Monitoring Biomolecular Interactions on a Digital Versatile Disk: A BioDVD Platform Technology

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ARTICLE

ABSTRACT A spinning-disk biosensor utilizing optical interference of reflected light from a multilayered structure, consisting of dielectric, metal, and optical phase-change thin films, is shown to have the potential to monitor various interactions on its surface. We refer to this platform as a BioDVD, since it utilizes the optical system of a digital versatile disk (DVD) to measure changes in reflected light intensity. Here, we demonstrated that nucleic acid hybridization and RNA-protein interactions can be analyzed efficiently, in a label-free environment, by measuring the reflected light intensity using a DVD-like mechanism. Moreover, our studies revealed that the detection sensitivity for the interactions on the BioDVD can be altered by shifting the state of the phase-change materials, where the amorphous state can be used for analysis and another state (crystalline) can be used both for recording information and selectively masking areas of the disk.

KEYWORDS: biomolecular interactions · nucleic acids hybridization · RNA-protein interactions · digital versatile disk · phase-change materials · blood coagulation proteins · human influenza virus · aptamers

Biomolecular interactions can be measured by many established techniques, such as, fluorescence and luminescence detection or radioactive labeling of the analyte. While these methodologies offer high sensitivity in establishing readout formats, the labeling of biomolecules is sometimes problematic, because the labeling molecule may occupy an important binding site or cause steric hindrance, resulting in false information regarding interactions. In some cases a labeling procedure is not possible, and an additional step is needed before the interactions can be analyzed. These limitations have stimulated interest in developing label-free techniques for monitoring biomolecular interactions, including nucleic acid hybridizations, and protein−protein, DNA−protein, and RNA−protein interactions. These technologies include: surface plasmon resonance,[1] microsurface-enhanced Raman scattering,[2] microsphere cavities,[3] Kelvin nanoprobes,[4] liquid crystal sensors,[5] calorimetry using enthalpy arrays,[6] and several interference methods such as microelectromechanical system cantilevers,[7] reflectometric interference spectroscopy,[8] interferometry,[9] and ellipsometry.[10] Among the different label-free affinity sensors, interferometric sensor systems are predicted to have the highest sensitivity.[11] However, in these interferometric approaches, the currently used static structures prevent repetitive scanning, and thus they lack high-speed capabilities. Therefore, the development of high-throughput multianalyte biosensors, especially in the areas of genomics and proteomics, is essential.

Recently, an optical biosensor based on spinning disk interferometry has been reported, which explored the well-established technology of the optical compact disk (CD) to develop ultrahigh-throughput immunological assays, dubbed as the BioCD.[12,13] The BioCD operates on the principle of microdiffraction quadrature, which achieves sensitive linear detection of bound molecules. The BioCD is implemented using microinterferometry, using light reflected at gold ridges fabricated on a dielectric mirror disk, although the gold ridges are formed in a radially symmetric structure, while the CD utilizes a spiral layout. These spinning disk interferometers are able to detect immobilized biomolecules with high speed and high sensitivity.[12−14] Furthermore, the costs of the components in the system, such as the pick-up lens and the laser diode have been greatly reduced in recent years, and therefore, it is quite feasible to create a small, inexpensive biosensing device. As an alternative to the BioCD strategy, we have recently proposed a new type of biosensor based on the optical interference of reflected light from the interfaces of a multilayered structure, which consists of dielectric, metal, and optical phase-change thin films fabricated on a disk substrate.[15] We refer to this biosensor as a

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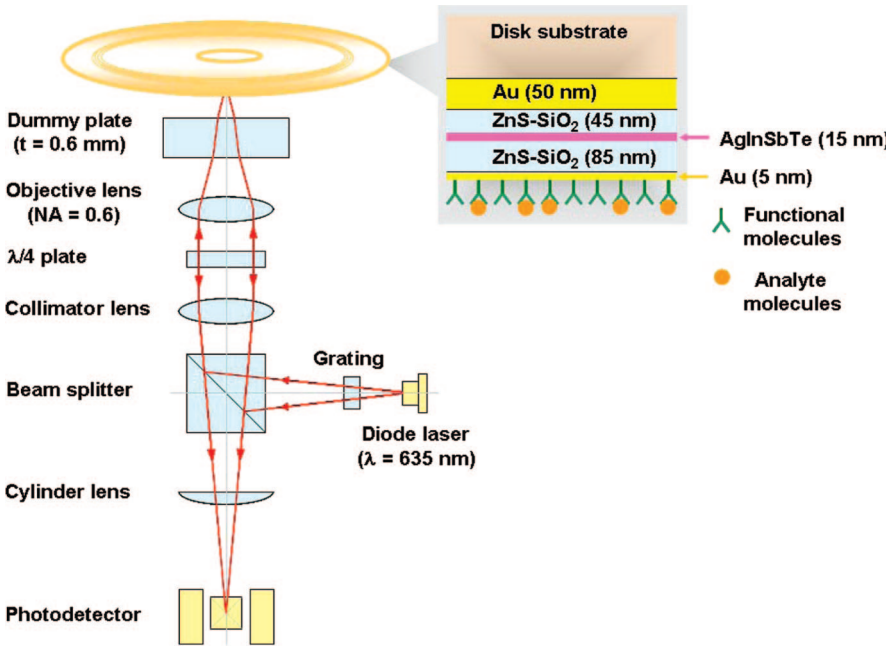

Figure 1. Optical system used for the BioDVD biosensor. The multilayered structure of the BioDVD was fabricated by the rf magnetron sputtering on a pregrooved polycarbonate disk substrate with a diameter of 12 cm and a thickness of 0.6 mm.

BioDVD (digital versatile disk). The BioDVD detects analytes by monitoring the changes in reflected light intensity due to analyte adsorption to the sensor surface, on which functional biomolecules are immobilized to bind specifically to the analytes. The BioDVD sensing instrument is based on a commercial digital versatile disk system, which allows the instrument to be small and inexpensive. The BioDVD platform can be fabricated utilizing mass production techniques with additional functional phase change layers that can serve both to enhance sensitivity by optimization of the interferometric cavity optical properties and also as a possible medium for the storage of test related information.

The basic operating principle of the BioDVD with an integral optical phase-change film relies on optical interferometry: two gold films (one thick and one thin) generate a standing wave between them, and the sandwiched dielectric film as well as the phase-change film thicknesses are designed to optimize the optical multi-interferometric response to be highly sensitive to a tiny refractive index change on the top thin gold film (Figure 1). Once a molecule binds to the surface, the optical standing waves generated in the structure are suppressed leading to a change in reflectivity. In our preliminary device design studies, the sensor surface was explored for analyzing biotin and streptavidin interactions, which revealed that the binding events can be detected on a rotating BioDVD at a scanning velocity of 4.0 m/s, a value typical of that used in conventional DVD readout.[15] In the present study, we show that widely analyzed biomolecular interactions (such as nucleic acid hybridization and RNA-protein interactions) commonly used in diagnosis can be analyzed efficiently and rapidly using the BioDVD platform. Furthermore, we report that the phase-shift multilayered disk has another advantage, as each phase (amorphous and crystalline) can be used for different purposes.

RESULTS AND DISCUSSION

Data Acquisition and Analysis of Biomolecular Interactions on the BioDVD. As described in the Methods section, we prepared the BioDVD as a multilayered disk containing five-layer structures that include a phase-change material layer. On the outer gold surface, we immobilized DNA oligo *via* a thiol-linker as an anchor (Supporting Information, Figure 1a) for analyzing various biomolecular interactions that include nucleic acids hybridization, RNA-protein interactions, and RNA-small ligand interactions. All samples were manually spotted on the BioDVD disks between 22.5 to 58.5 mm radial positions (Supporting Information, Figure 1b). Each sample spot consisted of 5 μL in volume, occupied a circular region about 1.0 mm in diameter, and was analyzed by disk drive unit (DDU-1000) at two different positions (25 and 45 mm) on the bioDVD. The area of the focused laser beam on the BioDVD surface was 1.2 μm and each track area was 1.6 μm, consisting of 0.8 μm groove and 0.8 μm land. Using the normal mode setting of the DDU, which allows for continuous acquisition of different tracks in a concentric manner, we have collected data from 50 tracks of the BioDVD for each sample and averaged along these tracks using LabVIEW 7.1 (National Instruments Corporation, TX). We used at least three different BioDVDs for each sample.

Nucleic Acid Hybridization Studies on the BioDVD. To observe whether the hybridization of nucleic acids can be analyzed using the BioDVD platform, we carried out experiments in which a thiolated (SH) dT_{20} oligo (~7000 daltons) was immobilized in spots containing 5 μL (1 μM stock) on the gold surface. The attachment of the thiolated DNA was analyzed by measuring the reflected light intensity after washing the BioDVD with water followed by drying. We observed approximately a 0.017 V change upon the immobilization of the thiolated DNA on the surface, a value corresponding to a signal-to-noise ratio of 10:1. To this, we added a complementary oligo $(dA)_{20}$ (~13600 daltons, upon duplex formation), which could hybridize to the immobilized oligo $(dT)_{20}$. The addition of a complementary oligo $(dA)_{20}$ increased the signal intensity further (an additional 0.004 V), suggesting that the observed in-

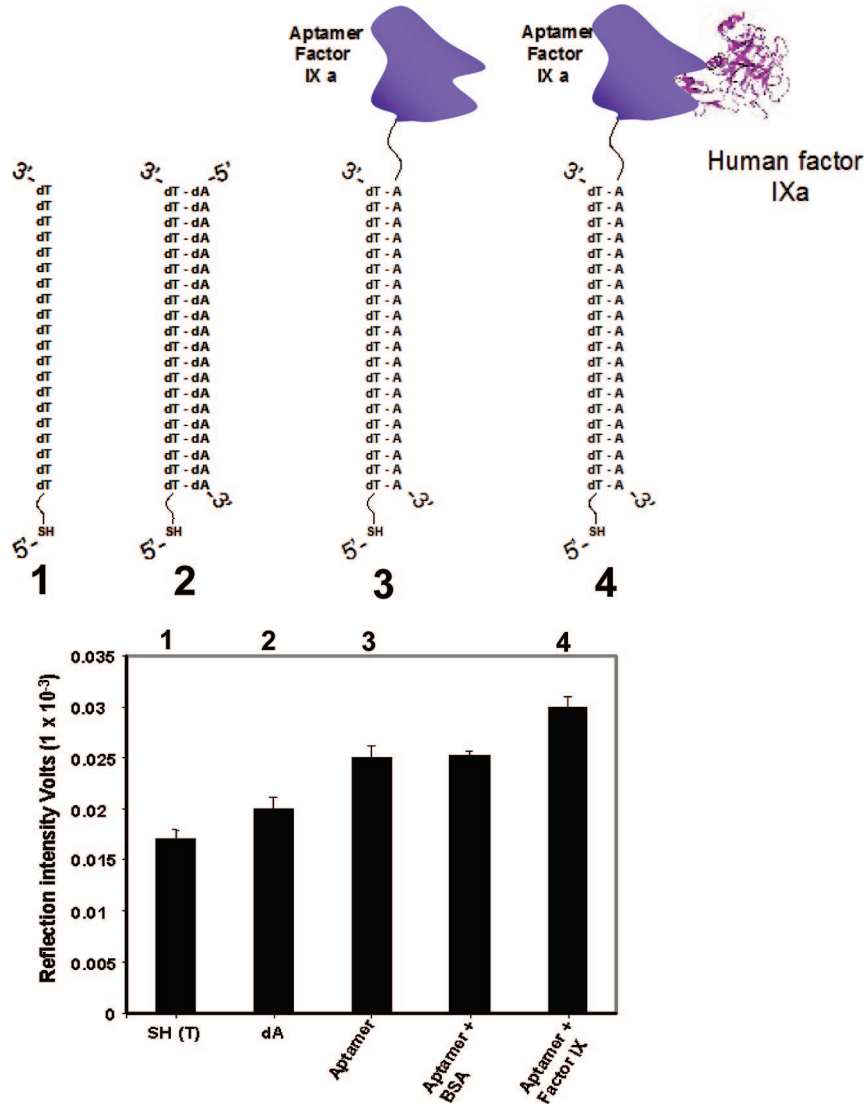

Figure 2. Observed reflection light intensities for thiolated nucleic acid, upon hybridized nucleic acids, and aptamer binding to the target protein. Each spot was about 1.0 mm in diameter, and the spots were placed on a concentric circle with a radius of about 25 mm.

crease in the intensity is primarily due to hybridization of $(dT)_{20}$ to the $(dA)_{20}$ oligo (Figure 2). In these analyses, the reflected light signal was averaged over 50 tracks, in order to reduce the noise level. However, with the addition of $(dT)_{20}$ to the immobilized $(dT)_{20}$ oligo, no further increases in signal intensity were observed (data not shown). In a similar manner, we also carried out hybridization studies between DNA and RNA. For this we used an aptamer RNA (that binds efficiently to the human factor IXa, 33-mer) with a poly $(A)_{24}$ tail at the 3′-end (total length of the RNA was 57 nucleotides; ~25800 daltons). The RNA was prepared as described in the materials section and was incubated with the immobilized $(dT)_{20}$ oligo for hybridization. After the incubation, we detected an intensity of reflection increased higher than $(dT)_{20}$ and $(dA)_{20}$ hybridization signal (0.009 V). Higher signal intensity observed here, could be due to the presence of extra noncomplementary sequence of 33 nucleotides at its 5′ end of the RNA, suggesting that reflection intensity change is sensitive even to the sequence length of the hybridizing molecule. The uniform attachment of aptamer to the thiolated oligo $(dT)_{20}$ was verified further by analyzing the change in reflectivity at the 25 mm track position for 10 spots. The change in reflection intensity response was within the error range of 5−6% for all spots, suggesting analysis was reproducible (Figure 3).

Monitoring RNA-Protein Interactions on the BioDVD. A number of high affinity RNA molecules, isolated from a random nucleic acid library (aptamers), that bind efficiently to various proteins and peptides have been reported,[16−18] including some from our laboratory.[19−21] These molecules displayed high affinity and specificity toward their targets, similar to that observed between an antigen and an antibody. Previously, we used these aptamers and developed platforms to detect target proteins both in solution[22] and on plastic chips.[23] However, in these two studies, fluo-

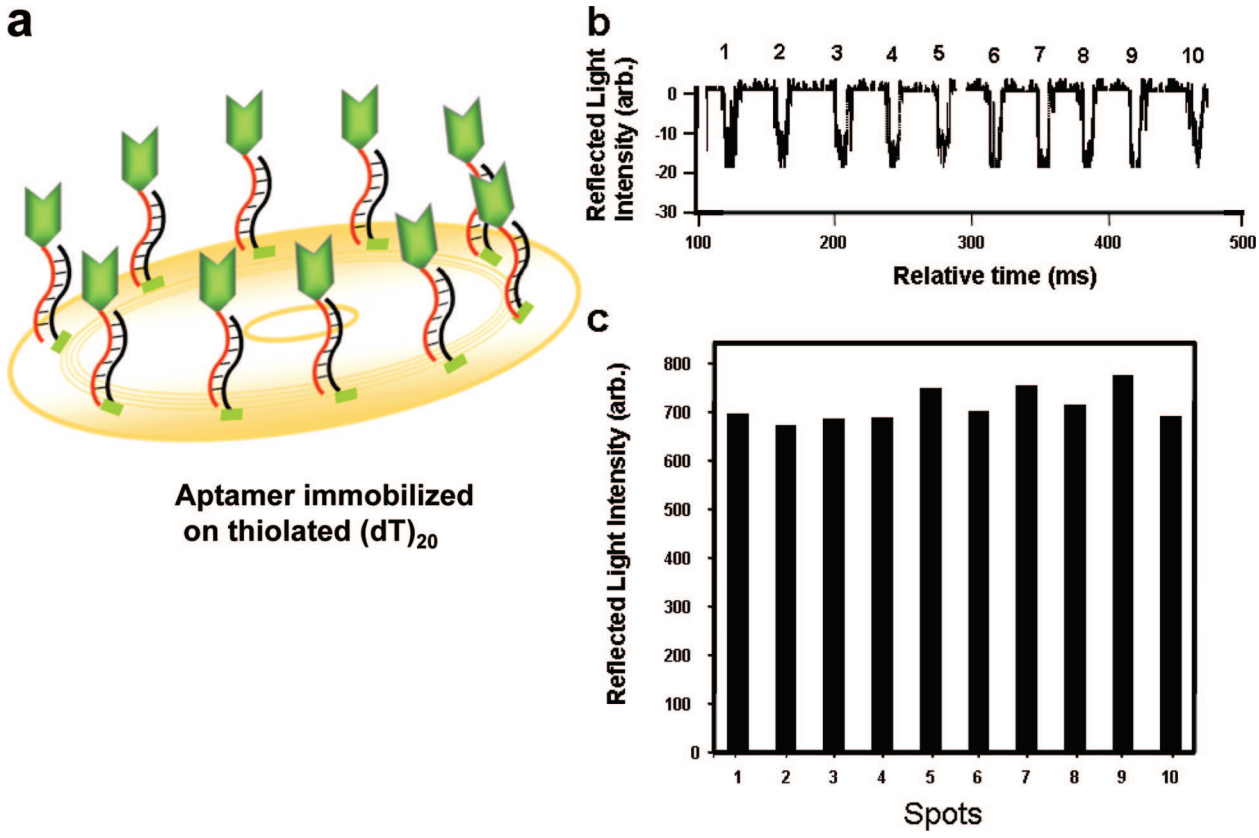

Figure 3. Analysis of ten different spots of DNA-RNA aptamer hybridization on BioDVD. (a) A cartoon showing ten spots of hybridized DNA-RNA aptamer; (b) The observed reflection intensity signal after normalizing with the software IGOR Pro Version 6.0 (WaveMatrics, Inc., USA); (c) Calculated peak area and plotted against ten different spots of hybridized DNA-RNA aptamer.

rescent labeling was essential for the detection and monitoring of the levels of viral proteins.

To develop a label-free detection method for a wide range of analytes using aptamers, we have explored the BioDVD platform for analyzing the RNA-protein interactions. To accomplish this, we used an aptamer that binds efficiently to the human coagulation factor IXa as mentioned above. Once the aptamer was immobilized, the human coagulation factor IXa was added to the same region where the aptamer was immobilized on the BioDVD. After an incubation, the BioDVD was washed and dried, and the intensity change was measured. We observed a further increase of about 0.005 V in the presence of the cognate ligand (Figure 2), however, no change in the reflected light intensity was detected in the presence of other ligands, such as bovine serum albumin (BSA). These results suggest that specific RNA−protein interactions can be analyzed using the BioDVD platform. To correlate these observed interactions with other known techniques, we also performed a surface plasmon resonance (SPR) analysis using a Biacore2000. The observed interactions between the nucleic acids and RNA-protein on the BioDVD were consistent with the Biacore analysis as well (Figure 4).

Next, to evaluate whether the signal intensity is dependent on the concentration of the ligand, we carried out the same experiments as described above, but with different concentrations of Factor IXa (~75800 daltons), ranging from 0.1 to 1000 nM. As seen in Figure 5a, the signal intensity became greater with increasing coagulation factor IXa concentrations over the range 1−1000 nM, suggesting an increase in the number of bound molecules. For quantification and measuring equilibrium dissociation constant (K_d) for the complex (aptamer-factor IXa), the observed reflection intensity areas for different concentrations of factor IXa were calculated and plotted against the concentration of factor IXa (Figure 5b). The obtained signal reflection intensity was normalized using the software IGOR Pro Version 6.0 (WaveMetrics, Inc., OR). As seen from Figure 5b, the reflection intensity area is directly proportional to the factor IXa concentrations (linear range concentrations up to 10 nM). The minimum concentration factor IXa detectable was found to be 10 nM, which is within the range of factor IXa present in the blood. In normal human plasma, the concentration of factor IXa is between 3 and 5 μg/ml (~80 nM).[24] Using the GraphPad Prism 2.0 program, a nonlinear regression curve fitting algorithm was used to calcu-

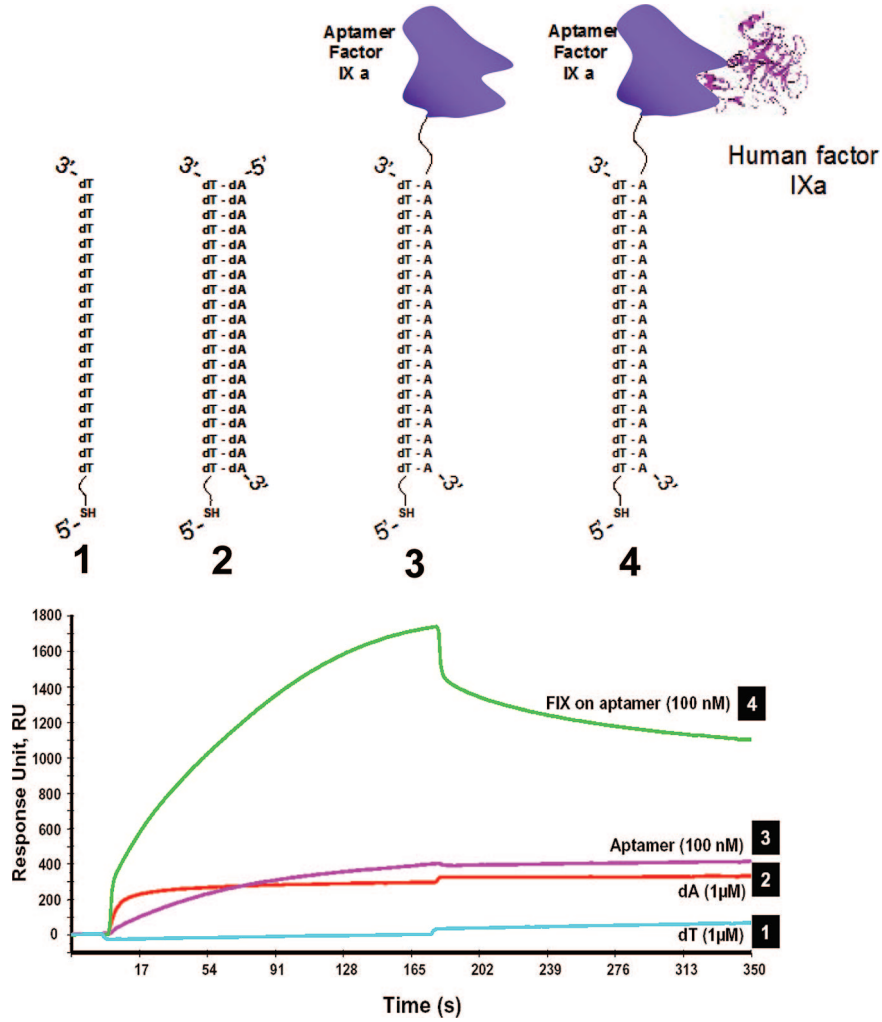

Figure 4. Analysis of nucleic acid hybridization and RNA−protein interactions using surface plasmon resonance (Biacore).

late an equilibrium dissociation constant (K_d) and it was found to be 360 nM (Figure 5b, figure insert). When a complementary aptamer was immobilized on the BioDVD, however, we could not detect a signal, suggesting sequence-specific recognition of the aptamer which was observed in our binding analysis (Figure 5a). As observed, a minimum concentration of 10 nM of factor IXa can be detected in fluids using the above platform, which is comparable to other methods. For example, in SPR analysis, for the same biomolecular interactions, we found that about 6.5 nM of factor IXa is required (Supporting Information, Figure 2). The above analyses also suggest that a wide range of molecular sizes interactions, ranging from about 7000 to 76000 daltons, can be analyzed on the BioDVD. Recently, we also evaluated interactions of the RNA-small ligand, Cyanocobalamin, on BioDVD and confirmed that about 0.5 μM of Cyanocobalamin is sufficient to detect binding to the RNA (Supporting Information, Figure 3).

Similar to the above analysis, we also analyzed our previously selected aptamer that binds with high affinity to the hemagglutinin (HA) of the influenza A virus.[21] This anti-HA aptamer has a high binding ability and can efficiently discriminate between HA-derived from influenza A and influenza B viruses.[21] We performed an experiment similar to that described above, by hybridizing an anti-HA RNA aptamer with thiolated dT_{20} on the gold surface of the BioDVD, and adding different concentrations (100−800 nM) of HA purified from influenza A. The reflected light intensity of the spotted complex was monitored by averaging over several tracks of the DVD structure. As expected, the reflected light intensity changed with increasing concentrations of HA-derived from the influenza A virus (Figure 6); however, when we used the HA-derived from influenza B (up to 1000 nM), no change in the reflected light intensity was observed. These results confirm our earlier observations using both filter binding assays and SPR-based analyses.[21]

Application of Phase-Change Materials in Analysis. To tune the sensitivity of the multi-interferometric system to local changes in the refractive index of the sensor surface, the inclusion of a phase-change layer offers a strong advantage. The phase-change layer ex-

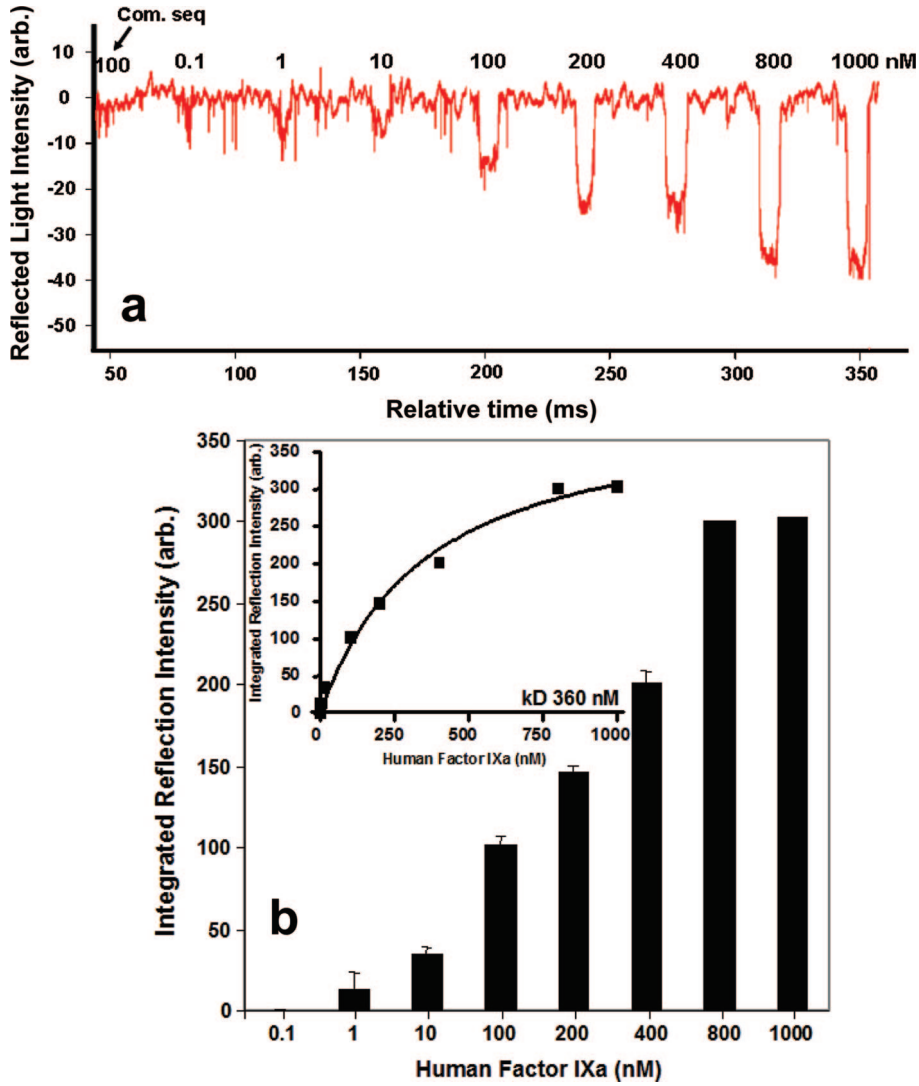

Figure 5. Analysis of interactions between an aptamer and coagulation factor IXa on the BioDVD. (a) Observed reflected light intensity for each concentration. The observed reflection intensity signal was normalized with the software IGOR Pro Version 6.0 (WaveMetrics, Inc., OR). (b) The reflected light intensity for each concentration was quantified and plotted as arbitrary units against different concentrations of coagulation factor IXa. Each bar represents the mean of two independent measurement results and used for determination of the equilibrium dissociation constant for the coagulation factor IXa−aptamer complex, using the software program GraphPad Prism 2.0 (figure insert).

hibits a large change in optical properties when switched from the amorphous to crystalline state which can be reversibly carried out by simple changes in the readout laser power. The underlying switching properties of the phase-change layers are well understood as the same materials are used in the form of rewritable DVD media as exemplified by DVD-RW or DVD-RAM. As these optical properties can be manipulated in well-defined spatial regions, well-defined areas of the BioDVD can be selectively masked with a high degree of precision and reproducibility allowing the fabrication of complicated disk structures for a wide range of biomolecular interactions while leaving other areas in an optimal state for multi-interferometric detection.

As described above, we analyzed both hybridization and RNA-protein interactions on the disk. The phase-change film state at all spotted points was initially in the amorphous state. The voltage drops for all these samples were quantified as area of reflection intensity and plotted for each sample. As observed before, the reflection intensity changes upon hybridizing to complementary oligo or binding aptamer to the factor IXa. (See labels 2 and 4 in Figure 7 and the corresponding structures labeled 2 and 4 in Figure 2). On the other hand, when we changed the phase-change state from the amorphous state (marked with a red sphere on Figure 7) to the crystalline state irradiation with 6 mW of dc laser power, little change in the signal intensity was observed among samples 1−4 (marked with green triangles in Figure 7), thus suggesting that by transformation to the crystalline state, the BioDVD can be made insensitive to biological interactions, because

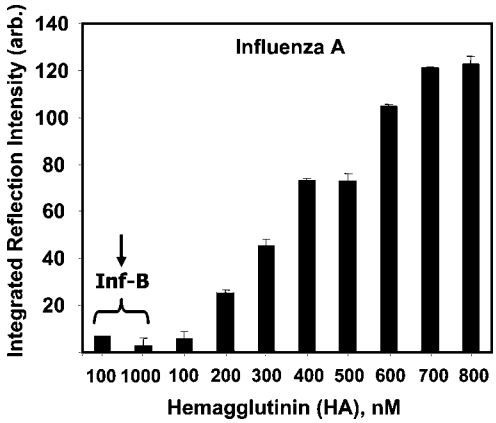

Figure 6. Anti-influenza aptamer to detect hemagglutinin of influenza A virus. Relative changes in the area of reflection intensity observed for different concentrations (100−800 nM) of HA from influenza A upon binding to the anti-HA aptamer. Similarly analyses were carried out with two concentrations of HA-derived from influenza B (100 and 1000 nM). Each bar represents the mean of two independent measurement results.

the multi-interferometric response is detuned. Interestingly, when we switched the phase-change layer back to the amorphous state (the recorded state in DVD-RAM or DVD-RW media) by irradiation with 15 mW (1 MHz) of pulsed laser power, we could observe again the same response at positions 2 and 4 (Figure 7). We confirmed that the process was reversible over many writing cycles without degradation of system response. This result suggests that regions in which the phase-change material is transformed into the crystalline state can be effectively and reversibly masked at will by the application of two different laser powers. These data show that the detection of biological molecules on a BioDVD can be precisely and quantitatively achieved using a combination of a phase-change thin film and optical interferometry (Figure 8). It is believed that further improvement of the $ZnS-SiO_2$ layer thickness may improve the sensitivity, as these manipulations may lead to a more ideal optical interferometric response due to optimization of the phase contrast of the aggregate structure.

The above-described BioDVD platform for measuring biomolecular interactions suggest that the analysis can be carried out with higher reproducibility at higher speeds (6.0 m/sec). The sensitivity of BioDVD was comparable to other known methods, for example, about a 10 nM concentration of factor IXa can be detected readily by BioDVD using higher affinity aptamer where as in the SPR analysis (using BIACore 2000), for the same interactions, the minimum concentration was 6.5 nM (Supporting Information Figure 2). Compared to the BioCD, the BioDVD platform has greater number of tracks per disk and thus allows us to analyze more samples per disk. In addition to this property, our disk has a multilayered structure consisting of phase-shifting material (AIST) which allowed us to reversibly mask the sensitivity of regions on the platform using currently available DVD technology. In the present study, we used 50 different tracks for analyzing biomolecular interactions, suggesting that only a fraction of the spotted area is being used, which represents 80 μm [50 tracks × 1.6 μm (groove and land) = 80 μm]. It is therefore, possible to reduce the spot size to about 1/10 of the present spot size (1.0 mm). Considering a spot size of 100 μm for each, it is possible to cast over 40000 spots per BioDVD, based on the total surface (4070 mm) available for the analysis and would require about 3.5 h (with disk speed 6.0 m/sec) for analyzing all these samples. On the other hand, the SPR analysis for each sample required about 30 min for the complete analysis of similar biomolecular interactions. However, such high precision spotting requires an automated system or an ink-jet array format combined with an addressing or sample coding for each spot. Presently, these spotting machines are commercially available and possible to use in our future studies. It is also interesting to note, from the above studies, that the BioDVD platform can be used for various kinds of biomolecular interactions having different sizes of ligands larger than 1000 daltons. Considering the above advantages, it is tempting to speculate that this platform can be readily useful not only in medical diagnosis (use at the bedside) but also in drug discovery. In the latter case specific binders can be screened from chemical libraries against important targets leading to an attractive label-free strategy (Figure 9), compared to other high throughput strat-

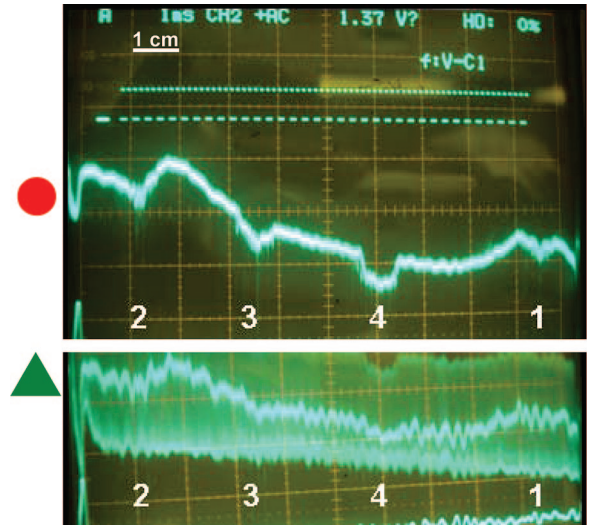

Figure 7. Detection of biomolecules in the states of amorphous and crystalline. Spots were made as single stranded DNA (1), duplex DNA (2), DNA with RNA-aptamer (3), and DNA, RNA-aptamer, and protein complex (4). Raw data obtained with amorphous state (marked as ●) and the crystalline state (marked as ▲) are overlaid.

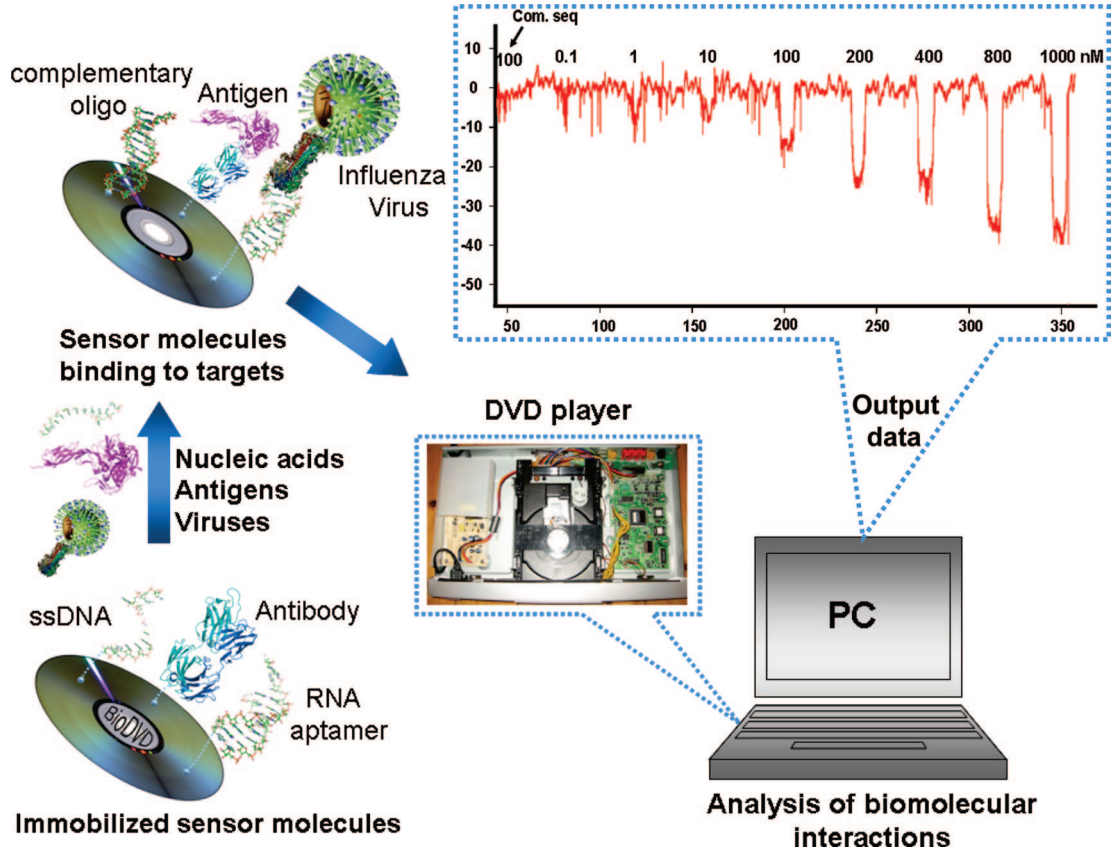

Figure 8. Summary of biomolecular interactions analysis using BioDVD platform technology.

egies with respect to the speed, reproducibility, sensitivity, and cost.

CONCLUSIONS

In this manuscript, we explored the BioDVD platform for the analysis of biological interactions, specifically, for the hybridization of nucleic acids and interactions between RNA and proteins. We found that these interactions can be measured with high sensitivity on this platform. The reflected light intensity was found to change linearly with increasing RNA-protein complex formation on the sensor surface for both coagulation factor IXa and HA of influenza A viral protein. We also found that the BioDVD fabricated with a phase-change layer offers the possibility for highly accurate and reversible masking of the sensitivity of regions on the platform using currently available DVD technology. In addition, the presence of the phase-change layer can be used to label different detection regions on the sensor surface, allowing flexible implementation of the biomolecular interactions used on a given disk structure in the field. Among the distinct advantage of the use of aptamers (which are targeted toward binding to a specific ligand with high-molecular discrimination) is both their insensitivity to the denaturing and renaturing processes and their ability to withstand the temperatures generated during the

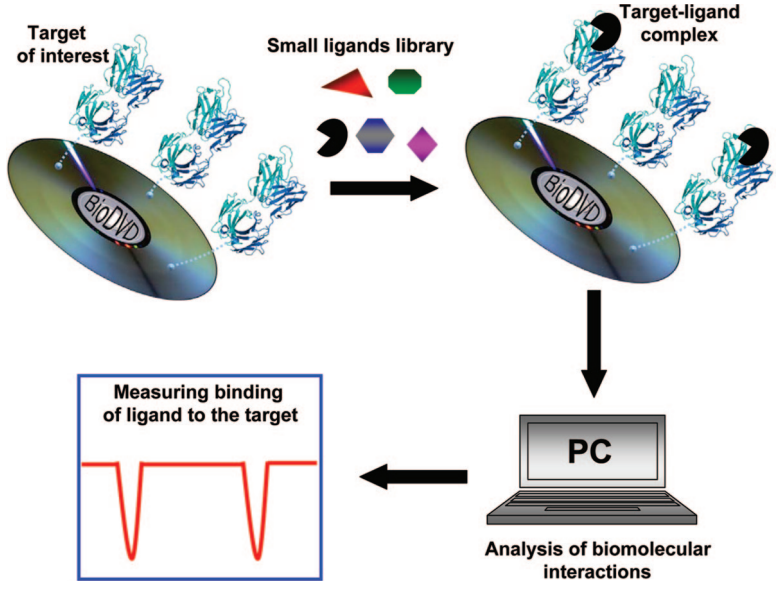

Figure 9. A proposed BioDVD platform for screening drug-leads against a target of interest.

writing process. Both of these attributes will allow the flexible use of BioDVD-based analysis systems in the field. In this way, the BioDVD serves to combine the best properties of both the biological and optical systems to yield a sensitive novel device that offers the capability of rapid analysis based upon well-established technology. These studies show that the BioDVD has the potential to become a label-free and high-throughput multianalyte platform for detecting a wide range of biomolecular interactions.

METHODS

Oligos and Proteins. Biotin dT_{20} was chemically synthesized and purchased from Sigma Genosys. Human factor IXa was purchased from American Diagnostica, Inc., CT, and hemagglutinin (HA) was purified from human influenza viruses, as described previously.[25] All sputtering materials were obtained as described.[15]

Fabrication of the Multilayered BioDVD. The multilayered structure of the BioDVD was fabricated by rf magnetron sputtering on a pregrooved polycarbonate disk substrate with a diameter of 12 cm and a thickness of 0.6 mm. On the surface, a spiral groove from the inner radius of 22.3 mm to the outer radius of 59.8 mm was prefabricated, with a 1.2 μm track-pitch and a depth of 60 nm. These specifications are nearly identical to those of a DVD-RW disk. Therefore, the signals obtained from the BioDVD can be retrieved using a conventional DVD drive. The only difference is in the multilayered structure deposited on the disk. The multilayered structure used here consisted of five layers of the structure: Au (5 nm)/$(ZnS)_{85}(SiO_2)_{15}$ (85 nm)/$Ag_{6.0}In_{4.4}Sb_{61.0}Te_{28.6}$ (15 nm)/$(ZnS)_{85}(SiO_2)_{15}$ (45 nm)/Au (50 nm), as illustrated in Figure 1. The thickness of each layer was computer-designed to optimize the sensitivity for 0 to 10-nm size target ligands located on the top Au layer. We assumed for the purposes of calculation, that the refractive index of the ligands was 1.45. Prior to the experiment, different thicknesses of SiO_2 layers were deposited on top of the Au layer, and we experimentally confirmed the simulated values.

Signal Read-Out. Before attaching the recognition molecules onto the BioDVD sensor surface, the desired track was marked with by a crystalline region (0.2 mm less than the desired track). All measurements were taken from a head position manually referenced from the center of the DVD, and the sensor surface faced the laser beam. The reflected light intensity was measured from the sensor surface by an optical disk drive tester (DDU-1000, Pulstec Industrial Co., Ltd.) equipped with a 635 nm diode laser and a pickup lens with an numerical aperture (NA) of 0.6. The laser beam was focused on the sensor surface of the BioDVD by inserting a dummy plate with a thickness of 0.6 mm between the BioDVD and the pickup lens, as shown in Figure 1. The readout laser power P_r was fixed at 1.0 mW, and the BioDVD was rotated at a scanning velocity of 6.0 m/s during the measurements. The measurements were carried out using the autofocus and autotracking modes available on the DDU unit. The reflected light intensity was measured as the output voltage from a photodetector located in the pickup unit using a sampling rate of 1 MHz and was smoothed using a 50-point boxcar average.

Preparation of DNA. The 5′ thiolated DNA was prepared chemically with 20 deoxythiamine residues $(dT)_{20}$ with a protected thiolated group. To deprotect the thiolated group, the oligos were treated with DTT (60 mM) and phosphate buffer (250 mM, pH 8.0) for 16 h at room temperature. After the reaction, the SH-poly dT_{20} oligo was subjected to purification by passage through a TSK-gel column in an HPLC system (Shimadzu, Japan), which was run with 2.5 mM TEAA, and eluted with a linear concentration of TEAA and acetonitrile in a ratio of 60:40. The solution from the peak area was collected and dialyzed for 5 h against double distilled water with two changes. The dialyzed sample was dried in a speed-vacuum, and after resuspension in water the DNA concentration was measured.

Preparation of RNAs. A 33-mer stable RNA-aptamer, previously selected against factor IXa,[26] was enzymatically synthesized by *in vitro* transcription, using T7 RNA polymerase on a synthetic DNA template. The template with the T7 promoter region (in italics) (5′-*AGTAATACGACTCACTATAGG*GATGGGGACTATA CCGCGTAATGCTG-3′) was synthesized to generate the double-stranded DNA. Using this template DNA and the primers (forward 5′-AGTAATACGACTCACTATAGG-3′; reverse 5′-$(T)_{24}$ATGGGGAGGCAGCATTACGCGGTATA-3′), a PCR reaction was performed with a commercial PCR kit (Ex *Taq* kit, Takara, Japan). The reaction mixture was cycled at 94 °C for 1 min 10 s, 55 °C for 50 s, and 72 °C for 1 min 10 s for 15 cycles. The PCR product was precipitated and used for RNA preparation by *in vitro* T7 transcription. Transcription was performed at 37 °C overnight, with a DuraScribe transcription kit (Epicenter Biotechnologies, WI). Afterward, the products were treated with 2 U of DNase I (RNase free) for 10 min at 37 °C, to remove the template DNA, and were mixed with an equal volume of 2X urea buffer (7 M urea, 50 mM EDTA, 90 mM Tris-borate containing 0.05% bromophenol blue). The reaction mixtures were denatured at 90 °C for 2 min and fractionated on a 15% polyacrylamide gel containing 7 M urea. The RNA band was excised, and the RNA was eluted from the gel. The RNAs were vacuum-concentrated, redissolved in water, and then quantitated by the absorbance at 260 nm. The resulting transcribed RNA (57-mer) had 33 nucleotides (which specifically recognize human factor IXa) and 24 nucleotides of A residues at its 5′ end and 3′ end, respectively. For the negative reaction, the complementary sequence of the selected aptamer was substituted. It was prepared according to the method mentioned above, from the DNA template 5′-*AGTAATACGACTCACTA TAGGG*TACCCCTGATATGGCGCATTACGAC-3′. The double-stranded DNA was made by using the T7 forward primer as above and the reverse primer 5′-$(T)_{24}$TACCCCTCCG TCGTAATGCGCCATAT-3′.

To prepare the anti-HA RNA, the template (5′-GGGTTAGC AGTCGGCATGCGGTACAGACAGACCC-3′) was synthesized to generate the double-stranded DNA. Using this template DNA and the primers (forward 5′-*AGTAATACGACTCACTATAGGG*TTAG-CAGTCGGCATGCGGTA-3′; reverse 5′-$(T)_{24}$GGGTCTGTCTGTACCGCA-3′), we prepared the dsDNA as described above. Transcription was performed at 37 °C for 3 h, with an AmpliScribe transcription kit (Epicenter Biotechnologies, WI) and the RNA was isolated in a similar manner as the antifactor IXa RNA. The resulting transcribed RNA (58-mer) had 34 nucleotides (which specifically recognizes HA-derived from influenza virus A, H3N2 subtype) and 24 nucleotides of A residues at its 5′ end and 3′ end, respectively.

For the preparation of RNA that binds to Cyanocobalamin, the oligomers 5′-GGG AAC CGG TGC GCA TAA CCA CCT CAG TGC GAG CAA-3′, 5′-AGT AAT ACG ACT CAC TAT AGG GAA CCG GTG CGC ATA ACC A-3′ and 5′-TTT TTT TTT TTT TTT TTT TTT TTT TTG CTC GCA CTG AGG TGG-3′ were used. The PCR product was amplified and transcribed and purified the Cyanocobalamin aptamer, as mentioned above.

Monitoring Hybridization of cDNA Oligos on the BioDVD. For analyzing the hybridization of cDNA oligos, to monitor the formation of base pairs between them, we prepared one oligo dT (20-mer) with a modified 5′ thiolated end. This oligo was attached to the gold surface of the BioDVD by spotting about 5 μL as the 1 μM level and incubating the disk for 1 h at room temperature. The disk was washed with either buffer or deionized water and then dried before measuring the change in the reflection intensity. After completion of this measurement, we added the second oligo, either complementary dA (20-mer) or noncomplementary (dT), as a negative control, in the same region as the thiolated oligo. We continued the incubation for about 15 min at room temperature, washed the disk with water, and measured the intensity, as described above. For DNA and RNA hybridization studies, we carried out ex-

periments similar to that mentioned above, except in this study we used an RNA containing polyA (24-mer) at its 3′ end, instead of the complementary dA (20-mer). Before hybridization, the RNA was denatured in annealing buffer (30 mM Hepes-KOH (7.4), 100 mM potassium acetate, and 2 mM magnesium acetate) at 90 °C for 2 min and slowly cooled at room temperature, to achieve proper folding. After the incubation, the spots were washed with deionized water and dried with an air-blower. The signal intensities were then measured as described above.

Monitoring RNA-Protein Interactions on the BioDVD. To monitor the RNA and protein interactions, a 1 μM solution of 5′ thiolated dT(20) was initially attached to the gold surface, as mentioned above, and was allowed to bind for 1 h at room temperature. After this incubation, the disk was thoroughly washed with deionized water and dried. On the same spot, we added the folded aptamer RNA (against the human coagulation factor IXa) at either a 100 nM or 1 μM concentration and incubated the disk at room temperature for 15 min. Upon completion of the incubation, the disk was washed and the reflection data were obtained. To observe the aptamer-factor IXa interactions on the disk, we added purified human coagulation factor IXa (1 μM concentration) in the same region, in the presence of calcium ions. The interactions were allowed for 15 min, and then the disk was washed and analyzed for changes in reflections, as mentioned above.

Similar to the RNA and protein interaction experiment above, to determine the concentration dependent reflection change, a 1 μM solution of 5′ thiolated dT(20) was attached to the gold surface and incubated for 1 h at room temperature. After this incubation, the disk was washed thoroughly with either buffer or deionized water, and the reflection signal was measured. On the DNA spot, the RNA aptamer with poly(A) was spotted at a similar concentration (1 μM) and the disk was incubated for 15 min. To clarify the interactions between the aptamer and the protein, different concentrations of factor IXa (0.1, 1, 10, 100, 200, 400, 800, and 1000 nM) were spotted on each DNA and RNA hybridized spot. To prove the specificity of the antifactor IXa aptamer, we also performed the negative reaction with the complementary sequences, using a similar concentration of binding sequences, and the binding reactions were carried out with 100 nM of factor IXa. Similar to the factor-IXa binding against its aptamer, we have also analyzed RNA aptamer interactions with viral proteins. In this study, we used our previously selected anti-influenza A aptamer.[21] As described for the previous experiments, we spotted the thiolated dT(20) on the BioDVD surface. The RNA aptamer was prepared with a poly-A tail to complement the polyd(T), and was attached to each experimental spot. On the DNA and aptamer hybridized spots, the viral protein hemagglutinin (HA) was spotted at different concentrations, in the range from 100 to 800 nM. After incubating and washing the disk, the reflection intensity was monitored. To confirm the ability of the aptamer to distinguish the HA of influenza A from that of influenza B, we also spotted the HA protein of influenza B (100 and 1000 nM).

Analysis of Biomolecular Interactions by Surface Plasmon Resonance. Aptamer binding was conducted using the SPR method on a BIAcore 2000, with a streptravidin-coated sensor-chip, from BIAcore, Sweden. The association and dissociation of the aptamer−protein complexes were evaluated. To determine the affinity constants of the aptamer, we prepared the aptamer with 24-mer poly(A) nucleotides at the 3′-end, which could anneal to the complementary biotinylated oligo(dT) (5′-biotin-$(T)_{24}$-3′). To prepare this 3′end extended RNA, we used two primers: a T7 forward primer similar to that used in the above study and a 3′ end primer [5′-$(T)_{24}$-ATGGGGAGGCAGCATTACGCGGTATA-3′]. The double-stranded DNA template was generated by PCR and transcribed *in vitro*, as described above. Initially, the biotinylated oligo $(dT)_{24}$ was attached to the streptavidin (SA chip, BIAcore, Sweden) by dissolving the oligo dT in binding buffer (5 μM final concentration) and injecting it for 12 to 24 s, to obtain a response of 1000 resonance units (RU), at a flow rate of 5 μL/min. The excess or unbound biotinylated oligo dT was removed with binding buffer containing 5 mM $CaCl_2$, at a flow rate of 20 μL/min for 10 min. To analyze the binding of the aptamer, 20 μL (50 nM final concentrations) of the aptamer was injected at a flow rate of 2 μL/min for 10 min, which resulted in an increase of about 1200 RU upon aptamer binding to the complementary biotinylated primer. After each measurement, the sensor chip was washed with a buffer solution, followed by 10 mM NaOH, before the next injection.

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Supporting Information Available: Supplementary figures. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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