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

Yu-Hsuan Lai,a Chang-Chun Lee,b Chwan-Chuen King,b Min-Chieh Chuang*ac and Ja-an Annie Ho*a

We herein report a strategy coupling DNA structural switches with an enzymatic cascade reaction for the specific and sensitive identification of dual-target nucleic acids toward the subtyping of influenza viruses. During the recognition process, a hairpin DNA was hybridized with dual target sequences at its stem and a partial section of the loop region to form duplexes. The concurrent presence of dual targets was reported as an explicit output interpreted by the enzymatic cascade reaction. The conformational changes of the hairpin–target duplex and the reaction kinetics upon dual hybridization were explored using a molecular beacon in conjunction with a FRET approach. The unzipping event was found to be predominated by a strand displacement which occurred in the stem of hairpins, and its rate constants were calculated as $3.1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (gHA alone) and $5.8 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (gHA & gNA). This strategy was further demonstrated on a multi-well electrochemical chip for the subtyping of influenza viruses (e.g., A/duck/Taiwan/DV30-2/2005 (H5N2)) through the simultaneous identification of genes encoding hemagglutinin (HA) and neuraminidase (NA). Moreover, this genosensor featured an effective capability to analyze unpurified amplicons generated from the genuine viral genome, as it could unambiguously discriminate between H5N2 and non-H5N2 viruses. To the best of our knowledge, this study is the first to demonstrate the leveraging of an individual hairpin DNA across concurrent analysis of dual target genes. Pursuant to the elegant characterizations with high applicability, extensible flexibility, and powerful utility, this platform holds great promise for use as a sophisticated molecular diagnostics tool against variable influenza viruses.

Introduction

Stem-loop folded deoxyribonucleic acids, or simply “hairpin DNA,” exhibit versatility when used to determine specific genetic sequences by virtue of their high thermodynamic stability and specificity for recognizing characteristic sequences of DNA/RNA fragments.1–7 Recently, a new class of reagentless electrochemical DNA (E-DNA) sensors with built-in surface-confined stem-loop DNA structures was developed.8,9 The principle behind the operation of an E-DNA sensor is the selective hybridization capability of the loop against a single target segment; this process unzips the stem duplex and provides an output display on a basis of either the distance-dependent electron transfer properties of a tagged redox molecule or an enzymatic amplification event.8–10 Among the diverse accomplishments reported, a particularly interesting approach is one that shields the affinity tag in the absence of a target; upon hybridization of the target to the loop, the hairpin DNA restores the accessibility of the tag to the reporter enzyme, thereby triggering the enzymatic amplification process.8,10 Such a design provides DNA detection with sensitivity on the femtomolar level.

Determination of a single genetic target could be an insufficient means to describe biological systems because of the tremendously complex genetic codes found in nature. Although many of the previously developed genosensors can be extended to various practical purposes, most were designed to recognize one targeted sequence at a time. Accordingly, they are not applicable to phenotype determination, where concurrent identifications of multiple nucleic acid sequences are often required for classification of pathogens, diagnosis of diseases, and clinical decision-making for personalized prescriptions.11–14 Herein we describe a new concept: the recognition of a pair of genes using a single hairpin DNA. Our strategy for the construction of the recognition scheme and the signal

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transduction system enhances the utility of hairpin DNA to interpret complex events, achieving dual-gene analyses that outperform previously reported single-gene measurements.

To demonstrate the feasibility of our platform, we systematically selected two characteristic nucleic acid sequences for use as targets for influenza viruses (see “sequence selection” in the ESI†). Influenza, an acute respiratory and highly contagious disease, has caused substantial morbidity and mortality globally since at least the Middle Ages.35-38 Most human pandemic influenza viruses are believed to originate from genetic reassortment—a process that is difficult to foresee, yet poses a persistent threat to public health. The re-assortment of viral genomes occurs when a single cell is infected by two distinct influenza viruses simultaneously, leading to the generation of a novel influenza viral strain through antigenic drift or shift of two surface proteins: hemagglutinin (HA) and neuraminidase (NA).35-38 Accordingly, influenza subtyping necessitates the simultaneous detection of both HA and NA. In light of the threat of influenza viruses and a demand for rapid diagnostics, the real-time reverse transcriptase polymerase chain reaction is widely accepted as the gold standard for subtyping of the influenza virus, even though its labor- and instrument-intensiveness hamper its feasibility in deployed diagnostics.39 It could be possible to circumvent these drawbacks through the development of electrochemical biosensing platforms, which are recognized as promising devices for point-of-care testing, particularly for clinical diagnosis and transmission surveillance. In this study, we designed a scheme combining an assembled hairpin DNA for the recognition of a pair of input genes (characteristic to HA and NA) with the subsequent amplification of an electrochemical signal through an enzymatic cascade, thereby expediting high-fidelity screening of influenza viruses.

Experimental section

DNA oligonucleotides

The DNA probes and molecular beacons (MBs) employed in this study were synthesized by Integrated DNA Technology (Coralville, IA). All oligonucleotides were purified through high-performance liquid chromatography (HPLC), examined using mass-analyzed laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and subsequently quantified through optical density (OD) measurements. MBs featured a 6-carboxyfluorescein (6-FAM) unit attached to the 5' end and a 4-((4'-dimethylaminophenylazo)benzoic acid (Dabcyl) quencher unit attached to the 3' end. The hairpin DNA for immobilization on gold electrodes featured a thiol modifier, 6-S, attached to the 5' end and a 6-FAM unit tagged on the 3' end. A biotin unit was conjugated to the 3' end of the reporter probe (rpDNA). The sequences of all oligonucleotides are provided in Table 1.

Reagents used for the genosensor

The buffers used in this study were (i) immobilization buffer (IB): 10 mM phosphate, 170 mM NaCl, 1 mM MgCl₂ (pH 7.6); (ii) hybridization buffer (HB): 2.5% bovine serum albumin (BSA) in IB; and (iii) binding buffer (BB): 10 mM phosphate, 1.25 M NaCl, 1 mM MgCl₂, 0.5% casein (pH 7.6). The substrate solution for horse radish peroxidase (HRP) contained 0.4 mM 3,3',5,5'-tetramethylbenzidine (TMB) and 0.8 mM H₂O₂ in 150 mM phosphate/citrate buffer (pH 5.0). The substrate solution for the glucose oxidase (GOx) and HRP cascade reaction contained 250 mM D- (+)-glucose and 0.4 mM TMB in phosphate/citrate buffer (pH 5.0). In addition, the activity of enzyme conjugates [i.e., HRP-conjugated FITC-antibody (z-FITC-HRP) and avidin-conjugated glucose oxidase (Av-GOX)] was further investigated using the Amplex Red assay as described in the ESI.†

Viral samples

Real genetic samples derived from influenza viruses, strains A/duck/Taiwan/DV30-2/2005 (H5N2) and A/chicken/Taiwan/CF16/2009 (H6N1), were kindly provided by the ID Public Health Lab (led by Professor Chwan-Chuen King) of the College of Public Health, National Taiwan University. The human influenza strains A/Hong Kong/JY2/1968 (H3N2) and A/Puerto Rico/8/1934 (H1N1) were provided by Professor/Dr Hung-Chih Yang of the College of Medicine, National Taiwan University.

Fabrication of the genosensor

Hairpin DNA (0.5 μM) was mixed with DTT (200 μM) in IB. Aliquots (6 μL) of this DNA/DTT solution were cast over each working electrode (2.5 mm² in diameter) of a 16-sensor Au array chip (Genefluorics, CA, USA), followed by incubation overnight at 4 °C in humidified surroundings. After washing with cold IB (4 °C) and drying under N₂, the modified Au chips were treated with 5 mM 6-mercapto-1-hexanol (MCH, 4 μL) in IB for 1 h at room temperature to avoid nonspecific binding of DNA to the Au surface of the chip. The washing and drying processes were repeated to provide sensors ready for functionalization. In addition, fabricated electrodes were subjected to characterization, and the surface coverage of the hairpin probe was estimated as described in the ESI (Fig. S9†).

Detection of multiple targets

Targeted DNA mixtures were mixed with the reporter probe in HB and incubated for 1 h. A aliquot of the DNA hybrid (4 μL) was then placed on the working electrode of the sensor and hybridized with the assembled hairpin DNA probe for 1 h. After rinsing with cold IB (4 °C) and drying under N₂, an aliquot (2 μL) of the solution containing the enzyme conjugates equilibrated in BB was added and incubated for 10 min at room temperature. The sensors were then washed and dried. To ensure reliable output signals throughout this study, the sensing chip was equipped with a tailor-designed adaptor system to facilitate multiple measurements. In each measurement, an aliquot (50 μL) of the substrate solution was cast to cover the three electrodes of the sensor, which was then left at ambient temperature for 10 min to expedite the enzymatic reaction. Chronoamperometric experiments were then conducted at −150 mV (a potential selected for efficient reduction of TMB, the cyclic voltammogram of TMB is shown in Fig. S1†) using a CHI 1030A Multi-Potentiotstat (CH Instruments, Austin, TX); the signaling current...
was sampled for 60 s. In the present study, the cathodic current is defined as “negative”; therefore, only the magnitude of the current signal (|i|) was used for comparisons.

**Laser scanning confocal microscopy for fluorescence detection on the Au surface of the chip**

Fluorescence measurements of the immobilized hairpin DNA tagged with 6-FAM (at the 3′ end) were performed using a Zeiss LSM 780 inverted microscope equipped with a liquid N2-cooled charge-coupled device (CCD). Prior to measurement, thiolated hairpin DNA was prepared and immobilized as described above. After self-assembly overnight at 4 °C in humidified surroundings, the hairpin-modified chip was washed with cold IB (4 °C) and dried in nitrogen. Subsequently, the modified Au chips were treated with 5 mM MCH in IB (4 °C) and dried under N2. Drops of aliquots of DNA targets were subsequently placed on the Au chip surface to hybridize with the modified probes for 1 h and then the samples were subjected to fluorescence measurement. The Au chip was covered by glass slips (thickness: 0.17 mm) and placed downward in the stage of the microscope; fluorescence images were captured upon exposure to excitation light (490 nm) that passed through a 60× oil objective. The optical signal was obtained at 520 nm to ensure that the fluorescence from 6-FAM was imaged by the CCD. The images obtained from the confocal microscope were analyzed using ZEN 2009 (Carl Zeiss) and Image J (National Institutes of Health, USA) software.

**Result and discussion**

We designed the hairpin DNA to consist of 43 nucleotides (43 nt) in length, labeled with a thiol group and a 6-FAM unit at its 5′ and 3′ ends, respectively. In its folded configuration, the hairpin DNA formed a stable (∆G = −9.41 kcal mol⁻¹; Tm = 61.4 °C; in 10 mM phosphate buffer containing 170 mM NaCl and 1 mM MgCl₂) 10-base-paired duplex stem (Fig. 1A, I) and 23-base single-stranded loop (Fig. 1A, II). The thiolated terminal unit enabled self-assembly of the hairpin DNA onto the surface of the Au electrode. In this hairpin DNA, the fragment 5′-CAG CTA TCA TGA TTG-3′, numbered 1–15 (Fig. 1A, III), from the 5′ end, hybridizes to a specific segment of the HA specific gene (gHA) in a complementary manner; likewise, a partial section of the loop (5′-GTT ACC CAA ATG-3′) numbered 22–33 (Fig. 1A, IV) hybridizes with its complementary sequence of the NA specific gene (gNA). For a scenario in which the targeted genes are absent (Fig. 1B, d), the hairpin DNA folds in its stable, native architecture (i.e., OFF state), thereby shielding the terminal 6-FAM unit from binding with the bulky HRP-tagged anti-FITC antibody (x-FITC-HRP). When gHA is present and hybridizes with the whole stem region as well as a proximal portion of the loop, the hairpin DNA unfolds as a result of strand displacement, making the 6-FAM unit accessible to conjugate specifically with x-FITC-HRP (Fig. 1B, c). Another scenario involves gNA hybridizing with a portion of the loop together with an additional 14-base dangling section (Fig. 1A, V), without destabilizing the stem region, thereby prohibiting the 6-FAM from interacting with x-FITC-HRP (Fig. 1B, b). In the presence of both gHA and gNA concurrently (Fig. 1B, a), two duplex regions form as the hairpin DNA is straightened from its folded configuration. In this state, the 6-FAM unit faces away from the electrode surface and becomes available for interaction with x-FITC-HRP. Subsequently, we introduced a biotin-labeled reporter probe (rpDNA), coded with a 13-base sequence complementary to the dangling section of gNA, to allow conjugation with glucose oxidase-tagged avidin (Av-GOx).

Upon addition of glucose and TMB(red) as substrates, GOx would catalyze the oxidation of glucose to yield H2O2, which could be further reduced by HRP, resulting in the production of

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence and modifications</th>
<th>Usage in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB</td>
<td>5′-/56-FAM/CCG CCA ATG ATT GAT CGT TAC CCA AAT AGT ATG CGG G/3Dab/-3′</td>
<td>Investigation of hairpin DNA in solution phase</td>
</tr>
<tr>
<td>TA</td>
<td>5′-ACC AAT CAT TGC CGG TC-3′</td>
<td>Target DNA to unzip the stem of MB</td>
</tr>
<tr>
<td>TB</td>
<td>5′-ACC TGG TGG GGA CAT TTG GGT AAC TC-3′</td>
<td>Target DNA to hybridize on the loop of MB</td>
</tr>
<tr>
<td>Hairpin DNA</td>
<td>5′-/5-ThioMC6-D/CAG CTA TCA TGA TTG ATC GAA GTT ACC CAA ATG ATG ATA GCT G/36-FAM/-3′</td>
<td>Specific probe for H5N2 identification</td>
</tr>
<tr>
<td>gH5</td>
<td>5′-CAA TCA TGA TAG CTG-3′</td>
<td>Specific sequence of H5 subtype</td>
</tr>
<tr>
<td>gN2</td>
<td>5′-TCT GCT GGT GGG GAC ATT TGG GTA ACA AG-3′</td>
<td>Specific sequence of N2 subtype</td>
</tr>
<tr>
<td>rpDNA</td>
<td>5′-CCC CAC CAG CAG ATT TTT/3BioTEG/-3′</td>
<td>Reporter DNA for recognition of N2 gene</td>
</tr>
<tr>
<td>H5F</td>
<td>5′-GCA GCG AGT TCC CTA GTA CTG-3′</td>
<td>The sense primer for asymmetric PCR of H5 gene</td>
</tr>
<tr>
<td>H5R</td>
<td>5′-CAT TGG AAC ACA TCC AGA AAG ATA GAC-3′</td>
<td>The antisense primer for asymmetric PCR of H5 gene</td>
</tr>
<tr>
<td>N2F</td>
<td>5′-CAA CTC AAT CCG GCT TTC-3′</td>
<td>The sense primer for asymmetric PCR of N2 gene</td>
</tr>
<tr>
<td>N2R</td>
<td>5′-CCC TTG TTA CCC AAA TGT C-3′</td>
<td>The antisense primer for asymmetric PCR of N2 gene</td>
</tr>
</tbody>
</table>

Table 1 Sequences of the DNA oligonucleotides employed in this study
Ultimately, the cathodic current of TMB(ox) is measured as the output signal. This novel concept of dual-target detection has three innate features: (i) gHA plays a prerequisite role in initiating the subtyping process—we obtained no output signal in the absence of gHA-mediated unzipping of the hairpin DNA (ON state); (ii) hybridization of gNA to the loop region does not rupture the stem-loop conformation—therefore, the output remained silent; (iii) the simultaneous presence of both gHA and gNA is essential to obtaining a positive output.

The success of such dual-target detection relies on a conformational switch of the hairpin DNA. Nevertheless, only limited efforts have been made previously to investigate the folding behavior of hairpin DNA upon hybridization with multiple complementary segments. We therefore commenced various approaches to address this issue. To validate our hypothesized mechanism, we examined the thermal denaturing profiles (Fig. S2†) of an MB (given with sequence similar to a conventional one and detailed in Table 1) through hybridizations with target A (TA) and/or target B (TB). Unambiguously TA was able to unzip MB, even at relatively low temperatures (e.g., <30 °C), yet TB did not. Moreover, in the circumstances with dual targets (TA + TB), there appeared an enhanced degree of hairpin opening, which inferred a straightened conformation of the MB loop upon the involvement of TB. Kinetic analysis was further carried out to gain insight into the mechanism of hairpin opening induced by the hybridization of dual targets (see the ESI† for details). Fig. 2 and S3† depict the fluorescence intensity, emitted from the 6-FAM labeled on 5′-end of MB, as a function of time, collected from different treatments with TA and/or TB. The pattern of the dynamic curve (as seen in Fig. S3†) acquired from the hybridization of MB with dual targets (TA + TB) was similar to that given by TA alone: the fluorescence intensity rose for the first 200 s, and reached a steady state (equilibrium) around 400 s. The rate constant for this unzipping reaction was estimated to be in the same order as that previously reported using...
conventional molecular beacons.⁴ We also noticed that the unzipping rate (correlated to the slope of the curves for the first 100 s, as shown in Fig. 2) of the molecular beacon treated with TA + TB (solid curves) was slightly higher than the one treated with TA only (dashed curves). This could be attributed to the fact that TB served as a helper toward TA-dominated hairpin opening by binding with the loop of MB, and rendered increased rigidity of the whole structure. It was also observed that treatment with TB alone resulted in little fluorescence emission, which coincided with the results obtained in the thermal denaturing profiles (as shown in Fig. S2†). To sum up, these observations endorsed the concept of our design, where the hairpin opening is predominated by TA. Particular attention was further paid to the effect of salt concentration and operating temperature on the unzipping kinetics in the first 100 s. A ~3-fold intensification of the unzipping rate constant was estimated while elevating the concentration of sodium chloride from 170 mM to 1 M (2.0 × 10⁴ M⁻¹ s⁻¹ vs. 5.8 × 10⁴ M⁻¹ s⁻¹, details shown in Table 2). As the unzipping of hairpin stem initiated by TA was based on a displacement reaction, our result was consistent with the evidence provided in previous studies,⁵,¹²,¹³ in which DNA strand displacement (exchange) has a strong positive dependence on the ambient salt concentration. Additionally, it was found that elevated temperature led to an increase of the rate constant (1.3 × 10⁴ and 5.8 × 10⁴ at 37 °C and 28 °C, respectively). According to the strand displacement theory proposed by Reynaldo et al.,²² hybridization of TA with MB was likely to proceed in two ways, the dissociative and sequential displacement pathways. Therefore, it could be expected that elevated temperature destabilizes the hairpin structure, resulting in the opening of the hairpin. The rate of the sequential displacement reaction, based on random walk theory,²⁵ was also positively correlated with elevated temperature.

Although the opening behavior and kinetics of DNA hairpins upon hybridization with dual targets have been investigated in a homogeneous phase, confining a DNA probe on a solid surface would presumably deteriorate its recognition capability to some extent. We therefore employed confocal microscopy to monitor the fluorescence signal, which was governed by the distance between the 6-FAM unit tagged on the MB and the surface of the thin-film Au electrode. When 6-FAM is excited by light from an Argon ion laser at 490 nm, its emission maximum appears near 520 nm; such light emission is quenched, however, by resonant intermolecular energy transfer from 6-FAM to Au, with an efficiency that decreases rapidly as the distance increases.²⁶ In the native configuration of hairpin DNA assembled on a Au electrode, the 6-FAM unit would reside in proximity to the Au surface, resulting in quenching of the fluorescence; in contrast, we would expect the 6-FAM unit to face away from the Au surface after any structural switching of the hairpin DNA and yield an observable fluorescence emission (Fig. 3A). In the simultaneous presence of both gHA and gNA (Fig. 3A(a)), the yielded fluorescence intensity was explicitly defined and higher than those in the other three scenarios (Fig. 3A(b–d)). This behavior reflects the fact that hybridization of the hairpin DNA with both targeted segments (gHA, gNA) led to the formation of a straightened duplex, thereby forcing the 6-FAM unit away from the Au surface. As expected, binding of gNA to the loop region led to minimal fluorescence (Fig. 3A(c)). Notably, the presence of gHA alone (Fig. 3A(b)) yielded slightly weaker fluorescence compared with that in Fig. 3A(c), but greater than that in Fig. 3A(b). Accordingly, we suspect that the flexible non-hybridized region (sequence numbered 16–43) of the hairpin DNA bent and coiled so that it was positioned close to the Au surface. This behavior is consistent with the fact that double-stranded DNAs comprising less than 150 base pairs are usually stiff (since the formation of sharp bends is not thermodynamically favored)²⁷–²⁹ whereas the persistence length of single-stranded DNA is approximately 1 nm (the length of ca. three nucleotides, in 1 M salt).²⁹ Fig. 3B presents quantitative analyses of the characteristic fluorescence intensities. The mean intensities displayed in the inset reveal that the signal acquired from the assembled hairpin DNA hybridized with both gHA and gNA simultaneously was approximately double that of the one coupled with gHA alone. These analytical results obtained using confocal microscopy correlate well with the tendencies observed in homogeneous solutions (Fig. S2†), confirming our hypothesized mechanism.

Because of the different signal transduction mechanism provided by chronoamperometry, the fluorescence quenching method described above may not fully reflect the actual statuses

Table 2 The opening rate constant of the DNA hairpin upon hybridization with target sequences under varied temperatures and salt concentrations

<table>
<thead>
<tr>
<th>DNA composition</th>
<th>Temperature (°C)</th>
<th>[NaCl], M</th>
<th>k₁ (M⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB, TA</td>
<td>28</td>
<td>0.17</td>
<td>3.1 × 10³</td>
</tr>
<tr>
<td>MB, TA, TB</td>
<td>28</td>
<td>0.17</td>
<td>5.8 × 10³</td>
</tr>
<tr>
<td>MB, TA</td>
<td>28</td>
<td>1</td>
<td>1.1 × 10⁴</td>
</tr>
<tr>
<td>MB, TA, TB</td>
<td>28</td>
<td>1</td>
<td>2.0 × 10⁴</td>
</tr>
<tr>
<td>MB, TA</td>
<td>37</td>
<td>0.17</td>
<td>9.6 × 10³</td>
</tr>
<tr>
<td>MB, TA, TB</td>
<td>37</td>
<td>0.17</td>
<td>1.3 × 10⁴</td>
</tr>
</tbody>
</table>

Fig. 2 Fluorescence restoration of MB upon hybridization with TA (dashed curves) or both TA and TB (solid curves) carried out under a medium salt concentration (170 mM NaCl) at 28 °C (black) and 37 °C (orange), as well as under a high salt concentration (1 M NaCl) at 28 °C (green).
of the interactions between the hairpin DNA and gHA. Therefore, we tested an HRP-amplified amperometric strategy to further identify the conformation of the hairpin DNA–gHA construct and its characteristic effect on the electrochemical signal (Fig. 3C). Upon an addition of 0.4 mM TMB and 0.8 mM \( \text{H}_2\text{O}_2 \) in 150 mM phosphate/citrate buffer (pH 5.0), we compared the output currents acquired at an applied potential of \(-150 \text{ mV}\) from solutions containing gHA and gNA. As indicated in Fig. 3C, the signal gain was elevated by approximately 2.5-fold when the system contained both targeted fragments together. By comparing this difference with that derived from fluorescence restoration on the surface (1.9 fold, Fig. 3B), it could be concluded that, in addition to the linearity and rigidity of the duplex DNA hybrid, the accessibility of the affinity tag also played an important role in determining the dynamic behavior of the various tested combinations. The current (310 nA) recorded in the sample containing only gHA was significantly lower than that of the sample containing both gHA and gNA (740 nA).


Notably, we have found that gNA, in the absence of gHA, potentially contributed to a “false positive” output since its hybridization to the loop occasionally destabilized the stem region. The probability of such an event is higher for a greater number of hybridized base pairs (i.e., IV in Fig. 1A) between gN2 and the loop of the hairpin DNA (Fig. S4†). The fluorescence intensity generated upon treatment with 15 nt gNA was substantially higher (2.8-fold) than that in the presence of 12 nt gNA. These results derived from confocal microscopic analyses related favorably with theoretical predictions of the thermodynamic tendencies toward hybridization.\(^{31-33}\) The simulated net free energy changes for the interactions of hairpin DNA with complementary targets in solution through 12- and 15-bp hybridization are \(-0.47\) and \(-1.17 \text{ kcal mol}^{-1}\), respectively, and no hairpin–target hybrid was expected to occur in the ensemble consisting of the probe and the 12 nt complementary sequence (Fig. S5†). Hence, we infer that 15-bp hybridization enhanced the probability of disrupting the hairpin structure, whereas the 12 nt complementary target has little inclination to form a rigid duplex with the folded hairpin. Furthermore, the difference in the signals between the “negative” and “positive” scenarios became less significant when the hybridization involved a greater number of base pairs (Fig. S6†)—a disadvantageous feature in the quest for high-fidelity viral subtyping. Thus, we conclude that an adequate number of base pairs and sequence specificity must both be taken into consideration to drive conformational switching of hairpin DNA, while avoiding excessive complementary pairs on the loop.

In light of the unpredictable orientation of the signaling tags (i.e., 6-FAM and biotin in current study) on the probe–target hybrids, we suspected that recognition of \( \alpha\text{-FITC-HRP} \) and Av-GOx by their counterparts might be challenging because of steric hindrance, thereby resulting in inadequate binding ratios and poor enzymatic amplification. Accordingly, we investigated the effect of enzyme conjugation at variable ratios to determine the optimal stoichiometry for the reaction (Fig. 4A). Because the output signal obtained in the presence of gNA alone could potentially lead to a false positive, we seek to maximize the difference in the output signal that could distinguish the positive case (gHA+, gNA+) from the scenario featuring only gNA (gHA−, gNA−). Interestingly, the current ratio for the positive and negative cases increased from 1.8 to 5.0 upon increasing the [Av-GOx]/[\( \alpha\text{-FITC-HRP} \)] concentration ratio from 1 to 25, reflecting the fact that undesired destabilization of the hairpin stem induced by gNA alone could be mitigated effectively by lowering the concentration of \( \alpha\text{-FITC-HRP} \). This phenomenon again underscores the significance of optimizing the density of hairpin DNA for maximum accessibility to both \( \alpha\text{-FITC-HRP} \) and Av-GOx (i.e., minimizing steric hindrance for enhanced recognition). It is reasonable to hypothesize that substantial retardation occurred in the pathway coupling the GOx and HRP enzymatic reactions (taking the current displayed in Fig. 3C into account). Nevertheless, an excessive load of HRP on the assembled system caused either undesired nonspecific binding on the electrode surface or unwanted interactions with the...
unshielded 6-FAM unit, thereby increasing the noise level in the presence of gNA alone (Fig. 4A, light gray bars).

To evaluate the viability and performance of the designed electrochemical genosensor based on the hairpin DNA-functionalized Au electrode, we tested synthetic oligonucleotides mimicking H5N2-specific genes in proof-of-concept experiments, acquiring amperometric sensing curves from the four possible combinations (Fig. 5A). The scenario featuring both gH5 and gN2 (gH5+, gN2+; red curve) yielded a current output of 25.8 nA after 60 s, substantially greater than the values obtained (ca. 4 nA) for the other three combinations; this dynamic difference was the largest we could obtain between the “positive” and “negative” groups. These findings suggest that the system was capable of unambiguous identification of the H5N2 virus using the current-sensing platform. Furthermore, we tested the responses of the genosensor toward variable concentrations of targeted genes. Fig. 4B reveals that the output signal from the group containing both gH5 and gN2 (filled circles) was dose-dependent; it was, however, independent of the concentration at which the (gH5+, gN2+) scenario was distinguishable from the negative combinations was approximately 50 nM.

We further tested the applicability of our developed genosensor toward genuine species from viral particles (A/duck/Taiwan/DV30-2/2005 (H5N2), inoculated in 9–11 day-old embryonated chicken eggs). Prior to testing our system, the viral RNA genome was reverse-transcribed to cDNA and then subjected to asymmetric polymerase chain reaction (aPCR) to produce single-stranded DNA (ssDNA) (see detailed preparation and characterization of amplicons in Fig. S7†). Fig. S8† displays the amperometric curves recorded from the unpurified amplicons; its inset reveals that the output currents for the three negative scenarios (curves a–c) were all less than 5 nA and were readily distinguishable from the “positive” case (coexistence of gH5 and gN2, curve d). Although this result verifies that our developed genosensor could also function successfully in the presence of amplicons, the dynamic differences between the (gH5+, gN2+) case and all of the negative combinations were lower than those for the synthetic probes in Fig. 5A. Furthermore, because the amplicon possessed two overhang sections (22 and 27 nt) next to the gH5-specific sequence, the overhanging 27 nt segment at the 3’ end may impede the initiation of strand displacement and subsequent stem unzipping due to steric hindrance.

We examined the specificity of the H5N2 genosensor by applying it to detection of the avian H6N1 strain (A/chicken/Taiwan/CF16/2009) and two human influenza strains, H3N2 (A/Hong Kong/JY2/1968) and H1N1 (A/Puerto Rico/8/1934). Gratifyingly, the H5N2 genosensor exhibited high specificity (Fig. 5B); as anticipated, samples containing amplicons templated from the cDNA of the H6N1, H1N1, and H3N2 strains resulted in low current signals (≤3 ± 0.5 nA). This result confirms the accuracy, specificity, and integrity of our gene screening process (for the detailed sequence selection procedures, see the ESI†).

Our system has been successfully demonstrated to implement accurate subtyping of influenza viruses using single hairpin DNA. In the present study, attention was also paid to the estimation of the hairpin density on the sensing surface and the geometry of the hairpin–targets complex. The optimal
fabrication process (i.e. co-immobilization of hairpin DNA, DTT and alkyl thiol) we employed herein was found to efficiently resist nonspecific binding of enzyme conjugates resulting in an improved signal-to-noise ratio. However, this approach led to a decreased density of probe DNA on the sensing surface (0.5 pmol cm$^{-2}$ vs. 3-4 pmol cm$^{-2}$ as reported by Steichen et al.,$^{24}$ Fig. S9†). Further improvement in assay performance will be pursued in future study. The assay presented herein circumvented the limitations of rapid influenza diagnostic tests (RIDT),$^{15-17}$ which suffer from insufficient sensitivity and specificity, and can only qualitatively identify the nucleoprotein. In addition, the present design potentially prevails over another category of influenza diagnostics,$^{18-20}$ which are based on the real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR) technique. Such RT-PCR assays often overlook the biological significance of neuraminidase and focus on identification of the hemagglutinin gene only. Our work has established a rapid subtyping system for influenza viruses (both hemagglutinin and neuraminidase can be recognized simultaneously) and provided a decisive interpretation based on molecular computation. This newly developed sensing platform can be used as an alternative strategy in rapid screening of specific influenza strains for surveillance of global transmission, especially during a pandemic season.

**Conclusion**

This work provides, to the best of our knowledge, the first example of the leveraging of a single hairpin-structured DNA probe for the concurrent analyses of dual-target genes with a digital interpretation via cooperative enzymatic processing. Through the tailored design of hairpin DNA with desirable thermodynamic properties, we successfully controlled the structural switching of the hairpin DNA upon hybridization with gH5 and gN2. Equilibrium and kinetics studies on the interaction of hairpin DNA and its complementary counterparts further revealed that the strand displacement reaction in the stem duplex played a pivotal role to set off the disruption of the hairpin structure. It was also found in our study that an elevated salt concentration and operating temperature were favourable for initiating the destabilization of the folded hairpin. Taking advantage of the specificity and selectivity of this unique detection scheme, the high-fidelity subtyping of a target influenza virus is offered, unambiguously discriminating between H5N2 and other prevalent viruses (e.g., H1N1, H3N2 and H6N1) in unpurified viral amplicons. Upon appropriate utilization of our concept, this screening platform technology can be readily extended toward rapid screening of other influenza viruses, thus holding considerable promise for use as a sophisticated and rapid tool for molecular diagnosis and surveillance.

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**References**


