Exploitation of stem-loop DNA as a dual-input gene sensing platform: Extension to subtyping of influenza A viruses

Yu-Hsuan Lai,[1] Chang-Chun Lee,[2] Chwan-Chuen King,[2] Min-Chieh Chuang,[3]** and Ja-an Annie Ho[1]*

[1]BioAnalytical Chemistry and Nanobiomedicine Laboratory, Department of Biochemical Science and Technology, National Taiwan University, Taipei, Taiwan; [2]Department of Public Health, National Taiwan University, Taipei, Taiwan; [3]Department of Chemistry, Tunghai University, Taichung, Taiwan

Electronic Supplementary Information

Experimental Section

Chemicals

6-Mercapto-1-hexanol (MCH, 97%), magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$, ≧99%), 3,3′,5,5′-tetramethylbenzidine (TMB, ≧98%), D-(+)-Glucose (≧99.5%), hydrogen peroxide solution (H_2O_2, 34.5-36.5%), dimethyl Sulfoxide (DMSO, ≥99.7%), albumin from bovine serum (BSA, ≧98%), casein from bovine milk, citric acid monohydrate (A.C.S. grade), boric Acid (A.C.S. grade), glycerol (A.C.S. grade) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium chloride (NaCl, ≧99%), potassium phosphate monobasic and dibasic (A.C.S. grade) were obtained from J. T. Baker (Phillipsburg, NJ). HRP-conjugated FITC-antibody (α-FITC-HRP) and avidin-conjugated glucose oxidase (Av-GOx) were acquired from NOVUS Biologicals (Littleton, CO) and Rockland (Gilbertsville, PA), respectively. Dithiothreitol (DTT) and ethylenediaminetetraacetic acid (EDTA, 99.5%) were purchased from PlusOne (Piscataway, NJ). 40% Acrylamide/Bis solution (29:1), ammonium Persulfate (APS), and N,N,N′,N′-Tetramethylethylenediamine (TEMED) were obtained from BIO-RAD (Hercules, CA). Phusion High-Fidelity DNA Polymerase (2U/μL) and GeneRuler Ultra Low Range DNA Ladder (0.5 μg/μL) were acquired from Thermo Scientific (Waltham, MA). Bromophenol blue (A.C.S. grade), dNTP (dATP, dCTP, dGTP, dTTP, 10 mM of each in 0.6 mM Tris-HCl, pH 7.5), and SYBR Gold (10,000X concentrated in DMSO) were purchased from AMRESCO (Solon, OH), GeneDirex (Las Vegas, NV), and Invitrogen (Carlsbad, CA), respectively.

Sequence Selection

The constituent sequences of the hairpin DNA probe were selected and designed on a basis of sequences acquired from the Influenza Virus Resource.[1] To identify contiguous conserved regions in the H5 and N2 gene segments of all influenza H5N2 viruses, the downloaded H5 and N2 sequences were aligned respectively using MUSCLE.[2] The identified regions possessed more than 90% conservation in the whole population of the specified H5N2 subtype. These candidate regions

were subsequently aligned to non-H5 and non-N2 subtypes separately with Bowtie[3] to eliminate the promiscuous sequences which can be identified in other subtypes. The selected candidate regions were analyzed by performing BLAST analysis against Nucleotide collection to assess the sequence specificity of influenza virus.[4]

Thermal Denaturing Profile of DNA Molecular beacons (MBs)

The actual melting temperature of hairpin DNA used in this study was verified in various buffers. DNA molecular beacons (MBs) with a quencher and a fluorescent dye were examined by CFX96 real time-PCR thermocycler (BioRad) to monitor the change in fluorescence signal as a function of temperature. Briefly, the thermal profile of each MB and the MB- targeted gene hybrids were obtained by mixing 0.2 μM MB with TA, TB, or both probes in characteristic buffers. The temperature was commenced at 10 °C for 5 min, and then increased from 10 to 85 °C at 1 °C increments. The lasting time in each temperature increment is 5 min.

Kinetics analysis

Dissociation constants for the hybridization between molecular beacon (MB) and its targets were obtained based on the approach described in Bonnet et al.[5] and Tsourkas et al.[6]. Briefly, in a solution containing MB and target DNA (T), there can be at least three distinct conformations: MB-T hybrid (phase 1, MB · T), stem-loop folded MB free from target (phase 2, MB_{closed} + T), and random coiled MB free from target (phase 3, MB_{open} + T). The reaction can be written as:

$$MB \cdot T \overset{K_{12}}{\rightleftharpoons} MB_{closed} + T \overset{K_{23}}{\rightleftharpoons} MB_{open} + T, \qquad \mathbf{1}$$

Following the model proposed by Bonnet et al.[5], the dissociation constant K_{23} describing the transition between hairpin conformation and random coils is given by

$$K_{23} = \frac{\theta - \beta}{\gamma - \beta}, \qquad \mathbf{2}$$

where θ is the fluorescence intensity as a function of temperature T (the thermal profile), β is the fluorescence of MB when they are all folded as hairpin, acquired from the intensity measured at 15°C, and γ is the fluorescence from MB when they are all randomly coiled, obtained from the intensity measured at 85°C. The dissociation constant K_{12} characterizing the transition between MB bound to target (MB · T) and unbound MB (MB_{closed}) can be derived from the thermal profile of MB with the existence of targets,

$$K_{12} = \frac{(\alpha - \phi)T_0}{(\phi - \beta) + (\phi - \gamma')K_{23}}, \qquad \mathbf{3}$$

where ϕ is the thermal profile for MB-target hybrid, β is the same as aforementioned, α and γ' are the fluorescence from MB in the presence of target at 15 and 85°C.

The rate constants which determine the opening kinetics of MB with corresponding targets were derived by mixing 0.2 μM MB with 1.2 μM of each target DNA using FlexStation 3 multimode microplate reader (Molecular Device, Sunnyvale, CA) with an integrated 8-channel pipettor. The excitation and emission wavelength were set at 495 and 525 nm, respectively, with a cutoff at 515 nm. The fluorescence as a function of time was observed before, during, and after sample mixing. According to Tsourkas et al.[6], opening kinetics of MB upon hybridization with target DNA can be analyzed by assuming a second-order reaction,

$$MB + T \underset{k_2}{\overset{k_1}{\rightleftarrows}} MB \cdot T, \quad \frac{d[MB \cdot T]}{dt} = k_1[MB][T] - k_2[MB \cdot T],$$

where k_1 is the opening rate constant, and k_2 is the closing rate constant of MB upon hybridization. Assuming that fluorescence could be normalized as following,

$$F_n = [F(t) - F_0]/[F_{eq} - F_0] = [MB \cdot T]_t/[MB \cdot T]_{eq},$$

in which F_{eq} is the fluorescence of the system when $t \to \infty$ and F_0 is the initial fluorescence intensity. After solving the above equation and rearranging the formula with normalized fluorescence F_n, an exponential function can be derived:

$$\frac{1 - F_n}{1 - \rho F_n} = e^{-\omega k_1 t}, \qquad \textbf{4}$$

where $\rho = ([MB \cdot T]_{eq})^2/MB_0T_0$, $[MB \cdot T]_{eq} = (MB_0 + T_0 + K_{12} - \omega)/2$, $\omega = \sqrt{(MB_0 + T_0 + K_{12})^2 - 4MB_0T_0}$, and $K_{12} = k_2/k_1$.

The opening rate constant k_1 could be derived by fitting the normalized fluorescence data to an alternative form of equation 4 (after taking natural log of it), resulting in a linear plot with a slope k_1,

$$\frac{1}{\omega}(1 - \rho F_n) - \frac{1}{\omega}\ln(1 - F_n) = k_1 t. \qquad \textbf{5}$$

It should be noted that though this model is suited for a bimolecular reaction (i.e. MB and TA), it can also be appropriately applied to the tri-molecular hybridization (MB, TA and TB) in our system for analysis of the "unzipping phenomenon" since the opening of the MB is dominated by TA. As indicated in the thermal profile, the dissociation behavior of MB-target hybrid in the solution

containing MB, TA and TB is similar to the bimolecular system consisting of MB and TA. The kinetics in the tri-molecular scenario also exhibits a similar curve pattern compared with that of the bimolecular reaction, reaching equilibrium nearly at the same time. These results echo our expectation that TA is responsible for opening the hairpin and TB is inconsequential for destabilizing the stem-loop structure.

Electrochemical Determination of Hairpin Coverage on Sensing Surface

The coverage of hairpin on the surface of gold electrode was estimated by an electrochemical method developed by Steel et al.[7] using chronocoulometry. Under equilibrium conditions, DNA density at the electrode surface was calculated from the number of cationic redox molecules, hexaammineruthenium(III), electrostatically associated with the anionic phosphate backbone, determined from the difference in chronocoulometric intercepts at t = 0, with and without $[Ru(NH_3)_6]^{3+}$. Cyclic voltammetry (CV) and chronocoulometry (CC) were performed using a CHI 1030A Multi-Potentiostat (CH Instruments, Austin, TX). The following parameters were employed: CV, 100 mV/s for sweep rate; CC, 0.5 s for pulse width.

The charge Q, as a function of time (t) in a chronocoulometric experiment is given by the integrated Cottrell Expression

$$Q = \frac{2nFAD_0{}^{1/2}C_0{}^{*}}{\pi^{1/2}}t^{1/2} + Q_{dl} + nFA\Gamma_0,$$

where n is the number of electrons per molecule for reduction, F the Faraday constant (C/equiv), A the electrode area (cm^2), D_0 the diffusion coefficient (cm^2/s), C_0* the bulk concentration (mol/cm^2), Q_{dl} the capacitive charge (C), and Γ_0 the amount of redox marker confined near the electrode surface. The saturated surface excess of redox molecule is then converted to surface density of DNA hairpin with the relationship

$$\Gamma_{DNA} = N_A\Gamma_0(z/m),$$

where Γ_{DNA} is the DNA surface coverage in molecules/cm^2, m is the number of bases in the probe DNA, z is the charge of the redox molecule, and N_a is Avogadro's number. In this study, the surface coverage of assembled DNA hairpin was estimated to be ~ $3x10^{11}$ molecules/cm^2 (Fig. S9).

Determination of enzymatic activities of enzyme conjugates

Activities of enzymatic conjugates (HRP-conjugated FITC-antibody (α-FITC-HRP) and avidin-conjugated glucose oxidase (Av-GOx)) employed in this study were determined using 10-acetyl-3,7-Dihydroxyphenoxazine (Amplex Red) reagent (Invitrogen). The working solution used in glucose oxidase assay contains 50 µM Amplex Red, 0.1 U/mL horseradish peroxidase (HRP), 50 mM D-(+)-glucose in 50 mM sodium phosphate buffer (pH 7.4), whereas the solution for peroxidase assay consists of 50 µM Amplex Red and 1 mM H_2O_2. The standard curve for enzymatic activity (U/mL) was established by measuring the fluorescence intensity of the product, Resorufin,

after 5-min enzymatic reaction (Ex: 530 nm; Em: 590 nm). Using this method, the enzymatic activities of avidin-GOx and α-FITC-HRP conjugate used in our system were estimated to be 25 U/mL and 0.6 U/mL, respectively.

Reverse Transcription and Asymmetric Polymerase Chain Reaction (aPCR)

The Genbank accession number is CY110933 for HA and CY110935 for NA gene. Viral RNA (vRNA) was extracted using QIAamp Viral RNA Mini Kit (Qiagen, Limburg, Netherlands) according to manufacturer's instructions. The extracted RNA were then reverse transcribed to cDNA ~~by~~ using an universal primer Uni12, which is complementary to 12 conserved contiguous nucleotides at the 3'-end of influenza vRNA[8]. Prior to reverse transcription, mixture of vRNA, Uni12 primer, and dNTP was heated at 65 °C for 5 min, followed by a 5-min incubation on ice. Subsequently, cDNA were generated from a 20 μL-solution composed of 5 μL vRNA, 500 μM dNTP, 500 nM Uni12 primer, 10 U Superscript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA), 10 mM DTT, 2U RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invotrogen) in the first-strand buffer. The solution was subsequently incubated at 50 °C for 1 hr and the reaction was inactivated by a 15-min incubation at 70 °C. Finally the cDNA products were purified with QIAquick PCR Purification Kit (Qiagen).

The sense and antisense primers (Table 1) for amplification of H5 and N2 genes from the cDNA genome of A/duck/Taiwan/DV30-2/2005 (H5N2) were designed by the Tm Calculator of Applied Biosystems. The aPCR product of H5 segment was generated from a 50 μL PCR mixture composed of 1X Phusion HF buffer / 0.2μM dNTP / 1.5 μM H5F / 0.15 μM H5R / 1μg viral genome (cDNA) / 2 units of Phusion High-Fidelity DNA polymerase. Reactions were performed with 40 cycles of aPCR in Biorad C1000^{TM} Thermal Cycler. The amplification cycles include 1 cycle of 2 min at 98 °C followed by 40 cycles of 98 °C for 30 sec, 60 °C for 20 sec, and 72 °C for 30 sec, and a final extension was executed at 72 °C for 10 min. In regard to the aPCR of N2 gene, the 50 μL PCR mixture consists of 1X Phusion HF buffer / 0.2μM dNTP / 1.5 μM N2F / 0.3 μM N2R / 1μg viral genome (cDNA) / 2 units of Phusion High-Fidelity DNA polymerase. The amplification cycles include 1 cycle of 2 min at 98 °C followed by 40 cycles of 98 °C for 30 sec, 57 °C for 20 sec, and 72 °C for 30 sec, and a final extension was executed at 72°C for 10 min.

Polyacrylamide Gel Electrophoresis (PAGE)

Products of the aPCR were verified by PAGE to determine the sizes of amplified fragments. The polyacrylamide gel was composed of 12% acrylamide (Acrylamide: Bisacrylamide, 29:1), 0.1% APS, and 0.1% TEMED in running buffer. The running buffer consisted of 89 mM Tris base, 89 mM boric acid, and 2 mM EDTA, pH 8.5. Before being loaded to the gel, all samples, including DNA ladders, were mixed with the tracking dye (0.08% bromophenol blue and 10% glycerol in running buffer). Gels were run on the Mini-PROTEAN electrophoresis system (BIORAD, CA, USA) at 14 V/cm for 70~80 min. After electrophoresis, gels were immersed and stained in 1X SYBR Gold solution (from stock diluted in running buffer) for 30 min. Stained gels were then photographed by FluorChem M (ProteinSimple, CA, USA) in which samples were excited by UV and detected at the

range of 537~563 nm.

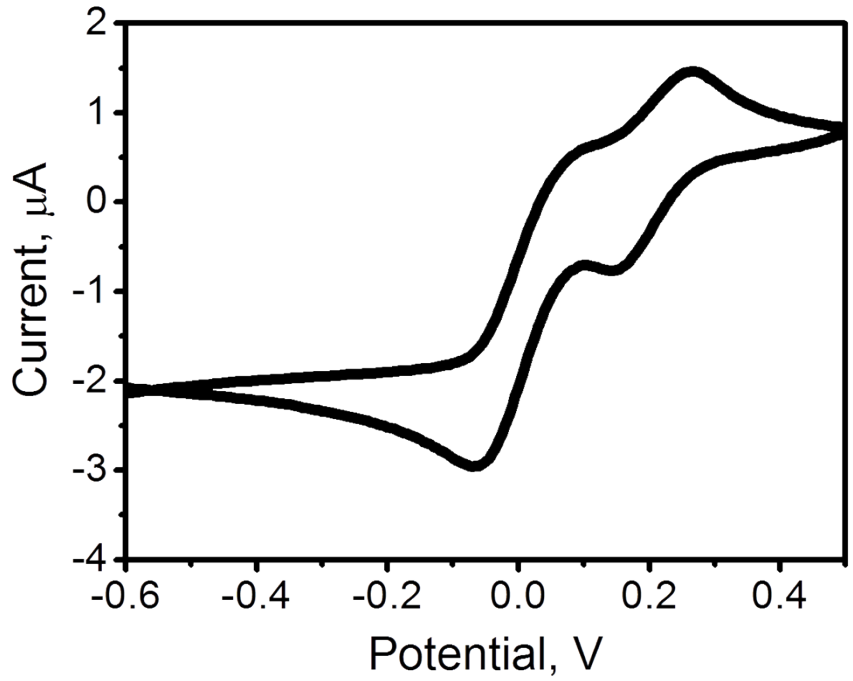


Figure S1. The cyclic voltammogram of the oxidized TMB produced by HRP on the gold surface modified with thiolated hairpin DNA, DTT, MCH, and blocking proteins including BSA and casein. The scan rate was 100 mV/s.

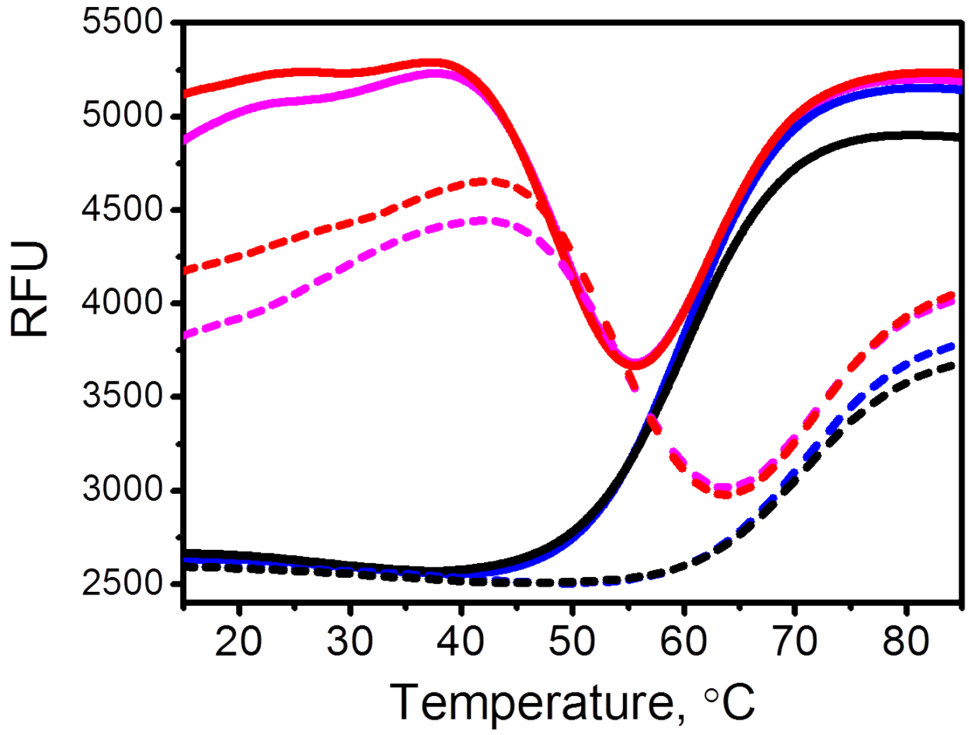


Figure S2. Thermal denaturing profiles of MB (black), MB treated with TA (magenta), MB treated with TB (blue), and MB treated with both TA and TB (red). The solid curves stand for groups in buffer with 170 mM NaCl and dotted ones represent those in buffer with 1 M NaCl.

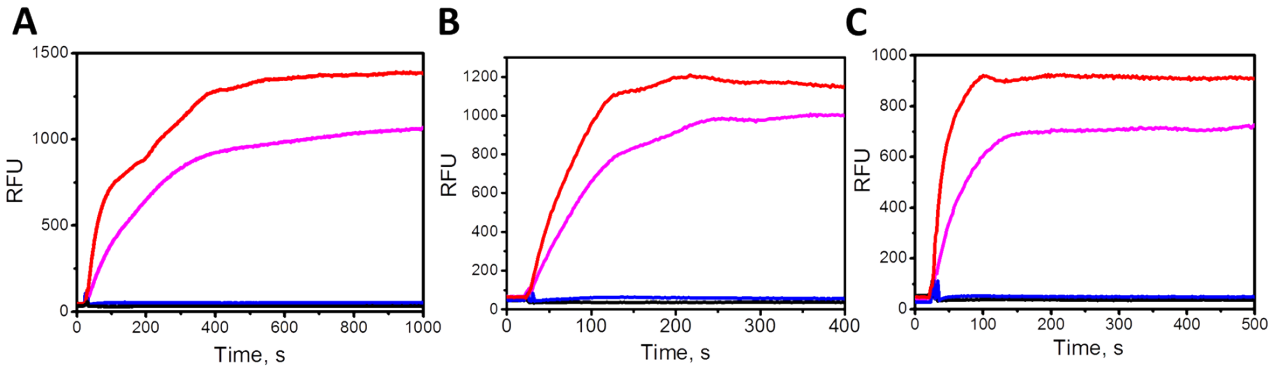


Figure S3. Observation of fluorescence change of MB (black), MB treated with TA (magenta), MB treated with TB (blue), and MB treated with both targets (red) as a function of time. Reactions were carried out under (A) a medium salt concentration (170 mM NaCl) at 28 °C, and (B) 37 °C, as well as under (C) a high salt concentration (1 M NaCl) at 28 °C.

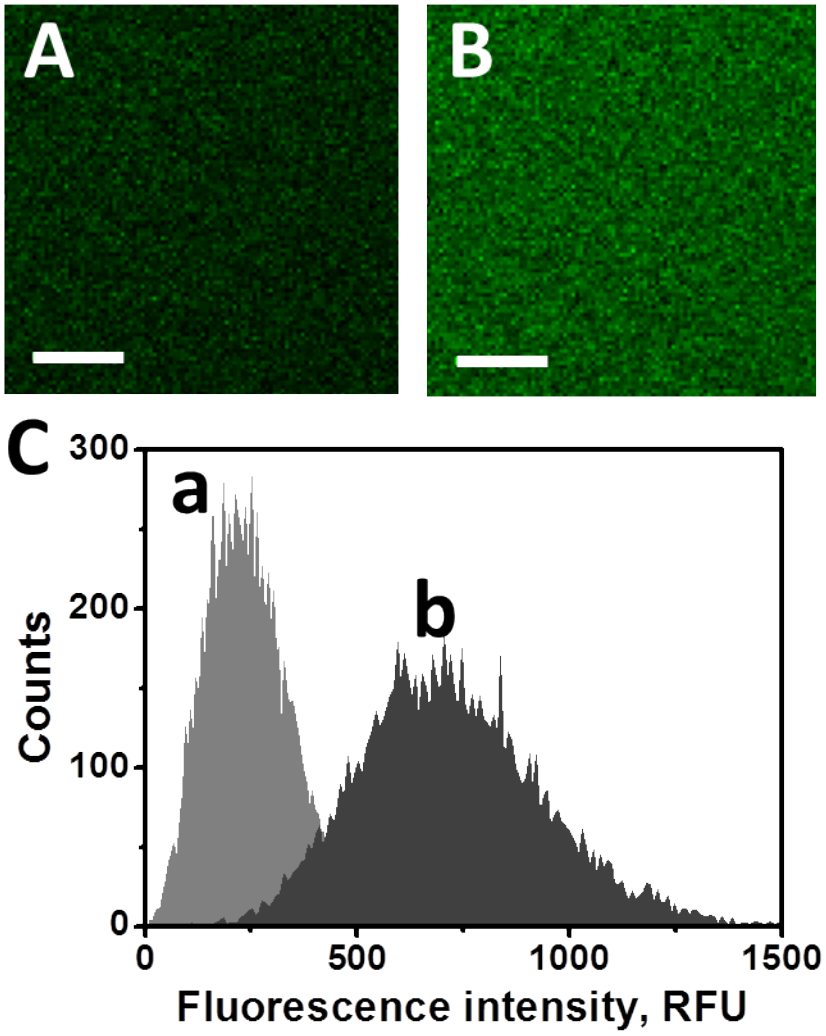
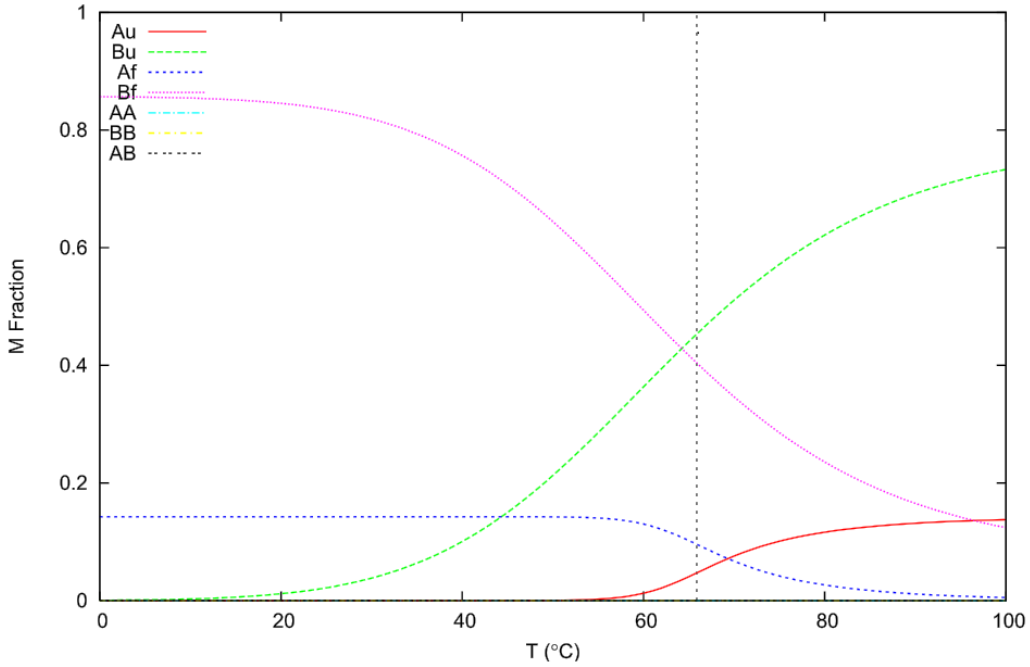


Figure S4. The fluorescence observed with confocal microscopy in the presence of N2 gene which hybridized with assembled hairpin DNA by (A) 12 base pairs or (B) 15 base pairs. Figure C is the fluorescent histograms of the pictured areas. The scale bar in (A) and (B) represents 10 μm.

The confocal microscopy images and the quantitative analysis of the measured fluorescence reveal that the fluorescence emission captured from the 15-bp gNA is unambiguously brighter than that from the 12-bp gNA. The mean intensity increased considerably, from approximately 257 (12-bp) to 735 (15-bp), underlining the significance of the number of hybridized base pairs of gNA on the structural changes of the immobilized DNA hairpin.

A



B

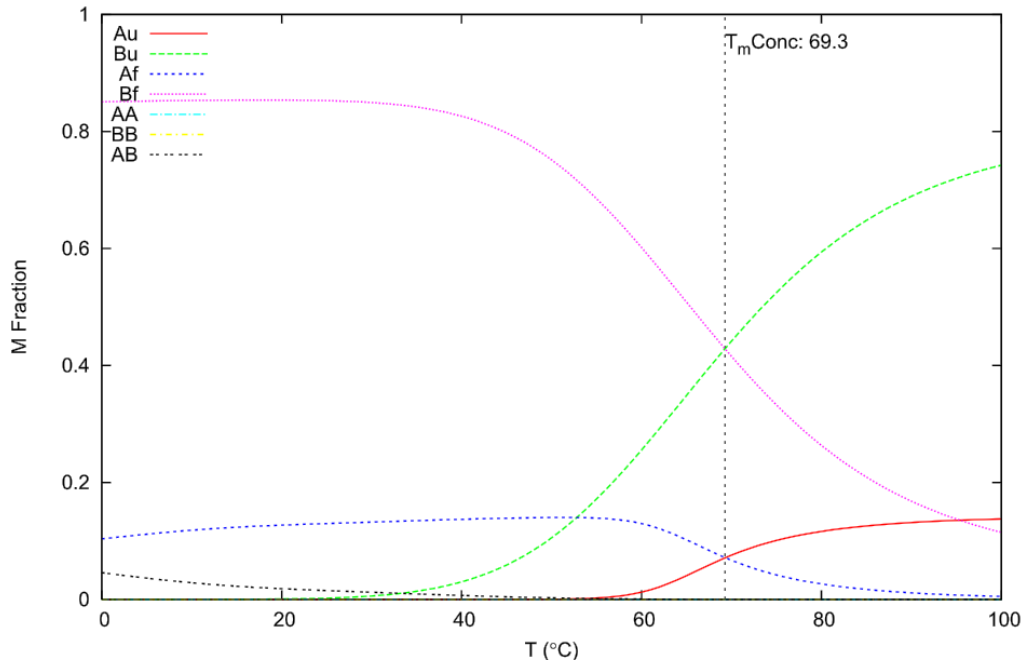


Figure S5. Simulated concentration plots of reactions containing hairpin DNA and gNA with hybridized base pairs of (A) 12, and (B) 15. These results were calculated using DINAMelt web server with the following inputted parameters: initial concentration of DNA hairpin ($[A_0]$), 0.2 µM; initial concentration of the target ($[B_0]$), 1.2 µM; salt conditions, 170 mM NaCl and 1 mM $MgCl_2$. The black line represents the concentration profile of duplex formed by DNA hairpin and its target as a function of temperature. No hybridization was predicted in Figure A.

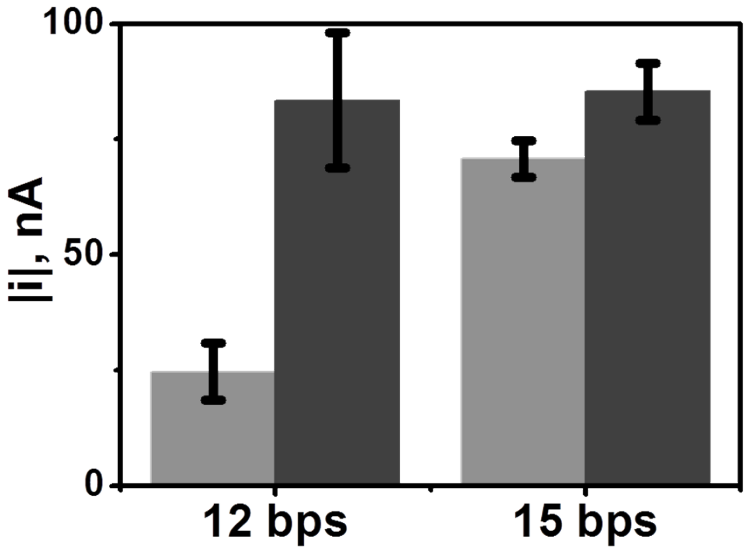


Figure S6. Comparison of the amperometric currents obtained from the hairpin DNA-assembled sensor which was treated with NA gene that hybridized with the hairpin loop by 12 bps or 15 bps and catalyzed by the enzymatic cascades of HRP and GOx. Gray bars represent groups added with N2 gene alone, and black bars are those with both HA and NA gene.

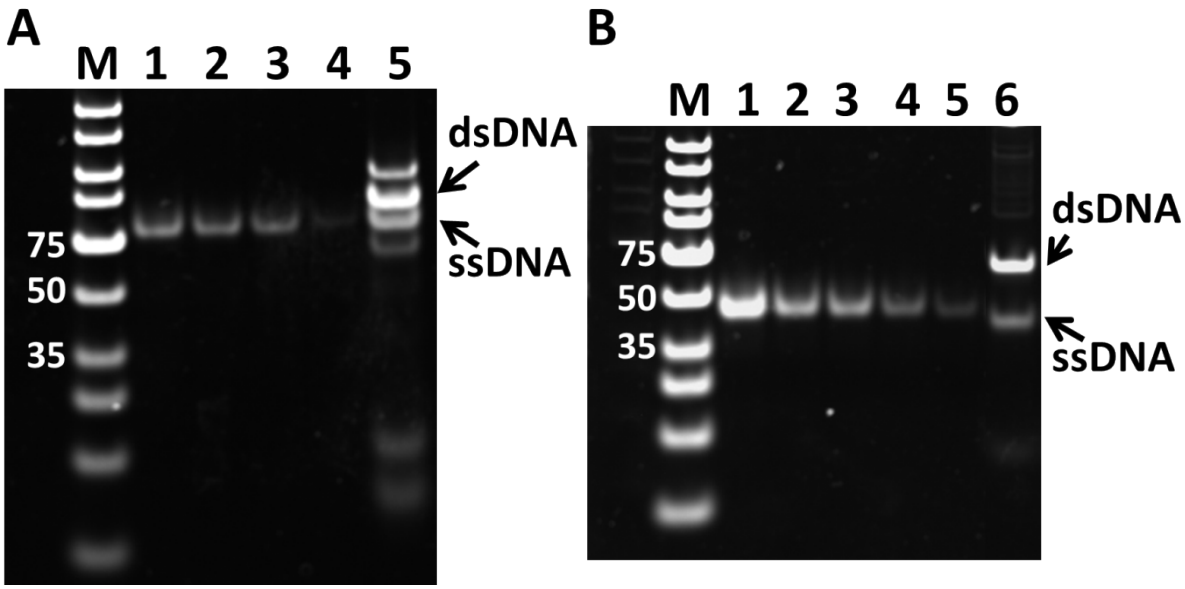


Figure S7. Polyacrylamide gel electrophoresis analysis of aPCR products generated from a real H5N2 viral genome. Bands in lane M are DNA ladders; sizes (bp) are indicated on the left side of the gel photos; pointed bands on gels are the dsDNA and ssDNA fragments expected to have been generated by the protocol. (A) Amplified DNA fragments of the H5 gene. Lanes 1–4: Synthetic DNA bearing the same sequence and length (64 nt) as that of the expected aPCR product for the H5 gene at 1, 0.75, 0.5, and 0.25 μM, respectively; lane 5: aPCR product of the H5 gene. (B) Amplified DNA fragments of the N2 gene. Lanes 1–5: Synthetic DNA bearing the same sequence and length (47 nt) as that of the expected aPCR product for the N2 gene at 2, 1, 0.75, 0.5, and 0.25 μM, respectively; lane 6: aPCR product of the N2 gene.

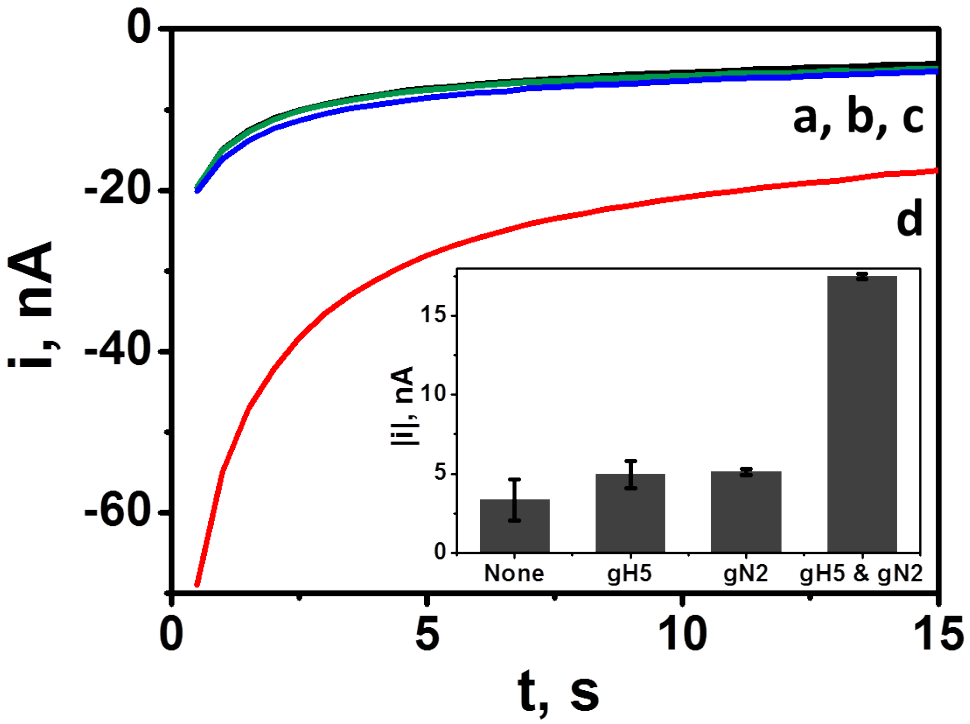


Figure S8. (A) Chronoamperograms of the genosensors corresponding to the conditions with (a) neither of the targeted genes, (b) the gH5 amplicon alone, (c) the gN2 amplicon alone, and (d) both gH5 and gN2 amplicons together. Inset: Magnitudes of the currents recorded after 60 s in the four scenarios of the inputs.

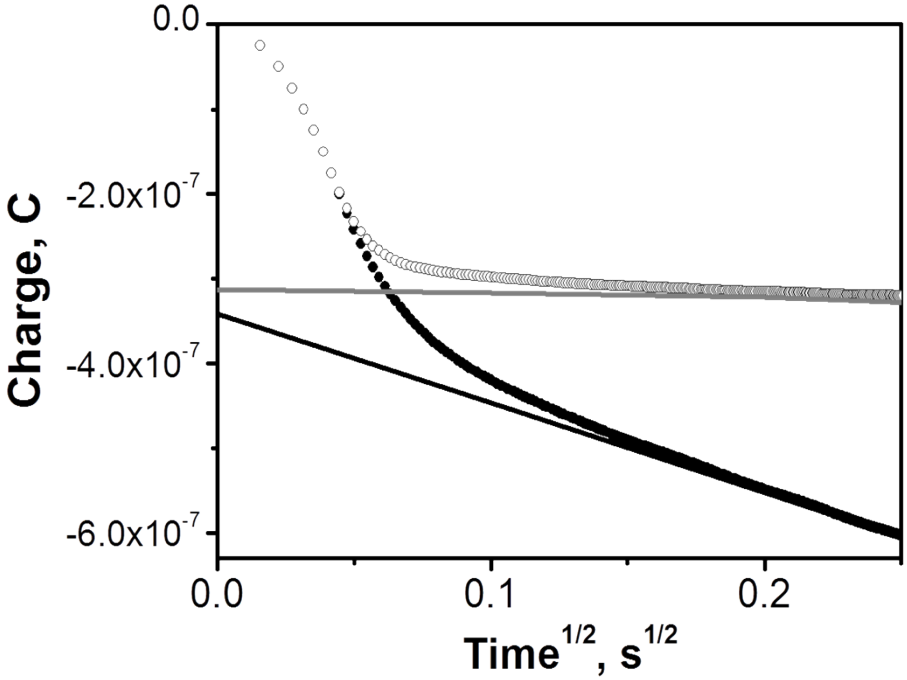


Figure S9. Chronocoulometric response curves for gold electrodes modified with hairpin DNA, DTT, and MCH in the absence (hollow circle) and presence (solid circle) of 50 µM RuHex. Fitting lines used to determine the intercept at t = 0 are displayed in gray and in black, for the group without and with $[Ru(NH_3)_6]^{3+}$, respectively.

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