/AuNPs nanocomposites. However, after addition of 1 ng mL\(^{-1}\) NoV-LPs, the enhancement of fluorescence is also observed which confirms the LSPR induced phenomenon. The TEM images of CdSeTeS QD/AuNPs nanocomposites are given in Fig. 3D where the agglomerated CdSeTeS QD/AuNPs nanocomposites are situated clearly on ~40 nm NoV-LPs surface. The higher magnification of the image of an isolated particle (inset of Fig. 3D) clearly demonstrates the formation of the NoV-LPs conjugated nanocomposites which support our hypothesis. From the aspect of the wide detection range, low LOD and short response time, our nanobiosensor shows much better performances compared with recently published reports, especially in the low detection range, listed in Table 1. In our previous study of LSPR detection, interaction of the target virus with Ab-conjugated AuNPs and other Ab-conjugated QDs induces an LSPR signal from adjacent AuNPs to trigger fluorescence-enhancement changes in the QDs in proportion to the concentration of the target virus. Though the excellent linearity have been achieved in that case, however due to the existence of free QDs in detection solution, the background signal is quite high, resulting higher LOD of 0.03 pg mL\(^{-1}\). In this present study, the covalent attachment between two nanomaterials initially exhibited quenched fluorescence, which could able to show fluorescence enhancement even in presence of small number of virus particles, ensuing low detection limit of 8 fg mL\(^{-1}\). In addition, the rapid detection with high sensitivity of this proposed sensor displays clear advantages over the conventional methods of enzyme immunoassays (EIAs), which requires ~15 – 20 min, and RT-PCR, which requires several hours for detection.

### 3.4. Selectivity test of the sensor

Selectivity is one of the most important parameters for real sensing application. The selectivity of the nanobiosensor for the detection of the target NoV-LPs was compared with
Influenza virus A (H3N2) and Zika viruses. Two percent BSA solution, human serum and 10 fold diluted human serum samples were treated as negative control to judge the matrix effect of the biosensor (Fig. 3E). Except the 100 % serum sample, the matrix effects are almost negligible. Due to the presence of huge inferring agents, the sensor cannot perform well in presence of 100 % human serum samples. Therefore, for further investigation in serum samples later, we have used 10 % diluted serum samples. Due to the structural similarities, the sensor has shown almost 32 % signal enhancement for influenza virus. However, the nanobiosensor for the targeted NoV-LPs is greater than that of other viruses, demonstrating the sufficient specificity of our biosensor for the target virus. In addition, some amino acids (2 mM mL\(^{-1}\)) and metal ions (100 µg mL\(^{-1}\)) which are common interferences for the real or clinical sample analysis are also investigated in higher concentrations and found ignorable for the detection analysis.

3.5. Sensing in serum sample

Human serum of 10 % was used as a detection medium to demonstrate the ability of the biosensor in a complex biological medium. The biosensor shows a similar trend of detection in the range of the spiked concentration of NoV-LPs which confirms the applicability of the sensor for the real sample monitoring (Fig. 4A). The calibration curve found from the NoV-LPs detection is plotted in Fig. 4B where the slope of linearity is little flattered with respect to the detection found in DI water samples, presented in Fig. 3B. Though the small interference of the serum matrix has lowered the slope of the calibration, decreasing of LOD value to 15.6 fg mL\(^{-1}\), however the sensitivity is quite appreciable with respect to other reports for its real application.

3.6. Detection of clinically-isolated NoV
The spiked amount of RNA of NoV from clinical sample was also detected by the CdSeTeS QD/AuNPs nanocomposites. The fluorescence intensity was gradually changed as a function of the NoV concentration, following the similar trend as NoV-LPs (Fig. 5A). A linear calibration curve was obtained in the range $10^2 - 10^4$ copies mL$^{-1}$ (Fig. 5B) and the detection limit was 87 copies mL$^{-1}$. This implies that the NoV from clinical sample was successfully detected by this proposed technique without compromising the efficiency. However, in case of higher NoV concentration of $10^6$ copies mL$^{-1}$, the enhancement turns to quenching of QDs fluorescence (data not shown). This may be due to the fact that in presence of excess virus particles, some viruses themselves can entrap on the QDs surface, resulting quenching. This limits the detection range up to $10^5$ copies mL$^{-1}$, however which is enough for its practical application.

For further confirmation, the three spiked samples of clinically-isolated NoV along with two NoV-LPs are tested by commercial ELISA kit (Denka Seiken Co Ltd., Model No. 324603, Niigata, Japan). It is clearly shown in Fig. 5C that the commercial NoV detection kit is unable to detect the virus as well as VLPs concentration in lower range though it is useful for higher concentration range of $10^4 - 10^6$ copies mL$^{-1}$. In contrast, our proposed sensor shows excellent detectability in the low NoV concentration of $10^2 - 10^5$ copies mL$^{-1}$.

4. Conclusion

In this study, we have successfully synthesized a new class of nanocomposites which can detect NoV in a single-step and rapid fluorescence based technique. In Ab-CdSeTeS QDs/AuNPs nanobiosensor, the adjacent AuNPs initially quench the fluorescence signal of the CdSeTeS QDs whereas after successful attachment of target NoV-LPs or NoV via antibody-antigen interaction, it triggers the fluorescence enhancement of QDs. The steric repulsion induced by the analyte causes the required distance replacement for the LSPR interaction which
is the key reason for obtaining higher sensitivity over other conventional LSPR based biosensors. The enhancement is proportionated with the concentration of the target NoV-LPs, maintaining a linear relationship from $10^{-14}$ to $10^{-9}$ g mL$^{-1}$ with a LOD of 8.2 fg mL$^{-1}$ in DI water and 15.6 fg mL$^{-1}$ in human serum, confirming proficient detection of the NoV-LPs. The clinically-isolated NoV from NoV-infected patients was also investigated, and the corresponding sensitivity was found 87 copies mL$^{-1}$. The easily applicable method of this proposed biosensor can be applied not only for the detection of NoV but also can be served as a general platform by changing the entrapped biomolecules, in the wide variety of other sensing application in future.

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**Appendix A: Supplementary data**

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Table 1. Comparison of the LSPR-based CdSeTeS QD/AuNPs biosensor with recently reported other virus detection methods in term of limit of detection, range of detection and response time.

Figure Captions:

Scheme 1. Schematic diagram for the preparation of CdSeTeS QD/AuNPs nanocomposites and its sensing mechanism towards NoV-LPs detection. The close covalent attachment of AuNPs with CdSeTeS QDs effectively quenched the fluorescence signal which has been recovered after NoV-LPs entrapment.

Fig. 1. (A) TEM image of CdSeTeS QDs (inset: particle size distribution where n=30), (B) TEM image of AuNPs (inset: particle size distribution where n=30 and a single AuNPs), (C) CdSeTeS QDs/AuNPs nanocomposites and (D) ELISA of CdSeTeS QDs, CdSeTeS QDs/AuNPs before and after NoV-LPs addition.

Fig. 2. (A) XRD of CdSeTeS QDs and CdSeTeS QDs/AuNPs nanocomposites, (B) Hydrodynamic diameter of AuNPs, AuNPs-MUDA, CdSeTeS QDs, Ab-CdSeTeS QDs, Ab-CdSeTeS QDs/AuNPs and NoV-LPs loaded Ab-CdSeTeS QDs/AuNPs, (C) Fluorescence spectra of Ab-CdSeTeS QDs/AuNPs nanocomposites in comparison with bare CdSeTeS QDs and NoV-LPs loaded Ab-CdSeTeS QDs/AuNPs, (D) Effect of different size of AuNPs on the quenching and LSPR effect of Ab-CdSeTeS QDs/AuNPs nanocomposites.

Fig. 3. (A) Fluorescence emission spectra showing the detection of NoV-LPs in the concentration range of 10 fg mL\(^{-1}\) to 100 ng mL\(^{-1}\) using the LSPR-induced Ab-CdSeTeS QDs/AuNPs nanobiosensor, (B) Corresponding fluorescence calibration curve for detection of the NoV-LPs. Error bars denote standard deviation of 3 replicate measurements, (C)
Fluorescence images of (i) bare CdSeTe QDs, (ii) Ab-CdSeTe QDs/AuNPs and (iii) 10 ng mL⁻¹ NoV-LPs loaded Ab-CdSeTe QDs/AuNPs nanocomposites in normal light and the UV lamp of 450 nm (D) TEM image of NoV-LPs loaded Ab-CdSeTe QDs/AuNPs nanocomposites (inset: isolated NoV-LP in higher magnification), (E) Selectivity test of the Ab-CdSeTe QDs/AuNPs nanobiosensor with 30 μg mL⁻¹ of Influenza, 10⁴ PFU mL⁻¹ of Zika viruses and other common amino acids and interfering metal ions.

**Fig. 4.** (A) Fluorescence emission spectra showing the detection of the NoV-LPs using Ab-CdSeTe QDs/AuNPs biosensor in 10 % human serum and its (B) corresponding calibration curve in presence of calibration line (black dots) found in DI water (shown in Fig. 3B). Errors bars denote standard deviation of 3 replicate measurements.

**Fig. 5.** (A) Fluorescence emission spectra showing the detection of clinically-isolated NoV in the concentration range of 10² copies mL⁻¹ to 10⁶ copies mL⁻¹ using the LSPR-induced Ab-CdSeTe QDs/AuNPs nanobiosensor, (B) Corresponding calibration curve for detection of the NoV, (C) Comparison of detection ability of the proposed method (red line) with commercial ELISA kit (blue line) (Lot No. 395121).
Table 1. Comparison of the CdSeTeS/AuNPs biosensor with recently reported LSPR-based other virus detection in terms of limit of detection, range of detection and response time.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Linear range</th>
<th>LOD</th>
<th>Response time</th>
<th>References</th>
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<tr>
<td>Influenza</td>
<td>10 – 100 pg mL(^{-1})</td>
<td>0.03 pg mL(^{-1})</td>
<td>5 min</td>
<td>Takemura et al., 2017</td>
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<td>1 ng – 10 µg mL(^{-1})</td>
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<td>-</td>
<td>Ahmed et al., 2016</td>
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<td>Influenza</td>
<td>5 – 50 ng mL(^{-1})</td>
<td>13.9 ng mL(^{-1})</td>
<td>15 min</td>
<td>Chang et al., 2010</td>
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<td>Norovirus</td>
<td>1.58 × 10(^5) – 7.9 × 10(^7) copies mL(^{-1})</td>
<td>9.5 × 10(^4) copies mL(^{-1})</td>
<td>10 min</td>
<td>Han et al., 2016</td>
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<td>Dengue</td>
<td>5 – 500 ng mL(^{-1})</td>
<td>5.2 ng mL(^{-1})</td>
<td>45 min</td>
<td>Linares et al., 2013</td>
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<td>NoV-LPs</td>
<td>10(^{-14}) – 10(^{-9}) g mL(^{-1})</td>
<td>8.4 fg mL(^{-1})</td>
<td>1 min</td>
<td>This work</td>
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<tr>
<td>Norovirus</td>
<td>10(^2) – 10(^5) copies mL(^{-1})</td>
<td>87 copies mL(^{-1})</td>
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