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a high sensitive peptide-based molecular beacon**

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Simple, rapid detection of influenza A (H1N1) viruses using a high sensitive peptide-based molecular beacon

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A peptide-based molecular beacon (PEP-MB) was prepared for the simple, rapid, and specific detection of H1N1 viruses using a fluorescence resonance energy transfer (FRET) system. PEP-MB exhibited minimal fluorescence in its “closed” hairpin structure. However, in the presence of H1N1 viruses, the specific recognition of the hemagglutinin (HA) protein of H1 strains by PEP-MB causes the beacon to assume an “open” structure that emits strong fluorescence. PEP-MB could detect H1N1 viruses within 15 min or even 5 min and can exhibit strong fluorescence even at low viral concentrations, with a detection limit of 4 copies.

Influenza viruses are a significant cause of morbidity and mortality worldwide and are therefore recognized as major threats to public health. All influenza pandemics in humans have been caused by influenza A viruses, which can also infect a wide range of animal species. In particular, among the several types of influenza A viruses classified based on 17 hemagglutinins (HAs) and 10 neuraminidases (NAs), the H1N1 subtype was first found in patients with febrile respiratory illness in 2009 (2009 H1N1) and has subsequently spread rapidly across the world by human-to-human transmission. This subtype causes “seasonal flu” in humans.^{1,2} The rapid detection of all types of influenza infections is important for the effective control and prevention of pandemics with appropriate antiviral therapy.³⁻¹⁰ The standard method of diagnosing influenza infection, which involves isolating viruses by culturing respiratory secretions, requires several days. A commercially available diagnostic kit based on antigen detection can be used to rapidly and simply detect influenza A viruses; however, this kit does not detect influenza B viruses and cannot distinguish among influenza A subtypes.¹¹⁻¹⁴ Traditional virus detection methods, such as real-time polymerase chain reaction (RT-PCR)/PCR and the branched-chain DNA (bDNA) test, have been proposed for the sensitive and specific detection of virus-derived nucleic acids, an approach that is more sensitive and specific than antigen detection.^{11, 15-22} However, these virus detection methods are not only time-consuming and expensive but also require trained personnel for sample preparation. In addition, other diagnostic procedures, such as enzyme immunoassay (EIA) and fluorescent antibody tests (FAT), require several hours to perform and suffer from poor sensitivity. Thus, more sensitive methods for the early and accurate detection of viral subtypes are

critically needed. As mentioned above, influenza viruses are characterized based on HA type (into categories such as H1N1 and H3N2). HA, which is coded by a genetic segment, is the most abundant surface antigen of the influenza virus and plays an important role in viral infection by specifically binding to glycan receptors of the host cell.²³⁻²⁶

In this study, we have developed a peptide-based molecular beacon (PEP-MB) that acts as a smart probe for the rapid, simple and sensitive detection of H1N1 viruses. PEP-MB included two complementary oligonucleotides in the stem region and a specific peptide sequence in the central loop region that targets a highly conserved region in the HA protein of H1 strains (HA1).^{24, 27-33} PEP-MB alone formed a hairpin structure that exhibited fluorescence quenching; however, the fluorescence of beacon molecules increased after these molecules specifically recognize the HA1 protein on H1N1 viruses (Fig. 1).

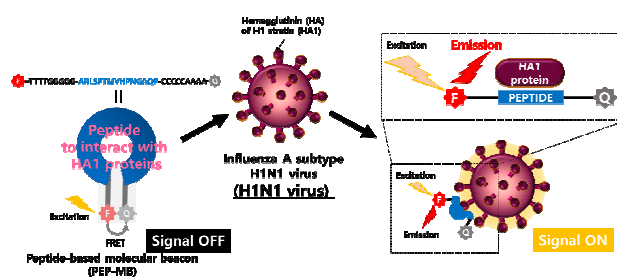


Fig. 1. Schematic illustration of a peptide-based molecular beacon (PEP-MB) for the simple and rapid detection of influenza A subtype H1N1 viruses (H1N1 viruses).

PEP-MB was prepared for specific H1N1 virus detection using a fluorescence resonance energy transfer (FRET) system in which a peptide that enabled the specific recognition of the HA1 protein was chemically conjugated with two oligonucleotides, an organic fluorophore (Cy3)-modified oligonucleotide (Oligo-Cy3) and a black hole quencher 2 (BHQ2)-modified oligonucleotide (Oligo-BHQ2); one oligonucleotide was placed on each side of the peptide, using sulfo-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-

carboxylate) (sulfo-SMCC) as a cross-linker (Fig. S1 and ESI†). The amine groups of the peptide were activated by maleimide groups; subsequently, the thiol groups of the oligonucleotides were conjugated with these amine groups.^{34, 35}

In particular, because the two oligonucleotides of PEP-MB consist of complementary sequences, PEP-MB formed a hairpin structure due to the base pairing of these oligonucleotides. The distance between the fluorophore donor (Cy3, maximal emission at 570 nm) and the fluorophore acceptor (BHQ2, absorption from 560-670 nm) was thus extremely small. The interaction between these oligonucleotides, which involved the excited Oligo-Cy3 fluorophore transferring its excitation energy to the BHQ2 group of Oligo-BHQ2, resulted in the strong quenching of fluorescence after annealing relative to before annealing (Fig. S2).³⁶⁻⁴⁷

In addition, the peptide sequence used in this study was known to have a high affinity for the HA proteins of H1 and H3 strains. However, PEP-MB, which was created by modifying both ends of this peptide with oligomers, was found to have higher affinity for H1N1 viruses (with a dissociation constant (K_d) of 4.9 nM) than for other strains (with K_d values of 326 nM, 289 nM, and 159 nM for H3N2, H5N2, and H6N5 viruses, respectively) (Fig. 2a).^{24, 48}

PEP-MB molecules were annealed to form hairpin loop structures and thereby reduce non-specific fluorescence signal. The quenching efficiency of PEP-MB was determined by annealing a fixed concentration of Oligo-Cy3 with increasing concentrations of Oligo-BHQ2. Fluorescence intensity were little difference overall before PEP-MB. However, their intensities were slightly increased over 4.0 nmol of Oligo-BHQ2 despite not annealing due to high concentration of quencher (Fig. 2b (i)). Comparisons of the fluorescence intensities before and after PEP-MB annealing ($\Delta F = F_{\text{before}} - F_{\text{after}}$) revealed that the maximal quenching efficiency was observed with 4 nmol of Oligo-BHQ2 (Fig. 2b (ii)). This value was chosen as the optimal condition for use in further experiments examining the specific detection of influenza A subtype H1N1 viruses (H1N1 viruses) by PEP-MB.

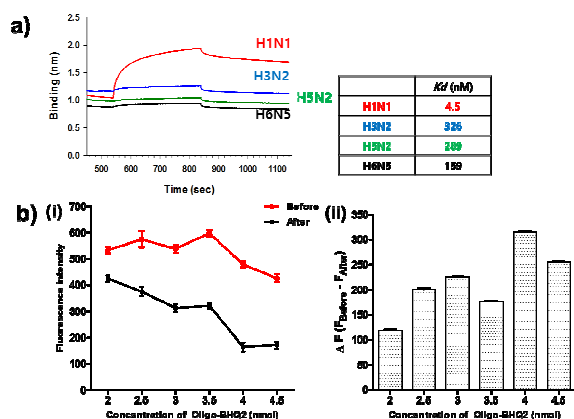


Fig. 2. (a) Binding affinities of PEP-MB for various viruses (specifically, H1N1, H3N2, H5N2 and H6N5 viruses). (b) Fluorescence intensity of PEP-MB molecules prepared by conjugating a fixed concentration of Oligo-Cy3 with increasing concentrations of Oligo-BHQ2 (2, 2.5, 3, 3.5, 4 and 4.5 nmol): (i) PEP-MB fluorescence before annealing (red) and after annealing (black) and (ii) changes in fluorescence intensity due to annealing ($\Delta F = F_{\text{Before}} - F_{\text{After}}$).

PEP-MB was designed for the rapid and simple detection of H1N1 virus; thus, minimal PEP-MB fluorescence emission was observed with the beacon in the “closed” hairpin structure in the absence of H1N1 viruses. However, strong fluorescence was emitted by beacons in the “open” structure, which was adopted in the presence of H1N1 viruses, because the distance between the fluorophore donor Cy3 and the quencher BHQ2 increased due to a spontaneous conformational change of peptide in the PEP-MB. Based on this phenomenon, we evaluated the specific detection ability of PEP-MB for H1N1 viruses relative to other viral subtypes (specifically, H3N2, H5N2 and H6N5 viruses). A control molecular beacon (Control MB) was designed with a 17-mer random peptide sequence that did not correspond to the target protein and with Oligo-Cy3 (donor) and Oligo-BHQ2 (acceptor) sequences identical to the sequences in PEP-MB.³⁰

The detection efficiency was measured by examining fluorescence intensities before and after virus treatment. All viruses obtained from the BioNano Health Guard Research Center (H-GUARD). The fluorescence of PEP-MB was largely quenched before virus treatment (non-treatment (NT)); however, this fluorescence changed after 30 min of virus treatment (with relative fluorescence intensities calculated as $\Delta F/F_{\text{NT}}$ (in %), where $\Delta F = F - F_{\text{NT}}$) (Fig. 3a). After the beacon was exposed to the H1N1 virus, fluorescence intensity was markedly increased by approximately 23-fold relative to the NT fluorescence (H1N1: 2275.9%) due to the binding of PEP-MB to the target HA1 protein. In comparison, PEP-MB fluorescence intensity was only slightly enhanced by incubation with other viruses (H3N2: 379.3%; H5N2: 17.2%; and H6N5: 220.7%) (Fig. 3b). The specific binding of the PEP-MB to the target viral HA1 protein induced a spontaneous conformational change in the beacon that promoted the opening of the hairpin structure and increased the distance between the Cy3 and BHQ2 groups. Control MB exhibited negligible fluorescence intensity enhancements in response to all tested viruses (Fig. 3b).

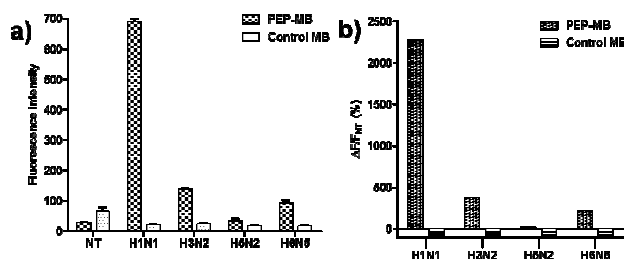


Fig. 3. Fluorescence detection of virus (10^3 copies/well) using PEP-MB and Control MB. (a) Fluorescence intensities of PEP-MB and Control MB after incubation with virus for 30 min (NT: non-treatment). (b) Relative fluorescence intensities (F) compared with the corresponding NT fluorescence intensities ($\Delta F = F - F_{\text{NT}}$).

We also examined the detection ability and sensitivity of PEP-MB (35 pmol) with respect to incubation time. Fluorescence was detected every 5 min for 15 min of incubation with viruses (10^3 copies/well). Minimal fluorescence was observed in the absence of viruses (the NT condition) (with relative fluorescence intensities $\Delta F/F_{\text{NT}}$ (in %), where $\Delta F = F - F_{\text{NT}}$). As expected, stronger

fluorescence was detected with H1N1 viruses than with other viruses (Fig. 4a). Within 5 min, H1N1 treatment produced a fluorescence intensity 5.7-fold higher than the NT intensity (571.9%). Fluorescence intensity gradually increased as incubation time increased (with an increase of 97.81% at 10 min relative to 5 min). PEP-MB was clearly able to detect the H1N1 virus after only 15 min of incubation, which produced a fluorescence intensity 16-fold higher than the NT intensity (1630 %) (Fig. S3a). No notable changes in PEP-MB fluorescence were observed after treatment with the other examined viruses (H3N2, H5N2 and H6N5 viruses), confirming that PEP-MB could be used for the rapid and specific detection of H1N1 viruses.

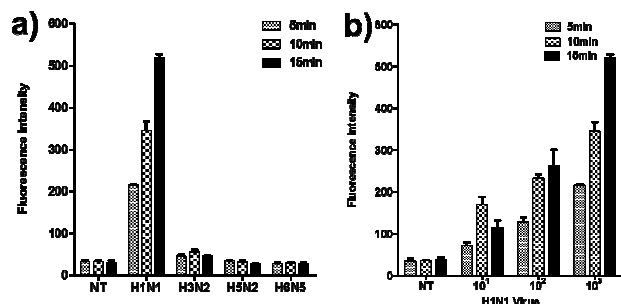


Fig. 4. Fluorescence intensities of (a) all viruses (10^3 copies/well) and (b) H1N1 virus under various concentrations (0 , 10^1 , 10^2 and 10^3 copies/well) after 35 pmol of PEP-MB incubation with different times (5, 10 and 15 min) (NT: non-treatment).

The sensitivity of PEP-MB was then investigated by measuring the fluorescence intensities obtained with different concentrations of H1N1 viruses (0 , 10 /well, 10^2 /well and 10^3 copies/well) and different incubation times (5, 10 and 15 min) at room temperature (Fig. 4b and ESI). PEP-MB fluorescence was detected every 5 min after the addition of the aforementioned concentrations of H1N1 virus. As indicated in Fig. 4b, fluorescence intensity was markedly increased after the addition of the virus. After short incubations (5 or 10 min), PEP-MB could be used to rapidly detect H1N1 viruses (at a concentration of 10^3 copies/well) after short incubations of 5 and 10 min, which produced high relative fluorescence intensities of 532.4% and 885.7%, respectively, compared with the NT intensity. Even at a low H1N1 concentration (10^1 copies/well), fluorescence intensities after treatment remained higher than NT fluorescence (by 111.8% at 5 min, 385.7% at 10 min and 200.0% at 15 min) (Fig. S3b). These differences in fluorescence intensity are sufficient to effectively detect the target (H1N1 viruses). Based on these results, we confirmed that PEP-MB could be used for the rapid, straightforward, and highly sensitive detection of H1N1 viruses.

In conclusion, we have described a simple, rapid and high sensitive detection method for H1N1 viruses using PEP-MB, a peptide-based molecular beacon with a peptide that specifically interacted with the HA1 protein. PEP-MB could be used to directly detect H1N1 viruses by producing intense fluorescence; thus, this approach constituted an alternative to genetic techniques, such as PCR assays. This was also

capable of detecting low concentrations (above 4 copies) of H1N1 viruses within just 15 min. Based on these results, it is likely that PEP-MB has great potential for use in multiplex assays of influenza viruses in which several fluorophores and peptide sequences allow for the detection of interactions with specific HA proteins.

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