Molecular Diagnostics
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Artificial Receptors in Serologic Tests for the Early Diagnosis of Dengue Virus Infection

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Background: Because of the range and nonspecificity of clinical presentations of dengue virus infections, we felt there was a need to create diagnostic tests. We used artificial receptors for the virus to develop serologic assays to detect dengue virus infection.

Methods: We coated a quartz crystal microbalance (QCM) with molecularly imprinted polymers specific for nonstructural protein 1 of flavivirus. These artificial receptors were specifically created on a QCM chip by polymerization of monomers and were cross-linked in the presence of the epitope site of nonstructural protein 1. We tested serum samples from patients with confirmed cases of dengue reported to the Center for Disease Control in Taipei. Samples were diluted 100-fold; no other sample pretreatment was used. The QCM response was compared with results of monoclonal ELISA.

Results: QCM signals were >15 Hz in 18 of 21 (86%) of dengue samples and in 0 of 16 control samples. The correlation (r^2) of the QCM response and the ELISA result was 0.73. Within-run and run-to-run imprecisions (CV) were 4%–28% and 10%–32%, respectively.

Conclusions: The described assay offers a serologic technique for diagnosis of early viremia. The results illustrate the potential of well-organized polymers on the highly sensitive sensor system for diagnostic and biotechnological applications.

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Dengue *(1, 2)* is the most important mosquito-borne viral infection in humans in the tropics worldwide *(3, 4)*. Dengue fever is caused by 1 of 4 closely related, but antigenically distinct, dengue virus serotypes (DEN-1 to DEN-4) belonging to the genus *Flavivirus*, family *Flaviviridae*. They also share characteristics of *Flavivirus* genus in that the genetic material is packaged in a typically spherical lipid envelope with the same flavivirus epitopes *(5, 6)*. Because of antigenic differences, infection with one virus does not provide cross-protective immunity against the others. Differentiation of primary and secondary dengue virus infections is critical *(7)*. Because dengue infections may suddenly become serious around day 4, after onset of fever, and can be fatal, early detection is important to alert physicians and parents to watch for early signs of increased vascular permeability.

We can detect acute dengue infections in acute phase sera by means of virus isolation or PCR *(8–10)* or by detection of circulating dengue nonstructural protein 1 (NS1)[4] *(11)* using an antigen-detection ELISA *(12–14)*; but commercially available methods *(15, 16)* are slow, cumbersome, and costly. They require research resources or simply are not available (NS1 ELISA). We have reported discrimination of peptides by use of a molecularly imprinted piezoelectric biosensor *(17)*. Previously, we generated a receptor mimic with specific binding to NS1 using the technology of molecular imprinting *(18)*. The artificial receptors were created specifically by polymerization of several monomers in the presence of the epitope site of NS1 on the quartz crystal microbalance (QCM) chip. The process resulted in the grafting of a polymeric thin film to the gold surface of the QCM, creating poly-amino acid-like cavities that exhibit highly selective recognition for the pentadecapeptide (epitope) and NS1 *(19)*. Fig. 1 illustrates the process for preparing a molecularly imprinted film for the detection of targeted protein. In fact, the response of molecularly imprinted polymer (MIP)-QCM chips to the NS1 using epitope-mediated imprinting demonstrated good sensitivity and specificity. It obtained a comparable frequency shift to chips immobilized with monoclonal antibodies *(20–22)*. Herein, we

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[4] Nonstandard abbreviations: QCM, quartz crystal microbalance; MIP, molecularly imprinted polymer; NS1, dengue nonstructural protein 1.
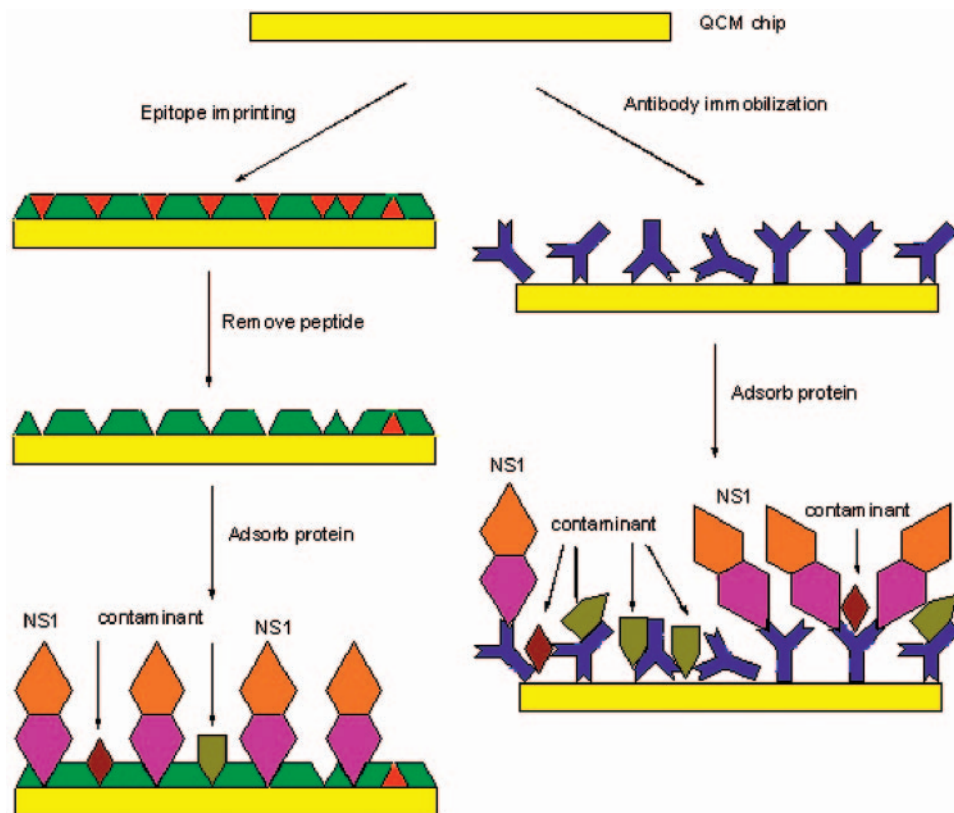
Fig. 1. Schematic representation of the fabrication of antibody-QCM and MIP-QCM for the detection of antigen.

describe proof-of-principle experiments on the applicability of this affinity matrix to an assay of clinical samples.

Materials and Methods

PATIENTS AND CONTROLS

The serum samples used in this study were collected from patients with confirmed cases of dengue reported to the Center for Disease Control, Department of Health, Taipei, Taiwan, from 1998 to 2003. Dengue virus infections were defined as febrile illness associated with the isolation of dengue virus, a positive RT-PCR test *(8)* (conventional nested reverse transcription (RT)-PCR or real-time one-step RT-PCR), or the detection of dengue virus-specific IgM and IgG antibodies (E/M-specific capture IgM and IgG ELISA and/or E/M-specific indirect IgG ELISA) *(7)*. We collected and analyzed a total of 26 antidengue-virus IgM-positive serum samples between days 1 and 36 of illness from confirmed dengue patients. Control serum samples were collected from 21 healthy donors without dengue virus infection. We performed the microchip analysis in a blinded fashion. Results of the ELISA were not available to us until the end of the validation procedure.

REAGENTS

We obtained $(Boc-L-Cys)_2$, acrylic acid, acrylamide, urea, acetic acid, Tween-20, *p*-nitrophenyl-phosphate, and ethylene glycol dimethylacrylate from Sigma-Aldrich. We

purchased *N*-benzylacrylamide from Lancaster. Alkaline phosphatase-streptavidin conjugate was from Kirkegard & Perry Laboratories Inc. The 15-mer peptide derived from NS1 of a Japanese encephalitis virus was synthesized by a peptide synthesizer. $(N-Acr-L-Cys-NHBn)_2$ was synthesized from $(Boc-L-Cys)_2$. The buffer used for all experiments was a PBS (20 mmole/L NaH_2PO_4, pH 4.0). The QCM was obtained from Tai-Tien Electronic Co. with an accuracy of ~1 Hz. The QCM consisted of an 8-mm diameter disk made from an AT-cut 9 MHz quartz crystal with gold electrodes on both sides (diameter: 4.5 mm, area: 15.9 mm^2) of the crystal. The AT cut refers to quartz wafers cut at an angle of ~35°10′ with respect to the optical axis.

NS1 ANTIGEN ELISA

A modified sandwich ELISA was used to analyze the NS1 antigen in the serum samples of infected dengue patients as previously described *(23)*. Briefly, each microtiter well was coated overnight at 4 °C with 100 µL/well of mAb D2/8-1, 5 mg/L, in 0.1 mol/L carbonate buffer of Na_2CO_3/$NaHCO_3$, pH 9.5. After being washed and blocked, the wells were washed and incubated with 100 µL of 1/10 diluted serum samples in PBS-Tween 20 containing 50 ml/L of healthy rabbit serum, for 1 h at 37 °C. After being washed, the wells were incubated with biotin-conjugated mAb D2/8-1 for 1 h at 37 °C at 1/500 dilution. The plates were then washed, and a 1/2000 dilution of alkaline

phosphatase-streptavidin conjugate was added and incubated for 1 h at 37 °C. Finally, the enzyme activity was developed with the addition of substrate p-nitrophenylphosphate, and absorbances were measured at 405 and 630 nm with a Dynatech MR700 microplate reader.

FORMATION OF A SELF-ASSEMBLY MONOLAYER (SAM) ON CHIP

The QCM disks were immersed in a 10 μmol/L solution of $(N$-Acr-L-Cys-NHBn$)_2$ in HPLC-grade acetonitrile for 16 h, then rinsed thoroughly with acetonitrile.

CONSTRUCTION OF A PROTOTYPE MIP-CHIP

A solution of acrylic acid (55 μmol), acrylamide (55 μmol), N-benzylacrylamide (110 μmol), and 3 μmol of 15-mer peptide was mixed in 0.3 mL of a solution made with equal volumes of acetonitrile and 20 mmol/L, pH 4.0 phosphate buffer = 1/1. After 4 μL of the aliquot was deposited on top of the $(N$-Acr-L-Cys-NHBn)-gold electrode, the chip was placed horizontally into a 20-mL vial containing acetonitrile (3 mL). The vial was screwed tightly and irradiated with ultraviolet (UV) light at 350 nm for 6 hrs. The polymer, which was formed as a thin film on the gold surface, was washed with 20 mmol/L phosphate buffer (pH = 3–4) to remove the template. This was followed by a wash with methanol and drying.

BIOSENSOR SYSTEM

The flow injection system contained an HPLC pump (Model L7110, Hitachi, flow rate = 0.1 mL min^{-1}), home-built flow cell, sample injection valve (Model 1106, OMNIFIT), home-built oscillation circuit (including oscillator and frequency counter) and a personal computer. The polymer coated QCM was fixed between 2 O-rings and inserted into the flow cell. Only one side of the QCM was in contact with the liquid. Sodium phosphate buffer (20 mmol/L, pH 4.0) was used for circulating, washing, and testing. To equilibrate the newly imprinted chips quickly, 100-μL solutions, including alkaline (pH 9 PBS), neutral (distilled water), and acidic (5% acetic acid in distilled water) mixtures, were injected into the flow cell during circulation.

FABRICATION OF RIGID MIP ON CHIP

A solution of acrylic acid (55 μmol), acrylamide (55 μmol), N-benzylacrylamide (110 μmol), ethylene glycol dimethylacrylate (220 μmol), and of 15-mer peptide (3 μmol) were mixed in 0.3 mL of solution containing equal volumes of acetonitrile and 20 mmol/L, pH 7.0 phosphate buffer. After 4 μL of the aliquot was deposited on top of the $(N$-Acr-L-Cys-NHBn)-gold electrode, the chip was placed horizontally into a 20-mL vial containing acetonitrile (3 mL). The vial was screwed tightly and irradiated with UV light at 350 nm for 6 h. The polymer, which was formed as a thin film on the gold surface, was washed with 20 mmol/L phosphate buffer (pH = 7) to remove the template. This step was followed by a wash with methanol and drying.

Results

Initially, N,N'-diBoc-L-cystine dibenzylamide was self-assembled onto the gold surface of a QCM chip *(17)*, and a frequency change of −600 Hz was measured. Because the change in quartz resonance frequency is in direct linear correlation with its mass, the amount of bound disulfide can be calculated from the frequency change according to the Sauerbrey equation *(24)* to be −0.85 ng per 1 Hz. Therefore, the formation of self-assembly monolayer resulted in fixing ~510 ng of disulfide (~1 nmol) on the gold surface.

FORMATION AND APPLICATION OF THE PROTOTYPE SYSTEM WITH SAMPLE PRETREATMENT

A monomer solution, containing acrylic acid/acrylamide/N-benzylacrylamide at a molar ratio of 1/1/2 was polymerized at pH 4 in the presence of 4 g/L concentration of a linear epitope (15-mer peptide) of Japanese encephalitis virus NS1 *(5)*. The 15-mer peptide (template) combined with functional monomers in solution and became spatially fixed in a solid polymer film with a thickness of ~70 nm after UV irradiation *(22)*. Subsequently, the resulting chip was treated with alkaline, neutral, or acidic solutions to remove the template and to effectively clean the chip surface.

Consequently, complementary sites were formed that possessed a size, shape, and chemical memory similar to the 15-mer peptide and NS1 protein.

An MIP-grafted chip was inserted into the flow-cell of QCM, and a flow injection system was set up and equilibrated with a solution of 20 mmol/L phosphate buffer, pH 4.0, in saline (PBS); the flow rate was 0.5 mL/min.

We studied the ability of this prototype system to bind NS1 in a serum sample. Human sera were diluted 1/1000 with PBS and incubated for 5 min at 95–98 °C in a boiling water bath, cooled to room temperature, and analyzed. Serum samples were then passed through the Cibacrown gel column *(22)* to reduce background. After the pretreatment, the clinical specimens (100 μL) were injected to the center of QCM disk for detection.

These artificial receptors retained their specificity toward NS1 protein in serum (Table 1). Four of 5 positive samples produced a frequency shift >13 Hz, and the 5 negative samples showed a frequency shift <5 Hz. The cutoff value is 6 to 7 Hz. The cutoff value for antibody-QCM *(22)* is much higher at 24 to 25 Hz. The high frequency shifts observed by antibody-QCM might result from the contaminant in serum samples, as proposed in Fig. 1.

We suspected that the proteins aggregated on the surface of these chips, especially at higher concentrations. The nonspecific binding kept increasing as higher concentrations of analyte were injected. Therefore, the frequency shifts continued to increase and no saturation was shown

Table 1. Information regarding the types of clinical serum specimens applied to various serological tests

Specimen code, n	Sex	Age, yr	Sampling time, day	PCR result	NS1 antigen ELISA, A (SD)	Antibody-QCM, Hz	MIP-QCM result, Hz
25114	M	41	1	DEN-2	1.68 (0.13)	37	21
25230	M	29	4	DEN-2	1.87 (0.19)	40	29
25339	M	60	4	DEN-2	1.64 (0.11)	48	14
25348	F	17	4	DEN-2	1.72 (0.09)	32	17
25433	F	14	4	DEN-2	1.02 (0.10)	26	8
26093	M	52	19	ND	Negative	21	5
26094	F	17	21	ND	Negative	17	0
26096	F	9	18	ND	Negative	17	3
26134	M	49	13	ND	Negative	23	6
26143	F	25	1	Negative	Negative	9	5

The definition of "dengue-positive" is that the specimen has been found to contain a detectable amount of dengue viral genomic materials for PCR assays. NS1 antigen ELISA was performed with mAb 8-1, and the cutoff value is 0.35A. Antibody-QCM tests were performed previously (22). MIP-QCM tests were performed after the pretreatment with Cibacrown gel. ND, not determined.

(19). To obtain optimum assay conditions for clinical specimens, a rigid molecularly imprinted thin film for the selective recognition of proteins was prepared onto QCM.

FORMATION AND APPLICATION OF THE RIGID MIP-QCM ASSAY WITHOUT PRETREATMENT

We adjusted the pH of the flow injection system to neutral. In addition, Tween-20 was added to the buffer solution to prevent serum proteins from forming nonspecific binding with MIP. Nonspecific binding was minimized.

To evaluate this modified assay we used blood samples collected by the Center for Disease Control (CDC) of Taiwan, 21 from confirmed dengue cases collected from day 1 to day 36 after onset of illness and 16 from patients who were NS1 protein negative. The dengue virus serotypes from the confirmed dengue cases were identified by PCR testing and/or virus isolation from the acute-phase serum samples. Dengue NS1 serotype-specific IgG ELISA was performed as previously described to differentiate primary and secondary infection (7).

Of the clinical specimens, 18 were detected as NS1 protein positive. These results correlated with the results of ELISA (Fig. 2), and negative control samples were negative by the new test. The correlation (r^2) with the ELISA result was 0.73, according to the following equation:

Correlation coefficient = [Covariance(MIP-QCM, ELISA)]/ [SD(MIP-QCM) × SD(ELISA)]

The run-to-run repeatability (CV) was 10%–32%, and within-run CVs were 4% to 28%. The CV of the quality control specimens in the run-to-run setup for a period of 20 days was <15% for frequency shifts <50 Hz and <32% for frequency shifts of 51 to 100 Hz. Again, aggregation of protein at higher concentrations was suspected.

Most of the confirmed specimens had signals > 18 Hz, and the negative cases had signals <12 Hz, values that could be used as criteria for distinguishing positive cases of dengue virus from negative cases. One confirmed specimen did not give a positive response, probably because of a secondary infection (7). Therefore, the cutoff value of the chip can be set at 15 Hz, which has a satisfactory CV of 10%–15%.

Compared with the 1000-fold dilution of the prototype system, the serum was diluted only 100-fold for rigid MIP-QCM. Therefore, the amount of NS1 in the specimen could be 10 times greater. Compared with the results presented in Table 1, the frequency shifts for the new test were ~4 times higher for a positive specimen, similar to that for negative clinical specimens. Although the amount of contaminant increased proportionally to 10 times that found in the serum sample, with the Tween-20, the negative samples, which formed nonspecific bonds with MIPs, showed only moderately higher frequency shifts to 12 Hz by forming nonspecific binding with MIPs.

The effect of sampling day on the frequency changes in the dengue viral antigen (NS1 Ag) obtained by the rigid NS1-imprint polymer is shown in Fig. 3. The signals for positive specimens were unrelated to sex, age, or sero-
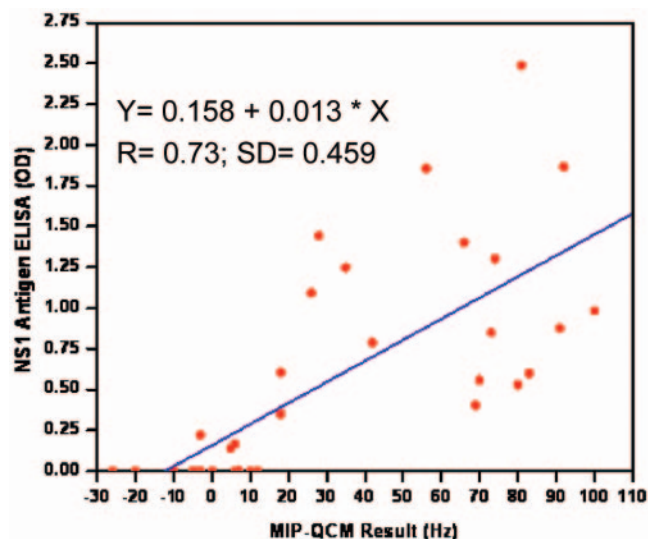

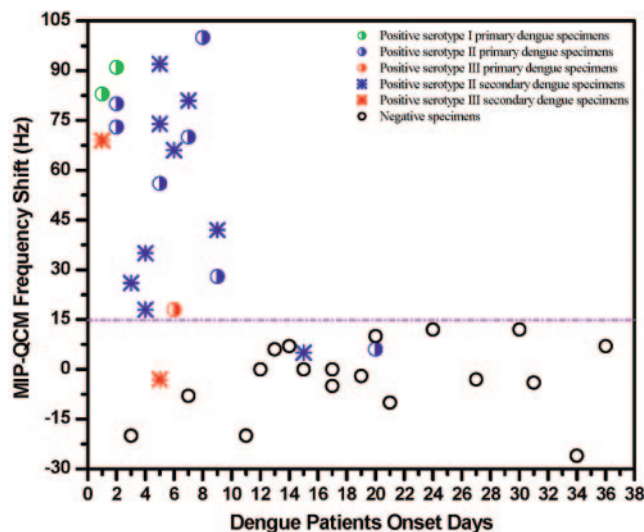Fig. 2. Correlation between rigid MIP-QCM results and ELISA results.

Fig. 3. The changes in frequency vs sampling time using rigid MIP-QCM.

type. No NS1 antigen was detected after 9 days. With some exceptions, most of the samples from newly infected patients showed high concentrations of NS1 protein.

The MIP chips were able to be regenerated repeatedly (5 times), and their affinity was stable during 30 days. To clean the surface of the MIPs efficiently, washing with 25 mmol/L urea and 3% acetic acid/0.1% Tween-20 was adopted as part of the activation/regeneration procedure. Over this period of time, the polymers lost 10% to 20% of their affinity. The decrease in affinity resulted mainly from the occupancy of the binding sites by contaminant. No evident decrease was observed for polymers that were kept intact after washing and used 1 month later.

Discussion

We demonstrated for the first time successful detection of the dengue virus using rigid MIP-QCM. The resulting chips enabled us to effectively distinguish dengue-infected specimens from reported cases in <1 h. The results correlated with the ELISA test and agreed with the clinical findings.

Previous studies on dengue NS1 antigen ELISA reported detection limits of 1 and 4 μg/L for affinity-purified dengue virus type 1 and 2 NS1 antigens and either a polyclonal mouse and rabbit antibody pair or a rabbit polyclonal antibody and mouse monoclonal antibody pair as capture and detection antibodies (12, 14). We have developed the sandwich dengue NS1 antigen ELISA with the same monoclonal antibody as the capture and developing reagents to detect NS1 antigens of all 4 dengue virus serotypes (23). The results showed that NS1 antigens could be detected from all 4 dengue virus serotypes in the acute-phase serum samples from both primary and secondary dengue virus infections (see the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol52/issue8 and our unpublished data). The detection limit of

our dengue NS1 antigen ELISA was estimated to be 1–10 μg/L with the use of dengue virus-infected Vero cell culture supernatant as control, which was reported to contain up to 7 mg/L in secreted culture media (14, 23). The exact detection limit of our dengue NS1 antigen ELISA, however, has yet to be determined with affinity-purified dengue virus NS1 antigens.

The MIP-QCM assay should be evaluated as a potential diagnostic test for dengue. Our rigid MIP-QCM system provided the first example of the application of molecularly imprinted polymers in the diagnosis of clinical specimens of dengue in its earlier clinical stages. The advantages of using MIP-QCM to detect dengue virus is high sensitivity ($\sim\mu$g/L scale), low technical demand, easily interpreted data, low cost, and short operation time (20–30 min per sample). The interferences existing in serum that caused larger background were minimized. Healthy human serum samples without pretreatment can be analyzed using these rigid NS1-imprinted chips. There is no need to reduce noise caused by serum proteins.

Artificial receptors may be valuable replacements for monoclonal antibodies. Generation of monoclonal antibody is not necessary, and nonspecific interaction between the test assay and the target protein is minimized. Pretreatment may not be needed. Dilution and the pretreatment of samples were not required for effective analysis by MIP-QCM sensors.

This assay could be used to detect many flaviviruses including all 4 dengue virus serotypes. Additional study will be required to determine the diagnostic accuracy of MIP-QCM-like assay for the detection of acute-phase dengue virus infection. An additional MIP-QCM-like assay using flavivirus subgroup-specific E-glycoprotein cross-reactive epitopes as the template might be useful for differentiating dengue serotypes.

In conclusion, synthetic materials with selective recognition are attractive candidates for further applications in the development of MIP-QCM-like assays for other virus proteins in serologic phase. They are well suited for a range of applications and appear to be broadly applicable.

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