Aptamer-Based ELISA Assay for Highly Specific and Sensitive Detection of Zika NS1 Protein

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S *Supporting Information*

ABSTRACT: We report here a few Zika NS1-binding ssDNA aptamers selected using the conventional SELEX protocol, and their application in an ELISA assay for sensitive diagnosis of Zika NS1 protein. Among the aptamers identified, aptamers **2** and **10** could recognize different binding epitopes of Zika NS1 protein. This complementary in binding site, when coupled with an extraordinarily high binding affinity by **2** (41-nt, K_D = 45 pM) and high specificity by **10**, was used successfully to construct an ELISA-based assay where **2** and **10** serve as the capture and detection agents, respectively, giving rise to a highly specific detection of Zika NS1 with a detection limit of 100 ng/mL in buffer. Further testing of a few in-house anti-Zika NS1 antibodies show that **2** could also pair with an anti-Zika NS1 antibody. Such aptamer-antibody pairing not only lowers the detection sensitivity by 3 orders of magnitude to 0.1 ng/mL in buffer but also enable highly sensitive detection of as low as 1 and 10 ng/mL of Zika NS1 to be carried out in 10% and 100% human serum, respectively. These results suggest that the selected aptamers would be useful for medical diagnosis of Zika virus infection in various aptamer-based diagnostic devices including ELISA assay.
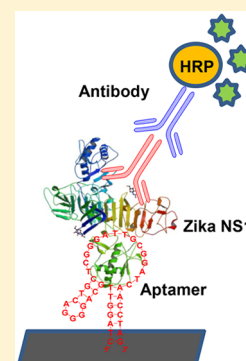
Zika virus poses great dangers to women at pregnancy as the virus can be passed from pregnant women to their fetus, resulting in microcephaly.[1] It has been also reported that Zika virus infection could be associated with Guillain-Barré syndrome (GBS) and meningoencephalitis in adult.[2] Given a lack of rapid and sensitive diagnostic tools for Zika virus at the present time, the recent outbreak of Zika virus infection in Brazil in 2015 and many others reported in more than 20 countries all over the world with an increase in cases[1] suggest the sensitive diagnostic tools for Zika infection are in urgent demand.

In clinical setting, early diagnosis of Zika infection would be more effective to control epidemic and for timely treatment. For this purpose, RT-qPCR assay using specific primers to detect viral RNAs is recommended as a preferred diagnostic method. This method however has some inherent disadvantages, including false-negative results from new strains or false-positive results arising from sample contamination.[3] Hence, the RT-qPCR results need to be further confirmed by other types of assays. Another preferred option is to use serological methods for detecting either Zika viral antigens (e.g., NS1) or immunoglobulins (e.g., IgG and IgM antibodies). Since IgG/IgM detection generally works in later stage of Zika fever (>7 days from symptom onset but variable from case to case),[1,3] early detection of Zika infection relies more on NS1 viral antigen (e.g., up to 9 days for Dengue NS1),[4,5] which is more sensitive than other Zika envelope proteins.[6,7] A combination of NS1- and IgG/IgM-based detections certainly would be very appropriate and could lead to higher accuracy and reliability of disease diagnostics.

For the diagnostic purpose, target-binding aptamers selected using SELEX (Systematic Evolution of Ligands by EXponential enrichment) technology[8,9] offer some advantages over anti-bodies in terms of high batch-to-batch consistency, lowered cost, better stability, the ease of modification, etc.[10−12] Therefore, in our pursuant in finding diagnostic agents for Zika infection, we opted to replace antibodies as much as possible with aptamers. Our motivation to use aptamers is further fueled by the fact that no useful aptamers for Zika NS1 antigen have been reported so far, while several anti-Zika NS1 antibodies for ELISA are presently commercially available in the market.

In our current work, we employed the SELEX protocol to discover a number of single stranded DNA (ssDNA) aptamers, exhibiting pico- to nanomolar binding affinities toward Zika NS1 antigen with one aptamer able to highly specifically recognize Zika NS1 but not any of four Dengue NS1 serotypes. Moreover, these selected ssDNA aptamers can pair with each other or with an anti-Zika virus antibody, enabling, for the first time, aptamer-mediated highly specific and sensitive detection of Zika NS1 protein in the sandwich complex-based applications such as ELISA assay. Moreover, the best sensitivity could reach as low as 0.1 ng/mL in binding buffer or 1 ng/mL in 10% human serum.

■ EXPERIMENTAL SECTION

Selection Conditions and Procedures for SELEX. Oligonucleotides including random aptamer library and primers were purchased from Integrated DNA Technologies (USA) or Sangon Biotech (China). All procedures were carried out at room temperature. Briefly, the random 60-nt nucleotide ssDNA

aptamer library (more than 10^{16} diversity (25 nmole), 5′-GATAGAATTCGAGCTCGGGC-N60-GCGGGTCGACAA-GCTTTAAT-3′) was heated at 95 °C for 5 min and then cooled to room temperature for >30 min to allow refolding in 200 μL of SELEX buffer (20 mM Tris at pH 7.5 with 150 mM NaCl, 1 mM $MgCl_2$, 2.7 mM KCl and 0.005% NP-40). The aptamer library was incubated with 1 μg of 6x histidine peptide and 40 μL of magnetic Ni-NTA beads (PureProteome nickel magnetic beads, Millipore) for 30 min with shaking (200 μL), followed by using a magnet rack to remove ssDNAs that bind to 6x histidine tag and beads. The recovered supernatant subsequently was incubated for 30 min with appropriate concentrations (500 nM for cycle 1, 100 nM for cycles 2−5, 10 nM of cycle 6 and 1 nM for cycle 7) of 6× histidine-tagged Zika NS1 protein (Acro Biosystems, Brazil strain, accession no. ALU33341), and further incubated with 30 μL of magnetic nickel beads for 30 min with shaking. After they were washed with SELEX buffer (1 mL) three times, the beads containing the sequences bound to Zika NS1 were incubated with a solution containing 1 M NaCl and 10 mM NaOH for 5 min with shaking and subjected to phenol extraction. The liquid layer was purified using G-25 microspin column (GE healthcare) and the eluted sequences were amplified by PCR using taq polymerase system (2× PCR master mix, first Base) using forward (5′-GATAGAATTCGAGCTCGGGC-3′) and 5′-phosphate containing reverse (5′-phosphate-ATTAAAGCT-TGTCGACCCGC-3′) primers [95 °C for 5 min, 10 to 20 cycles (95 °C for 30 s, 55 °C for 30 s and 72 °C 30 for seconds) and 72 °C for 3 min]. After ethanol precipitation and centrifugation to recover PCR product, ssDNA aptamers were prepared using lambda nuclease (Thermo Fisher Scientific) reaction for digesting the complementary sequences containing 5′-phosphate with manufacturer's protocol. ssDNAs were purified by phenol extraction and ethanol precipitation with 20 μg of glycogen, and then used for the next cycle of SELEX selection after quantification.

Deep Sequencing. Sequence analysis of PCR product from the seventh SELEX cycle was carried out using Ion PGM sequencer system and kits (Life Technologies) using manufacturer's protocol.

Determination of Binding Affinity (K_D) using BLItz Label-Free Biosensor System. Biotinylated or nonmodified ssDNA aptamers were purchased from Integrated DNA technologies. BLItz label-free biosensor system for biolayer interferometry assay (ForteBio, USA) was used for the binding test and the determination of binding affinity (K_D). Briefly, 5′-biotinylated (5′-biotin-TEG) ssDNA aptamer (100 nM) was loaded onto streptavidin (SA) sensor (10 to 20 s) for immobilization. Various concentrations of Zika NS1, which mainly exist in the form of dimer or hexamer in the membrane-bound or solution state, respectively,[1] were incubated with the aptamer-immobilized SA sensor or control SA sensor in binding buffer (30 mM Tris at pH 7.5 with 150 mM NaCl, 1 mM $MgCl_2$ and 0.1% bovine serum albumin (BSA)). Both association and dissociation events were recorded for 300 s for the kinetic analysis after a waiting time of 60 s for baseline to stabilize. Binding kinetics data were analyzed using BLItz Pro 1.2 software.

For binding specificity test, a solution containing Zika NS1, Interferon-gamma (IFN-γ, Sino Biological) or any of four Dengue NS1 serotypes (10 nM for **2** or 500 nM for **10**, BioRad) was loaded onto a SA sensor immobilized with the aptamer. After a waiting time of 60 s for baseline to stabilize,

both association and dissociation events were recorded for 300 s for the kinetic analysis.

For screening of aptamer−aptamer pair or aptamer-antibody pair for sandwich ELISA assay, biotinylated **2** (100 nM) was loaded onto SA (Streptavidin) sensor (150 or 20 s) for immobilization after a waiting time of 30 s for baseline to stabilize. 100 nM of Zika NS1 protein was then incubated with the aptamer-immobilized SA sensor for 300 s in binding buffer. After 120 s (or 60 s for antibody screening) for baseline to stabilize, 1 μM of identified aptamer or in-house anti-Zika NS1 monoclonal antibody (rabbit IgG) was loaded to check if these aptamers or antibodies show competitive binding or additional binding to the preformed **2**-Zika NS1 complex. Both association and dissociation events were monitored for 300 s, respectively.

Sandwich ELISA for Detecting Zika NS1 Antigen in 96-Well Plate Format. For the sandwich complex-based ELISA assay using **2** for capturing NS1 protein and **10** for detection, amine-reactive maleic anhydride activated plate (Pierce, >125 pmol/well of binding capacity) was used to immobilize 5′-amino C6-modified **2** (60 pmole in 100 μL) to the plate using manufacturer's protocol. The plate was then blocked using SuperBlock reagent (Thermo Fisher Scientific) with manufacturer's protocol. After incubating Zika NS1 protein at various concentrations in binding buffer or human serum in a 96-well plate, the plate was washed with binding buffer containing 0.1% Tween 20 (200 μL), and 500 nM of 5′-biotinylated **10** (100 μL) was added and incubated for 30 min. After washing, streptavidin-HRP conjugate (Jackson Immu-noResearch, 1 μg/mL) was added and incubated for 30 min. After washing the plate three-times using binding buffer containing 0.1% Tween 20, color development was carried out using TMB-based substrate and stop solution with manufacturer's protocol (KPL, USA), and absorbance was measured at 450 nm using Cytation3 multimode plate reader (BioTek, USA). The same procedure was followed for the sandwich complex-based ELISA assay using **2** for capture and rabbit anti-Zika NS1 monoclonal antibody for detection with streptavidin-coated plate (Kaivogen, >15 pmol/well of binding capacity) used to immobilize 5′-biotin-containing **2** (20 pmole in 200 μL) to the plate.

■ RESULTS AND DISCUSSION

SELEX was carried out to identify Zika NS1-binding ssDNA aptamers using a random 60-nt ssDNA aptamer library consisting of ∼10^{16} sequences. Negative selection against 6x histidine tag peptides and magnetic Ni-NTA beads were carried out to remove nonspecific bead-bound binders for every SELEX cycle, and the enrichment of the Zika NS1-binding aptamer pool was monitored using BLItz label-free biolayer interferometry system (Figure S1). After 7 rounds of selection with increasingly reduced amounts of the target Zika NS1 protein (500 nM for first cycle, 100 nM for second to fifth cycle, 10 nM for sixth cycle and 1 nM for seventh cycle), the enriched aptamer pool binds to 100 nM of Zika NS1 protein more significantly than the initial random ssDNA library, suggesting that Zika NS1-binding aptamers have been successfully enriched after 7 rounds of SELEX selection.

Therefore, the enriched aptamer pool was sequenced using an Ion Torrent deep sequencing system to identify the specific binders for Zika NS1. A total of 20313 clones were sequenced and sequences with >1.5% occurrences were further charac-terized (Table 1). Among sequenced clones, we noticed that

Table 1. Sequences of the 60-nt Random Region for Selected Aptamers with >1.5% Occurrences[a]

Clone	Sequences on random 60-nt region (5' to 3')
1 (42.9%)	ATACCGGTGCCATATTCCACAAGGGGGACTGCTCGGGATTGCGGATTTGTGGAATTGTTG
2 (7.4%)	ACTAGGTTGCAGGGGACTGCTCGGGATTGCGGATCAACCTAGTTGCTTCTCTCGTATGAT
3 (6.5%)	CACAGACTCCATCTTGGATTGCAAAGGTCTGCTGTGTGGTAGTCTGTGGAGGCCATGTCT
4 (5.0%)	ACTCCGCGATAGACGGTTCTGCATGCACGTTCCTCCGACGTCCCGCCTCTGGTTGCTATC
5 (4.3%)	ACTATGGAGTAGATCAAACATCGGTAGATCATGCTTGTCGGGGGATTGCCATTCCGGTCT
6 (3.8%)	GGTATGGTATTCAACCAGGCTCTAGGTACCAGCTTGTCCTGGCGTGAAGAGGGTTTGGCT
7 (3.7%)	CCTCTTGTAGACCTGAAGCTGACAGAGGGGGCTGCTCGGGATTGCGAATATCTGGTGGGT
8 (3.2%)	CCCCCAGTTAGGACAGATCTTAGGGTCTGCTCGGGATTGCGGAGGATCTGTTCCACTCGC
9 (2.0%)	ATTCAGTTGACGTCGGCCTTGACCAAGCTCATATAGGACTGCTTAGGATTGCGAAGTTGA
10 (1.5%)	GGCTGTTGTTGTTACCTATTGCGTGGCGATCGGACTTTCGATTCCGATTAACGCCGGAGG

[a]Values in parentheses refer to the occurrence of clones with the same sequence among a total of 20313 clones. Underlined sequences are the consensus sequence shared by clones **1**, **2**, **3**, **7**, **8** and **9**. Red letters refer to mutations in the consensus sequence.

Figure 1. Possible secondary structures of the minimum binding domains for aptamers **2** (41-nt, a) and **10** (54-nt, b) as predicted by M-fold program. 3' region of **10** contains some of fixed sequences for the reverse primer binding (5'-GCGGGTCGACAAGC-3').

clones **1**, **2**, **3**, **6**, **8**, and **9** share the whole or part of the consensus sequence (Table 1). The result suggests that the specific sequences, which might have a specific structure preferred by the target, were enriched after 7 rounds of SELEX cycles.

Binding affinities and specificities for the selected aptamers were determined using BLItz label-free biolayer interferometry system. After preliminary screening for the affinity and the specificity for Zika NS1 protein (data not shown), aptamers **2** (100-nt in length including a 60-nt random region and two 20-nt fixed sequences, Figure S2), which has the best binding affinity (K_D = 24 pM, Figure S3a), and **10**, which has the best specificity but relatively weaker in binding (K_D = 134 nM, Figure S3b), were chosen for structure optimization and further analysis. K_D values of selected clones were also summarized in Table S1.

To determine the minimum binding domain responsible for the observed binding affinity, several truncated versions of **2** and **10** were designed based on their secondary structures predicted using M-fold web server.[13] Binding affinities determined using biolayer interferometry show that **2** and **10** can be truncated from 100-nt (Figure S2 and Table S1) to 41 and 54-nt (Figure 1), respectively, with comparable binding affinities (24 and 45 pM for 100-nt and 41-nt **2**, and 134 and 240 nM for 100-nt and 54-nt **10**, respectively). Therefore, these two truncated versions containing minimum binding sequences were used for further analysis. It might be worth pointing out that the truncated **10** of 54-nt in length contains the 3' fixed sequence, suggesting that the fixed sequence plays an important role in target binding (Figure 1b).

Next, the specificities of the truncated aptamers were assessed since full-length **2** binds to Dengue NS1 protein while **10** does not (data not shown). As shown in Figure 3a, although the truncated **2** does not bind to unrelated proteins such as interferon-γ (IFN-γ) and bovine serum albumin (BSA,
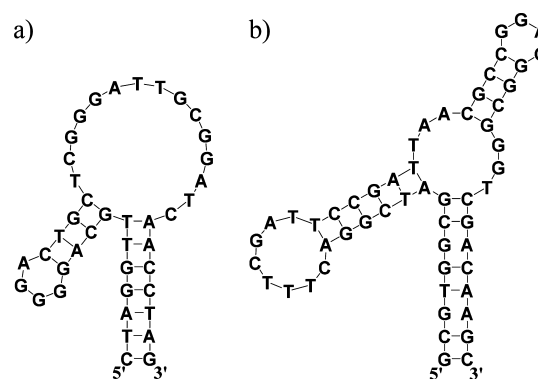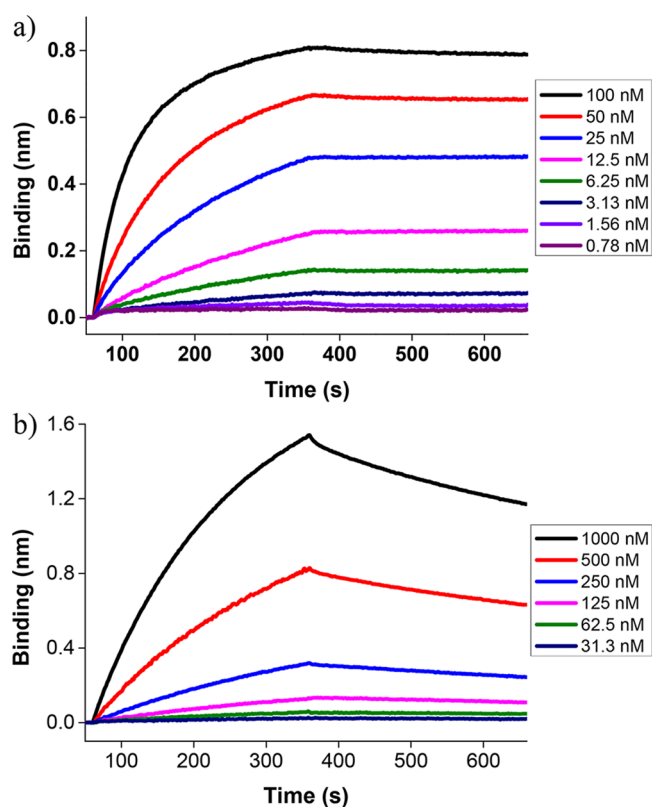

Figure 2. Binding affinity determination for (a) **2** (41-nt) and (b) **10** (54-nt) using BLItz label-free biolayer interferometry system. 100 nM of 5'-biotinylated aptamers was immobilized onto SA sensor for 20 s. After 60 s of baseline stabilization, various concentrations of Zika NS1 protein were added. Both association and dissociation events were monitored for 300 s. Data were analyzed using BLItz Pro 1.2 software. Binding (nm) refers to changes in optical interference (e.g., shift of the wavelength).

~ 15 μM in binding buffer), it does bind to all four Dengue NS1 serotypes, ranked in the order of Zika NS1 ≥ Dengue NS1 serotype 4 > serotype 1 > serotype 2 ≥ serotype 3. On the other hand, the truncated **10** does not bind to any of Dengue NS1 serotypes as well as IFN-γ and BSA, confirming that **10**-mediated recognition of Zika NS1 proceeds in a highly specific fashion (Figure 3b).

Despite advances in molecular diagnostics, the false-negative or -positive results, which could arise from a high degree of
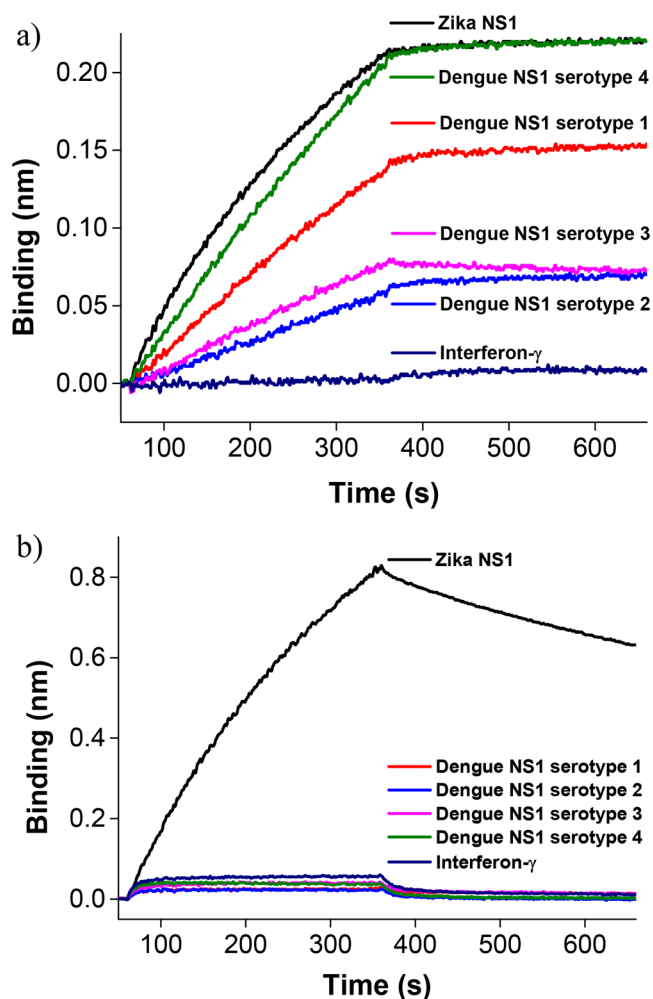
Figure 3. Binding specificity of (a) **2** and (b) **10** against unrelated proteins using BLItz label-free biolayer interferometry system. 100 nM of 5′-biotinylated **2** or **10** was immobilized on SA sensors for 20 s. After 60 s of baseline stabilization, 10 (in the case of **2**) or 500 nM (in the case of **10**) of each protein (Zika NS1, Dengue NS1 serotypes or IFNγ) was added and incubated in binding buffer containing 0.1% (~15 μM) BSA. Both association and dissociation events were monitored for 300 s. Data were analyzed using BLItz Pro 1.2 software.

sequence conservation among related viruses, sequence variations from new strains, etc., are still common issues in terms of sensitivity for Zika infection.[1] For these reasons, serological assays are still generally considered as important diagnostic tool. For example, ELISA[14] is the gold standard assay with high sensitivity while the paper-based assay[15] is rapid and of lower costs. For these methods, finding a pair of ligands with complementary binding sites able to sandwich the target protein is the prerequisite.

To identify the aptamer or the antibody that could pair with **2** for forming the sandwich complex for detecting Zika NS1, a number of Zika NS1-binding aptamers, commercial antibodies or our in-house rabbit anti-Zika NS1 monoclonal antibodies were screened in label-free interferometry system. Briefly, 5′-biotinylated **2** was immobilized on streptavidin (SA) sensor, and Zika NS1 protein from Brazil strain[16] was added and the solution was incubated to preform the **2**-Zika NS1 complex. Next, aptamers or antibodies to be screened were added, and the association and dissociation of the preformed **2**-Zika NS1 complex were monitored. From the screening, aptamer **10**
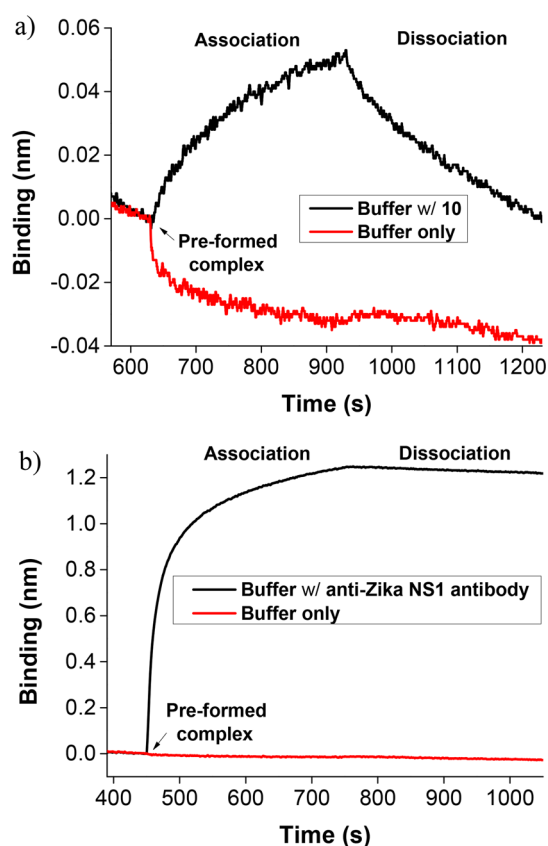

Figure 4. Sandwich complex formation of (a) **2**-Zika NS1-**10** and (b) **2**-Zika NS1-antibody complexes) was monitored using BLItz label-free biolayer interferometry system. (a) 100 nM of 5′-biotinylated **2** was immobilized on streptavidin (SA) sensor for 150 s. After baseline stabilization for 30 s, 100 nM of Zika NS1 protein was added and incubated for 300 s to preform the **2**-Zika NS1 complex. After baseline stabilization for another 120 s, 1 μM of **10** was added to check the binding to the preformed **2**-Zika NS1 complex. Both association and dissociation events were monitored for 300 s and normalized based on Figure S4a. Data were analyzed using BLItz Pro 1.2 software. In panel b, 1 μM of rabbit anti-Zika NS1 monoclonal antibody was used for binding to the preformed **2**-Zika NS1 complex.

(Figure S4a and 4a) and our in-house anti-Zika NS1 monoclonal antibody (Figure S4b and 4b) were found to be capable of pairing with **2** to form a Zika NS1-sandwiched complex. Accordingly, we chose **2**/**10** or **2**/anti-Zika NS1 antibody as the pair in the ELISA assay using a 96-well plate (Figure 5).

For **2**/**10** pair, ELISA was carried out in the binding buffer (Figure 5a) with **2** immobilized onto the plate as the capture agent, 5′-biotin-modified **10** as the detection agent and streptavidin-HRP conjugate for signal amplification. Our data show that Zika NS1 can be reliably detected at a concentration ranging from 100 ng/mL to 1 μg/mL.

For **2**/anti-Zika NS1 antibody pair, ELISA assay was carried out in both binding buffer and human serum (Figure 5b) with **2** as the capture agent and the antibody as the detection. In binding buffer, this aptamer-antibody pair excitedly lowers the detection sensitivity to 0.1−1 ng/mL, which is comparable with those reported using immunoassays (e.g., 0.2 ng/mL and 3 ng/mL in PBS buffer using impedimetry and capacitance detection modes, respectively[3,4]). Even in 100% human serum containing many other types of proteins, protease, small molecules and ions, Zika NS1 of as low as >10 ng/mL can be detected. This is
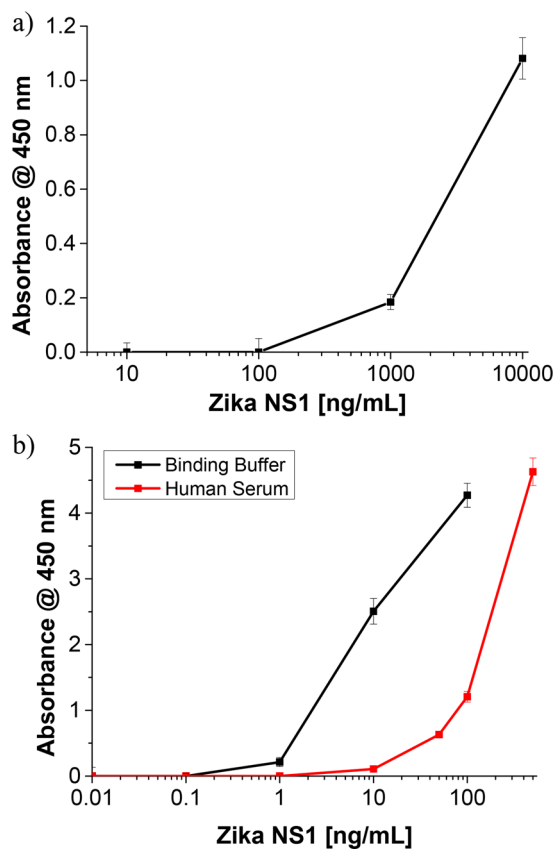
Figure 5. Sandwich complex-based ELISA assay for Zika NS1 detection. (a) ELISA assay results using the truncated **2** immobilized on 96-well plate as the capture agent and 5′-biotin-modified **10** as the detection agent in binding buffer. Streptavidin-HRP conjugate was used for the color development. (b) ELISA assay results using the truncated **2** as the capture agent and anti-Zika NS1 monoclonal antibody as the detection agent in binding buffer or 100% human serum. Antirabbit IgG-HRP conjugate was used for the color development. After color development using TMB-based substrate and stop solution, absorbance was measured at 450 nm using Cytation3 multimode plate reader (BioTek, USA). Background absorbance from the well without Zika NS1 was subtracted. X axis is in log10 scale. Error bars are ± SD.

significant because nucleic acid aptamers often are not stable or easily deform in structure[17] in human serum. In our case, after incubation in human serum for 30 min at room temperature, **2** still could function properly and bind to Zika NS1 protein. In fact, we could not observe any significant degradation of **2** in gel after 1 h (Figure S5).[18] Upon diluting 100% human serum by 10 folds using binding buffer (Figure S6), the detection limit could be increased by 10 folds to ~1 ng/mL, which is comparable to the detection limit of 0.5−30 ng/mL obtained using capacitive or impedimetric immunoassays[3,4] and of 0.2 ng/mL for commercially available antibody-based Zika NS1 ELISA kits.[19,20] For comparison, under the identical conditions, this aptamer-antibody pair does not detect any of four Dengue NS1 serotypes, West Nile Virus NS1 and Yellow Fever Virus NS1 (Figure S7). Lastly, although we have tested **2**/antibody-mediated detection of Zika NS1 protein in human serum or diluted 10% human serum with high selectivity and no cross-reactivity from other unrelated proteins (IFN—γ and BSA), further testing on various clinical specimens including plasma, blood or urine samples is required before the performance of this assay on true clinical samples can be validated.

Even though the detection limit using **2/10** pair is worse than **2**/antibody pair, **2/10** pair constitutes one of very rare aptamer−aptamer pairs with successful applications in the sandwich ELISA format[21−26] and/or other sandwich assays.[27] Further research and optimization using such as doped library[28] is needed to discover aptamers that are complementary to **2** in binding site and that come with higher binding affinity, selectivity and slower dissociation rate.

We have further compared our current aptamer/antibody approach with the other two alternative approaches (e.g., antibody/aptamer and antibody/antibody) where antibody was used as the capture agent.

In the reversed antibody/aptamer approach, we encountered some technical problems such as low efficiency of antibody immobilization and lack of defined orientation for antibody, which are general issues for antibody immobilized as a capture agent. Likely because of these problems, significantly lowered signals were observed for Zika NS1 (data not shown). In addition to overcoming this sensitivity issue by using aptamer **2** as the capture agent, other added values of using aptamer, when compared to antibody, include the relatively lower cost in aptamer production and high batch-to-batch consistency in product quality.

As for antibody/antibody approach, screening some commercial antibodies identified antibody SQAb1610 (Arigo Biolaboratories, Taiwan) as the complementary antibody for pairing with our in-house antibody to form a sandwich complex with Zika NS1 protein (Figure S8). Using this antibody/antibody pair and the same ELSIA assay protocol, a detection limit of 10 ng/mL was obtained in binding buffer (Figure S9), which is 100-fold less sensitive than the hybrid aptamer/antibody approach with a detection limit of 0.1 ng/mL (Figure 5b).

■ CONCLUSIONS

In summary, we have successfully discovered several consensus sequence-sharing Zika NS1-binding ssDNA aptamers, and developed ELISA-based assay for highly specific and sensitive detection of Zika NS1 antigen in buffer using a pure aptamer−aptamer pair (e.g, **2/10**) and in human serum using a hybrid aptamer/antibody pair (e.g., **2**/antibody). With the use of **2**/antibody pair, detection limits could reach 0.1−1, 1−10 and >10 ng/mL in buffer, 10% human serum and 100% human serum, respectively. In particular, aptamer **2** of 41-nt in length exhibits exceedingly high binding affinity of 45 pM toward Zika NS1 antigen, **2/10** pair is one of limitedly available aptamer−aptamer pairs with proven applications for antigen detection with a detection limit of >100 ng/mL in buffer, and hybrid **2**/antibody pair might find clinically relevant applications in medical diagnosis of Zika virus infection.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

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> Binding assays for the enriched pool and aptamers using label-free interferometry assay, predicted secondary structures of **2** and **10**, sandwich complex formation test using label-free interferometry assay, control ELISA assays, stability test of **2** in human serum, ELISA using **2**/

anti-Zika NS1 antibody pair in 10% human serum, binding affinities of other aptamer clones (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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(16) In addition to Brazil strain, **2** also binds NS1 protein from Suriname and Uganda strains with comparable binding affinities (data not shown). These two strains carry 99% and 97% sequence similarities with Brazil strain, respectively.

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