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Influenza A Virus Molecularly Imprinted Polymer and Its Application in Virus Sub-type Classification

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Materials and Methods

Influenza A Viruses:

Avian influenza H5N1 (A/open-billed stork/Nakhonsawan/ BBD0104F/04) and H6N1 viruses were isolated from an open-billed stork and duck, respectively. The original stock of reassortant vaccine virus A/California/7/2009 (H1N1) like strain (NYMC X-179A) was obtained from WHO Collaborating Centre in CDC, USA. Reverse genetic H1N3 and H5N3 viruses were kindly provided from Dr.Anan Jongkaewwattana (National Center for Genetic Engineering and Biotechnology, Thailand). The internal genes were A/PR/8/34, while the H1 HA, H5 HA and N3 viruses were derived from H1N1 (A/PR/8/34), H5N1 (A/Vietnam/DT-036/2005), and H2N3 (A/duck/Germany/1215/ 1973), respectively.

Preparation of Beta-propiolactone inactivated viruses:

All viruses were propagated in MDCK according to a WHO protocol as previously described.[1] Virus stock was measured by hemagglutination with 0.5% goose red blood cells. The virus at HA titer more than 32 HAU were inactivated with Beta-propiolactone followed CDC protocol with a minor modification. Briefly, the viruses were thawed and mixed with disodium phosphate solution pH7.0 prior to adding Beta-propiolactone at a final concentration of 0.1%. The viruses were inactivated at 37 ^{o}C for 2 hours. The inactivated viral fluids were adjusted to pH7.4 by adding drop wise with 7%$NaHCO_3$ before incubating at 4 ^{o}C overnight. The inactivated viruses were investigated for HA titer by hemagglutination assay and viral infectivity by passaging in MDCK cells for 6 days.[2]

Polymer synthesis and molecular imprinting:

For synthesizing copolymers, 13.0 mg of acrylamide (AMM, purchased from Acros), 10.6 mg of methacrylic acid (MAA, purchased from Sigma-Aldrich), 6 mg of methylmethacrylate (MMA, purchased from Sigma-Aldrich) and 6.3 mg of *N*-vinylpyrrolidone (VP, purchased from Sigma-Aldrich) monomer were mixed with 48 mg of *N, N*-(1,2-dihydroxyethylene) bisacrylamide (DHEBA, obtained from Alfa Aesar) as cross-linker. This mixture was dissolved in 300 µl of dimethylsulfoxide (DMSO, obtained from Merck in analytical gread) containing 1 mg of *2, 2′*-azobis(isobutyronitrile) (AIBN, purchased from Sigma-Aldrich) as initiator. Afterwards, this was followed by pre-polymerization at 70 °C until it reached a gel point as sol-gel polymerization in approximately 40 minutes.

In parallel, the template stamp was prepared on a 5x5 mm^2 glass plate by dropping 5 µl of influenza virus on a bare glass plate and keeping it at 4 °C for 30 minutes for sedimentation and avoiding participation of media and buffer. This was then followed by spinning off at 3000 rpm for 3 seconds in order to remove an excess of solution.

The sol-gel polymer was then dropped on both QCM electrodes and spun off at 3000 rpm to obtain the thin layer of 350 to 450 nm in thickness. Immediately, the virus stamp template was pressed on thin polymers and polymerized under 254 nm UV light overnight. Finally, the template was removed from the polymer surface by rinsing it with 10% hydrochloric acid to denature the virus and then stirred in water at 45 °C for 3 hours to remove the virus particles left on the surface. This resulted in a contained virus imprinted cavity in rigid polymers that were ready to be applied on QCM as biosensor.

QCM preparation and measurement:

The dual gold electrodes were generated on AT-cut quartz water of 15.5 mm in diameter and 168 µm in thickness with screen-printing procedure as described in previous work (Fig. S1).[3] Briefly, the gold electrode was generated on a quartz wafer by screening printing of the electrode structure. The first side of electrode was printed and then burned at 400˚C for 2 hrs. The second side was generated subsequently using the same procedure and was burned at 400˚C for 4 hrs. Undesirable organic solvent was then removed from surface, to yield a highly robust electrode that can withstand mechanical stress. Afterwards, the quality of dual gold electrodes quartz crystals were verified by their damping value under a network analyzer. Quartz wafers with damping values less than 3 were used to create all MIPs used in this study. The resulting 10 MHz quartz was measured with a custom made measuring cell with 75 µl volumes of poly(dimethylsiloxane) cast. The QCM electrodes were connected to a frequency counter (Agilent 53131A) and read out to computer via GPIB USB interface with custom-made LabView routine. The measurements were carried out in stop-flow mode at room temperature. A typical measurement consists of putting PBS (1 mM, pH 7.2) into a measuring cell and running it until

reaching a constant value. Then, we paused the measurement and removed the PBS solution from the measuring chamber and flushed the cell with 100 µl of virus sample. After this, we filled the measuring chamber with a virus sample and started running again. After a stable signal was reached, we removed the virus sample and followed the washing steps, including 10% of acetic acid, 4 times of water and 2 times of PBS (1 mM, pH 7.2) and then started recording again with PBS (1 mM, pH 7.2). When the signal was going back to the initial constant value, the new sample could be applied into the measuring cell and the same procedure was followed.

AFM measurement:

All AFM images were recorded with a Veeco Nanoscope IVa operated in contact mode using a Veeco SNL-10 silicon tip with spring constant of 40 Nm and onset pressure corresponding to differential signal 1 V on photo diode. All samples including influenza virus template stamps, non-imprinted polymers (NIPs), molecular imprinted polymers (MIPs) and viruses rebinding on MIPs were put onto the sample disk and AFM scanned with a 2.4 Hz scan rate at room temperature. In order to count the virus, 1 µl of influenza virus from stock solution was sedimentation on 5 x 5 mm^2 glass plates which could be directly counted by AFM.

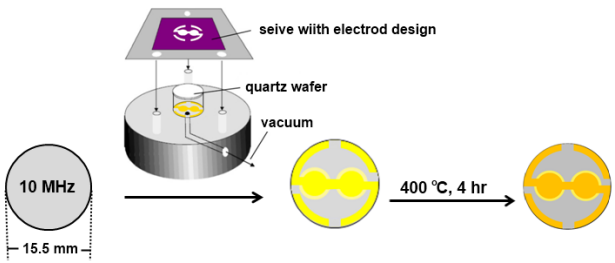

Fig.S1 Screen printing process of dual gold quartz crystal microbalance.

Table S1. The absolute Δf/Hz values (top) and the %response relative to the standard (bottom).

		Virus Type				
	Average (absolute)	**H1N3**	**H5N3**	**H5N1**	**H1N1**	**H6N1**
MIP	**H1N3**	459.59	186.98	60.91	73.92	65.56
	H5N3	269.82	333.30	39.84	21.45	43.74
	H5N1	172.01	77.22	463.17	260.21	77.04
	H1N1	226.61	287.62	371.58	569.43	418.72
	H6N1	200.13	157.46	174.13	241.42	346.59

		Virus Type				
	Average (%)	**H1N3**	**H5N3**	**H5N1**	**H1N1**	**H6N1**
MIP	**H1N3**	100%	41%	13%	16%	14%
	H5N3	81%	100%	12%	6%	13%
	H5N1	37%	17%	100%	56%	17%
	H1N1	40%	51%	65%	100%	74%
	H6N1	58%	45%	50%	70%	100%

Table S2. The sequence similarity between the HA and NA domains of influenza-A virus

HA similarity	**H1N1**	**H1N3**	**H5N1**	**H5N3**	**H6N1**
H1N1	100%	79%	66%	66%	62%
H1N3	79%	100%	65%	65%	62%
H5N1	66%	65%	100%	98%	62%
H5N3	66%	65%	98%	100%	62%
H6N1	62%	62%	62%	62%	100%

NA similarity	**H1N1**	**H1N3**	**H5N1**	**H5N3**	**H6N1**
H1N1	100%	52%	83%	52%	87%
H1N3	52%	100%	53%	100%	53%
H5N1	83%	53%	100%	53%	88%
H5N3	52%	100%	53%	100%	53%
H6N1	87%	53%	88%	53%	100%

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