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A Novel Approach to Identify Molecular Binding to Influenza Virus N5N1: Screening using Molecularly Imprinted Polymers (MIPs)

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Thipvaree Wangchareansak[a], Arunee Thitithanyanont[b], Daungmanee Chuakheaw[a], M. Paul Gleeson[a], Peter A. Lieberzeit[c] and Chak Sangma[*a]

In this report we investigate whether a molecularly imprinted polymer (MIP) of an inactivated influenza A H5N1 strain could be used to help identify molecules capable of binding to, and inhibiting the function of the virus, via either competitive or allosteric mechanisms. Molecules which bind to the virus and induce a conformational change are expected to show reduced binding to the H5N1 specific MIP. Given the importance of molecular recognition in virus replication, such conformational change might also reduce the effectiveness of neuraminidase (N1) to cleave the sialic groups necessary for virus replication. We show that the method can indeed differentiate between potent neuraminidase inhibitor, H1 and H5 antibodies, and N1 specific and non-specific monosaccharide substrates. We suggest that such a method could potentially be used in conjunction with traditional biochemical assays to facilitate the identification of molecules functioning via novel modes of action.

Introduction

The discovery of novel hits for a target are predominantly performed using high throughput phenotypic and binding assays.[1] More low throughput biophysical methods such NMR, X-ray crystallography or iso-thermal calorimetry can then be used to shed more light on the mode of action, binding kinetics and thermodynamics.[2, 3] The benefit of this collection of information is that it facilitates the development of strategies to rigorously probe the link between target modulation and effficacy. For example the identification of non-competitive, allosteric inhibitors is a well known strategy, and methods that facilitate their identification are highly desirable.

Allosteric inhibitors, such as the NNRTIs of HIV1-RT,[4] bind to a pocket adjacent to the orthosteric site occupied by the less effective 1st generation NRTIs competitive inhibitors. NNRTI binding lead to a signifcant conformational change in the hetero-dimer structure as observed by X-ray crystallography and this prevent DNA replication from occuring. The key challenge in the identification of allosteric inhibitors is that the methods used to assess binding are time and resourse intensive.

In this paper we discuss the application of H5N1 influenza molecularly imprinting polymers (MIPs) to facilitate the identification of molecules that can induce sizeable changes in the target conformation on the virus surface such that it alters its structural recognition features. MIPs consist of a polymer matrix formed in the presence of a template.[5, 6] Once the template is removed, binding cavities remain that are highly selective to the template in question. MIPs can therefore be used to selectively identify specific chemicals, proteins or virus particles[7, 8], and their concentration.[9, 10]

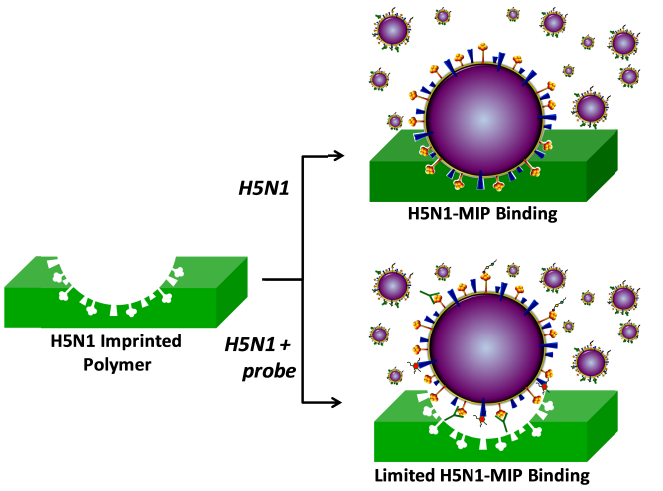

Fig.1 Schematic of H5N1 virus binding to an MIP (top) and the inhibition of the process due to a conformational change in the N5N1-probe complex.

In Fig. 1, we show a schematic representation of H5N1 virus binding to its corresponding MIP. H5N1 has two major proteins on its surface, hemagglutinin (HA) and neuraminidase (NA). HA binds to host cell receptor when the virus is initially

entering the host cell. NA, then, cleaves the terminal of sialic acid from cell receptor and pushes itself into inner cell. The binding of a probe molecule(s) to the virus particle will interrupt binding to the MIP (bottom) if it induces a sufficiently large conformational change. Therefore, recognition is expected to show concentration dependence while the use of a control system to taken into account non-specific molecular binding to the template is employed.

Results and discussion

Recently, we have succeeded in influenza A virus imprinting where the MIP was able to discriminate among virus subtypes using a quartz crystal microbalance (QCM)[11]. The QCM method relies on the principle that the resonance frequency of a QCM in an oscillator will change depending on the mass removed from or added to the quartz surface. The relationship between mass and frequency can be determined by the Sauerbrey equation:[12]

$$\Delta f = -c\Delta m$$

Where c is a constant value detemined by sensitive area and fundamental frequency. We have coated a QCM electrode with influenza virus MIP and used it to measure the recognition to virus sample in term of frequency change. The result suggests that recognition of a unique pattern of the specific area by the MIP is possible. The QCM can differentiate influenza A subtypes having identical structure but a different number of distinctive amino acids on the surface.

In this work, we try to confirm this hypothesis by using a similar experiment to monitor absorption of H5N1 virus before and after reacting with molecules known to bind with H5N1 at different sites (Fig. 1). According to this scheme, the virus absorption on the H5N1 MIP should decrease when a probe molecule is attached to the virus surface, thereby preventing it from binding to the MIP. In this experiment, the probe molecules and its binding sites on the influenza A virus subjected to this experiment are anti-H5 and anti-H1 antibody–influenza A hemagglutinin (HA) antibody, sialic acid and N-acetylglucosamine (GlcNAc) derivatives[13]–parts of the influenza A receptor targeting to HA protein at receptor binding pocket, and oseltamivir–an anti-neuraminidase drug.

The influenza A based MIP used in this experiment was prepared as co-polymer using acrylamide (AAM), methacrylic acid (MAA), methymethacrylate (MMA) and N-vinylpyrrolidone (VP) based on our previously reported method published elsewhere.[11] The polymer surface was stamped using an inactivated virus template (H5N1).

Our MIP uses not only one but four monomers with different side-chains during preparation. These side-chains allow the MIP to have both polar and hydrophobic functionality to interact with protein surfaces. Imprinting generates recognition sites for molecules or microorganisms in synthetic polymers by adding a template to the respective reaction mixture just before polymerization. The monomers still have the possibility to align themselves around the template where they can pre-form a specific non-covalent interaction network. After hardening and removal of the template, the polymer surface of the MIP retains information of the template's shape, size and the surface properties caused by self-organization[14]. This explains how minute details are detectable and how the decrease in QCM signal can occur as a result of the molecular binding.

The ability of the MIP to absorb H5N1 was subsequently undertaken using a QCM device as illustrated in Fig. 2. The detection limit of assay was found to be equivalent to 1 HA titer unit (HAU). In subsequent screening applications, all experiments used the following procedure. First, the probe molecules at various concentrations were mixed with fixed concentration of H5N1 virus (8 HAU). Each mixture was injected into the QCM measuring cell (containing both MIP and NIP) and the relative frequency change was recorded (see experimental part for details). After each experiment the absorbed H5N1 particles were washed off the surface of the MIP. Each experiment was performed in triplicate.

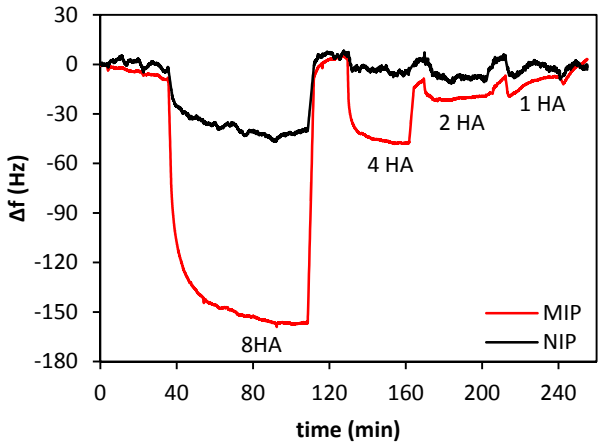

Fig.2 H5N1 MIP QCM measurement using an MIP (red), NIP (black) with differing concentrations of H5N1. More extensive binding leads to a larger frequency change between the MIP and the reference NIP.

To assess the ability of the MIP approach to differentiate between molecular binders of different types and different affinity, we used the following probes; H5 antibody[15] (high affinity), Oseltamivir (high affinity)[16], sialic acid[17] (moderate affinity), GlcNAc[13] (low affinity) and H1 antibody (low affinity).

The probes used in this study are of different size, shape, binding affinity. Sia and GlcNAc respectively, are constituents of the pentasaccharide, Sia-Gal-GlcNAc-Gal-Glc (linkage-type omitted), which is a cell receptor of influenza A.[18, 19] Sia is the terminal portion of the molecule that enters into the Sia binding pocket of HA while the GlcNAc substructure is located outside of the binding pocket. The latter is therefore expected to be of low overall affinity without the attached Sia head group.[17]

In Fig. 3, the results obtained for high and moderate affinity probes are reported, while those for low affinity probes are shown in the Fig. 4. Firstly, it should be noted that the resolution of MIP towards large macroscopic virus particles is clearly lower than for small molecules.[20] This can be seen in Fig. 3 where we were unable to fully prevent binding of H5N1 to the MIP, even at the highest probe concentrations. This is proposed to be due to the fact that binding to the MIP can occur via different surfaces of the virus and some of these may be slightly less susceptible to conformational changes on probe binding. This is in line with previous experiments with erythrocytes, where different glycolipid surface concentrations even lead to subgroup selectivity with MIP[21] or other virus serotype selectivity, as shown with human rhinovirus (HRV)[7].

Nevertheless, given that high potency oseltamivir (IC50 ~1 nM)[22] more strongly inhibits the binding of H5N1 to the polymer than anti-H5 (IC50 ~5-34 ng/ml)[23] which is in turn more effective than sialic acid (3-10 mM)[24], the method is clearly capable of distinguishing between ligands of different affinity and type. Crudely, the points where 50% response was reached in the MIP based experiments were approximately 10nM, 36 ng/ml and 2.4 mM respectively which is in reasonable agreement with the experimental values. Furthermore, analysis of the results for the H1 anti-body (Fig. 4) show a maximum response at ~10% while that for the low affinity substrate (GlcNAc) reaches ~25% at the highest concentrations.

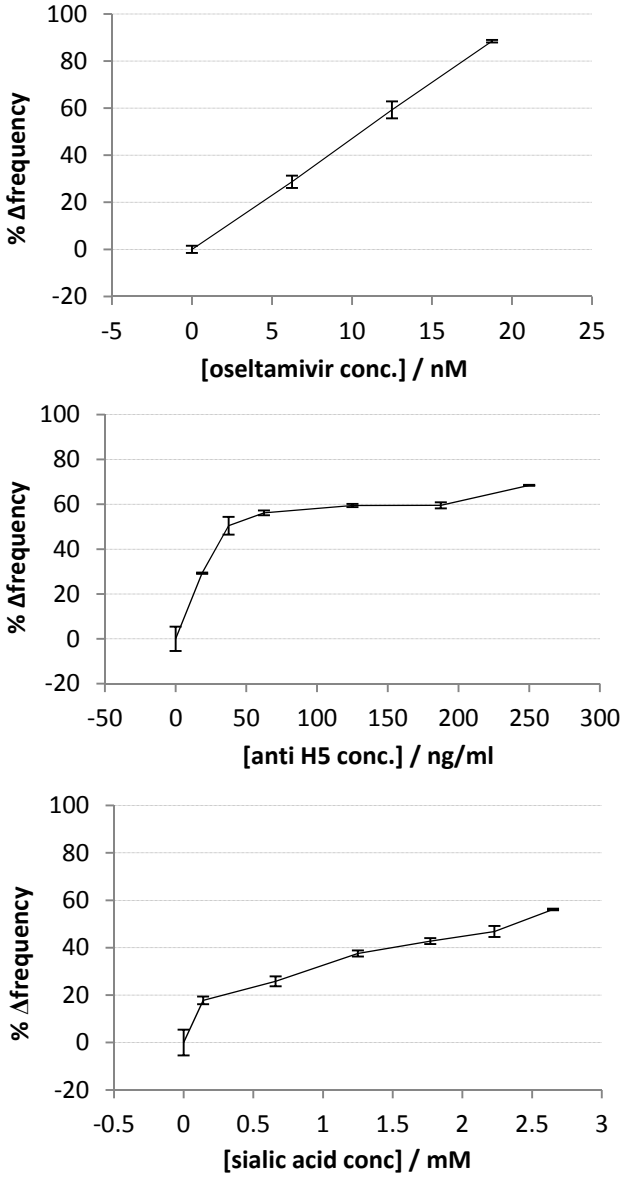

Fig.3 Effect of probe binding on H5N1 binding to the H5N1 based MIP. The greater the affinity of the probe for H5N1, the lower the binding as given by the Δfreq with respect to H5N1 alone. All QCM experiments were carried out at 25°C.

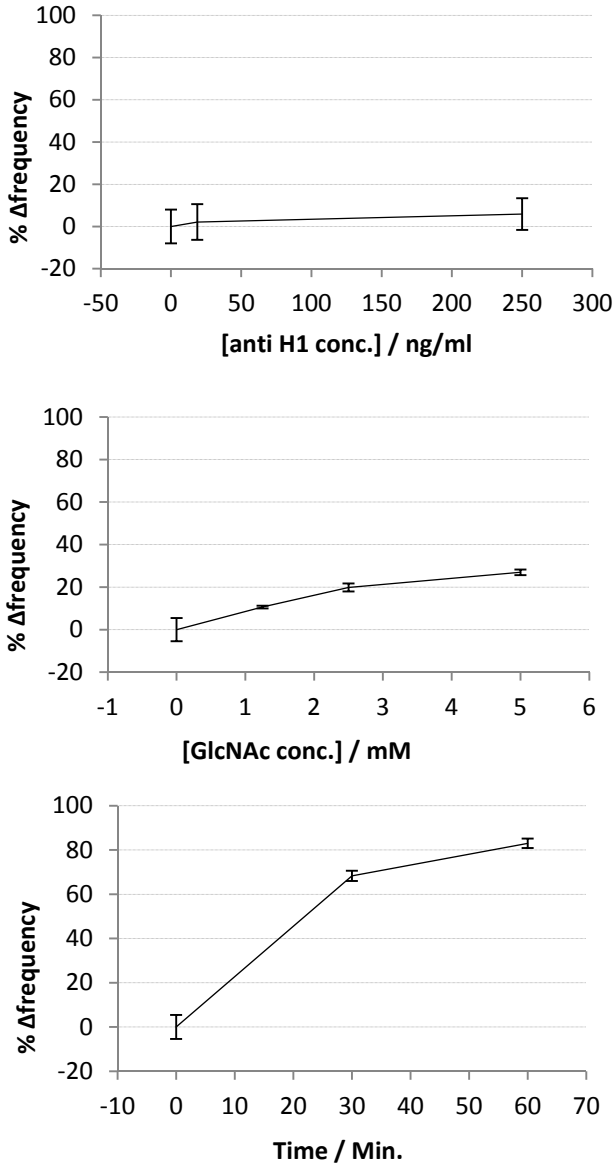

Fig.4 Effect of probe binding and temperature on H5N1 binding to the H5N1 based MIP. This figure shows the results from low affinity probes (GlcNAc) (top), non-specific binding probe (anti-H1) (middle) and temperature effect (bottom) (by time used to heat virus sample at 90°C) for H5N1 binding samples. All QCM experiments were carried out at 25°C.

We use a separate control experiment to confirm that the signal observed comes from H5 absorption not other binding agents. For that purpose, heat was applied to denature the viral protein, and it was observed that this reduces the signal to approximately 50% of its initial value due to denaturing (Fig.4). In addition, we observe a much smaller and non-specific response in QCM signal from probe binding to the NIP and MIP suggesting that each probe does indeed bind to the virus to limit binding to the MIP (see supporting information).

These results suggests that the method is clearly capable of differentiating between induced conformational effects from small inhibitors and substrates, to large macro-molecules and

could certainly be used a rapid screening tool to estimate the approximate binding affinity and whether binding results in a noticeable conformational effect (otherwise the method will not provide discrimination). Furthermore, to the very best of our knowledge this is one of the first attempts to actually characterize the effect of drugs on their target bio-species in situ.

Experimental

Virus Preparation:

The H5N1 (A/open-billed stork/Nakhonsawan/BBD0104F/04) was isolated from an open-billed stork and propagated in MDCK at P3 biosafety laboratory, Department of Microbiology, Mahidol University, Bangkok, Thailand. In this experiment we used only inactivated virus and carried out all experiments in strictly controlled laboratory.

Virus Imprinting:

The virus imprinting protocol and copolymer conditions followed our previous work[11]. Briefly, the polymer system consisted of: acrylamide (13.0 mg): methacrylic acid (10.6 mg): methymethacrylate (6 mg): N-vinylpyrrolidone (6.3 mg) was dissolved in 300 µl of dimethylsolfoxide containing 2, 2′-azobis(isobutyronitrile) initiator. Afterwards, this was pre-polymerized at 70 °C for 40 minutes to reach a gel point. These pre-polymerization polymers were dropped on QCM electrode and spinning off to obtain thin layer. The template stamp was prepared on glass substrate by adding 5 µl of H5N1 virus sample and kept at 4 °C for 30 minutes. Then, the template stamp was pressed onto the polymer layer and polymerized under UV light (254 nm) overnight. The imprinted cavities of H5N1 virus were obtained by removing the virus template from rigid polymer with 10% hydrochloric acid and stirred in water at 45 °C for 3 hours. Finally, the H5N1 imprinted polymer is installed into the measuring cell for using in QCM measurement.

QCM measurement:

The QCM with dual gold electrodes was placed into the custom-made poly(dimethylsiloxane) cell (75 µl volume). This measuring cell was connected to oscillator circuit and frequency counter (Agilent 53131A) for real-time frequency read out through computer via GPIB interface and LabView software. The QCM experiment is measured 2 channels in the same time at 25 °C. It was started by injecting the PBS buffer (pH 7.2) into the measuring cell to obtain the baseline. After the frequency change in both channels is reach at the stable value, the PBS was replaced by H5N1 sample and waited for equilibrium. Then, this was followed by the washing step with 10% acetic acid, 3x water and 3xPBS buffer and start to re-measurement. After the frequency was going back to the initial value, this was already used for the next sample.

Virus Binding Assay:

The molecules used in virus binding assay experiments were CMP-sialic acid (Acros), p-nitrophenyl-N-acetylglucosamine (Sigma-Aldrich), Oseltamivir (obtained from the Thailand Government Pharmaceutical Organization), antibody H5 and antibody H1 (Immune Tech). For each binding assay, we collected QCM signals from different ligand/antibody concentrations at fixed H5N1 (8HAU) using the same procedure for QCM measurement. For binding assay sample, we prepared the samples by mixing 8 HAU of H5N1 with the interested molecule such as antibody or drug into the small tube then left it for 30 minutes for the binding process. Then, this mixture sample is ready to use for QCM measurement. Heat was used as the negative control to denature virus. The virus samples were heated at 90 °C for 30 minutes and 90 minutes before left to cool down to the room temperature. Then, this virus sample was injected into measuring cell and started record the signal changed.

Conclusions

We have used an MIP based approach to screen molecular probes of differing size and affinity for H5N1. We find that H5N1-based MIPs can successfully differentiate between high and low affinity probe molecules due to conformational changes induced by binding. This conformational change reduces the affinity of the H5N1-probe complex for the MIP produced using H5N1 alone. The net reduction in binding, as given by the weight of virus bound to the MIP is proportional to their known binding constants. These initial results suggest that an MIP based methodology could be used to screen for inhibitors capable of inducing conformational change in the target protein. While the resolution of the process is somewhat limited by the fact that the MIP recognition sites may be formed by surfaces unchanged by probe binding, it is possible with the diverse probes used here to see an effect proportional to their know affinities. Such a method could prove useful for other protein such as HIV1-RT or EGFR where binding within an allosteric pocket/region is known to produce a sizeable conformational change in the protein structure and confer a considerable therapeutic advantage over non-allosteric inhibitors.

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Notes and references

[a] Department of Chemistry, Faculty of Science, Kasetsart University, Chatuchak, Bangkok, Thailand. Fax: +66-2-5793955; Tel: +66-2-562-5555 extn 2192; E-mail: fscicsm@ku.ac.th

[b] Department of Microbiology, Faculty of Science, Mahidol University, Rama 6 road, Phaya Thai, Bangkok, Thailand.

[c] Department of Analytical Chemistry, University of Vienna, Vienna, Austria.

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