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Label-free electrochemical detection of neuraminidase activity: A facile whole blood diagnostic probe for infectious diseases

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ABSTRACT

Neuraminidase has been found in many pathogenic bacteria and influenza viruses, and it plays important roles in the spreading of pathogens; thus, it is an important biomarker for infectious diseases. We developed a simple, rapid, sensitive, and label-free electrochemical assay to detect neuraminidase activity, by employing a newly synthesized latent probe AP-Neu5Ac that acts as a substrate for neuraminidase. The release of *p*-aminophenol, resulting from the neuraminidase-catalyzed hydrolysis of AP-Neu5Ac, was monitored using linear sweep voltammetry. Our assay was highly sensitive with a limit of detection of 5.6 ng mL^{-1}, which is comparable to those of other currently available sensitive methods such as ELISA and luminescence-based assays. The AP-Neu5Ac probe is a non-protein reagent, and was shown to be robust, highly stable at room temperature and durable. Analysis of whole blood samples containing neuraminidase revealed the reproducibility of our method in colored and cloudy samples. Our sensor of neuraminidase activity was shown to be superior to those used in previously reported methods The significance of these results showed the potential application of this method for monitoring biological status or progress in the recovery from infectious diseases caused by pathogenic neuraminidase-producing bacteria or influenza virus.

1. Introduction

Neuraminidase (or sialidase), which cleaves terminal sialic acids of glycoproteins, glycolipids, and oligosaccharides, has been found in many pathogenic bacteria and influenza viruses [1]. Viral neuraminidase plays important roles in the spreading of viral particles, as this enzyme facilitates release of the newly formed virus from the surface of the infected host cell to attack new target cell [2]. In addition, bacterial neuraminidase has been implicated in the virulence of several bacterial pathogens. Neuraminidase produced by bacteria has been shown to enhance the pathogenesis of disease through synergistic effects with other bacterial factors; for example, *Vibrio cholerae* neuraminidase enhances the activity of cholera toxin [3], *Pseudomonas aeruginosa* neuraminidase has an important role in biofilm formation, which contributes to initial colonization of the respiratory tract [4], and NanA and NanB neuraminidase of *Streptococcus pneumoniae* are essential for respiratory tract infection and sepsis [5]. Neuraminidase is an important biomarker for infectious diseases; thus developing a rapid, simple, highly sensitive diagnostic tool to detect neuraminidase is essential toward infectious diseases prevention and control [1].

The most common methods that have been used to detect neuraminidase, such as enzyme-linked immunosorbent assay (ELISA) [6], reverse transcription polymerase chain reaction (RT-PCR) [7] and liquid chromatography-tandem mass spectrometry (LC–MS/MS) [8], are laborious, bulky, expensive, and time-consuming; these methods also need to be performed by highly skilled operators, and usually rely on the use of antibodies or other biomolecules or other reagents. Some of these reagents have a short shelf life and lack thermal stability, which may undermine their application in resource-limited settings [9,10]. Circumventing these limitations by developing technically simple, rapid, and effective methods for monitoring neuraminidase activity is necessary for realizing early diagnoses of neuraminidase-based diseases. Simple colorimetric tests such as the ZstatFlu test produce a visual readout [11], but the readout of optical tests is prone to human

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error and is not sensitive. Recently, luminescence-based assays were demonstrated to detect neuraminidase activity by employing chemical probes as substrates. The luminescence intensity of the enzymatic reaction product was recorded as the assay results and this method showed high sensitivity and specificity towards neuraminidase [12,13]. The disadvantages of luminescence-based assays are that they require equipment that is not so easy to miniaturize and integrate into low-cost portable devices and they give misleading results when performed in colored, autofluorescent, or cloudy samples (Table S1) [14]. Moreover, commercially available luminescence-based diagnostic kit such as Amplex Red Neuraminidase requires the use of a protein as a reagent, which may reduce the stability and the usability after long period time of storage.

Over the past few years, electrochemical devices have garnered considerable attention in biosensor development. An electrochemical biosensor is a molecular sensing device that couples a biological recognition event with an electrode transducer to produce a useful electrical signal [15]. The high sensitivity levels of electrochemical transducers together with their amenability to being miniaturized or microfabricated have made electrochemical sensors attractive and have offered a simple, low-cost, accurate, rapid and sensitive platform for early diagnosis of various diseases [16]. To date, however, very few reports have been published regarding the electrochemical detection of neuraminidase. Relatively recently, an electrochemical detection of neuraminidase based on its interaction with zanamivir using gold nanoparticle-modified boron-doped diamond electrodes was reported [17]. Unfortunately, the fabrication of modified electrodes is laborious, and the fabricated electrodes show poor selectivity, stability issues, and nanoparticle aggregation, and require expensive materials [18,19]. Another report demonstrated the use of an electrochemical biosensor to detect neuraminidase activity, specifically based on monitoring the interactions between neuraminidase and fetuin A, followed by the addition of peanut agglutinin (PNA) lectin to monitor the cleavage of fetuin A by neuraminidase [20]. However, the use of a protein as a reagent might diminish the stability of the sensor when stored over a long period of time. In particular, improper immobilization of protein to a solid electrode surface may cause the protein to denature and hence lead to a very large loss in shelf life and overall sensing performance.

Developing and exploring electrochemical latent redox probes is an ongoing research interest of our research group [21]. Such latent chemical probes constitute a class of highly selective and stable probes equipped with unique triggering groups and masked electroactive reporters. These probes selectively interact with the analyte of interest *via* user-designated chemical or biochemical events to unmask their inherent reporter signal [22]. These latent probes are highly selective and specific, suffer little from interference and background noise, and are highly suitable for monitoring hydrolase enzyme activity [21,23]. We envision by combining the detection advantages of electrochemical devices with highly selective latent chemical probes may improve many of the limitations that current diagnostic tools encountered for sensing neuraminidase. In the current work, we exploited small-molecule derivatives of *N*-acetylneuraminic acid coupling with the electroactive species *p*-aminophenol to rapidly monitor neuraminidase activity in a simple sensing platform (Scheme 1).

We developed a facile synthesis and application of a latent electrochemical probe for a rapid, simple, inexpensive, highly sensitive, and label-free detection of neuraminidase activity. Our label-free method was developed based on the latent redox activity of *N*-acetyl-2-*O*-(4-aminophenyl)-α-neuraminic acid (AP-Neu5Ac), as a substrate for the analyte neuraminidase. The aim of this study was to evaluate the use of the AP-Neu5Ac probe for the electrochemical detection of neuraminidase activity and the applicability of this method in biological media. We used a simple preparation of AP-Neu5Ac, a one-step synthesis from the commercially available compound **1** (Scheme 1). To the best of our knowledge, this is the first report of a label-free electrochemical assay employing a latent electrochemical probe to directly detect neuraminidase activity. Our probe was shown to have a long shelf life, and reproducible results were obtained after the probe was stored for a long time. The developed sensor also yielded highly reproducible results in applications involving real samples, including colored and cloudy samples. The ability of the sensor to detect neuraminidase activity in colored and cloudy blood samples revealed the great potential of our sensor for the screening of infectious diseases caused by the presence of neuraminidase-producing bacteria or influenza virus in the bloodstream. The procedure used to operate the sensor is inexpensive and convenient, due to absence of electrode modification or immobilization of a reagent on the surface of the electrode. This promising approach has great potential applicability in clinical testing.

2. Experimental section

2.1. Chemical and instrumentation

All of the electrochemical measurements were carried out with a CH Instrument 612d workstation in a single compartment cell with three electrodes. Glassy carbon electrode (GCE) was used as the working electrode, while platinum wire and Ag|AgCl electrode were used as the counter electrode and the reference electrode, respectively. GCE surface was pre-cleaned by polishing it with 0.05 μm alumina slurry using a Buehler polishing kit, then washed with water and dried.

The synthesized compounds were characterized by using ^{1}H and ^{13}C nuclear magnetic resonance (NMR) on a Bruker Avance 300 or 500 MHz spectrometers. Mass spectra were obtained by a Finnigan TSQ 700 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) ion source. Optical rotations were obtained by a Jasco digital polarimeter. Neuraminidase from *Clostridium perfringens* and β-galactosidase (β-Gal) from *Aspergillus oryzae* were purchased from Sigma-Aldrich and zanamivir was supplied by AK Scientific. Saliva, urine, and nasal samples were collected from healthy human. A nasal swab samples were obtained with a dry sterile cotton tip and diluted in PBS buffer. Human blood samples were acquired from Taipei Medical University, Taiwan and the experimental protocols were approved by the institutional Ethic Committee. *N*-acetyl-2-*O*-(4-nitrophenyl)-α-neuraminic acid (compound **1**) was prepared from the commercially available *N*-acetylneuraminic acid, according to the literature procedure [24] or directly purchase from Sigma-Aldrich.

2.2. Synthesis of N-acetyl-2-O-(4-aminophenyl)-α-neuraminic acid (AP-Neu5Ac)

AP-Neu5Ac was synthesized by one-step hydrogenation reaction of compound **1**. The mixture of compound **1** (0.4 mmol) and 10% palladium on carbon (0.4 mmol) in 5 mL of H_2O/MeOH (1:4) were placed in high pressure reactor. The reactor was purged with H_2 three times to remove air, and the reaction mixture was stirred at room temperature under H_2 atmosphere (50 psi) for 15 h, after which the catalyst was filtered off and washed with methanol. The filtrate was evaporated to obtain the title compound AP-Neu5Ac (69%, 0.28 mmol). $[\alpha]_D^{20}$ +23.2 (*c* 0.1, CH_3OH). ^{1}H NMR (300 MHz, D_2O) δ 1.86 (t, 1H, J = 12.2 Hz, H-3 ax), 2.02 (s, 3H, NAc), 2.87 (dd, 1H, J = 12.5, 4.7 Hz, H-3 eq), 3.57-3.87 (m, 9H, H-4, 5, 6, 7, 8, 9, 9′, $-NH_2$), 6.88 (d, 2H, J = 8.7 Hz, Ar-H), 7.04 (d, 2H, J = 9 Hz, Ar-H). ^{13}C NMR (125 MHz, D_2O) δ 22.11, 40.69, 52.23, 62.63, 68.19, 71.98,