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Acrylic-based genosensor utilizing metal salphen labeling approach for reflectometric dengue virus detection

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ABSTRACT

An optical genosensor based on Schiff base complex (Zn^{2+} salphen) DNA label and acrylic microspheres (AMs) as polymer support of the capturing DNA probe (cpDNA) was developed for dengue virus serotype 2 (DEN-2) detection via reflectance spectrophotometric method. The solid-state optical DNA biosensor showed high selectivity and specificity up to one-base mismatch in the target DNA sequence owing to the salphen chemical structure that is rich in localized electrons, and allowed π-π stacking interaction between stacked base pairs of double-stranded DNA (dsDNA). The reflectometric DNA microsensor demonstrated a broad linear detection range towards DEN-2 DNA from 1×10^{-15} M to 1×10^{-3} M with a low limit of detection (LOD) obtained at 1.21×10^{-16} M. The DNA biosensor gave reproducible optical response with a satisfactory relative standard deviation (RSD) at 3.1%, ($n = 3$), and the reflectance response was stable even after four regeneration cycles of the DNA biosensor. The optical genosensor was proven comparable with standard reverse transcription polymerase chain reaction (RT-PCR) in detecting DEN-2 genome acquired from clinical samples of serum, urine and saliva of dengue virus infected patients under informed consent. The developed reflectometric DNA biosensor is advantageous in offering an early DEN-2 diagnosis, when fever symptom started to manifest in patient.

1. Introduction

Dengue viruses are members of genus *Flavivirus* in the family *Flaviviridae*. There are four antigenetically distinct serotypes, namely dengue virus serotype 1 (DEN-1), dengue virus serotype 2 (DEN-2), dengue virus serotype 3 (DEN-3) and dengue virus serotype 4 (DEN-4), which are the causative agents in inflicting symptomless infection, undifferentiated fever, dengue fever (DF) and severe dengue hemorrhagic fever or dengue shock syndrome (DHF/DSS) in humans [1–3]. However, the pathogenesis of dengue virus in human has yet to be clearly confirmed despite its persistency [4]. One thing the researchers could agree on is that the infection severity may depend on the immunity response of different hosts and variety types of cells involved [5]. Usually, the incubation phase takes between two days and two weeks' time following viremia inoculation (i.e. mosquito biting) into humans by Aedes vectors (e.g. *Aedes aegypti* or *Aedes albopictus*) [6]. From illness onset (i.e. fever) perspective, viremia usually peaks at this time or shortly after that, and could be detected within two to twelve days' time, whereby the quantity may vary according to the immune status of the patient and virus serotype [7–10]. For instance, it was found that viremia varies, in titer [quantified as 50% mosquito infection doses (MID_{50})], between 10^3 MID_{50} (which is almost undetectable) and $10^{8.5}$ MID_{50}, from patients' serums having viremia on the 2nd to 12th day of illness onset. Another study detected viremia at 10^6 minimal infection doses per mL in human serum acquired within few hours after illness onset [11,12].

Although the required specimen or sample size for serological test is small in order to identify non-structural dengue proteins (NS1) and

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specific immunoglobulin dengue antibodies (IgM and IgG), false positive results can be yielded due to antigenic cross-reactivity among different dengue virus serotypes and different *flavivirus* members, e.g. Japanese encephalitis viruses, tick-borne encephalitis (TBEV), West Nile and yellow fever [13,14]. Furthermore, at least five days interval (since the illness onset) is needed to gain enough quantity of antibodies before serological tests can be carried out [15,16]. DNA or RNA-based detections have been employed worldwide for dengue clinical diagnostic as to compensate the poor selectivity and sensitivity of serology tests, namely the virus isolation and reverse transcription polymerase chain reaction (RT-PCR) methods [17,18]. The cultivation and isolation of viruses normally takes from days to weeks' interval, and sometimes could encounter failures due to the small quantity of viable viruses present in the inocula [19]. On the other hand, RT-PCR approach has undoubtly able to detect dengue viruses rapidly in viremia phase of patients' serum [20,21]. Many improvisations have been reported to place RT-PCR technique on par with virus isolation in terms of dengue serotyping [22,23]. Sadly, not all dengue-affected countries are that fortunate to afford this high performance facilities, which require experienced and well-trained personnel [24,25].

The incidence of dengue fever has been shown to keep rising regardless of age, races, location and time [26]. A tetravalent chimeric dengue vaccine developed by Sanofi Pasteur called Dengvaxia® (CYD-TDV) has become one of the trending issues worldwide where its Phase III clinical trial had been completed successfully in several Latin American and Southeast Asian countries [27,28]. Despite its growing recognition in public health and medical fields, it is found that Dengvaxia® causes complication in those without prior dengue infection, and protection is limited to those currently having dengue infection or those who had a previous infection, aged within 9–45 years old, living in highly endemic areas [29,30]. Besides, it is also known as a fact that Dengvaxia® only exhibits around 40% efficacy towards DEN-2 protection [31–33]. To overcome the growing health concern for those outside of Dengvaxia® protection range, having rapid, specific and sensitive early detection tools can greatly assist medical practitioners to quickly detect dengue virus infection in human followed by fluid replacement therapy given in the early stage, while waiting pathogenesis severity to be fully understood in near future [34,35].

Schiff bases are generally imines or azomethines ($R^1R^2C=NR^3$), where R^1 and R^2 can be of hydrogens, whilst R^3 can be of an alkyl or aryl group [36]. Schiff base salphen tetradentate ligands containing aromatic rings are often coordinated to a transition metal (e.g. Zn^{2+}, V^{4+}, Cu^{2+}, Ni^{2+} or Al^{3+}) [37], and have been extensively explored due to their notable optical behaviour, chemical stability and biological activity via electrostatic or π-π stacking interaction with double-stranded DNA (dsDNA), which could be directly detected by the naked-eye [38–40]. The flexible conformational tunability of Schiff bases forming unique geometries i.e. square-planar and square-based pyramidal geometries [41] have been demonstrated to have affinity towards G-quadruplex and duplex DNA structures [42].

With recent advances in micro/nanomaterials, new strategies are emerging to design and develop robust optical chemical sensor or biosensor as visual detection systems [43] for application in biological, analytical and environmental monitoring or diagnostic purposes, depending on the nature of the matrix platform on which it is constructed [44–49]. Various advanced materials, such as graphene and graphene oxide [50], carbon nanotubes [51], and metallic and magnetic nanomaterials [52], have been successfully exploited for designing sensors relying on different optical signal transduction systems. These advanced materials can lead to significant improvement in the analytical performance of the resulting optode with regard to sensitivity, selectivity and portability. The most widely used techniques employed in chemical sensors are optical absorption [44–48], fluorescence [39,43,49], reflectance, etc. for fast track screening of cation, anion, organic and inorganic compound of interest.

In this study, an optical genosensor for sensing of DEN-2 genome has been constructed based on the employment of Zn^{2+} Schiff base complex as dsDNA label, and poly(*n*-butylacrylate-*co*-N-acryloxysuccinimide) [poly(*n*BA-NAS)] microspheres or simply referred as acrylic microspheres (AMs) as the immobilization phase of DNA. The proposed novel DNA bio-optode permits naked eye observation of DEN-2 because salphen complex is rich in localized electrons, therefore allowing π-π stacking mode of interaction between stacked base pairs to take place, and AMs possess hydrophobicity character that hinders non-specific interaction towards polar Zn^{2+} salphen label, thus giving a distinct yellow colouration on the microsensor surface. The optical DNA biosensor provided a new approach in the diagnosis of DEN-2, whereby the detection of DEN-2 genome extracted from the clinical serum, urine and saliva have been validated with RT-PCR standard method. Consequently, the proposed reflectance DEN-2 biosensor would offer a non-invasive approach to dengue diagnosis especially in dealing with children and the elderly having fear of needles and blood. Thus, the proposed DNA optode is favourable to be an integrated point-of-care dengue diagnostic tool for naked eye and non-invasive surveillance of dengue virus serotype.

2. Experimental

2.1. Instrumentation

Nuclear Magnetic Resonance (^{1}H-NMR and ^{13}C-NMR) spectra of Zn^{2+} salphen complex were obtained by using Bruker Avance III 400 MHz NMR spectrometer. Here, TMS (tetramethyl silane) was used as an internal standard and DMSO-d_6 (deuterated dimethyl sulfoxide) as dissolving solvent. Fourier Transform Infrared spectroscopy (FTIR) spectra of metal complex and AMs were acquired from Perkin Elmer Spectrum 400 FT-IR with germanium (Ge) ATR crystal top-plate within the wavenumber range of 4000–400 cm^{-1}. Electrospray Ionization-Mass Spectrometry (ESI-MS) spectra of metal salphen complex were recorded on BRUKER MicroTof-Q with DIONEX Ultimate 3000 LC. Field Emission Scanning Electron Microscopy (FESEM) image of AMs was captured on Zeiss Merlin/Merlin Compact/Supra 55VP. Branson ultrasonic cleaner and Mettler Toledo pH meter were used in sonication process of AMs precursors and buffer pH determination, respectively. Photopolymerization of AMs was carried out in an UV box installed with four UV light tubes (wavelength of 350 nm) and gaseous nitrogen line. The reflectance spectra of the fabricated genosensor were visualized on an Ocean Optics USB4000 Spectrometer coupled with a bifurcated optical fiber probe and DH-2000-BAL UV–VIS–NIR light source in the wavelength range of 200–1099 nm.

2.2. Chemicals and reagents

All the required chemicals were ordered from Merck, Fluka and Aldrich which were used directly without further purification. The synthetic 16-mer oligonucleotides of capturing DNA probe (cpDNA), complementary target DNA (tcDNA), 1-base mismatched DNA (1mDNA) and non-complementary DNA (ncDNA) were synthesized by Sigma-Aldrich and their base sequences are listed in Table 1. All four single-stranded DNAs (ssDNA) obtained were made into 100 μM using Milli-Q water to serve as stock solutions for further usage. Zn^{2+} salphen complex solution was prepared by dissolving as little as 3 mg of the

Table 1
Base sequences of single-stranded DNA (ssDNA) used in this study.

16-mer ssDNA	Base sequences
cpDNA	5′-NH_2-CAT GGC CCT TGT GGC G-3′
tcDNA (DEN-2 cDNA)	5′-CGC CAC AAG GGC CAT G-3′
1mDNA	5′-CGC CAC **C**AG GGC CAT G-3′
ncDNA (*Escherichia coli*)	5′-CGT CGC GGT ATA AGT A-3′

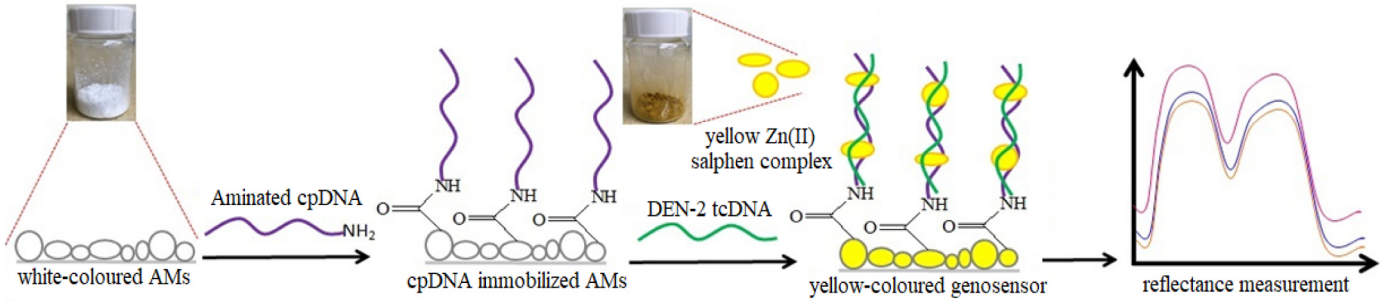
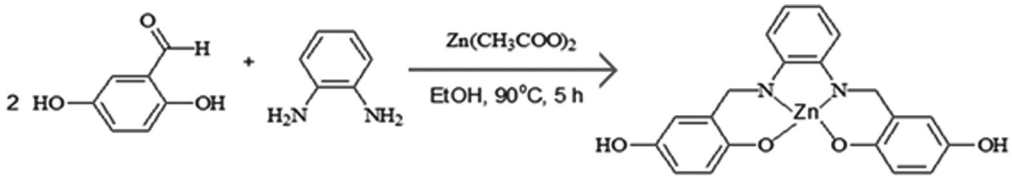


Fig. 1. The schematic representation of the fabrication steps of the reflectometric DEN-2 DNA biosensor based on poly(*n*BA-NAS) nucleic acid immobilization matrix and Schiff base metal complex DNA intercalating agent.

metal complex in 1457 µL of dimethyl sulfoxide (DMSO, ≥99%, Merck) to produce a 5 mM stock solution. Upon reaction with DNA, 5 mM metal salphen complex stock solution was further diluted to 1 mM with appropriate buffer solution, thus rendered them mixable with buffered DNA solutions. The AMs suspension was prepared by dissolving 50 mg of these particles in 500 µL ethanol (EtOH, 99.8%, A.R. R&M). The different buffer types used were prepared by mixing together potassium di-hydrogen phosphate (KH_2PO_4, 99.5%, Merck) and di-potassium hydrogen phosphate (K_2HPO_4, > 99%, Systerm) for potassium phosphate buffer (PPB); sodium di-hydrogen phosphate dihydrate ($NaH_2PO_4{\cdot}2H_2O$, ≥99%, Fluka) and di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4{\cdot}2H_2O$, 99.5%, QReC) for sodium phosphate buffer (SPB); 2-(N-morpholino)ethanesulfonic acid (MES, ≥99%, Sigma) and sodium hydroxide (NaOH, ≥97.0%, Merck) for MES-NaOH buffer (MNB); and tris(hydroxymethyl)aminomethane (Tris, ≥99.8%, Merck) and hydrochloric acid (HCl, 37%, Aldrich) for Tris-HCl buffer (THB).

2.3. Synthesis and characterization of Schiff base Zn^{2+} salphen complex

The N,N′-bis-5-(hydroxysalicylidene)phenylene-diamine-zinc(II) (Zn^{2+} salphen) complex was synthesized following the previously reported procedure [42], as demonstrated in Eq. (1).



(1)

The one-pot reaction involved 2,5-dihydroxybenzaldehyde (2 equivalents, 0.69 g, 5 mmol, 98%, Aldrich), *o*-phenylenediamine (1 equivalent, 0.27 g, 2.5 mmol, 99.5%, Aldrich) and zinc(II) acetate (2 equivalents, 0.9174 g, 5 mmol, 99.99%, Aldrich) mixed together in EtOH solvent (40 mL), under reflux condition (90 °C) with continuous stirring for 5 h. The yellow coloured powder of Zn^{2+} Schiff base complex (1.029 g, 90.8%) was then obtained after filtration with filter paper pulp (Whatman® No. 1) to remove EtOH solvent followed by vacuum filtration of trapped particulates to remove contaminants with the use of washing solvents e.g. EtOH (50 mL), Milli-Q water (50 mL) and diethyl ether (50 mL), and lastly air-dried at ambient temperature (25 °C). The physical and chemical properties of the as-synthesized metal salphen complex was later characterized by ^{1}H-NMR, ^{13}C-NMR, FTIR and ESI-MS analyses for molecular structure confirmation of the resulting metal complex.

2.4. Synthesis of succinimide functionalized polyacrylate microspheres

Poly(*n*-butylacrylate-*co*-N-acryloxysuccinimide) [poly(*n*BA-NAS)] microspheres were prepared based on the previously reported method with slight changes [53]. Sonication was carried out for 10 min for a mixture of 15 mL Milli-Q water, 7 mL *n*BA (*n*-butyl acrylate, 99%, Aldrich), 6 mg NAS (N-acryloxysuccinimide, 99%, Aldrich) monomers, 0.1 g DMPP (2–2-dimethoxy-2-phenylacetophenone, 99%, Merck), 0.01 g SDS (sodium dodecyl sulphate, Fluka) and 450 µL HDDA (1,6-hexanadiol diacrylate, 90%, Aldrich) in a 20 mL scintillation vial. The white emulsion formed was then poured into a petry dish to be photocured for 10 min under ultraviolet light with continuous nitrogen gas flux. After that, the acrylic microspheres (AMs) suspension was pipetted into 15 mL centrifuge tube and subjected to centrifugation (3760 rpm, 10 min). This washing step was repeated thrice with Milli-Q water, and the as-prepared AMs was subsequently left for air-drying at room temperature. Finally, as much as 50 mg AMs were suspended in 500 µL of EtOH prior to DNA biosensor fabrication procedure. Chemical structure elucidation of the as-synthesized AMs was later done by using FTIR, and their size and surface morphology were determined with FESEM.

2.5. Fabrication of optical DEN-2 DNA biosensor

The step-wise fabrication of the optical solid-state reflectance DNA biosensor was carried out as illustrated in Fig. 1. In brief, some 80 µL of AMs suspension in EtOH was first loaded into a round cap of 200 µL Eppendorf microcentrifuge tube, and air-dried at 25 °C. About 3.2 µL of cpDNA in 0.1 M PPB (pH 7.5) was then added onto the AMs surface to allow spontaneous reaction between extended amine groups of cpDNA and succinimide groups of AMs to form amide covalent bonds for 24 h. Next, the DNA biosensor was rinsed with abundant 0.1 M PPB at pH 7.5 to wash off the cpDNA strand that was loosely bound to the microspheres substrate. 3.2 µL of tcDNA and 16 µL of Zn^{2+} salphen complex were then dispensed onto the DNA biosensor surface, and left for 15 min followed by washing with copious amounts of 0.1 M PPB (pH 7.5) before reflectance response was measured with the fiber optic reflectance spectrophotometer.

2.6. Optimization of optical reflectance DEN-2 DNA biosensor

The selectivity of 1 μM immobilized cpDNA towards the detection of 1 μM tcDNA, 1mDNA and ncDNA was carried out in PPB (0.05 M, pH 7.0). The effect of buffer type study was performed by using different types of buffers i.e. PPB, SPB, MNB and THB at 0.05 M and pH 7.0 as the DNA hybridization media for the determination of 1 μM tcDNA at the maximum reflectance wavelength (λ_{max}) of 670.06 nm. pH effect study was conducted by varying the 0.05 M PPB pH from pH 6.0–8.0 in the detection of 1 μM tcDNA using the proposed optical DNA biosensor. PPB buffer capacity study was done within the buffer concentration range of 0.01–0.30 M, whilst the buffer pH was fixed at pH 7.5 in the determination of 1 μM tcDNA at 670.06 nm. Optimizations of AMs, cpDNA and Zn^{2+} salphen complex loadings on the biosensor platform were performed in the concentration ranges of 20–50 mg AMs, 1–6 μM cpDNA and 0.2–3.0 mM Zn^{2+} salphen complex, respectively using 0.10 M PPB at pH 7.5. The dynamic linear range of the reflectometric DEN-2 DNA biosensor was determined by using a series of tcDNA concentrations from 1×10^{-21} M to 1×10^{-2} M tcDNA under optimal conditions with 45 mg AMs, 3 μM cpDNA and 1 mM Zn^{2+} salphen in 0.10 M PPB at pH 7.5 and 670.06 nm. DNA hybridization duration was investigated within the time period of 0–120 min towards the reflectance biosensing of 1×10^{-2} M, 1×10^{-6} M and 1×10^{-18} M tcDNA, whereby the reflectance measurement was taken every 15 min interval for 2 h. Repeatability test of the DNA biosensor was carried out by soaking the DNA biosensor in 1 μM tcDNA for 15 min, and the DNA biosensor response was measured at 670.06 nm followed by immersing the DNA biosensor in 0.1 M NaOH regeneration solution for another 15 min before subsequent rehybridization of the DNA biosensor with 1 μM tcDNA (15 min) was performed. This step was repeated until a substantial decline in the biosensor reflectance response with 1 μM tcDNA was observed at 670.06 nm.

2.7. Clinical dengue sample collection and ethics approval

Blood samples (6 mL) were collected into plain red-top VACUTAINER® tubes (for serum, no anticoagulant, non-evacuated tubes) in a manner that any froth formation was minimized [54,55]. The midstream urine (MSU) method was employed in the urine specimen collection, which defined as the urine that flows in the middle of urination process [56,57]. As for saliva samples, prior to its collection, patients were asked to drink, gargle and swallow Milli-Q water for three times so as to eliminate food debris from their mouths. Saliva samples were then acquired via the spit method, whereby the subjects were asked to lower their heads for 5 min to allow saliva to accumulate behind the lips. Any saliva accumulated was then spitted out into the collection containers [58–62]. All the samples were immediately processed upon arrival at the Molecular Biology Lab, Universiti Kebangsaan Malaysia, Bangi, Selangor, Malaysia. These processes involved the addition of EDTA to every specimen to a final concentration of 40 mM in 1.5 mL microcentrifuge tube followed by flash freezing in liquid nitrogen (-196 °C) before long-term storage at −80 °C freezer until further usage. Each subject signed an informed consent for inclusion before participating to the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the National University of Malaysia Research Ethics Committee (UKMREC) [JEP-2017-406] and Ministry of Health Medical Research and Ethics Committee (MREC) [NMRR-17-1574-36144].

2.8. RNA isolation and first strand cDNA synthesis

The total RNAs were extracted from the respective human bodily fluid samples (i.e. blood, urine and saliva) from patient 1 (P1), patient 2 (P2), patient 3 (P3), patient 4 (P4), patient 5 (P5) and healthy volunteer (PH) using components of QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the method described by the manufacturer's protocol with a starting material volume of 200 μL for each sample. All samples were centrifuged at 3000 rpm for 10 min prior to RNA extraction. The total RNA obtained was eluted with elution buffer (TE buffer: 10 mM Tris-CL and 1 mM EDTA) using QIAamp mini column at a final volume of 60 μL and was stored at −80 °C when not in use. The quality and quantity of the isolated RNA were then determined using NanoDrop spectrophotometer to fulfil all the requirements for cDNA synthesis and RT-PCR. The first strand cDNA synthesis kit ReverTra Ace qPCR RT Master Mix Kit (Toyobo, Japan) was used for cDNA synthesis. An amount of RNA at 100 ng was used for RT-PCR with the addition of 4 μL RT mix and the nuclease free water to a total volume of 20 μL. The reaction mixture was incubated at 37 °C for 15 min followed by heating at 98 °C for 5 min. The resulting cDNA was directly used for PCR amplification or stored at −20 °C.

2.9. PCR amplification of cDNA, gel electrophoresis and DNA sequencing

The amplification of cDNA was performed by conventional PCR Top Taq Master Mix (QIAGEN, Hilden, Germany) using primer pair of DENV_F (5′-CATGGCCCTGGTGGCG-3′) and DENV_R (3′-CCCCATCTCT TCAATATCCCTG-5′) (300 nM), Top Taq DNA polymerase (6.25 μL), 1.65 μL nuclease free water, 2 μL CoralLoad Gel loading dye and 2 μL of cDNA template in a total reaction volume of 12.5 μL. PCR amplification was carried out by initial denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 56 °C for 30 s, extension at 72 °C for 1 min and final extension step at 72 °C for 5 min. The PCR product was later confirmed by gel electrophoresis analysis on 4% agarose gel stained with RedSafe Nucleic Acid Staining Solution in 1 × TBE buffer (Tris/Borate/EDTA). The DNA agarose gel electrophoresis was run at 90 V for 60 min.

The DNA fragments required for subsequent DNA sequencing procedure was cut off from the agarose gel using sterile surgical blade, and kept in sterile microcentrifuge tube. Gel extraction was performed using QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations. Cloning of the resulting PCR product was done using the CloneJet PCR cloning kit (Thermo Scientific) based on the sticky end cloning protocol for ligation into pJET1.2 cloning vector in a total reaction volume of 20 μL. The sequence of the cloned materials was confirmed using DEN-2 specific forward and reverse primer pairs synthesized by Integrated DNA Technologies (IDT).

2.10. Detection of DEN-2 genome in urine, saliva and blood samples with optical DNA biosensor

Detection of DEN-2 genome in dengue infected human fluid samples (e.g. blood, urine and saliva) with the developed reflectometric DNA biosensor were carried out in triplicate for each sample type, and the results obtained were statistically compared with standard RT-PCR method using paired *t*-test (Student's *t*-test) for statistical consideration at 3 degrees of freedom (df) and 95% confidence level ($p = 0.05$). Paired *t*-test, also known as paired samples *t*-test or dependent samples *t*-test, the mean results acquired from the optical DNA biosensor and RT-PCR techniques were based on the null hypothesis, whereby the pairwise difference between the two methods was equal (H_0:$\mu_d = 0$) [63].

3. Results and discussion

3.1. Physical and chemical characterizations of Zn^{2+} Schiff base complex and polyacrylate microspheres

The prominent peaks for Zn^{2+} Schiff base complex ^{1}H-NMR (400 MHz, TMS, DMSO-d_6, 25 °C) were observed at δ = 6.57–6.59 (d, ^3J(H,H) = 9 Hz, 2 H, arom, H_9), 6.76–6.77 (d, ^4J(H,H) = 3 Hz, 2 H, arom, H_6), 6.82–6.85 (dd, ^3J(H,H) = 9 Hz, ^4J(H,H) = 9 Hz, 2 H, arom, H_8),

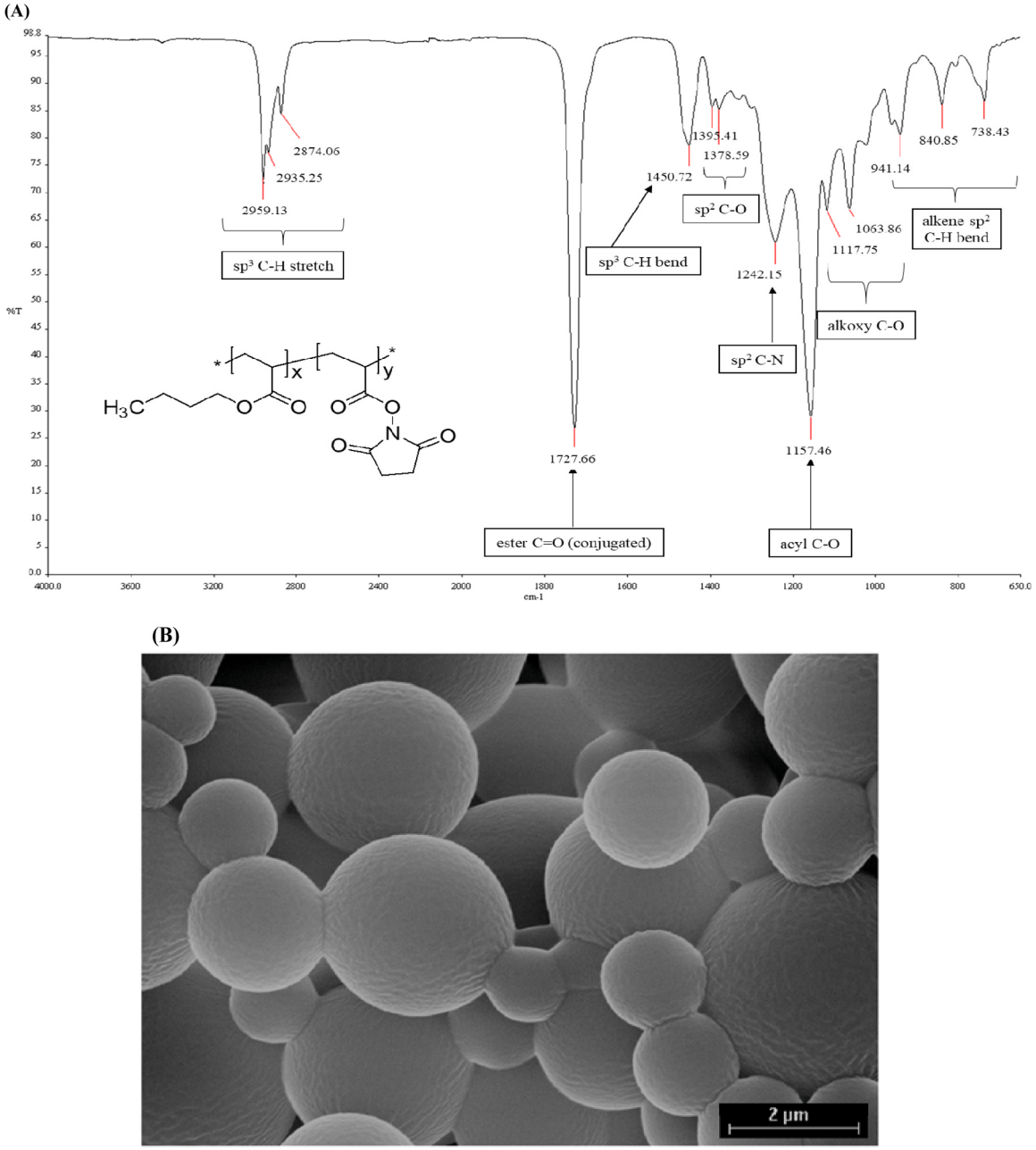


Fig. 2. (A) The FTIR spectrum showing the main functional groups across the AMs, poly(*n*-butylacrylate-*co*-N-acryloxysuccinimide) samples. (B) The FESEM image shows smooth and spherical shape of the polyacrylate microspheres at 10k× magnification with an average diameter of ~2 µm.

7.34–7.36 (dd, ^{3}J(H,H) = 6 Hz, ^{4}J(H,H) = 3 Hz, 2 H, arom, H_1), 7.86–7.88 (dd, ^{3}J(H,H) = 6 Hz, ^{4}J(H,H) = 4 Hz, 2 H, arom, H_2), 8.52 (s, 2H, Ar-OH, H_7) and 8.87 (s, 2H, N = C-H, H_4) (Fig. A.1). In addition, the Zn^{2+} salphen complex ^{13}C-NMR (400 MHz, TMS, DMSO-d_6, 25 °C) exhibited prominent peaks at δ = 116.83, 118.39, 118.58, 123.92, 125.55, 127.49, 139.79, 145.10, 162.35, 167.20 (Fig. A.2). FTIR (Germanium) spectrum of the metal salphen complex shows absorption bands at ν = 3333 cm^{-1} (Ar-OH), 3023 cm^{-1} (aromatic C-H), 1611 cm^{-1} (HC=N), 1539–1363 cm^{-1} (Ar C=C), 1291 cm^{-1} (C-N) and 1217–1151 cm^{-1} (C-O) (Fig. A.3). Mass-spectrometry MS (ESI) analysis confirmed the Schiff base compound molecular weight at m/z = 432.9974 $[M + Na]^+$ (Fig. A.4).

FTIR (Germanium) spectrum of the as-synthesized poly(*n*BA-NAS) microspheres, on the other hand, exhibited absorption bands at ν = 2959–2874 cm^{-1} (sp^3 C-H stretch), 1728 cm^{-1} (conjugated ester C=O), 1451 cm^{-1} (sp^3 C-H bend), 1395–1379 cm^{-1} (sp^2 C-O), 1242 cm^{-1} (sp^2 C-N), 1157 cm^{-1} (acyl C-O), 1118–1064 cm^{-1} (alkoxy C-O) and 941–738 cm^{-1} (alkene sp^3 C-H bend) (Fig. 2A), which were consistent with those functional groups reported in the previous study [64]. The micro dimensional structure of AMs was also confirmed through FESEM image showing an average size of ~2 µm at 10k× magnification (Fig. 2B). The AMs micro particles are spherical in shape having smooth surfaces to allow high loading capacity of DNA molecules during immobilization process.

3.2. Selectivity of DEN-2 genosensor via optically active Zn^{2+} Schiff base complex label

As Fig. 3 indicates, the bare AMs loaded in the microcentrifuge tube's cap gave the highest reflectance reading at λ_{max} = 670.06 nm (reflectance intensity, a.u. = 1497.72) ascribed to its bright white spheres' background that reflected all seven colours of the rainbow

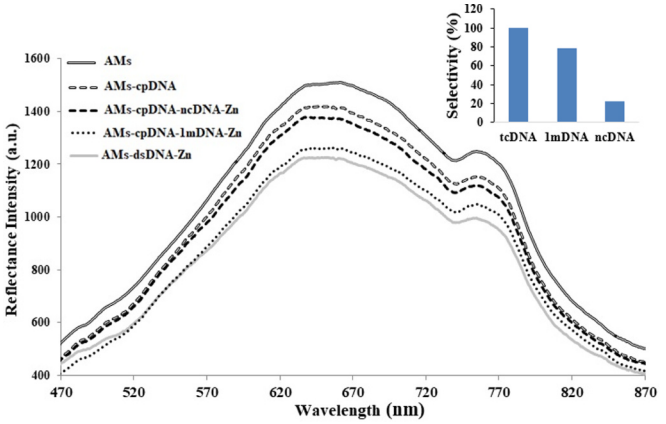


Fig. 3. Selectivity profile of the optical reflectance DEN-2 DNA biosensor towards the determination of 1 μM tcDNA, 1mDNA and ncDNA in 0.05 M PPB at pH 7.0. The inset shows the selectivity of immobilized cpDNA towards tcDNA, ncDNA and 1mDNA.

spectrum. Upon covalent immobilization of the amine ($–NH_2$) functionalized capture probe to the succinimide-modified poly(*n*BA-NAS) microspheres (AMs-cpDNA), the DNA biosensor response showed slight decrement in terms of reflectance intensity (λ_{max} = 670.06 nm, reflectance = 1396.02) as it absorbed some photons from the feed fiber optical light. However, the DNA biosensor exhibited no colour change, and remained white in colour. Hybridization of the immobilized cpDNA with target strand followed by intercalation of the immobilized double-stranded DNA (dsDNA) with optically active Zn^{2+} Schiff base complex (AMs-dsDNA-Zn), the DNA biosensor displayed an obvious change of colour from white to yellow, and demonstrated the lowest light reflectance intensity at 670.06 nm as dark object (i.e. yellow coloured microspheres) absorbs more photons than a lighter-coloured object (i.e. white coloured microspheres). This has clearly shown the role of Zn^{2+} salphen optical properties in showcasing the selectivity of immobilized cpDNA towards its tcDNA, in which it gave the highest relative intensity of 192.7 (100%) compared to ncDNA (relative intensity = 42.73, 21.5%) and 1mDNA (relative intensity = 151.27, 77.8%). Herein, the relative intensity is defined as the reflectance difference of the DNA biosensor reflectance response before and after reaction with tcDNA and Zn^{2+} intercalator at the working reflectance wavelength of 670.06 nm. The DNA hybridization was indicated via intercalation of Zn^{2+} salphen complex through π-π stacking between π-electron delocalization in nucleobase stacked aromatic rings and azomethines (HC=N) as well as benzenes (C=C) groups of Zn^{2+} labels. Zeglis et al. [65] have recently revealed that octahedral metal complexes could bind DNA non-covalently, and target reactions to specific sites as well as target single base mismatches in duplex DNA through metallo-insertion. The highest recognition of immobilized cpDNA towards tcDNA was attributed to the maximum base-pairing between 16 complementary bases of immobilized cpDNA and tcDNA that enabling the full hybridization process to take place, in order to yield the immobilized double helical structure of dsDNA. Detection towards 1mDNA (AMs-cpDNA-1mDNA-Zn) with the proposed reflectometric DNA biosensor showed some 77.8% decrement in the relative reflectance intensity (i.e. relative intensity = 151.27) as 93.8% of its bases were complementary to the immobilized cpDNA. Boon et al. [66] described an electrocatalytic method for the detection of single-base mismatches in fully hybridized duplexes, based on electrocatalytic signal of redox-active methylene blue DNA intercalator coupled to with $[Fe(CN)_6]^{3-}$. A substantially decline in the electrocatalytic signal was monitored upon detection of mismatched or damaged DNA bases, which provides a sensitive method for probing the integrity of DNA sequences.

3.3. Optimizations of reflectometric DNA optode

Fig. 4 depicts the different parameters studied in order to determine the best working conditions for the newly developed DEN-2 optical DNA biosensor. In Fig. 4A, PPB was chosen as the best DNA hybridization buffer compared to three other buffers (i.e. SPB, MNB and

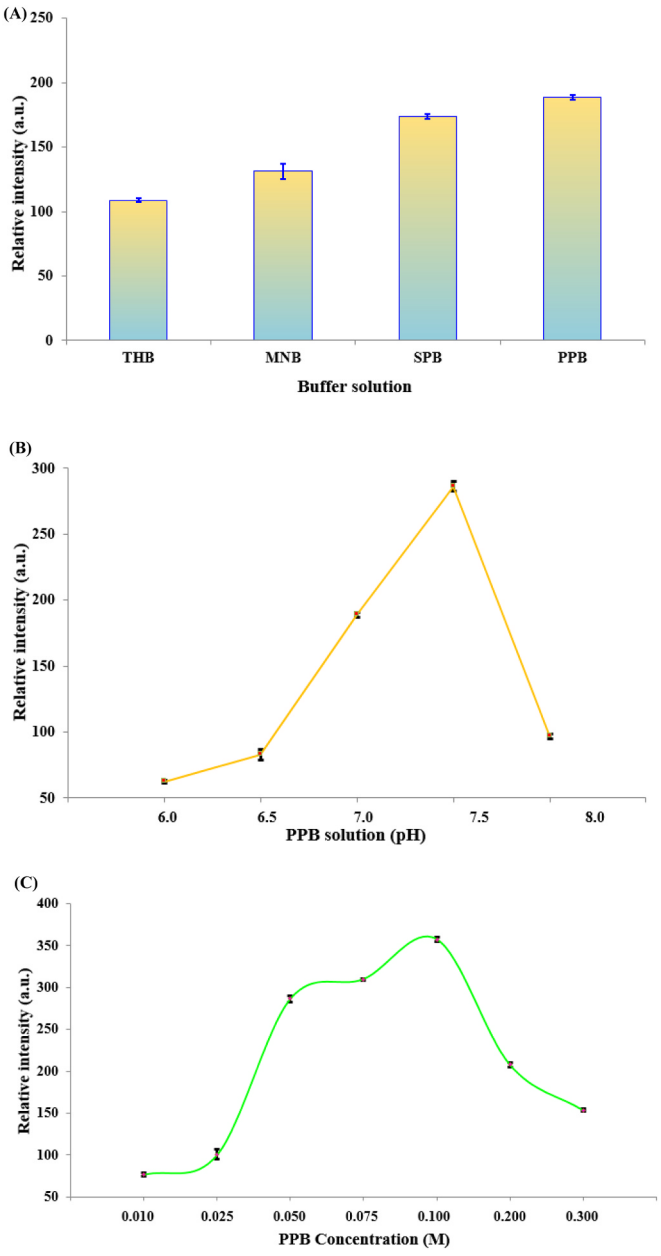


Fig. 4. (A) The reflectometric response of the DEN-2 DNA optosensor towards different buffer types i.e. PPB, SPB, MNB and THB (0.05 M, pH 7.0) using 50 mg AMs, 4 μM cpDNA, 1 μM tcDNA and 1 mM Zn^{2+} salphen complex. (B) The pH profile of the DEN-2 DNA biosensor between pH 6.0 and pH 8.0 using 0.05 M PPB, 50 mg AMs, 4 μM cpDNA, 1 μM tcDNA and 1 mM Zn^{2+} intercalator. (C) Buffer concentration effect on the reflectance DNA biosensor using various PPB concentrations from 0.01 to 0.30 M at pH 7.5 whilst AMs, cpDNA, tcDNA and Zn^{2+} label loadings remained constant at 50 mg, 4 μM, 1 μM and 1 mM, respectively. (D) Effect of AMs loading on the optical DNA biosensor response using 4 μM cpDNA, 1 μM tcDNA and 1 mM Zn^{2+} salphen complex in 0.10 M PPB (pH 7.5). (E) The cpDNA loading effect of the DEN-2 DNA biosensor using 45 mg AMs, 1 μM tcDNA and 1 mM optical Zn^{2+} label in 0.10 M PPB at pH 7.5. (F) The optical DEN-2 genosensor response trending at different Zn^{2+} salphen complex concentrations from 0.2 to 3.0 mM using 45 mg AMs, 3 μM cpDNA and 1 μM tcDNA.

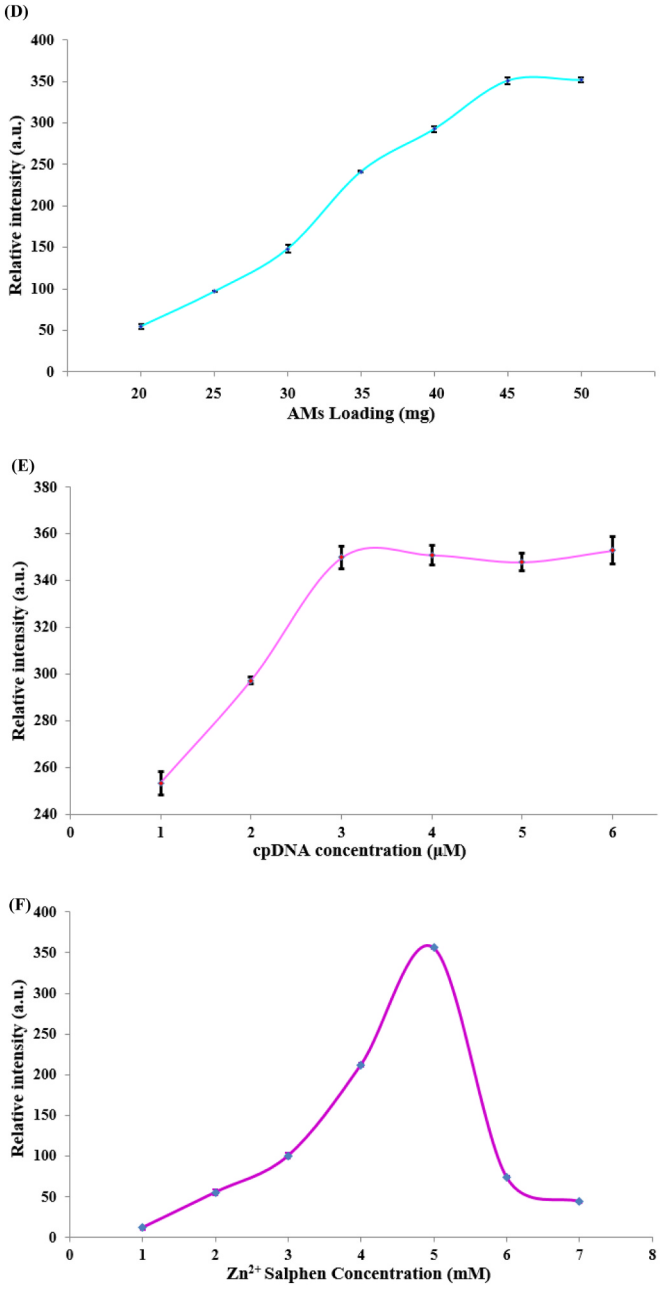


Fig. 4. (*continued*)

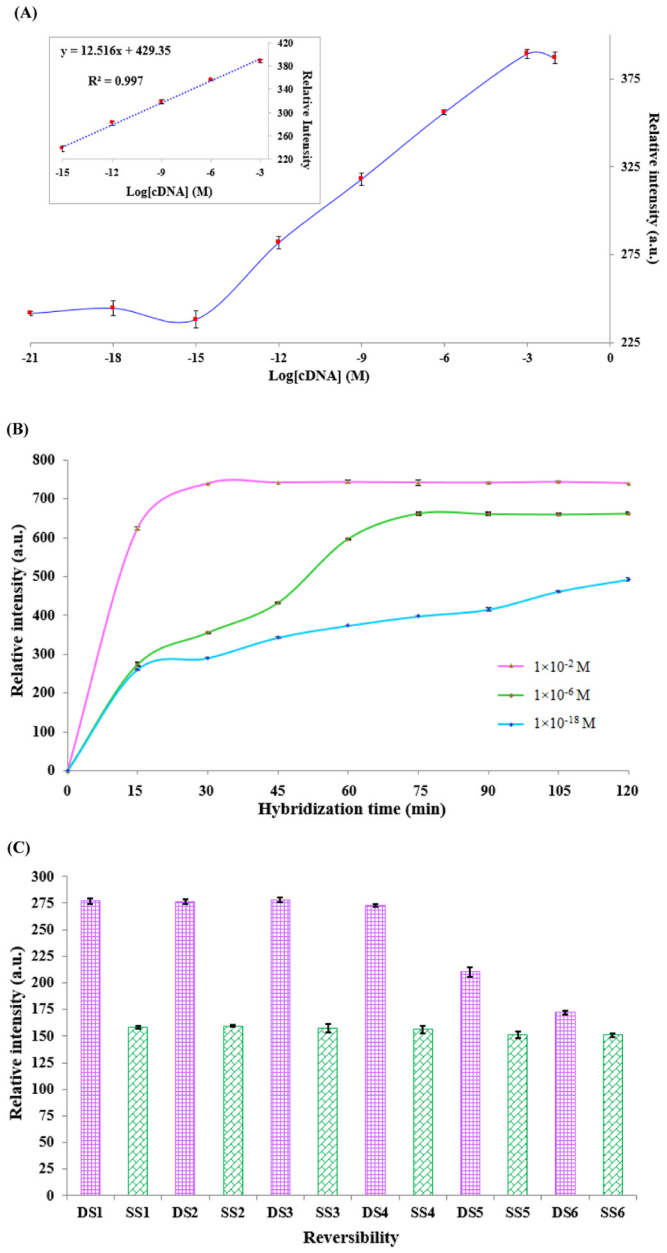


Fig. 5. (A) The sigmoid response curve of the reflectance DNA biosensor towards DEN-2 tcDNA concentration determination between 1 zM and 0.1 cM. The inset shows the linear response range of the optical DNA biosensor from 1 fM to 1 mM DEN-2 tcDNA. (B) The DNA hybridization time of the reflectometric DEN-2 biosensor towards 1 aM, 1 μM and 1 cM tcDNA detection in the presence of 1 mM Zn^{2+} intercalator. (C) The reversibility profile of the DEN-2 biosensor using 0.1 M NaOH as the DNA biosensor regeneration solution (SS1-SS6) and rehybridization of the DNA biosensor using 1 μM tcDNA and 1 mM Zn^{2+} salphen complex in 0.10 M PPB at pH 7.5 (DS1-DS6).

THB) used in the determination of 1 μM tcDNA owing to the highest relative reflectance response obtained at 670.06 nm, which could provide high sensitivity in the subsequent DEN-2 DNA detection experiments. The use of PPB rendered a benign environment for DNA hybridization reaction by enhancing the electrostatic interaction between the DNA bases of immobilized dsDNA to allow later intercalation of optical Zn^{2+} label into the hybridized dsDNA. The optimum pH of the DNA hybridization medium at pH 7.5 (Fig. 4B) is in agreement with the PPB pH used by other researchers in extracting viral dengue DNA from cell cultures i.e. at pH 7.2 [67] and pH 7.4 [68]. Besides, an intense yellow colouration was also perceived when PPB buffer capacity at 0.10 M was used (Fig. 4C). This can be explained by the fact that PPB could provide less steric repulsion, and hence a greater electrostatic interaction between negatively-charged DNAs. The monovalent cation of PPB i.e. K^+ ion neutralizes the negatively charged DNA sugar-phosphate backbone, and reduces the electrostatic repulsions between DNA molecules to promote DNA hybridization reaction. Additionally, the high salt content could also facilitate to stabilize the configuration of the immobilized DNA double helix.

With respect to AMs loading effect, when more than 45 mg AMs were loaded, a horizontalized reflectance response was attained due to the over packed arrays of the microspheres, which has prevented further covalent binding of their succinimide functional groups with amine functional groups of cpDNA (Fig. 4D). The cpDNA loading of more than 3 μM resulted in a plateau state response of the DNA biosensor, which suggests a saturated DNA hybridization state has achieved with 1 μM tcDNA and 1 mM Zn^{2+} salphen complex (Fig. 4E). By referring to Fig. 4F, the DNA optode response gradually increased with the

increasing of the Schiff base metal complex DNA intercalating agent from 0.2 to 1.0 mM, and 1.0 mM of Zn^{2+} salphen complex optical DNA label gave the highest relative reflectance response. The optical DNA biosensor response decreased significantly when the Zn^{2+} salphen complex concentration increased to 2.0 mM and 3.0 mM as a greater DMSO volume was used in preparing the metal solution, and that rendering it not miscible in aqueous buffered DNA solutions, thus preventing effective intercalation of metal salphen complex into immobilized DNA duplexes. Therefore, the concentration of Zn^{2+} intercalative complex was maintain at 1 mM in the next DNA biosensor optimization studies.

Fig. 5A refers to a sigmoidal response trend of the reflectometric DNA biosensor in determining a series of DEN-2 DNA concentrations, ranging from 1×10^{-21} M (1 zM) to 1×10^{-2} M (1 cM). As expected, by increasing the concentration of DEN-2 tcDNA from 1×10^{-15} M (1 fM) to 1×10^{-3} M (1 mM) the relative reflectance response increased proportionally as the yellow colouration gradually built up on the micro-biosensor surface, which can be observed by a human naked eye. This was attributed to the growing number of immobilized DNA duplexes formed on the DNA biosensor surface, thereby promoting a higher number of yellow-coloured Schiff base complexes intercalated into the immobilized dsDNA, and that reduced the amount of light reflected from the darker DNA biosensor background. The correlation coefficient (R^2) of the linear response range of the optical DNA biosensor (i.e. 1 fM-1 mM) was obtained at $R^2 = 0.9978$ with a linear equation of y = 12.516 × + 429.35. And, the reproducibility of each data point obtained within the dynamic linear range gave an average relative standard deviation (RSD) of about 3.1% ($n = 3$). The response curve of the DNA biosensor was eventually levelled off from 1×10^{-3} M tcDNA and onwards due to the saturation phase has achieved on the DNA biosensor surface. The DNA biosensor reflectance response towards DEN-2 DNA concentrations at 1×10^{-18} M (aM) and 1 zM was found to show a negligible over-response compared to 1 fM tcDNA as it gave only a slightly higher response of not > 3% than the target DNA at fM concentration level. The limit of detection (LOD) of the DNA biosensor was estimated to be at 1.21×10^{-16} M DEN-2 DNA based on the limit of blank (LOB) formula given in Eq. (2) in terms of relative reflectance intensity, where SD refers to the standard deviation of blank, and converted into molar concentration using the biosensor linear equation followed by substituting the LOB value into Eq. (3).

$$\mathrm{LOB} = \mathrm{mean}_{\mathrm{blank}} + 1.645(\mathrm{SD}_{\mathrm{blank}}) \quad (2)$$

$$\mathrm{LOD} = \mathrm{LOB} + 1.645(\mathrm{SD}_{\mathrm{low\ concentration\ sample}}) \quad (3)$$

The response time trending in Fig. 5B illustrates the DNA hybridization duration of the solid-state genosensor in the determination of three different DEN-2 DNA concentrations i.e. 1×10^{-2} M, 1×10^{-6} M and 1×10^{-18} M tcDNA in the presence of 1 mM Zn^{2+} salphen complex at pH 7.5 and 670.06 nm. It was noticed that a longer time period was needed for the determination of low target DNA concentration at 1×10^{-18} M due to the low DNA hybridization reaction rate at low DNA concentration range detection. The DNA hybridization reaction rate increased with increasing tcDNA concentration at 1×10^{-6} M, but still levelled off at a much lower rate as it required as long as 75 min for the DNA hybridization process to go to completion. By increasing the tcDNA concentration to 1×10^{-2} M, the maximum reaction rate has greatly increased, and the DNA biosensor become saturated at 30 min of DNA hybridization time. However, a significant colour change can already be observed at min-15 of DNA hybridization reaction, and therefore it was chosen as the optimal response time of the proposed optical genosensor.

The bar chart in Fig. 5C exhibits the optical DNA biosensor reversibility in detecting 1 μM tcDNA. The DNA biosensor was found capable to be reused for optical DNA bioassay of DEN-2 up to four times (repeatability RSD = 2.5%, $n = 3$) after regeneration of the DNA biosensor with 0.1 M NaOH solution for 15 min. Upon exposure of the DNA biosensor to NaOH regeneration solution, the hydrogen bonds between nitrogenous bases of the immobilized dsDNA broken up leading to the unwinding of double-helical structure of dsDNA, and consequently causing intercalated label complexes to fall off from the DNA biosensor surface. The repetitive regeneration steps allowed subsequent re-hybridization of the DNA biosensor with the same tcDNA concentration for further reflectometric evaluation until the DNA biosensor response considerably declined. After four consecutive cycles of re-hybridization of the solid-state DNA biosensor, the optical genosensor response decreased to about 76% of its initial response, and on the 6th cycle of re-hybridization step, the DNA biosensor only remained about 62% of its original response. The large decrement in the optical reflectance response of the DNA biosensor was attributable to the desorption of immobilized cpDNA from the AMs immobilization matrix after several dehybridization and re-hybridization cycles of the DNA biosensor.

3.4. The purity and quantity of PCR product

Absorbance analysis of micro volume sample has become of paramount importance as there are many molecular biology techniques (e.g. gene cloning, PCR and DNA sequencing) require accurate quantitation of nucleic acids with minimum sample amount and smallest measurement time in order to eliminate traditional containment devices such as cuvettes [69]. Absorption of the nucleic acid samples is measured at specific wavelengths in order to assess the purity and concentration of nucleic acids, namely the ratio of absorbance at 260/230 nm and 260/280 nm that could verify the nucleic acid purity [68]. Ratios of 1.9–2.0 indicate highly purified preparations of RNA. The presence of contaminants from residual especially protein molecules has always been reported to lower the absorbance ratio [69,70].

In the present study, we examined five enrolled patients suspected with dengue virus infection aged 23–42 years old. The collection of blood, urine and saliva samples were performed from 17th of November 2017 until 5th of January 2018. Total RNAs of blood, urine and saliva were successfully obtained, and the quantity and quality of the respective extracted RNAs were evaluated by NanoDrop spectrophotometer at the wavelength of 260 nm and 280 nm with the concentration obtained in the range of 5.6–48.8 ng/μL RNA. In addition, we included RNA samples isolated from healthy volunteer's blood, urine and saliva as negative control (Table A.1). The absorption of reagent blank i.e. Milli-Q water was also taken prior to the RNA measurement using UV–Vis spectrophotometer. The absorbance readings obtained from the RNA samples showed inconsistent values ranging from 0.00 to 3.00 in the A260/230 and A260/A280 ratios indicating the presence of contaminants such as protein, residual phenol and chloroform from the extraction buffer, resulting in impurity of the RNA samples. Furthermore, Mackey and Chomczynski [71] had demonstrated in their study and revealed that both pH and ionic strength of the solutions may significantly reduce the RNA absorbance upon spectrophotometric assessment of nucleic acid purity. Moreover, RNA itself is an unstable molecule and has a very short half-life once extracted from the cell or tissues [72], and that special care and precautions are required during RNA isolation step as it is susceptible to degradation process. Most importantly, it is crucial to obtain purified biomolecules such as DNA, RNA and protein in sufficient quality and purity for the study related to clinical sample for further use in the downstream testing applications [73,74].

3.5. Detection of DEN-2 in blood, urine and saliva by PCR electrophoretic analysis

In order to obtain DNA molecule, RNA must first be converted to a double-stranded molecule. The product would contain a complementary strand (first, antisense or minus-strand cDNA) that is hybridized to what remains of the original RNA template in a reaction catalyzed by enzyme reverse transcriptase together with a suitable

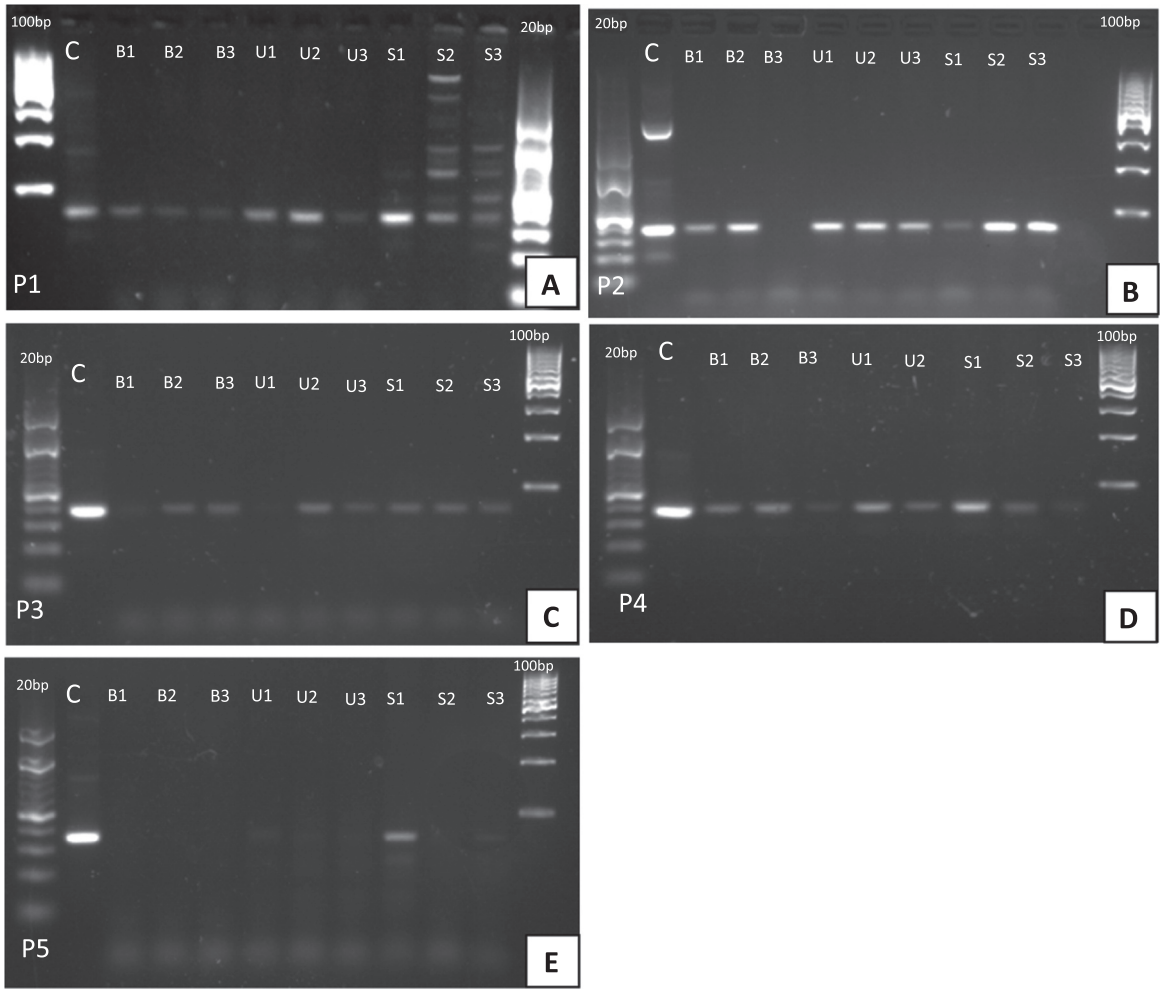


Fig. 6. (A–E) Detection of DEN-2 by PCR analysis in blood (B), urine (U) and saliva (S) samples for patient 1 (P1), patient 2 (P2), patient 3 (P3), patient 4 (P4) and patient 5 (P5). The lane numbers indicate patients' sample codes. Positive control of amplification was included and denoted as 'C'. Amplicon size: 69 bp. DNA ladder used: O'Range Ruler 20 bp and O'Range Ruler 100 bp. PCR products were detected on 4% Agarose in TBE buffer at 90 V for 60 min.

primer and addition of deoxyribonucleoside triphosphates (dNTPs) [75]. Then, the RNA will be degraded, and cDNA will act as a template for further amplification using PCR into dsDNA employing specific primer pair. And, since the yields depend on the targets and the choices of reverse transcriptase, it is imperative to use the same enzyme, priming strategy and experimental conditions if the results are to be comparable [75,76].

PCR analysis has been done for blood (B), urine (U) and saliva (S) samples collected from patient 1 (P1) to patient (P5) to confirm the presence of DEN-2 in these samples. As can been in Fig. 6A–D, the PCR electrophoretic results show the human fluid samples from P1 to P4 were all positive, discerning with approximately 69 base pairs of the expected amplicon bands, demonstrating the presence of DEN-2 in these samples. However, it was noticed that faint bands were observed for urine and saliva samples of P5, and no band was observed in the blood samples (Fig. 6E). No visible or barely discernable bands were observed for lanes P2B3, P2S1, P3B1, P3U1, P4S3 and P5S2 relative to the other samples within the triplicate might be due to poor primer annealing process, which gave no PCR product or primers bound non-specifically to the template within the same samples or pipetting errors, and resulted in weak bands observation. For these amplifications, positive control of 500 base pairs gBlocks of synthetic gene DEN-2 (C) was included in the PCR electrophoretic analysis. However, genomic DNA contaminations were discernible for saliva samples from P1 as multiple bands can be observed during PCR amplification (Fig. 6A). Additionally, no band was observed for the DNA of healthy volunteer (PH) used as negative control in the DEN-2 detection (Fig. 7A). In order to validate these negative results, β-actin DNAs were amplified using the corresponding cDNA and amplicons were detected in all samples (Fig. 7B), which indicates no inhibition in the PCR reaction. It is imperative to note that β-actin is expressed constitutively and involved in the basic housekeeping functions required for cell maintenance. It has a more stable expression level compared with other internal controls and also known as one of the commonly used reference genes in quantifying gene transcription [77].

3.6. DEN-2 sequencing analysis

The presence of DEN-2 was further verified with DNA sequencing using genomic DNA isolated from one of the patient samples (i.e. P1S1). List of Basic Local Alignment Search Tool (BLAST) hits that produced significant sequences homology with the query sequence is exhibited in Fig. A.5 in the Supplementary Data. By default, the results were sorted in ascending order according to the Expect value (E-value). Based on the list of BLAST hits, the sequence alignments indicate the query sequence was well-matched with the subject sequences in the database, which then resulted in significant alignments (99% sequence identity) with dengue type 2 (AY744150.1) (Fig. A.6).

Based on the results obtained in this study, infective DEN-2 virus particles were found to exist in both urine and saliva of dengue virus infected patients besides those commonly found in the blood samples. The isolation of viral RNA from blood, urine and saliva samples were

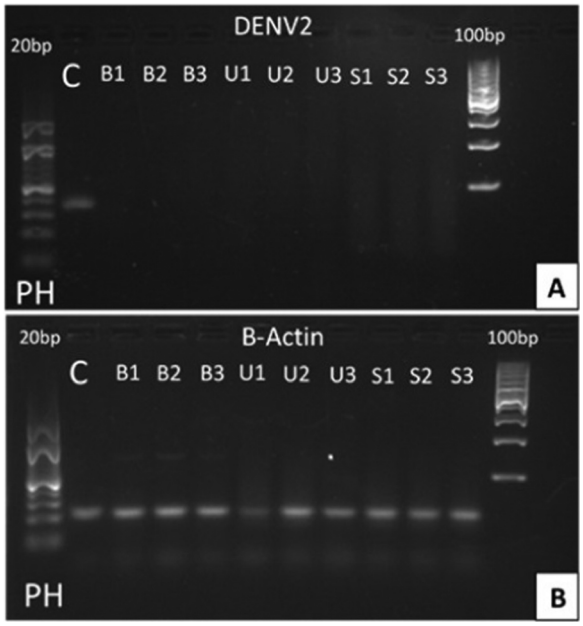


Fig. 7. (A) Detection of DENV-2 and (B) β-actin by PCR electrophoretic analysis in blood (B), urine (U) and saliva (S) samples for healthy volunteer (PH). The lane numbers indicate patient's sample codes. Positive control of amplification was included (denoted as 'C'). Amplicon size: 69 bp (DENV-2) and 50 bp (β-actin). DNA ladder used: O'Range Ruler 20 bp and O'Range Ruler 100 bp. PCR products were detected on 4% Agarose in TBE buffer at 90 V for 60 min.

associated with the dengue virus infected patients from day 1 to day 5 of fever. As dengue viremia only last for a few days after the onset of fever, the samples selected for the detection of viral genome were mainly those collected during patient hospitalization period. Previously reported studies had recognised the use of urine and saliva samples for the detection and diagnosis of dengue virus infection in human via real-time RT-PCR (RT-qPCR), the laboratory diagnostic method [55,78,79]. Furthermore, the detection of another *flavivirus* genome in urine has been described earlier for yellow fever [80] and Japanese encephalitis [81] using this method. Owing to the occurrence of viable dengue virus in saliva, hence a large range of viruses may potentially occur in this type of non-invasive specimen, such as Ebola, HIV, human papillomavirus (HPV), rubella, hepatitis B and hepatitis C viruses [82,83]. In fact, most of the current laboratory diagnostic methods require blood specimens that need medically-trained staffs for specimen collection.

In this study, we assessed the possibility to employ saliva and urine samples as alternatives to the blood specimen in the diagnosis of dengue virus infection. Urine and saliva samples can be easily collected via non-invasive procedures as they require only limited laboratory facilities, and rendering the patients to easily agreed with the procedures to diagnosis. Furthermore, a large volume of urine can be collected from the patients for subsequent diagnosis purposes. In some cases, dengue virus genome was still able to be determined in urine samples even when the serum sample collected from the same dengue virus infected patient already showed negative result by qPCR assay [55,80]. This was in agreement with a previously reported study on DEN-2 detection in human by RT-qPCR assay, which revealed a full DEN-2 genome sequence from the urine sample on day 18 of fever, hence providing the longest time frame ever reported for DEN-2 complete genome detection [84].

3.7. Validation of DEN-2 reflectometric DNA biosensor with standard RT-PCR method

Typically, the quality of cDNA can be accessed through the use of primer pairs of reference genes that are stably expressed in the samples by qPCR analysis, and that the purity and concentration of cDNA concentration can also be determined by modern OD (optical density) measurement via NanoDrop spectrophotometric analysis. The OD A260/A280 ratio greater than 1.8 is usually considered as an acceptable indicator of good cDNA quality [85]. Herein, the quality of cDNA obtained from the patients' fluid samples were found to give values of OD A260/A280 ratio within the range of 1.74–1.86, which is an indication of good cDNA quality (Table A.2). And, the concentration of RT-PCR products in ng/μL were converted into molarity (mol/L) by dividing their concentrations in mg/L with the molecular weight (M_W) of DEN-2 gBlocks (308,799.7 g/mol) (Table A.3).

In order to determine the accuracy and reliability of the developed optical DNA biosensor for DEN-2 detection, the results obtained from the DNA optode were compared with an established standard method of RT-PCR for nucleic acid detection [86–89]. Since blood, urine and saliva samples from the 5th patient showed some rather faint bands on the agarose gel due to lack of RT-PCR product was obtained, therefore only the first four patients were necessary in the validation study of the reflectometric DNA biosensor. As summarized in Table 2, the t values obtained for all sample types are less than the critical t value of $t = 3.182$ with p values larger than 0.05, hence there were no significant difference in the DEN-2 cDNA concentration detections by both measurement methods. This implies the proposed DNA biosensor based on optical Zn^{2+} label was providing results that were statistically similar with RT-PCR at 95% confidence level. Apart from this, the developed genosensor also proven to be capable in detecting DEN-2 cDNA in an accurate manner regardless of the complexity of blood components. The statistical analysis results are provided in Table A.4, Table A.5 and Table A.6 in the Supplementary Data.

Table 2
Validation of the reflectance DNA biosensor with RT-PCR method using DEN-2 cDNA at micromolar (μM) concentration levels.

Sample type	Patient code	cDNA concentration by DNA biosensor (μM)	cDNA concentration by RT-PCR (μM)	t value
Blood	P1B1	5.1 ± 0.869	4.6 ± 0.227	2.127
	P2B1	1.6 ± 1.123	1.3 ± 0.046	
	P3B1	2.8 ± 1.576	2.3 ± 0.350	
	P4B1	1.6 ± 2.599	1.7 ± 0.258	
Urine	P1U1	4.2 ± 0.384	4.8 ± 0.217	1.879
	P2U1	2.8 ± 1.146	2.6 ± 0.210	
	P3U1	2.4 ± 1.263	2.2 ± 0.167	
	P4U1	1.8 ± 0.464	1.7 ± 0.036	
Saliva	P1S1	1.2 ± 1.222	1.4 ± 0.053	0.197
	P2S1	1.3 ± 0.936	1.3 ± 0.093	
	P3S1	1.3 ± 0.359	1.1 ± 0.019	
	P4S1	1.7 ± 0.998	1.7 ± 0.122	

*$t_4 = 3.182$ ($p = 0.05$).

3.8. Performance comparison with other reported dengue-targeted biosensors

Table 3 tabulates the analytical performance of the developed reflectometric DEN-2 genosensor in terms of dynamic linear range, LOD and response time in comparison with other previously reported biosensors with different design bases developed by other researchers in detecting dengue virus particles. It is interesting to note that most researchers designed their dengue-targeted biosensors based on electrochemical approaches, such as potentiostat-differential pulse voltammetry (DPV) [90], potentiostat-electrochemical impedance spectroscopy (EIS)-DPV [34], potentiostat-cyclic voltammetry (CV)-DPV [91,92] and potentiostat-galvanostat-CV [93], nevertheless, there have been no one reported on optical sensing of dengue virus particles. Along with incorporation of hydrophobic AMs as the nucleic acid solid support material and Schiff base metal complex DNA intercalating

Table 3
Analytical performance comparison between the proposed DEN-2 optical microsensor with various reported dengue-targeted sensor.

Ref.	Sensor/approach	Design basis	Probe/target	Linear range	LOD	Response time (min)	Sample validation
Present study	Genosensor/optical	AMs-salphen	Synthetic DEN-2 DNA/cDNA	1×10^{-15} M to 1×10^{-3} M	1.21×10^{-16} M	15	Serum, saliva, urine
[90]	Genosensor/electrochemical	probe-modified PGE	Synthetic DEN-3 DNA/cDNA	$10–100 \times 10^{-9}$ M	3.09×10^{-9} M	10	Spiked serum
[34]	Genosensor/electrochemical	Pt electrode-AAO-Glutaraldehyde	Synthetic DEN DNA/cDNA	1×10^{-12} to 1×10^{-6} M	2.7×10^{-12} M	60	N/A
[91]	Genosensor/electrochemical	SiNWs/AuNPs- ITO-MB	Synthetic DEN probe/cDNA	9.0–178 ng/mL	3.5 ng/mL	120	N/A
[92]	Genosensor/electrochemical	SPGE-SiNWs-DTPA-AuNPs	Synthetic DEN probe/cDNA	1×10^{-11} to 1×10^{-7} M	1.63×10^{-12} M	120	N/A
[93]	Immunosensor/electrochemical	CNT-SPE	DEN NS1 antibody/antigen	40 ng/mL to 2 mg/mL	12 ng/mL	30	Spiked serum, spiked buffer

*AAO = anodic aluminum oxide, AuNPs = gold nanoparticles, CNT-SPE = carbon nanotube-screen printed electrodes, DTPA = dithiodipropionic acid, ITO = indium tin oxide, MB = methylene blue, NS1 = non-structural 1, PGE = pencil graphite electrode, Pt = platinum, SiNW = silicon nanowire, SPGE = screen-printed gold electrode.

agent, the proposed optical DEN-2 genosensor design basis was proven to be far more superior than the electrochemical-based biosensors, for instance, the developed optical reflectance biosensor was managed to achieve a more sensitive DEN-2 DNA linear detection range from 1 fM to 1 mM with a lower detection limit at 0.12 fM. Furthermore, validation study of both electrochemical immunosensor and nucleic acid biosensor merely involved recovery testing over spiked synthetic cDNA in serum and buffer media without any application demonstrations using clinical samples of whole blood, urine and saliva from dengue virus infected patients. Recovery analysis done to validate NS1 immunosensor, on the other hand, showed better accuracy in detecting spiked synthetic cDNA in buffer medium than in serum medium ascribed to the complexity of blood medium that restricted the efficiency of the electrochemical immunosensor's antigen/antibody assay [93].

Even though the electrochemical genosensor based on pencil graphite electrode (PGE) has been reported to show fast DNA hybridization time at 10 min for DEN-3 DNA detection [90], however, the DNA hybridization reaction required stirring (300 rpm) and annealing (52 °C) steps to be done in a thermomixer. Moreover, it needed as much as 70 µL of DEN-3 DNA for hybridization reaction to take place. In contrast, the developed optical DNA microsensor required much less amount of DEN-2 DNA at 3.2 µL to be simply loaded at the microcentrifuge cap at room temperature to provide a visible colour change without any pre-stirring and pre-heating steps were involved. Thus, the developed reflectometric DNA biosensor is advantageous in offering clinicians an early DEN-2 diagnosis in human through naked-eye observation as early as onset of illness, i.e. when fever symptom started to manifest in patient.

4. Conclusions

We have designed a novel optical genosensor to detect DEN-2 cDNA with the utilization of Zn^{2+} salphen as optical nucleotide label and AMs polymer as DNA immobilization matrix. This design basis has provided a very good selectivity in distinguishing one-base variation in nucleotide sequence, high sensitivity screening at low tcDNA concentrations and rapid hybridization time to give observable colour change at the DNA biosensor surface. In addition, demonstration of the DNA biosensor in testing of DEN-2 genome in clinical samples of blood, urine and saliva from dengue-positive patients have been conducted in comparison with RT-PCR reference method with no statistical significant different in the results generated by both methods. Thus, this genosensor can potentially be applied in the future as DEN-2 epidemiological surveillance tool in larvae and mosquitoes.

Acknowledgements

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Appendix B. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2019.02.036.

References

[1] S. Sekaran, A. Rathakrishnan, A. Yeo, Dengue: an overview, J. Health Transl. Med. 17 (2017) 23–32.
[2] A. Shankar, Indigenous composit in management of dengue fever, Int. J. Ayurveda 1 (2017) 17–24.
[3] E.C. Shekar, B. Kishan, S. Kumar, B. Rao, A prospective study to analyze the clinical, biochemical and hematological parameters of dengue fever in Mahatma Gandhi Memorial Hospital, Warangal, Telangana, India, Int. J. Adv. Med. 4 (2017) 218–224.
[4] I. Sanchez-Vargas, J.C. Scott, B.K. Poole-Smith, A.W.E. Franz, V. Barbosa-Solomieu, Dengue virus type 2 infections of *Aedes aegypti* are modulated by the mosquito's RNA interference pathway, PLoS Pathog. 5 (2009) 1–11.
[5] B.D. Lindenbach, C. Rice, Flaviviridae: the viruses and their replication, in: D.M. Knipe, P.M. Howley (Eds.), Fields Virology, Lippincott-Raven Publishers, Philadelphia, 2001, pp. 1101–1152.
[6] A. Guzman, R.E. Isturiz, Update on the global spread of dengue, Int. J. Antimicrob. Agents 36 (2010) S40–S42.
[7] S. Eram, Y. Setyabudi, T.I. Sadono, D.S. Sutrisno, D.J. Gubler, J. Sulianti-Saroso, Epidemic dengue hemorrhagic fever in rural Indonesia: clinical studies, Am. J. Trop. Med. Hyg. 28 (1979) 711–716.
[8] D.J. Gubler, D. Reed, L. Rosen, J.C.J. Hitchcock, Epidemiologic, clinical and virologic observations on dengue in the Kingdom of Tonga, Am. J. Trop. Med. Hyg. 27 (1978) 581–589.
[9] T.T. Kuberski, L. Rosen, A simple technique for the detection of dengue antigen in mosquitoes by immunofluorescence, Am. J. Trop. Med. Hyg. 26 (1977) 533–537.
[10] D.W. Vaughn, S. Green, S. Kalayanarooj, B.L. Innis, S. Nimmannitya, S. Suntayakorn, A.L. Rothman, F.A. Ennis, A. Nisalak, Dengue in the early febrile phase: viremia and antibody responses, J. Infect. Dis. 176 (1997) 322–330.
[11] D.J. Gubler, Dengue and dengue hemorrhagic fever, Clin. Microbiol. Rev. 11 (1998) 480–496.
[12] D.J. Gubler, W. Suharyono, R. Tan, M. Abidin, A. Sie, Viraemia in patients with naturally acquired dengue infection, Bull. World Health Organ. 59 (1981) 623–630.
[13] D. Burke, T. Monath, Flaviviruses, in: D.M. Knipe, P.M. Howley (Eds.), Fields Virology, Lippincott Williams and Wilkins, Philadelphia, 2001, pp. 1043–1126.
[14] I.M. Mackay, Real-time PCR in the microbiology laboratory, Clin. Microbiol. Infect. 10 (2004) 190–212.
[15] Z. Fatima, M. Idrees, M.A. Bajwa, Z. Tahir, O. Ullah, M.Q. Zia, A. Hussain, M. Akram, B. Khubaib, S. Afzal, Serotype and genotype analysis of dengue virus by sequencing followed by phylogenetic analysis using samples from three mini outbreaks-2007–2009 in Pakistan, BMC Microbiol. 11 (2011) 200–208.
[16] P. Jahanshahi, E. Zalnezhad, S.D. Sekaran, F.R.M. Adikan, Rapid immunoglobulin M-based dengue diagnostic test using surface plasmon resonance biosensor, Sci. Rep. 4 (2014) 3851–3858.
[17] B.-T. Teoh, S.-S. Sam, K.-K. Tan, M.B. Danlami, M.-H. Shu, J. Johari, P.-S. Hooi, D. Brooks, O. Piepenburg, O. Nentwich, Early detection of dengue virus by use of reverse transcription-recombinase polymerase amplification, J. Clin. Microbiol. 53 (2015) 830–837.
[18] N.T. Toan, S. Rossi, G. Prisco, N. Nante, S. Viviani, Dengue epidemiology in selected endemic countries: factors influencing expansion factors as estimates of underreporting, Trop. Med. Int. Health 20 (2015) 840–863.
[19] K.-I. Yamada, T. Takasaki, M. Nawa, I. Kurane, Virus isolation as one of the diagnostic methods for dengue virus infection, J. Clin. Virol. 24 (2002) 203–209.
[20] H.P. Nascimento, M.D. Oliveira, C.P. de Melo, G.J. Silva, M.T. Cordeiro, C.A. Andrade, An impedimetric biosensor for detection of dengue serotype at picomolar concentration based on gold nanoparticles-polyaniline hybrid composites, Colloids Surf. B Biointerfaces 86 (2011) 414–419.
[21] M.L. Moi, D. Kobayashi, H. Isawa, T. Sasaki, M. Saijo, I. Kurane, K. Sawabe, T. Takasaki, Dengue virus isolation in mosquito *Aedes albopictus* captured during an outbreak in Tokyo, 2014, by a method relying on antibody-dependent enhancement mechanism using FcγR-expressing BHK cells, Vector Borne Zoonotic Dis. 16 (2016) 810–812.
[22] Y.Y. Kong, C.H. Thay, T.C. Tin, S. Devi, Rapid detection, serotyping and quantitation of dengue viruses by TaqMan real-time one-step RT-PCR, J. Virol. Methods 138 (2006) 123–130.
[23] M. Parida, K. Horioke, H. Ishida, P.K. Dash, P. Saxena, A.M. Jana, M.A. Islam, S. Inoue, N. Hosaka, K. Morita, Rapid detection and differentiation of dengue virus serotypes by a real-time reverse transcription-loop-mediated isothermal amplification assay, J. Clin. Microbiol. 43 (2005) 2895–2903.
[24] S. Bustin, Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems, J. Mol. Endocrinol. 29 (2002) 23–39.
[25] V. Kumarasamy, A.A. Wahab, S. Chua, Z. Hassan, M. Mohamad, K. Chua, Evaluation of a commercial dengue NS1 antigen-capture ELISA for laboratory diagnosis of acute dengue virus infection, J. Virol. Methods 140 (2007) 75–79.
[26] J.P. Messina, O.J. Brady, D.M. Pigott, N. Golding, M.U.G. Kraemer, T.W. Scott, G.R.W. Wint, D.L. Smith, S.I. Hay, The many projected futures of dengue, Nat. Rev. Microbiol. 13 (2015) 230–239.
[27] S. Flasche, M. Jit, I. Rodríguez-Barraquer, L. Coudeville, M. Recker, K. Koelle, G. Milne, T.J. Hladish, T.A. Perkins, D.A. Cummings, The long-term safety, public health impact, and cost-effectiveness of routine vaccination with a recombinant, live-attenuated dengue vaccine (Dengvaxia): a model comparison study, PLoS Med. 13 (2016) e1002181.
[28] J. Lourenco, M. Recker, Dengue serotype immune-interactions and their consequences for vaccine impact predictions, Epidemics 16 (2016) 40.
[29] S.B. Halstead, Critique of world health organization recommendation of a dengue vaccine, J. Infect. Dis. 214 (2016) 1793–1795.
[30] Lyon, Sanofi Pasteur: First dengue vaccine approved in more than 10 countries, ⟨http://www.sanofipasteur.com/en/articles/first_dengue_vaccine_approved_in_more_than_10_countries.aspx⟩, October, 2016. (Accessed February 2017).
[31] Y. Liu, J. Liu, G. Cheng, Vaccines and immunization strategies for dengue prevention, Emerg. Microbes Infect. 5 (2016) e77.
[32] A. Sabchareon, D. Wallace, C. Sirivichayakul, K. Limkittikul, P. Chanthavanich, S. Suvannadabba, V. Jiwariyavej, W. Dulyachai, K. Pengsaa, T.A. Wartel, Protective efficacy of the recombinant, live-attenuated, CYD tetravalent dengue vaccine in Thai schoolchildren: a randomised, controlled phase 2b trial, Lancet 380 (2012) 1559–1567.
[33] L.M. Schwartz, M.E. Halloran, A.P. Durbin, I.M. Longini, The dengue vaccine pipeline: implications for the future of dengue control, Vaccine 33 (2015) 3293–3298.
[34] J. Deng, C.-S. Toh, Impedimetric DNA biosensor based on a nanoporous alumina membrane for the detection of the specific oligonucleotide sequence of dengue virus, Sensors 13 (2013) 7774–7785.
[35] D.H. Libraty, P.R. Young, D. Pickering, T.P. Endy, S. Kalayanarooj, S. Green, D.W. Vaughn, A. Nisalak, F.A. Ennis, A.L. Rothman, High circulating levels of the dengue virus nonstructural protein NS1 early in dengue illness correlate with the development of dengue hemorrhagic fever, J. Infect. Dis. 186 (2002) 1165–1168.
[36] C.M. da Silva, D.L. da Silva, L.V. Modolo, R.B. Alves, M.A. de Resende, C.V. Martins, Â. Fátima, Schiff bases: a short review of their antimicrobial activities, J. Adv. Res. 2 (2011) 1–8.
[37] A. Arola-Arnal, J. Benet-Buchholz, S. Neidle, R. Vilar, Effects of metal coordination geometry on stabilization of human telomeric quadruplex DNA by square-planar and square-pyramidal metal complexes, Inorg. Chem. 47 (2008) 11910–11919.
[38] A. Terenzi, C. Ducani, L. Male, G. Barone, M.J. Hannon, DNA interaction of Cu II, Ni II and Zn II functionalized salphen complexes: studies by linear dichroism, gel electrophoresis and PCR, Dalton Trans. 42 (2013) 11220–11226.
[39] V.K. Gupta, A.K. Singh, L.K. Kumawat, Thiazole Schiff base turn-on fluorescent chemosensor for Al^{3+} ion, Sens. Actuators B Chem. 195 (2014) 98–108.
[40] S. Karthikeyan, V.K. Gupta, R. Titus, G. Sekaran, A new approach for the degradation of high concentration of aromatic amine by heterocatalytic Fenton oxidation: kinetic and spectroscopic studies, J. Mol. Liq. 173 (2012) 153–163.
[41] M. Benjamin, D. Manoj, K. Thenmozhi, P.R. Bhagat, D. Saravanakumar, S. Senthilkumar, A bioinspired ionic liquid tagged cobalt-salophen complex for nonenzymatic detection of glucose, Biosens. Bioelectron. 91 (2016) 380–387.
[42] N.H.A. Karim, O. Mendoza, A. Shivalingam, A.J. Thompson, S. Ghosh, M.K. Kuimova, R. Vilar, Salphen metal complexes as tunable G-quadruplex binders and optical probes, RSC Adv. 4 (2014) 3355–3363.
[43] V.K. Gupta, N. Mergu, L.K. Kumawat, A.K. Singh, Selective naked-eye detection of Magnesium (II) ions using a coumarin-derived fluorescent probe, Sens. Actuators B Chem. 207 (2015) 216–223.
[44] S.K. Srivastava, V.K. Gupta, S. Jain, Determination of lead using a poly(vinyl chloride)-based crown ether membrane, Analyst 120 (1995) 495–498.
[45] K. Suresh, S.K. Srivastava, V.K. Gupta, M.K. Dwivedi, S. Jain, Caesium PVC–crown (dibenzo-24-crown-8) based membrane sensor, Anal. Proc. 32 (1995) 21–23.
[46] S.K. Srivastava, V.K. Gupta, S. Jain, Determination of lead using a poly(vinyl chloride)-based crown ether membrane, Analyst 120 (1995) 495–498.
[47] V.K. Gupta, L.P. Singh, R. Singh, N. Upadhyay, S.P. Kaur, B. Sethi, A novel copper (II) selective sensor based on dimethyl 4, 4′ (o-phenylene) bis(3-thioallophanate) in PVC matrix, J. Mol. Liq. 174 (2011) 11–16.
[48] S.K. Srivastava, V.K. Gupta, S. Jain, PVC-based 2,2,2-cryptand sensor for zinc ions, Anal. Chem. 68 (1996) 1272–1275.
[49] V.K. Gupta, N. Mergu, L.K. Kumawat, A.S. Singh, A reversible fluorescence "off–on–off" sensor for sequential detection of aluminum and acetate/fluoride ions, Talanta 144 (2015) 80–89.
[50] M.L. Yola, V.K. Gupta, T. Eren, A.E. Sen, A novel electro analytical nanosensor based on graphene oxide/silver nanoparticles for simultaneous determination of quercetin and morin, Electrochim. Acta 120 (2014) 204–211.
[51] H. Karimi-Maleh, F. Tahernejad-Javazmi, N. Atar, M.L. Yola, V.K. Gupta, A.A. Ensafi, A novel DNA biosensor based on a pencil graphite electrode modified with polypyrrole/functionalized multiwalled carbon nanotubes for determination of 6-mercaptopurine anticancer drug, Ind. Eng. Chem. Res. 54 (2015) 3634–3639.
[52] V.K. Gupta, N. Atar, M.L. Yola, Z. Ustundag, L. Uzun, A novel magnetic Fe@Au core–shell nanoparticles anchored graphene oxide recyclable nanocatalyst for the reduction of nitrophenol compounds, Water Res. 48 (2014) 210–217.
[53] A. Ulianas, Y.H. Lee, S.A. Hanifah, L.L. Tan, An electrochemical DNA microbiosensor based on succinimide-modified acrylic microspheres, Sensors 12 (2012) 5445–5460.
[54] M. Gupta, D. Yadav, S. Mishra, P. Sharma, Identification of preanalytical errors in the clinical laboratory of North Indian tertiary care hospital, Biochem. Physiol. 4 (2015) 1–6.
[55] T. Hirayama, Y. Mizuno, N. Takeshita, A. Kotaki, S. Tajima, T. Omatsu, K. Sano, I. Kurane, T. Takasaki, Detection of dengue virus genome in urine by real-time reverse transcriptase PCR: a laboratory diagnostic method useful after disappearance of the genome in serum, J. Clin. Microbiol. 50 (2012) 2047–2052.
[56] N. Shahfiza, H. Osman, T.T. Hock, K. Shaari, A.-H.Z. Abdel-Hamid, Metabolomics for characterization of gender differences in patients infected with dengue virus, Asian Pac. J. Trop. Med. 8 (2015) 451–456.
[57] N. Shahfiza, H. Osman, T.T. Hock, A.-H.Z. Abdel-Hamid, Metabolomics approach for multibiomarkers determination to investigate dengue virus infection in human patients, Acta Biochim. Pol. 64 (2017) 1–5.
[58] J.M. Jones, C.A. Watkins, J.S. Hand, J.J. Warren, H.J. Cowen, Comparison of three salivary flow rate assessment methods in an elderly population, Community Dent. Oral Epidemiol. 28 (2000) 177–184.

[59] Y. Li, M.A.R. St. John, X. Zhou, Y. Kim, U. Sinha, R.C.K. Jordan, D. Eisele, E. Abemayor, D. Elashoff, N.-H. Park, D.T. Wong, Salivary transcriptome diagnostics for oral cancer detection, Clin. Cancer Res. 10 (2004) 8442–8450.
[60] P. Lopez-Jornet, F. Camacho-Alonso, A. Bermejo-Fenoli, A simple test for salivary gland hypofunction using Oral Schirmer's test, J. Oral Pathol. Med. 35 (2006) 244–248.
[61] M. Navazesh, M. Christensen, A comparison of whole mouth resting and stimulated salivary measurement procedures, J. Dent. Res. 61 (1982) 1158–1162.
[62] K.W. Stephen, Methods for collecting individual components of mixed saliva: the relevance to clinical pharmacology, Br. J. Clin. Pharmacol. 3 (1976) 315–319.
[63] Statistics How To. T Test (Student's T-Test): Definition and Examples. Available at: ⟨http://www.statisticshowto.com/probability-and-statistics/t-test⟩. (Accessed 28 May 2018).
[64] A. Ulianas, L.Y. Heng, M. Ahmad, A biosensor for urea from succinimide-modified acrylic microspheres based on reflectance transduction, Sensors 11 (2011) 8323–8338.
[65] B.M. Zeglis, V.C. Pierre, J.K. Barton, Metallo-intercalators and metallo-insertors, Chem. Commun. 44 (2007) 4565–4579.
[66] E.M. Boon, D.M. Ceres, T.G. Drummond, M.G. Hill, J.K. Barton, Mutation detection by electrocatalysis at DNA-modified electrodes, Nat. Biotechnol. 18 (2000) 1096–1100.
[67] K.A. Hanley, J.J. Lee, J.E. Blaney, B.R. Murphy, S.S. Whitehead, Paired charge-to-alanine mutagenesis of dengue virus type 4 NS5 generates mutants with temperature-sensitive, host range, and mouse attenuation phenotypes, J. Virol. 76 (2002) 525–531.
[68] V. Stollar, R.W. Schlesinger, T.M. Stevens, Studies on the nature of dengue viruses: III. RNA synthesis in cells infected with type 2 dengue virus, Virology 33 (1967) 650–658.
[69] M.H. Simonian, J.A. Smith, Spectrophotometric and colorimetric determination of protein concentration, Curr. Protoc. Mol. Biol. 76 (2006) (10.1.1-10.1A.9).
[70] F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, K. Struhl (Eds.), Current Protocols in Molecular Biology, John Wiley and Sons Inc, New York, 1999.
[71] W. Wilfinger, K. Mackey, P. Chomczynski, Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity, Biotechniques 22 (1997) 474–481.
[72] Z. Li, S. Reimers, S. Pandit, M.P. Deutscher, RNA quality control: degradation of defective transfer RNA, EMBO J. 21 (2002) 1132–1138.
[73] S.R. Gallagher, P.R. Desjardins, Quantitation of DNA and RNA with absorption and fluorescence spectroscopy, Curr. Protoc. Mol. Biol. 76 (2006) (A.3D.1-A.3D.21).
[74] T. Maniatis, E.F. Fritsch, J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1982, p. 545.
[75] A. Ståhlberg, J. Håkansson, X. Xian, H. Semb, M. Kubista, Properties of the reverse transcription reaction in mRNA quantification, Clin. Chem. 50 (2004) 509–515.
[76] A. Ståhlberg, M. Kubista, M. Pfaffl, Comparison of reverse transcriptases in gene expression analysis, Clin. Chem. 50 (2004) 1678–1680.
[77] A. Radonić, S. Thulke, I.M. Mackay, O. Landt, W. Siegert, A. Nitsche, Guideline to reference gene selection for quantitative real-time PCR, Biochem. Biophys. Res. Commun. 313 (2004) 856–862.
[78] E.M. Korhonen, E. Huhtamo, A.-M.K. Virtala, A. Kantele, O. Vapalahti, Approach to non-invasive sampling in dengue diagnostics: exploring virus and NS1 antigen detection in saliva and urine of travelers with dengue, J. Clin. Virol. 61 (2014) 353–358.
[79] T.R. Poloni, A.S. Oliveira, H.L. Alfonso, L.R. Galvão, A.A. Amarilla, D.F. Poloni, L.T. Figueiredo, V.H. Aquino, Detection of dengue virus in saliva and urine by real time RT-PCR, Virol. J. 7 (2010) 22.
[80] Y. Mizuno, A. Kotaki, F. Harada, S. Tajima, I. Kurane, T. Takasaki, Confirmation of dengue virus infection by detection of dengue virus type 1 genome in urine and saliva but not in plasma, Trans. R. Soc. Trop. Med. Hyg. 101 (2007) 738–739.
[81] A. Mathur, N. Khanna, R. Kulshreshtha, S. Maitra, U. Chaturvedi, Viruria during acute Japanese encephalitis virus infection, Int. J. Exp. Pathol. 76 (1995) 103–109.
[82] C. Streckfus, L. Bigler, Saliva as a diagnostic fluid, Oral Dis. 8 (2002) 69–76.
[83] P.L. Corstjens, W.R. Abrams, D. Malamud, Saliva and viral infections, Periodontol 70 (2016) 93–110.
[84] X. Ma, W. Zhen, P. Yang, X. Sun, W. Nie, L. Zhang, H. Xu, K. Hu, First confirmation of imported dengue virus serotype 2 complete genome in urine from a Chinese traveler returning from India, Virol. J. 11 (2014) 56.
[85] S.M. Peterson, J.L. Freeman, RNA isolation from embryonic zebrafish and cDNA synthesis for gene expression analysis, J. Vis. Exp. (2009) 1470.
[86] K. Bunroddith, N. Viseshakul, K. Chansiri, P. Lieberzeit, QCM-based rapid detection of PCR amplification products of *Ehrlichia canis*, Anal. Chim. Acta (2017) 1–6.
[87] L. Chang, J. Li, L. Wang, Immuno-PCR: an ultrasensitive immunoassay for biomolecular detection, Anal. Chim. Acta 910 (2016) 12–24.
[88] D.J. O'Shea, P.J. O'Sullivan, G.V. Ponomarev, D.B. Papkovsky, Post-PCR detection of nucleic acids using metalloporphyrin labels and time-resolved fluorescence, Anal. Chim. Acta 537 (2005) 111–117.
[89] L. Yang, F. Du, G. Chen, A. Yasmeen, Z. Tang, A novel colorimetric PCR-based biosensor for detection and quantification of hepatitis B virus, Anal. Chim. Acta (2014) 1–7.
[90] N. Oliveira, E. Souza, D. Ferreira, D. Zanforlin, W. Bezerra, M.A. Borba, M. Arruda, K. Lopes, G. Nascimento, D. Martins, M. Cordeiroand, J. Lima-Filho, A sensitive and selective label-free electrochemical DNA biosensor for the detection of specific dengue virus serotype 3 sequences, Sensors 15 (2015) 15562–15577.
[91] J.I.A. Rashid, N.A. Yusof, J. Abdullah, U. Hashim, R. Hajian, The utilization of SiNWs/AuNPs-modified indium tin oxide (ITO) in fabrication of electrochemical DNA sensor, Mater. Sci. Eng. C 45 (2014) 270–276.
[92] J.I.A. Rashid, N.A. Yusof, J. Abdullah, U. Hashim, R. Hajian, A novel disposable biosensor based on SiNWs/AuNPs modified-screen printed electrode for dengue virus DNA oligomer detection, IEEE Sens. J. (2015) 1–8.
[93] A.C.M.S. Dias, S.L.R. Gomes-Filho, M.M.S. Silva, R.F. Dutra, A sensor tip based on carbon nanotube-ink printed electrode for the dengue virus NS1 protein, Biosens. Bioelectron. 44 (2013) 216–221.