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Electrochemical Assay to Detect Influenza Viruses and Measure Drug Susceptibility**

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Dedicated to Professor Malcolm Chisholm on the occasion of his 69th birthday.

Abstract: *An electrochemical assay has been designed to rapidly diagnose influenza viruses. Exposure of a glucose-bearing substrate to influenza viruses or its enzyme, neuraminidase (NA), releases glucose, which was detected amperometrically. Two methods were used to detect released glucose. First, we used a standard glucose blood meter to detect two viral NAs and three influenza strains. We also demonstrated drug susceptibility of two antivirals, Zanamivir and Oseltamivir, using the assay. Finally, we used disposable test strips to detect nineteen H1N1 and H3N2 influenza strains using this assay in one hour. The limit and range of detection of this first generation assay is 10^2 and 10^2–10^8 plaque forming units (pfu), respectively. Current user-friendly glucose meters can be repurposed to detect influenza viruses.*

Influenza virus is a highly contagious virus. The US Centers for Disease Control (CDC) estimate that seasonal influenza is responsible for over 200000 hospitalizations and 30000 deaths in the US.[1] Pandemics, although infrequent, can cause significant devastation. In 2009, the H1N1 "swine flu" outbreak infected people in more than 200 countries within weeks of the initial outbreak.[2] Measuring drug susceptibility is equally important since antivirals are most efficacious when administered before onset of infection[3] and the virus has a high mutation rate, approximately 1.5×10^{-5} mutations per nucleotide per infectious cycle.[4]

Diagnostics for influenza viruses include nucleic acid amplification tests (NAAT) and antibody and fluorescence tests. NAATs such as the Xpert Flu tests are highly selective and sensitive but require sophisticated instruments; the correct primers and can be cost prohibitive for use in primary care facilities, resource poor areas, and homes.[5] Chemiluminescence-based tests such as the Amplex Red Neuraminidase kit require a laboratory setting.[6] Antibody-based tests can be variable as it is highly dependent on antibody purification, bioconjugation, and the quality controls established by the manufacturer. Indeed, the CDC does not recommend the use of these tests unless it is supported by more accurate techniques.[7] Colorimetric tests such as the ZstatFlu test provide a visual readout, but as is the case with several optical tests, the readout is prone to human error and is not sensitive.[8] All these tests are expensive and none of them can measure drug susceptibility rapidly in a point-of-care setting. The lack of good rapid diagnostic tests leads to asymptomatic treatment and overuse of drugs, which increases drug resistance. Indeed, reports of resistance to Oseltamivir, the preferred antiviral for influenza, have been reported.[9]

Influenza has two major surface glycoproteins, hemagluttinin (HA) and neuraminidase (NA). Hemagluttinnin is implicated in viral entry and neuraminidase is the enzyme that cleaves *N*-acetyl neuraminic acid (sialic acid) from the surface of host cells to release viral progeny.[10] There are approximately 50–100 copies of NA on the viral surface as determined by immunogold labeling and cryoelectron tomography.[11] Since NA is present as a tetramer, there are approximately 200–400 individual units capable of cleaving sialic acids, which makes it a suitable target for biosensing applications.

Our strategy was to develop and expose substrates that would release glucose upon action of NA (Figure 1a). The released glucose can be measured amperometrically. A similar approach has been used to detect other enzymes, for example β-galactosidase[12] and α-amylase.[13] Herein, we demonstrate detection of viral NA and nineteen unique strains of influenza and demonstrate drug susceptibility of the two antivirals, Zanamivir and Oseltamivir (Figure 1b). These results were validated using rRT–PCR (real-time reverse transcription–polymerase chain reaction) and plaque assays. We choose to measure glucose concentration after 1 hour of incubation using disposable test strips, however, a continuous measurement system can also be designed.

We synthesized a sialic acid derivative (SG1) where sialic acid is attached to glucose at the 6-position (Scheme 1). Briefly, benzyl 2,3,4-tri-*O*-benzyl-α/β-D-glucopyranoside (**1α,β**) was synthesized using a modified procedure and reacted with the known *N*-acetyl-5-*N*,4-*O*-carbonyl-protected thiosialoside donor (**2**)[14] to yield **3α,β**. Exclusive α sialoside was obtained, which was confirmed by NMR spectrosco-

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Supporting information for this article (including the synthesis of SG1, characterization data of intermediates and the final compound, and details of the assays) is available on the WWW under http://dx.doi.org/10.1002/anie.201412164.

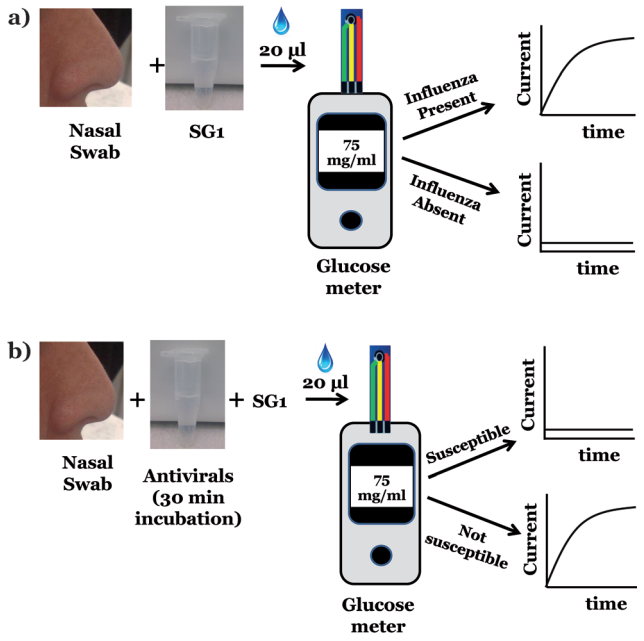
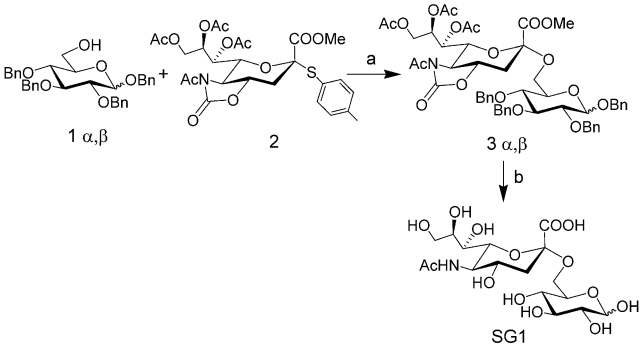


Figure 1. Detection of influenza virus using SG1. a) Workflow and scheme for electrochemical detection of influenza virus. b) Measurement of drug susceptibility. c) Influenza NA cleaves SG1 to release glucose.



Scheme 1. Synthesis of SG1. Reagents and conditions: a) TfOH, NIS, CH_2Cl_2, −60 °C, 2 h, 75 %; b) i) NaOMe, MeOH, RT, 30 min; ii) $Pd(OH)_2/C/H_2$, EtOH, RT, 12 h; iii) 0.05 N NaOH in H_2O, RT, 4 h, 93 % yield over three steps. TfOH = trifluoromethanesulfonic acid. NIS = *N*-iodosuccinimide.

py.[14a,15] Next, a three-step procedure was performed. First, Zemplén deacetylation conditions were used to remove the acetates and regioselectively open the oxazolidinone ring to obtain the *N*-acetamido group.[21] This was followed by hydrogenation to remove the benzyl groups and the resulting product was saponified to produce SG1 in excellent yield. To measure glucose, we developed a three-electrode electrochemical cell comprising a reference, working and counter electrode and used this electrochemical cell to develop a standard curve[16] (see Figure S1 in the Supporting Information).

SG1 (0.5 mM) was dissolved in PBS buffer and tested for the presence of glucose. In the absence of enzymes, there is no glucose released (Figure 2 a; sample N, negative control, no NA or virus added). Membrane-free influenza viral NA from two different strains (N1 from H5N1 A/Anhui/1/2005 and N2 from A/Babol/36/2005) was incubated for 2 h at room temperature with SG1. The sample was analyzed for the presence of glucose directly without further sample preparation. Glucose was released as determined by the current (*i*) measured amperometrically, which indicated that complete cleavage of SG1 had occurred (Figure 2 a, samples A, B). The positive control (Figure 2 a, sample P) was glucose as the only analyte in PBS buffer. Next, we tested three influenza strains, H1N1 A/Brisbane/59/2007, H3N2 A/Aichi/2/1968, and H3N2 A/HongKong/8/68, which were quantified by plaque assays and rRT-PCR (Figure S2). Introduction of UV-inactivated virus (100 µL) to SG1 and incubation for 2 h

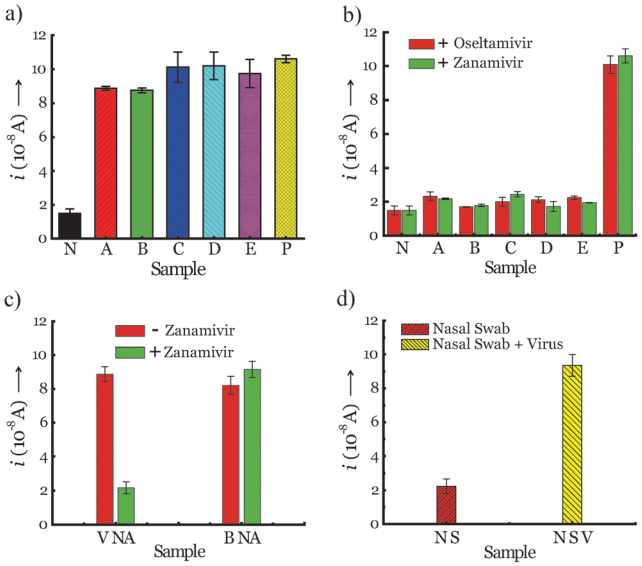


Figure 2. a) Detection of influenza virus or viral NA. SG1 (0.5 mM) was incubated with membrane-free soluble N1 NA (sample A, strain H5N1 A/Anhui/1/2005) or N2 NA (sample B, strain H3N2 A/Babol/36/2005) or three different UV-inactivated influenza strains, H3N2 A/Aichi/2/1968 (sample C), H1N1 A/Brisbane/59/2007 (sample D), or H3N2 A/HongKong/8/68 (sample E) for two hours. The negative control where no virus or NA was added (sample N) did not show any noticeable current and the positive control (sample P) was D-glucose at 0.5 mM. b) Drug susceptibility studies. 10 ng of Zanamivir or Oseltamivir (Carbosynth, USA, San Diego, CA) were premixed with the strains (as detailed in (a)) for 30 min at RT before addition of SG1. c) Studies with bacterial NA (BNA). BNA cleaves SG1 to release glucose, however, Zanamivir does not inhibit BNA and glucose is released when BNA was premixed with Zanamivir and incubated with SG1. d) Studies with human samples. NS denotes nasal swab only, and shows that no glucose is present. NSV denotes a nasal swab spiked with 10^5 pfu of H1N1 A/Brisbane/59/2007 and added to SG1. The positive signal indicates there are no matrix effects. In (a–d), the y axis shows current (*i*) in amperes measured after 100 s using an amperometric *i*–*t* curve at a working potential of 0.00 V. All experiments were performed in triplicate independently on different days.

resulted in the release of glucose (Figure 2a, samples C, D, and E). The cleavage was confirmed by mass spectral analysis. The mass spectra of the control where there is no virus or NA revealed a peak at m/z 494.1497 (M+Na, positive ion) corresponding to uncleaved SG1. A new peak emerges at m/z 181.0711 (M+1, positive ion) corresponding to glucose when virus or NA is added.

To test for drug susceptibility, we premixed FDA-approved NA inhibitors Zanamivir or Oseltamivir with the virus or NA for 30 min before introducing SG1. If the strains are not resistant to the antivirals, they are expected to block the action of NA and a signal for glucose should not be detected. As seen in Figure 2b, the three strains and NA are completely inhibited by the antivirals. Thus, we determined drug susceptibility of these strains within a sample results in a test time of less than 2 hours. This is highly significant because drug susceptibility for influenza viruses using genotypic and/or phenotypic methods requires sophisticated instruments, trained personnel, and several hours to complete.[17]

To differentiate between bacteria/human NA that are also expected to cleave SG1, we exploited known differences in the binding pocket of NAs. Bacteria/human NA have a smaller binding pocket and cannot accommodate larger groups at the 4-position of sialic acid, as confirmed by X-ray structures and functional assays with Zanamivir and Oseltamivir.[10] We, and others, have exploited this feature to develop inhibitors[10,18] and substrates[19] that are highly specific for influenza NA. As expected, bacterial NA (BNA) from *Clostridium perfringens* and viral NA cleave SG1 (Figure 2c, sample BNA). However, when Zanamivir was premixed with both NAs, only BNA cleaves SG1 and not the viral NA (VNA) because the antiviral is specific for VNA (Figure 2c). Therefore, we can distinguish between BNA and viral NA using the antivirals. An alternate approach is to introduce larger groups at the 4-position of sialic acid to make the substrate highly specific for viral NA instead of using Zanamivir; the syntheses of these next-generation substrates are ongoing.

Next, we were interested in determining if nasal or throat swabs, the standard source of clinical samples for influenza viruses, have a background level of glucose. We found glucose is absent in the nose or throat by testing samples obtained from healthy human volunteers. Glucose is released when the samples are spiked with known concentrations of the virus (Figure 2d). Thus, this assay could be used to test influenza in nasal and/or throat swabs.

To improve assay performance, we used disposable printed electrodes (CH instruments, Austin, Texas) for the next set of experiments (Figure S3). This experimental setup requires only 20 μL of solution, similar to commercial disposable test strips used in blood glucose meters. We obtained 19 H1N1 and H3N2 influenza strains that spanned eighty years, from 1933 to 2011, including strains from the most recent 2009 pandemic. All strains were detected in one hour, which demonstrates broad specificity. These results were validated using rRT–PCR and plaque assays (Table 1). Some strains cleaved SG1 slowly; we corroborated the results by measuring the slow release using a fluorescent substrate, 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA; Figure S4). As expected, the rate of cleavage of A/PuertoRico/8/1934 (H1N1), A/California/07/2009 (H1N1), and A/New Caledonia/20/1999 (H1N1) strains are slower than the A/Beijing/262/1995 (H1N1) strain. We note that, despite variations in printed electrodes from different manufacturer or different batches from the same manufacturer, the assay detects all strains. We also determined the analytical sensitivity using one of the strains using this rudimentary setup (Figure S3). The limit of detection and range is 10^2 and 10^2–10^8 pfu, respectively. As multiple studies have reported that patients ($n > 50$) suffering from influenza typically harbor 10^3–10^8 pfu mL^{-1} in the nose/throat,[20] this assay could be useful for rapid detection in a primary-care setting.

Table 1: Electrochemical detection of 19 influenza strains and validation with rRT–PCR and cell-culture plaque assays.

Influenza strains	Plaque Assay[a]	C_t[b]	i [10^{-8} A][c]
No virus + SG1	n/a	n/a	3.6 ± 2.2
No glucose	n/a	n/a	3.4 ± 1.4
β-Galactosidase	n/a	n/a	4.2 ± 1.9
α-Mannosidase	n/a	n/a	4.7 ± 1.6
Glucose (1 mM)	n/a	n/a	124.9 ± 2.3
A/Wilson-Smith/1933 (H1N1)	4.2×10^6	18	99.5 ± 4.4
A/PuertoRico/8/1934 (H1N1)	1.4×10^4	15	13.4 ± 2.0
A/HongKong/8/1968 (H3N2)	1.5×10^5	22	64.4 ± 5.2
A/Aichi/2/1968 (H3N2)	1.0×10^5	15	31.3 ± 5.3
A/Beijing/262/1995 (H1N1)	3.5×10^5	21	118.1 ± 11.6
A/Nanchang/933/1995 (H3N2)	7.0×10^5	18	15.1 ± 2.8
A/Sydney/5/1997 (H3N2)	8.0×10^3	29	115.7 ± 4.3
A/NewCaledonia/20/1999 (H1N1)	4.4×10^7	12	17.7 ± 2.6
A/SolomonIslands/3/2006 (H1N1)	1.1×10^9	11	79.7 ± 5.0
A/Uruguay/716/2007 (H3N2)	1.2×10^7	20	132.4 ± 6.3
A/Brisbane/59/2007 (H1N1)	2.5×10^7	12	106.2 ± 5.1
A/Brisbane/10/2007 (H3N2)	2.2×10^6	21	78.6 ± 4.9
A/California/07/2009 (H1N1)	3.6×10^6	13	13.4 ± 1.5
A/New York/18/2009 (H1N1)	3.5×10^5	17	74.4 ± 5.6
A/San Diego/1/2009 (H1N1)	1.2×10^5	19	108.5 ± 12.0
A/Wisconsin/629-D02452/2009 (H1N1)	6.0×10^4	13	98.9 ± 5.7
A/Wisconsin/15/2009 (H3N2)	5.0×10^3	26	107.8 ± 5.9
A/Brownsville/31H/2009 (H1N1)	4.0×10^3	22	91.7 ± 9.7
A/Victoria/361/2011 (H3N2)	7.0×10^6	26	94.5 ± 2.3

[a] Reported in pfu mL^{-1}. [b] rRT–PCR was performed using 100 μL of virus to measure C_t, that is, the cycle number at which fluorescence is above the threshold (background). C_t is inversely proportional to the number of amplicons. [c] Measured by electrochemical assay. 100 μL of virus was mixed with 100 μL of PBS buffer with SG1 for 1 hour at 37°C. Glucose concentration was determined using 20 μL of this solution.

To summarize, we have developed an electrochemical assay that releases glucose upon introduction of influenza viruses. We successfully detected 19 influenza strains. The assay can be used to measure drug susceptibility rapidly, a significant advantage over current genotypic and phenotypic methods that take time, resources, and a laboratory environment.[17] The assay can be integrated into current glucose meters by repurposing the instruments to test nasal or throat swabs for influenza. As glucose meters with disposable test strips are user friendly, ubiquitous, and inexpensive, this

method has great potential to improve clinical decisions and minimize disease burden. Further optimization of the lead compound, developing conditions to maximize the enzyme-cleavage rate, constructing disposable strips with better quality control, and integrating the assay into existing glucose meters is ongoing.

Keywords: biosensors · carbohydrates · electrochemistry · glycosides · influenza virus

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