RNA aptamer-based sensitive detection of SARS coronavirus nucleocapsid protein

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Severe acute respiratory syndrome coronavirus (SARS-CoV) is the etiological agent of a newly emerged disease SARS. The SARS-CoV nucleocapsid (N) protein is one of the most abundant structural proteins and serves as a diagnostic marker for accurate and sensitive detection of the virus. Using a SELEX (systematic evolution of ligand by exponential enrichment) procedure and recombinant N protein, we selected a high-affinity RNA aptamer capable of binding to N protein with a dissociation constant of 1.65 nM. Electrophoretic mobility shift assays and RNA competition experiments showed that the selected aptamer recognized selectively the C-terminal region of N protein with high specificity. Using a chemiluminescence immunosorbent assay and a nanoarray aptamer chip with the selected aptamer as an antigen-capturing agent, we could sensitively detect N protein at a concentration as low as 2 pg/ml. These aptamer–antibody hybrid immunoassays may be useful for rapid, sensitive detection of SARS-CoV N protein.

1. Introduction

Aptamers are DNA or RNA oligonucleotides selected from random-sequence, single-stranded nucleic acid libraries by an *in vitro* selection and amplification procedure known as SELEX (systematic evolution of ligands by exponential enrichment).[1,2] The selected aptamers are capable of adapting unique tertiary structures and recognizing target molecules with high affinity and specificity.[3,4] Binding affinities of aptamers are known to be comparable to those of antibodies.[5] Moreover, aptamers can be modified chemically at defined positions and linked stably to solid surfaces. In addition, aptamers are easy to screen and can be reproducibly obtained in large quantities and at low cost; thus, they have been considered as potentially useful diagnostic agents.[6,7]

Severe acute respiratory syndrome (SARS)-associated coronavirus (SARS-CoV) emerged in November 2002 in Guangdong, China and rapidly spread to over 30 countries all over the world, culminating in over 8000 cases of SARS.[8] Since the SARS outbreak, various diagnostic assays have been developed. Reverse-transcription (RT)-PCR has been used for detection of the viral RNA genome with a sensitivity of 50–80%.[9] Positive PCR results, however, do not necessarily mean that infectious virus is present. Other alternative tools have also been developed for the detection of SARS-CoV, and one of the most accurate methods is the detection of nucleocapsid (N) antibodies in convalescent-phase samples from SARS patients by an enzyme-linked immunosorbent assay (ELISA). This serological test cannot detect antibodies against the virus until 2–3 weeks after infection.[10,11] To overcome this, antigen capture ELISA using monoclonal antibodies against the SARS-CoV N protein has been used to directly detect N protein.[12] The positive detection rate for N protein in serum within the first 10 days of infection is higher than that of RT-PCR assays,[13,14] indicating that N protein is a good diagnostic marker for SARS-CoV. Nevertheless, N protein detection sensitivity decreases to less than 30% when testing is performed more than 11 days after the onset of symptoms.[15] Therefore, sensitive diagnostic assays still remain to be established to detect N protein during the course of infection for improved treatment and prognosis of SARS.

In this work, we have used a SELEX procedure to isolate RNA aptamers binding specifically to SARS-CoV N protein. We demonstrate that the selected aptamers act as antigen-capturing molecules in an aptamer-based chemiluminescence immunosorbent assay and in a nanoarray aptamer chip assay to sensitively detect low levels of N protein.

2. Experimental

2.1 Plasmids

Total RNA was extracted from SARS-CoV (Urbani strain)-infected Vero E6 cells using Trizol LS reagent (Invitrogen) according to the manufacturer's instructions and used for reverse-transcription (RT) using Superscript II reverse transcriptase (Invitrogen). The cDNA encoding the full-length N protein and its N-terminal (residues 1–199) and C-terminal (residues 200–422) domains were PCR-amplified with appropriate primer sets, digested with *Xba*I and *Bam*HI, and inserted into an *Nhe*I- and *Bam*HI-digested pTrcHisB vector (Invitrogen). For expression of the Japanese encephalitis virus (JEV) methyltransferase (MTase),[16] the cDNA encoding the N-terminal region (residues 1–265) of NS5 protein was

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PCR-amplified from pTrcHisB-JEVNS5B[17] and cloned into the pTrcHisB vector.

2.2 Expression and purification of SARS-CoV N protein

SARS-CoV N protein and its derivatives were expressed in Top10 *Escherichia coli* cells (Invitrogen) at 25 °C overnight by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside. The recombinant N proteins were purified by metal affinity chromatography using a Ni-nitrilotriacetic acid (NTA)-agarose (Qiagen) column as described previously.[17] The bound N proteins were further purified using heparin-Sepharose (Amersham Biosciences) and SP-Sepharose columns (Amersham Biosciences) sequentially, as described previously.[18] Similarly, JEV MTase was expressed in *E. coli* cells and purified by affinity chromatography using a Ni-NTA agarose column, followed by chromatography using a heparin-Sepharose column, as described previously for JEV NS5.[17] Protein concentrations were determined using a Bio-Rad protein assay kit with bovine serum albumin (BSA) as a standard.

2.3 Selection of RNA aptamers and RNA secondary structure prediction

An RNA aptamer library was generated by *in vitro* transcription of synthetic DNA templates by T7 RNA polymerase using 2′-deoxy-2′-fluoro CTP and UTP (Epicentre Technologies) and normal GTP and ATP. The 2′ position of the RNA aptamers was modified to increase stability.[19] The sequence of the resulting RNA library is 5′-GGGAGAGCGGAAGCGUGCUGGGCCN$_{40}$CAUAACCCAGAGGUCGAUGGAUCCCCCC-3′, where N$_{40}$ represents 40 nucleotides (nt) with equimolar incorporation of A, G, C, and U at each position. SELEX was performed as described previously. The RNA library (330 pmol) was pre-cleared by incubation with 20 μl of Ni-NTA agarose beads to remove any RNA binding non-specifically to the beads. The pre-cleared RNA was incubated with 75 pmol of purified recombinant N protein with a hexahistidine tag at the N-terminus in 200 μl of binding buffer (30 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 2 mM DTT, 1% BSA). Bound RNA was recovered and amplified by RT-PCR for four more rounds of selection. For the sixth to ninth round of selection, the amount of N protein was reduced by 5-fold to isolate aptamers under more stringent conditions. After a total of nine rounds of selection, the amplified DNA was cloned and several randomly picked clones were sequenced. RNA secondary structures of the selected aptamers were analyzed using the Mfold program.[20]

2.4 Surface plasmon resonance analysis

Surface plasmon resonance (SPR) experiments were performed using a BIAcore 2000 biosensor system to measure the binding affinity of selected aptamers. The carboxymethylated sensor chip (BIAcore) was pre-equilibrated with running buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2.7 mM KCl) and activated with 100 mM *N*-hydroxysuccinimide and 400 mM of *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide to immobilize the $(His)_6$-tagged N protein on the chip surface. After immobilization of the protein, the chip surface was deactivated with 1 M ethanolamine hydrochloride, pH 8.5, to protect the remaining activated groups. After stabilizing the baseline, at least five different concentrations of the selected RNA aptamer were injected into the flow cells. The protein surface was regenerated with 10 mM NaOH after injection of each sample and re-equilibrated with the running buffer. The SPR sensograms were analyzed to calculate the association rate constant k_a and the dissociation rate constant k_d using BIAevaluation software (Version 3.2, BIAcore).

2.5 Electrophoretic mobility shift assay

For 5′-end ^{32}P-labeling of RNA aptamers, dephosphorylated *in vitro* transcripts were phosphorylated using [γ-^{32}P]ATP (Izotop) and T4 polynucleotide kinase (Takara Bio Inc.), and purified as previously described.[17] The 5′-end ^{32}P-labeled RNA probe (30 fmol) was incubated with increasing amounts of N protein in a total volume of 8 μl of binding buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM DTT, 0.5% BSA). Unlabeled competitor aptamer RNA or yeast tRNA was used in the competition assays. Unless otherwise specified, assay mixtures were analyzed on non-denaturing 5% polyacrylamide gels (10 × 11 cm), as previously described.[3]

2.6 Aptamer-based chemiluminescence immunosorbent assay

Streptavidin-coated 96-well plates (Perkin Elmer) were coated with 10 pmol of biotin-labeled oligo$(dT)_{16}$ in 50 μl of PBS per well by incubation at 4 °C overnight. Plates were washed twice with PBS and then incubated with the aptamer flanked by oligo$(A)_{16}$ at the 3′-end (2 pmol per well) at room temperature for 1 h. After washing with PBS, each well was blocked with 200 μl of blocking solution (5% BSA in PBS) and washed with PBST (PBS containing 0.05% Tween 20). Then, 50 μl of the protein samples diluted in binding buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM DTT, 1% BSA) was added to each well. After incubation for 1 h, samples were discarded and wells were washed four times with 200 μl of PBST. Then, polyclonal anti-SARS-CoV N protein antibody (ab34716, Abcam) in 50 μl of PBST containing 2.5% BSA was added to each well. After incubation for 1 h, wells were washed four times with PBST. Alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Sigma-Aldrich; diluted 1 : 50 000) in PBST containing 2.5% BSA was added to each well and incubated for 1 h. Wells were washed four times with PBST and twice with an assay buffer (20 mM Tris-HCl, pH 9.8, 1 mM $MgCl_2$). Luminescence was measured using disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.13,7]decan}-4-yl)phenyl phosphate substrate (0.4 mM, Applied Biosystems) in a TopCount NTX Microplate Luminescence Counter (Perkin Elmer Life Science and Analytical Sciences).

2.7 Nanoarray aptamer chip assay

A commercially available atomic force microscope (AFM) tip (silicon nitride cantilever, 0.27 N/m, Veeco Instruments) and glass plate surface were coated with mercaptopropyltrimethoxysilane (MPTMS), which was then evaporated at 120 °C for 30 min in a closed jar. Dip-pen nanolithography (DPN) writing was performed by Bio-AFM (Nanoscope IIIa, Digital

Instruments) in contact mode using maleimide-PEO_2-biotin (1 mg/ml, Pierce) with 0.05% Tween-20 (Sigma-Aldrich) as ink. The patterns were imaged by tapping-mode AFM immediately after writing. The non-patterned areas of the mercaptosilanized glass surface were passivated with 50 μl of PEG-maleimide (ID Biochem) in PBS (1 mg/ml) for 10 min at room temperature to prevent non-specific protein binding. After passivation, 10 μl of streptavidin (10 μg/ml in PBS, Sigma-Aldrich) and 10 pmol of biotinylated-oligo$(dT)_{16}$ in 10 μl PBS were serially added to the biotin-patterned glass for 1 h each at room temperature. The patterned glass was washed with PBS and dried with nitrogen gas at every following step. Then, 2 pmol of aptamer 1 with oligo$(A)_{16}$ at the 3′-end was applied to the patterned plate and annealed to the immobilized oligo$(dT)_{16}$ by incubating for 1 h at room temperature. The plate was incubated with serially diluted N protein for 2 h at room temperature. N protein captured by the aptamer was then recognized by a polyclonal anti-SARS-CoV N protein antibody (ab34716). After washing and drying with nitrogen gas, FITC-labeled anti-rabbit IgG (Sigma-Aldrich) antibody was applied to detect the fluorescence emission signal using an inverted epi-fluorescence microscope with an Hg/Xe arc lamp (Eclipse TE300, Nikon). Images of fluorescence patterns were captured with a high-resolution, Peltier-cooled CCD camera (Cool-Snap-HQ, Roper Scientific).

3. Results and discussion

3.1 *In vitro* selection of RNA aptamers to SARS-CoV N protein

We screened an RNA aptamer pool with approximately 10^{14} random, 40-nt long aptamers by SELEX to isolate those binding to SARS-CoV N protein. The N protein used for the affinity RNA selection experiments was purified to near homogeneity by sequential chromatography using Ni-NTA agarose, heparin-agarose, and SP-Sepharose columns (Fig. 1A). During selection of high-affinity RNA aptamers, RNA quantities captured by empty resin and resin containing N protein were compared by real-time RT-PCR. The amount of RNA selected by N protein was 4-fold higher than that captured by the resin after five cycles, and 400-fold higher after nine cycles (data not shown). After a total of nine cycles of selection, cDNA for the selected pools of RNA was cloned and 17 independent clones were sequenced. Binding affinities of the selected RNA aptamers to N protein were estimated by quantitative real-time RT-PCR in order to select two aptamers, termed aptamer 1 and aptamer 2 (Fig. 1B), with enhanced binding activity compared with other selected aptamers. Both aptamer 1 and aptamer 2 exhibited high-affinity binding to N protein, with apparent dissociation constants of 0.81 nM and 3.35 nM, respectively. Like other coronavirus N proteins, SARS-CoV N protein was postulated to recognize a specific sequence, termed the packaging signal (PS), for the viral RNA genome packaging.[21] The 63-nt PS and flaking sequences, which represent SARS-CoV genomic RNA from 19 715 to 20 294, were experimentally shown to contain a functional PS important for the packaging of the viral RNA.[22] Sequence analysis revealed that the sequences of the selected RNA aptamers are not similar to those of SARS-CoV PS and other parts of the viral genome.

The binding ability of the selected aptamers was further assessed by electrophoretic mobility shift assay (EMSA). As shown in Fig. 1C, aptamer 1 formed a complex with N protein at an aptamer-to-protein ratio of 1 : 1 (lane 6). The multiple bands, which are likely the complexes formed with intermediate forms of N protein, were converted into a higher molecular weight complex (lanes 7 and 8) due to the oligomerization of N protein.[23,24] In contrast, aptamer 2 formed less amounts of intermediate complexes with the same amount of N protein (compare lanes 2 and 6), suggesting that aptamer 1 has a slightly higher binding affinity to N protein than aptamer 2. We therefore chose aptamer 1 for further analysis. SPR experiments revealed that aptamer 1 bound strongly to N protein, with an equilibrium

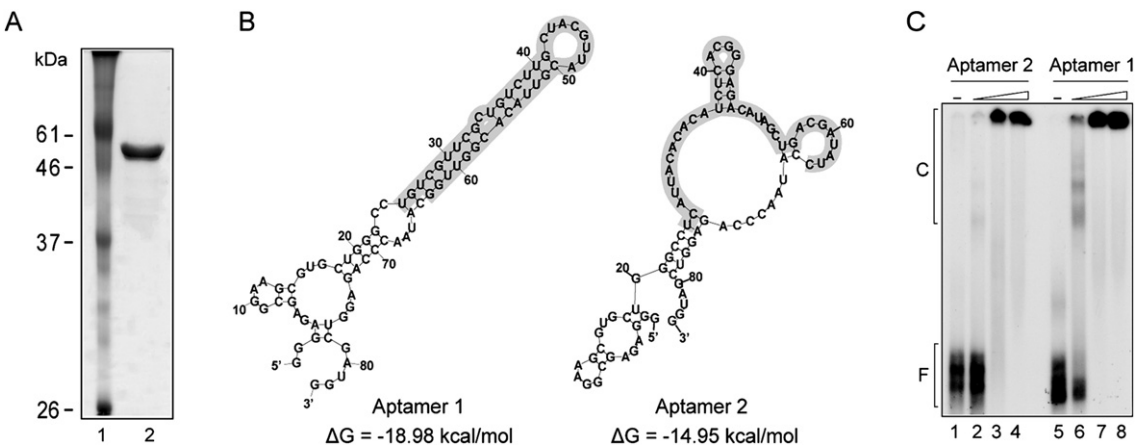

Fig. 1 Selection of RNA aptamers that bind specifically to SARS-CoV N protein. (A) Coomassie blue staining of purified N protein (1 μg, lane 2) resolved by SDS-PAGE. Lane 1, protein size markers. (B) Sequences and secondary structures of RNA aptamers that bind to N protein. The most stable secondary structures of the selected aptamers, predicted using the Mfold program,[30] are shown with their ΔG values of formation. Random-sequence regions (40 nt) flanked by 5′ and 3′ primer sequences for PCR amplification are shaded in gray. (C) EMSA was performed with the indicated probe, which was incubated without (lanes 1 and 5) or with increasing amounts of purified N protein (lanes 2–4 and 6–8; 1-, 5-, 10-fold molar excess over the probe) to form aptamer/protein complexes. Mixtures were resolved on a 5% non-denaturing polyacrylamide gel (20 × 20 cm) by electrophoresis at 100 V. Gels were dried for autoradiography. C: aptamer/protein complex; F: free form of probe.

Table 1 Binding affinity of aptamer 1 to SARS-CoV N protein[a]

Protein	k_a (1/M·s)	k_d (1/s)	k_d/k_a (K_D, nM)
SARS-CoV N	$(2.63 \pm 1.20) \times 10^6$	$(4.59 \pm 3.08) \times 10^{-3}$	1.65 ± 0.41
JEV MTase	9.89 ± 2.57	$(9.67 \pm 0.47) \times 10^{-6}$	1008 ± 215

[a] The association rate constant (k_a), dissociation rate constant (k_d), and equilibrium dissociation constant (K_D) were determined by SPR analysis.

dissociation constant (K_D) of 1.65 nM, whereas it bound very weakly to the JEV MTase, with a K_D of approximately 1 μM (Table 1). The binding affinity of aptamer 1 appears to be superior to that of the selected single chain variable region fragment antibody against N protein, which was recently isolated by phage display technology and shown to bind to the target with a K_D of 2.1 μM.[25]

3.2 Specific binding of SELEX aptamer 1 to N protein

To assess the binding specificity of aptamer 1 to N protein, increasing amounts of unlabeled aptamer RNA or yeast tRNA were added to the probe–protein mixtures to compete with the probe RNA. As shown in Fig. 2A, when as little as 10-fold molar excess of cold aptamer 1 over the probe was added to the N protein–probe complex (lane 3), most of the aptamer 1 probe was dissociated from the complex and migrated to the free probe position. In contrast, tRNA could not dissociate the labeled probe from the complexes as effectively as the same unlabeled probe (lanes 6–8). Lower bands indicated by the asterisk are likely to be a monomeric form of N protein bound to the probe and were observed when a higher amount of tRNA was added (compare lane 6 with lane 7). These results suggest that aptamer 1 binds to N protein with sequence specificity.

It has been reported that SARS-CoV N protein has two RNA-binding domains in both the N-terminal and C-terminal regions,[24] as depicted in Fig. 2B. Nuclear magnetic resonance spectroscopy analysis of the structure of the N-terminal domain (residues 45–181) revealed that the long flexible β hairpin with its positively charged surface is involved in RNA binding.[26] The crystal structure and biochemical analysis of the C-terminal domain of N protein showed that the residues 248–280 form a positively charged groove, which also represents a likely site for RNA binding.[24] These results suggested that more than one region of N protein might be involved in interaction of N protein with RNA for packaging of the viral RNA genome. To demonstrate that the affinity-selected aptamer 1 can recognize

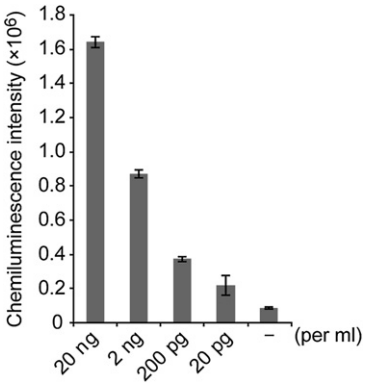

Fig. 3 Detection of the SARS-CoV N protein by an aptamer-based chemiluminescence immunosorbent assay. Serially diluted N protein was captured by aptamer 1 bound to a streptavidin-coated 96-well plate *via* hybridization between oligo(A)$_{16}$ tagged to the 3′-end of the aptamer and biotinylated oligo(dT)$_{16}$. Captured N protein was detected using polyclonal anti-N antibody and alkaline phosphatase-conjugated secondary antibody. The luminescence intensities and standard deviations are from triplicate experiments.

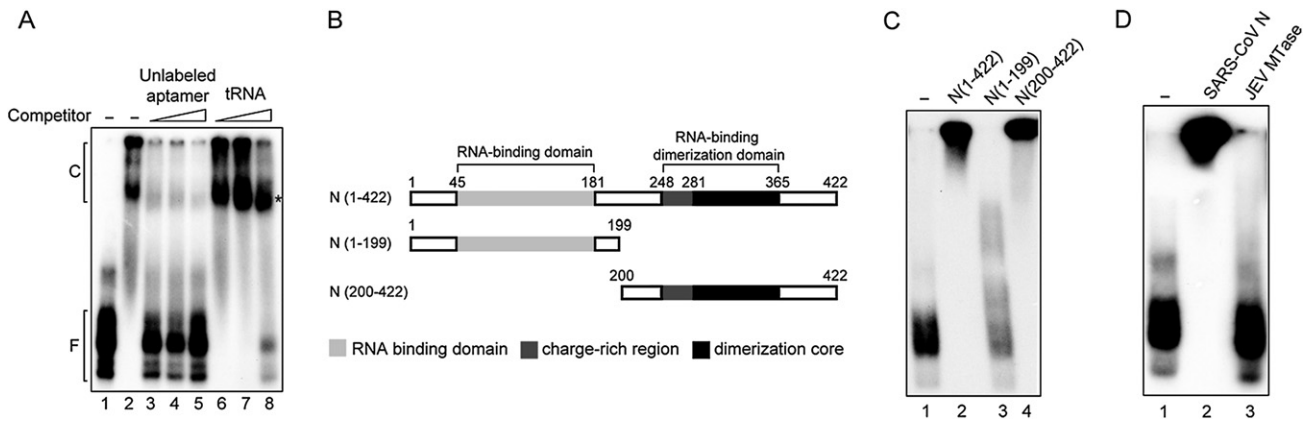

Fig. 2 Specific recognition of N protein by aptamer 1. (A) The 5′-end ^{32}P-labeled RNA probe (30 fmol) was incubated with 75 fmol of N protein. For competition, unlabeled aptamer 1 (10-, 25-, and 50-fold molar excess over probe) or yeast tRNA (10-, 50-, and 100-fold molar excess over probe) was added to the aptamer/N protein mixture. Mixtures were resolved on a 5% non-denaturing polyacrylamide gel (10 × 11 cm) by electrophoresis at 70 V. The asterisk indicates the complex formed between the probe and monomer or intermediate multimer form of N protein. (B) Schematic representation of the functional domains of N protein. (C) The aptamer-binding domain of N protein was determined by EMSA. The end-labeled, radioactive aptamer 1 probe was incubated with each domain of the N protein (10-fold molar excess over a probe) in the presence of yeast tRNA (50-fold molar excess over the probe). The incubation mixtures were analyzed as in part (A). (D) The N protein or JEV MTase domain (300 fmol) was incubated with 30 fmol of aptamer 1 probe. The incubation mixtures were analyzed as in part (A).

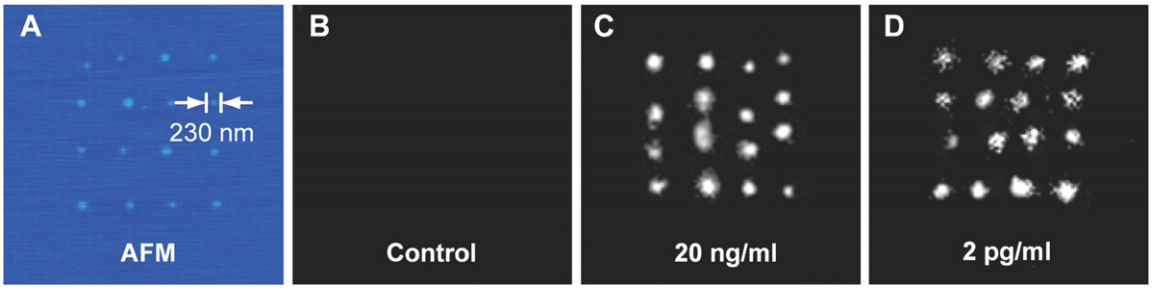

Fig. 4 Detection of N protein by a nanoarray aptamer chip. (A) AFM section analysis image of DPN-patterned maleimide-biotin on an MPTMS-coated cover glass. (B–D) Fluorescence images of detected N protein. Serially diluted N protein (B: no N protein; C: 20 ng/ml; D: 2 pg/ml) was captured by aptamer 1 immobilized on a nanoarray aptamer chip and detected using a polyclonal anti-N antibody and FITC-labeled anti-rabbit IgG secondary antibody.

the target protein specifically, we sought to determine which domain of N protein is recognized by aptamer 1. Full-length (residues 1–422) N protein and its N (residues 1–199) or C (residues 200–422) terminal domains were incubated with end-labeled aptamer probe 1 in the presence of excess tRNA (50-fold molar excess over the probe), and protein–aptamer probe complexes were then resolved on a non-denaturing polyacrylamide gel. As shown in Fig. 2C, aptamer 1 probe strongly bound to the full-length N protein as well as the C-terminal domain (lanes 2 and 4) and weakly to the N-terminal domain (lane 3). In addition, consistent with our results from SPR analysis, EMSA also revealed that the JEV MTase domain did not show a mobility shift in the presence of the SARS-CoV N protein-specific aptamer 1 (Fig. 2D, lane 3). These results suggest that the affinity-selected aptamer 1 does not bind to N protein by non-specific charge interactions and that the C-terminal domain or dimer form N protein is specifically recognized by the aptamer 1. However, further analysis of the specificity of the selected aptamers using other related coronavirus capsid proteins is needed to determine whether the aptamers recognize a common motif of coronavirus capsid proteins or a SARS-CoV capsid-specific epitope.

3.3 Sensitive detection of N protein by an aptamer-based chemiluminescence immunosorbent assay and on a nanoarray aptamer chip

Because the RNA aptamer can be engineered *in vitro* for immobilization on a solid surface in an ordered manner, our results raised the possibility of using aptamer 1 in conjunction with antibodies to detect SARS-CoV N protein. We used aptamer 1 as an N protein-capturing molecule in an aptamer-based chemiluminescence immunosorbent assay. By immobilizing aptamer 1 onto a streptavidin-coated 96-well plate through hybridization of the 3′-$(A)_{16}$ tail of aptamer 1 to oligo$(dT)_{16}$, we could detect as little as 20 pg/ml (420 fM) of N protein (Fig. 3). This detection limit is comparable to the detection limit achieved by ELISA using a polyclonal anti-N antibody as the capture agent and a monoclonal antibody as the detection antibody.[8]

Recently, aptamer-based microarray chips have been suggested as a new diagnostic tool.[27,28] Although the nanoarray technology has various advantages over the microarray chips, such as requirement of a smaller volume and faster and more sensitive detection,[29] the RNA aptamer nanoarray chip has not yet been developed for the detection of proteins or chemical target molecules. We have established a nanoarray chip assay to test whether the affinity-selected aptamer 1 can be used as an antigen-capturing molecule to sensitively detect N protein. The aptamer 1 with oilgo$(A)_{16}$ at the 3′-end was immobilized onto the streptavidin-patterned glass plate *via* hybridization to the biotinylated-oligo$(dT)_{16}$. Then serially diluted N protein was applied on the plate for detection of the aptamer-captured N protein using a polyclonal anti-N antibody and FITC-conjugated secondary antibody. With this nanoarray aptamer chip, we were able to detect femtomolar concentrations of N protein (42 fM) with no background signal from the passivated portions of the chip (Fig. 4). The detection limit was improved at least 10-fold compared to the aptamer-based chemiluminescence immunosorbent assay (Fig. 3).

Altogether our results suggest that the RNA aptamers capturing the N protein could be further developed as sensitive diagnostic agents. Furthermore, an aptamer nanoarray chip might have application potential for rapid diagnosis of small-volume clinical samples.

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