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Epitope-Cavities Generated by Molecularly Imprinted Films Measure the Coincident Response to Anthrax Protective Antigen and Its Segments

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A molecularly imprinted film was fabricated, in the presence of epitope-peptides, onto a quartz crystal microbalance (QCM) chip. These five peptides are known linear or conformational epitopes of the anthrax protective antigen PA_{83}. Imprinting resulted in an epitope-cavity with affinity for the corresponding template. With the use of a basic monomer, the binding-effect was further enhanced increasing the affinity to nanomolar levels. The affinities of the peptide to their corresponding molecularly induced polymers (MIPs) were more closely related to the molecular weight of the analyte than to the number of residues. All epitope-cavities differentiated their epitope region on the protective antigen PA_{83} as well as the corresponding furin cleavage fragments PA_{63} and PA_{20}. The QCM chip differential response to the protective antigen fragment was observed in the picomolar range, thus demonstrating a method to manipulate protein on the surface with defined orientation.

Anthrax,[1] the acute infectious disease, is caused by the spore-forming *Bacillus anthracis*. The necessary quarantine and inspection for this pathogen[2] is a hazardous problem in airports, customs, and battlefields. When cells are infected with anthrax, they secrete three proteins: protective antigen (PA_{83}, 83 kDa), lethal factor (LF, 90 kDa), and edema factor (LF, 90 kDa). The protective antigen (PA_{83}) is found bound to a receptor on most mammalian cells. The PA_{83} is cleaved by furin to fragments PA_{20} and PA_{63}. Then, PA_{63} is converted to a heptameric ring-shaped oligomer to form a pore,[3] allowing LF or EF to enter the cell.[4] Since PA_{83} has an important role in triggering the disease, PA_{83}, PA_{63}, and PA_{20} are all considered to be biomarkers of anthrax.

The active component of human anthrax vaccine is also protective antigen.[5−7] Its crystal structure[8] has been determined. In addition, the anti-PA_{83} antibodies possess the ability to protect animals against anthrax exposure.[7,9,10] It is crucial to diagnose anthrax early with high accuracy, short operational time, and labor-free processing. A novel detection system for protective antigen recognition would be of great value. So far, several antibodies were applied for detecting protective antigen.[11−13]

Molecularly imprinted polymers (MIPs) have already been successfully formulated to mimic natural receptors.[14,15] Nowadays, more and more attempts to directly imprint proteins have been reported.[16,17] However, this approach is limited due to the cost; proteins are generally expensive. Therefore, the development of a cheap/disposable chip is almost impossible. Previously, the epitope approach has been demonstrated.[18] The advantages to develop these linear epitope-cavities are (1) the epitopes of protein antigens are abundant and accessible through revealed crystal structure and epitope mapping. (2) Epitope-imprinting results have demonstrated that the cavities function as their mother protein binding sites.[19,20] (3) Epitope-cavities are flexible enough[21] to incorporate a protein and maintain the native protein conformation

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for subsequent attachment (e.g., antibody).[19] (4) Epitope-cavities are able to manipulate protein onto the surface with defined orientation; the fabrication is easier and less expensive than using histidine tags,[22] biotin tags,[23] or specific antibody complexation.[24,25]

Herein, we designed and synthesized polymeric film to form several epitope-cavities to further explore the influence of the size and position of the epitope-cavities. Their ability to correctly detect the existence of anthrax PA_{83}, PA_{63}, and PA_{20} was demonstrated.

EXPERIMENTAL SECTION

(Boc-L-Cys)$_2$, acrylic acid, acrylamide, tyramine, and Boc-L-His were obtained from Sigma-Aldrich (St. Louis, MO). *N*-Benzylacrylamide was purchased from Lancaster (Lancashire, U.K.). The peptides derived from protective antigen (PA) of anthrax were synthesized by a peptide synthesizer (Discover SPPS) using microwave.[26] Acrylation of tyramine afforded *N*-Acr-tyramine, and (*N*-Acr-L-Cys-NHBn)$_2$ was synthesized from (Boc-L-Cys)$_2$.[27] The buffer used for all experiments was a phosphate buffered saline (PBS) (20 mM NaH_2PO_4, pH 7.0). The QCM was obtained from Tai-Tien Electronic Co. (Taipei, Taiwan) with a reproducibility of ±1 Hz. The QCM consisted of an 8 mm diameter disk made from an AT cut 10 MHz quartz crystal with gold electrodes (diameter, 4.2 mm) on both sides of the crystal. The protective antigens(PA_{83}) and its fragaments (PA_{63} and PA_{20}) used for the evaluation procedure was purified rPA obtained commercially from List Biological Laboratories (Campbell, CA) as a lyophilized preparation.

Synthesis of Acr-L-His-NHBn. Boc-L-His was esterified with methanol using papain as a catalyst to form Boc-L-His-OMe.[28] Then Boc-L-His-OMe was converted to Boc-L-His-NHBn with benzylamine in the presence of papain.[29] Deprotection of the Boc group with trifluoroacetic acid, followed by acrylation, furnished Acr-L-His-NHBn.

Preparation of Imprinted Polymer-Coated QCM. The QCM disks were immersed in a 10 mM solution of (*N*-Acr-L-Cys-NHBn)$_2$ in HPLC-grade acetonitrile for 16 h and then rinsed exhaustively with acetonitrile. Either acrylic acid or Acr-L-His-NHBn (55 μmol), acrylamide (55 μmol), *N*-benzylacrylamide, or *N*-Acr-tyramine (110 μmol), *N*,*N*′-ethylene bisacrylamide (220 μmol), and 3 μmol of peptide were mixed in 0.3 mL of solution (acetonitrile/20 mM, H_2O = 1:1). After depositing 3 μL of the aliquot 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 closed 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 mM 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 a HPLC pump (model L7110, Hitachi, flow rate = 0.1 mL min^{-1}), home-built flow cell, sample injection valve (model 1106, OMNIFIT), QCM sensor (ANT P-Sensor2000, Taiwan), and a personal computer. The polymer coated QCM was fixed between two O-rings and inserted into the flow-cell. Only one side of the QCM was in contact with the liquid. Sodium phosphate buffer (20 mM, pH 7.0) 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), were injected into the flow cell during circulation.

RESULTS AND DISCUSSION

PA_{83} contains many binding sites. The antigenic epitopes recognized by the response were distributed throughout the PA_{83}. Previously, an antigenic region (671−721) within domain 4 was recognized as the epitope of PA_{83} to bind cells while region 581−601 within domain 3 binds LA.[11,30] A more narrow peptide region, sequence 686−694, was reported to directly interact with the cell receptor.[31] A chymotrypsin-sensitive site (312−315) was also reported within domain 2.[32] Nevertheless, the antibodies were found to recognize determinants located at domain 1 (PA_{20}).[6,33] Sequence 680−692 was also indicated as the conformational epitope of PA_{83}.[7,34] To apply these results, a 12-mer peptide containing 683−694 of the protective antigen (DKLPLYISNPNY) was chosen as the template to generate epitope-cavity.

At first, *N*,*N*′-diBoc-L-cystine dibenzylamide was self-assembled onto the gold surface of a QCM chip. A solution, containing monomers, cross-linker, and the epitope-peptide was added onto the QCM chip, irradiated with UV to form polymeric thin films, and washed as previously described.[20]

Peptides−MIP Chips Interaction. The MIP-grafted peptide chips were then tested for their ability to rebind templates. *N*-Acryltyramine (ATA) was found to be more suitable than *N*-benzylacrylamide due to its solubility and for providing an acidic recognition site for analyte. As shown in Table 1, ATA raised the sensitivity of the sensor.

To further improve the MIP fabrication, it is also necessary to produce a basic, preorganized region for binding with the acidic component of templates. The chiral amino acid derivative Acr-L-His-NHBn (AHB) was then introduced as a basic monomer. As shown in Table 1, it helped to increase specific binding; higher affinity for the analyte at a lower concentration was observed. Meanwhile, adding cross-linker formed more rigid MIPs and resulted in a more stable and durable QCM chip.

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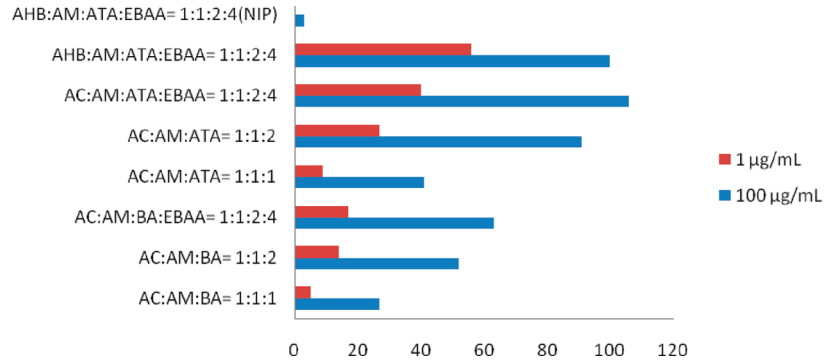
Table 1. Formula Effect on the MIP-QCM Chip Frequency Shifts[a]



[a] Abbreviations used: AC, acrylic acid; AM, acrylamide; BA, *N*-benzylacrylamide; ATA, *N*-acryltyramine; EBAA, ethylene bisacrylamide; AHB, *N*-acryl-L-histidine benzylamide.

Table 2. Amino Acid Sequences for Epitope-Peptides As a Template

QCM chip	sequence no.	epitope sequence	region[8]	CD structure[a]
PA_{71-79} chip	71−79[36]	VKKSDEYTF	$1\beta_5$	type I β-turn
$PA_{659-672}$ chip	659−672[36]	RYDMLNISSLRQDG	$4\beta_7$	random-coil
$PA_{681-694}$ chip	681−694[34]	YNDKLPLYISNPNY	$4\beta_8$> <$4\beta_9$	random-coil
$PA_{683-694}$ chip	683−694[31]	DKLPLYISNPNY	$4\beta_8$> <$4\beta_9$	random-coil
$PA_{713-722}$ chip	713−722[36]	NGDTSTNGIK	$4\beta_9$> <$4\beta_{10}$	random-coil

[a] CD: Circular dichroism.

Table 3. Comparison of the Affinities (K_d) of Epitope-Peptides to Their QCM Chip[a]

QCM chip	analyte	K_d [nM]	no. of residue	p*I*[37]	M_w [Da]
PA_{71-79} chip	VKKSDEYTF	10	9-mer	6.01	1115.55
$PA_{659-672}$ chip	RYDMLNISSLRQDG	5	14-mer	5.57	1666.81
$PA_{681-694}$ chip	YNDKLPLYISNPNY	3	14-mer	5.45	1712.84
$PA_{683-694}$ chip	DKLPLYISNPNY	7	12-mer	5.88	1435.73
$PA_{713-722}$ chip	NGDTSTNGIK	20	10-mer	5.64	1005.47

[a] AHB/AM/ATA/EBAA = 1:1:2:4.

The p*I* of PA_{83} from Sterne was previously reported as 5.5 from protein-stained horizontal isoelectric focusing (IEF) gels.[35] It is necessary to adjust the epitope-peptide to a similar p*I* value to obtain the epitope-cavity with oppositely charged infrastructures. Since the protective antigen (PA_{83}) is cleaved by furin to PA_{20} and PA_{63}, different epitope-peptides of PA_{20} and PA_{63} were used to construct epitope-cavities for screening these fragments. As a confirmation, four other peptides belonging to different epitope sites of PA_{83} were chosen as the template.[34,36] As shown in Table 2, the selective epitope-peptides were also adjusted to a p*I* point near 5.5. The synthesized peptides served as the templates to fabricate MIPs for evaluation.

From the binding experiments, the polymers imprinted with an epitope-peptide efficiently recognized the template. The frequency shifts of these QCM chips were showing a tendency of saturation in all cases near the concentration of 500 μg/mL.

The data obtained were plotted with the saturation equation for specific binding[16] ($B = (B_{max}c)/(K_d + c)$, $B = H/M_w$, where c is the concentration of protein, B is the fraction of sites bound, M_w is the molecular weight of the analyte, and H is the frequency shifts in the QCM).

The affinities of the peptides to their MIPs were thus measured and shown in Table 3. It was found that the $PA_{683-694}$ chip had a higher K_d value than the $PA_{681-694}$ chip. As the number of residues was longer, the K_d value was smaller.

Therefore, at the same epitope position and with the similar p*I* point, the greater the number of peptide residues in the template and the better was the observed binding effect. For the 14-mer peptide, the dissociation constant was 3 and 5 nM ($PA_{659-672}$ chip): better affinity than the 12-mer, 10-mer, and 9-mer peptide.

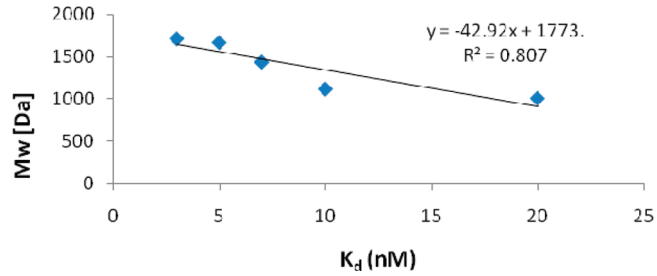


Figure 1. Template molecular weight effect on the MIP-QCM chip frequency shifts.

However, comparison of these epitope-cavities, Figure 1, shows the affinities of the peptide to MIPs were more closely related to the molecular weight of the analyte instead of the number of residues. For example, the 9-mer had a better cavity than the 10-mer in our case. It was also demonstrated that a heavy weight 14-mer ($PA_{681-694}$ chip) was a better selection than the lighter one ($PA_{659-672}$ chip). Apparently, the weight of the epitope-peptide, p*I* point, solubility, conformation, and its charge distribution affected the imprinting efficiency. Perhaps, there is a tendency for a large peptide to maintain a more stable conformation during the polymerization process.

Proteins−MIP Chips Interaction. The MIP-grafted peptide chips were then tested for their ability to bind their mother protein

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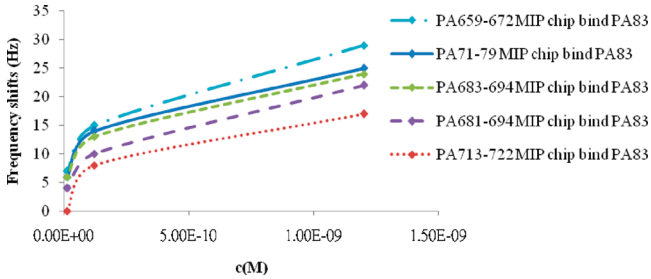

Figure 2. Comparison of the binding effects of PA_{83} to epitope-cavity.

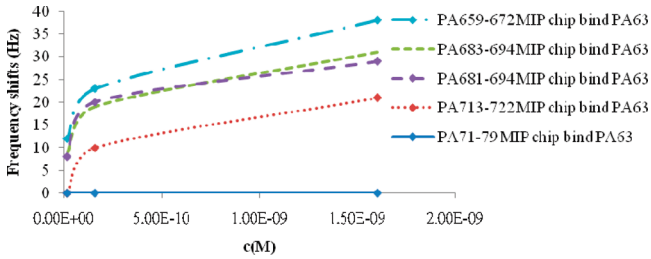

Figure 3. Comparison of the binding effects of PA_{63} to epitope-cavity.

Table 4. Comparison of the Affinities (K_d) of PA_{83}, PA_{20}, and PA_{63} to QCM Chips

	protein		
QCM Chip	PA_{83} [pM]	PA_{63} [pM]	PA_{20} [pM]
PA_{71-79} chip	20		100
$PA_{659-672}$ chip	10	10	
$PA_{683-694}$ chip	20	20	
$PA_{681-694}$ chip	30	30	
$PA_{713-722}$ chip	200	200	

(PA_{83}) and its fragment proteins PA_{20} and PA_{63}. As shown in Figure 2, all the epitope-cavities efficiently recognized the protein PA_{83}.

The frequency response of the PA_{63} on each epitope-cavity binding is shown in Figure 3. All the epitope-cavities except for the PA_{71-79} chip efficiently recognized the protein PA_{63} that possessed the same epitope part of the structure.

On the contrary, it was found that PA_{20} was only bound to the PA_{71-79} chip. The adsorption of PA_{20} to other epitope-cavities was almost undetectable. Therefore, each chip remarkably recognized the protective antigen specifically adsorbed at the different regions. The complexation thus induces the protein to possibly settle in certain orientations using the designed epitope-cavity.

From the affinity measurements shown in Table 4, PA_{83} exhibited slightly lower K_d values than PA_{63}. The differences were probably resulting from the conformational change of the epitope region and the mass difference. The size of PA_{83} is larger, and they must occupy a larger area on the surface. Once the protein−MIPs interaction occurred, the size and the shape of the PA_{83} prevents the further binding of other PA_{83} to the MIP-chip. Moreover, the molecular weight of the PA_{83} is larger; thus, they are actually less concentrated. In fact, because of the aggregation occurring on the chip at the higher concentration of the antigen, there are less available binding sites for PA_{83}. Several proteins (pepsin, trypsin, and H-albumin) were checked, and no cross-reactivity was observed.

All K_d values were in the picomolar range, indicating a strong polymer−protein interaction. From the chip point of view, the $PA_{659-672}$ chip had the lowest K_d values. Surprisingly, the $PA_{683-694}$ chip performed better than the $PA_{681-694}$ chip, which is inconsistent with our peptide detection in Figure 2. It seems this result correlates with a previous report that sequence 686−694 was the epitope to interact with the cell receptor,[30] and sequence 680−692 was a linear conformational epitope.[7,34]

CONCLUSIONS

We demonstrated the ability to detect the protective antigen of anthrax using MIP-QCM. It provides a convenient in vitro assay for quantitatively recognizing proteins at the nanogram scale within 30 min. High affinity toward template molecules and target proteins were observed at the picomolar range. This technical platform can also be used as a rapid and specific immunoassay to detect other bacterial antigens and various protein toxins.

Our results also suggest certain advantages of these complex epitope-cavities. First, it is normally applicable to apply linear peptides on the surface to generate molecularly imprinted epitope-cavities. Second, we were able to employ functionalized epitope-cavities in the screening of antigen proteins and effectively catch the antigen in its epitope-cavity. Finally, these fabricated epitope-cavities on a surface conveniently provide information to indicate attached peptides' and proteins' conformation and orientation. These epitope-cavities could also be used for epitope-mapping and identifying conformationally relevant antigen proteins, therefore providing a unique tool to trace proteomics and biomarker assembly.

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