Discrimination of Peptides by Using a Molecularly Imprinted Piezoelectric Biosensor

Chung-Yin Lin,[a] Dar-Fu Tai,*[a] and Tzong-Zeng Wu[b]

Abstract: Based on the direct formation of a molecularly imprinted polymer on gold electrodes, we have developed a peptide sensor for the detection of low-molecular-weight peptides. A new cross-linking monomer, (N-Acr-L-Cys-NHBn)$_2$, was employed to attach the surface of the chip and to copolymerize with other monomers. Interestingly, N-benzylacrylamide participates in the polymerization and recognition is carried out in an aqueous environment. By using quartz crystal microbalance detection, short peptides can be monitored by their interaction with plastic antibodies specific for the target peptides. The selectivity of molecularly imprinted polymers and the sensitivity of such artificial biosensors have been combined to differentiate between traces of oxytocin and vasopressin to the ng mL^{-1} scale.

Keywords: imprinting · molecular recognition · peptides · polymerization · sensors

Introduction

Molecular imprinting (MIP)[1–4] is a process for synthesizing organic polymers that contain recognition sites for small molecules. The imprinting process consists of a template molecule that organizes functional and cross-linking polymerizable monomers during the polymerization process. The template is extracted from the insoluble network material leaving behind domains that are complementary in size, shape, and functional group orientation to the template molecule. The preparation of molecularly imprinted polymers as the stationary phase for selective separation of amino acids and small peptides is known.[5] Some of these systems have utilized protected peptides in organic solvents.[6–8] These formulations employ free-radical polymerization and rely on the use of hydrogen-bonding interactions between the template and functional monomers as the selectivity-providing interaction.[9]

Direct detection of a peptide–macromolecule interaction is rare and is currently under investigation. The preparation of artificial binding sites for such peptides may provide insight into recognition processes. Examples include sensing of enkephalins,[10] tripeptides,[8] helical peptides,[11] and oxytocin and its derivatives[12, 13] by imprinted macromolecular receptor. Recent progress was the recognition of His peptides[14] by using peptide–metal interactions. These artificial receptors may also facilitate the screening of peptide mixtures and proteins, or assist in the evaluation of peptidomimetics that can be used to either enhance or inhibit receptor responses.

Our strategy for creating peptide receptors using molecular imprinting takes advantage of quartz crystal microbalance (QCM).[15–17] The QCM is a kind of bulk-acoustic wave (BAW) resonator, as derived by Saurbrey.[18] In 1980, Konash and Bastiaans[19] developed a QCM apparatus fixed between two spacers that allowed the liquid to flow through from side to the other in contact with air. This permits the oscillation to occur in the liquid and also the measurement of the liquid with the QCM. Due to the high sensitivity, simple operation, easy interpretation, and "real-time" measurement, QCM allows the label-free detection of molecules with applications to the study of kinetics,[20, 21] peptide binding to immobilized oligonucleotides,[11, 22] protein binding to immobilized receptors,[23] medical diagnosis,[24, 25] the detection of pathogenic microorganisms,[26] and other molecular discrimination events. An MIP-QCM sensor has been reported for the detection of (*S*)-propanolol[27] and terpenes[28] in organic solvent.

We now report protocols for molecular imprinting that create macromolecular receptors for small peptides. Oxytocin is a nonapeptide that is synthesized in hypothalamic neurons and transported down axons of the posterior pituitary for secretion into blood. We chose oxytocin and another nonapeptide vasopressin as the template target for capturing molecular imprint sites. The availability of the water soluble

[a] Prof. D.-F. Tai, C.-Y. Lin
Department of Chemistry
National Dong-Hwa University, Hualien (Taiwan)
Fax: (+886) 3-8663570
E-mail: dftai@mail.ndhu.edu.tw

[b] Prof. T.-Z. Wu
Institute of Biotechnology
National Dong-Hwa University, Hualien (Taiwan)

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form of both peptides was an advantage that could be used to establish the specificity of the interaction.

Results and discussions

To investigate the interaction of molecular imprinting to discriminate the target peptides vasopressin and oxytocin, we chose three other peptides for comparison (Figure 1).

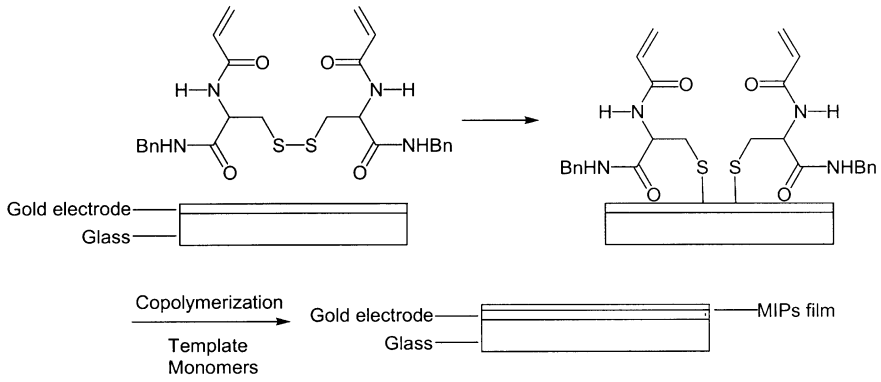
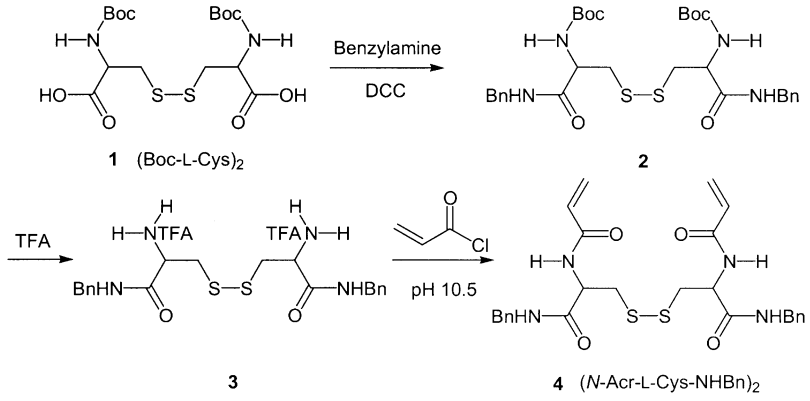


Figure 2. Schematic representation of the peptide imprinting process.

A new cross-linking monomer in neutral form, containing a chiral center as well as disulfide bond was designed and prepared. As shown in Scheme 1, synthesis of (*N*-Acr-L-Cys-NHBn)$_2$ is straightforward with a total yield of 50% from *N*,*N*′-diBoc-L-cystine ((Boc-L-Cys)$_2$).

Angiotension II	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Oxytocin	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2
Vasopressin	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2
15-mer	Thr-Glu-Leu-Arg-Tyr-Ser-^{90}Trp-Lys-Thr-Trp-Gly-Lys^{95}-Ala-Lys-Met

Figure 1. Amino acid sequence of the tested peptides. The 15-mer peptide contains the 90 – 95 sequence of Japanese encephalitis virus non-structureal protein 1.[29]



Scheme 1.

Figure 2 illustrates our strategy for preparing highly cross-linked polyacrylamides containing binding sites that incorporate a (*N*-Acr-L-Cys-NHBn)$_2$-Au complex. The QCM employed in this work consisted of a disk of crystalline quartz with gold electrodes on the upper and lower surfaces. The use of water in the polymer synthesis and recognition steps has evident over organic systems. Although protic solvents such as alcohols and water are compatible with free-radical polymerization, they have been largely excluded from use in imprinting due to their abilities to compete with hydrogen-bonding interactions. However, as lack of solubility of peptides in organic media and more subtle effects such as peptide conformation, a water/acetonitrile mixture was made the solvent of choice.

The polymerizable (*N*-Acr-L-Cys-NHBn)$_2$-Au complex was prepared by combining aqueous solutions of (*N*-Acr-L-Cys-NHBn)$_2$ on 4.5 mm diameter gold electrodes.[30, 31] The disulfide functional group was used as a "glue" to attach (*N*-Acr-L-Cys-NHBn)$_2$ to the electrode; this functioned as an asymmetric molecule to provide chirality[32] to the QCM surface, and a cross-linker to copolymerize with other monomers. The benzylamide of (*N*-Acr-L-Cys-NHBn)$_2$ also prevented displacement of the polymer by self-assembly of *N*-benzylacrylamide (BAA) or a template to form a hydrophobic layer. All the monomers and cross-linkers were thus attached to the surface to formulate MIPs in a more organized manner after copolymerization.

To avoid imbedding too great amounts of the template, copolymerization of the (*N*-Acr-L-Cys-NHBn)$_2$-Au complex was carried out without adding other cross-linking monomer. The polymerization complex was then formed by irradiation with BAA, acrylic acid, acrylamide (at a ratio of 2:1:1), and the template in a water/acetonitrile mixture (water/acetonitrile = 1:1). The polymer, which was formed as a thin film, was washed with phosphate buffer (20 mM, pH 3 – 4) to remove 70 to 80% of template, followed by washing with acetonitrile and drying. The frequency shifted to -750 ± 44 Hz after coating with (*N*-Acr-L-Cys-NHBn)$_2$ and shifted further to -3400 ± 800 Hz after copolymerization. It shifted back to 300 ± 50 Hz after the removal of the template. The thickness of the polymer films were measured as 92 ± 15 nm.

The peptide recognition sites were formed by incorporating two types of interactions, which are established during the polymerization. One consists of ionic binding between acrylic

acid and the N terminus of the peptide. This binding is compromised by water or other protic solvents. The second bonding frame is composed of multiple weaker interactions between the network polymer chains and the imprinting peptide molecule. We find that the hydrophobic interactions between the peptide and *N*-benzylacrylamide are very important. Without *N*-benzylacrylamide, the polymer matrices that are developed during the polymerization are not sufficient to provide sequence selectivity between the imprinted peptide and other amino acid sequences. Compared to only one equivalent of *N*-benzylacrylamide, the frequency shifts were larger as the monomer ratio increases to 2:1:1 (Figure 3).

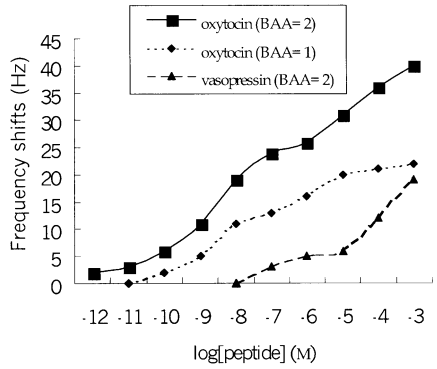


Figure 3. The frequency changes of oxytocin and vasopressin obtained by using oxytocin-imprinted QCM.

Binding studies were performed to evaluate uptake of the template and non-template peptides. Aqueous solutions (PBS, pH 7) were allowed to flow through the system. After equilibration, aqueous solutions of the tested peptide were injected and the change of frequency was measured by QCM. Binding isotherms were obtained for the template peptide (oxytocin) (Figure 3) as well as vasopressin (Figure 4).

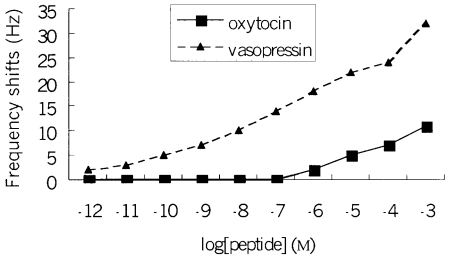


Figure 4. The frequency changes of oxytocin and vasopressin obtained by using vasopressin-imprinted QCM.

As shown in Figures 3 and 4, the adsorption of non-template peptides was not observed until the concentration of other peptides reached 1 ng mL^{-1}. The frequency shifts of three other peptides, angiotensin II, bradykinin, and 15-mer peptide were compared in the same concentration. No trace was detected at 1 ng mL^{-1}. However, nonspecific adsorption of these peptides began to be visible when the concentration reached the level of 1 ng mL^{-1}. To clearly demonstrate the binding abilities of MIPs, B_{max} is set as the maximun frequency shift observed and B is the frequency shift obtained at the indicated concentration of peptide.[33] The data obtained are plotted in Figures 5 and 6. Thus, K_d values were calculated

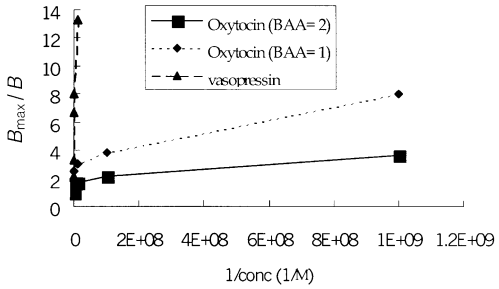


Figure 5. The binding effects of oxytocin-imprinted QCM.

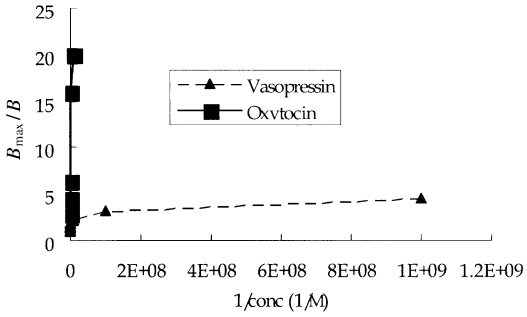


Figure 6. The binding effects of vasopressin-imprinted QCM.

from the slope of curves. The best MIP K_d value for oxytocin was about 1.1×10^{-8} M (Figure 5). The best MIP K_d value for vasopressin was about 2.0×10^{-8} M (Figure 6). In general, MIP demonstrated a marked 10–100 times enhancement in K_d value toward the template peptides relative to their non-specific adsorptions to non-template peptide.

In conclusion, we present data that show it is possible to directly and sensitively discriminate peptides by using a combination of molecular imprinting and QCM. Interestingly, *N*-benzylacrylamide participates both polymerization and recognition is carried out in an aqueous environment. Thus, we have developed protocols for creating macromolecular receptors for peptides using molecular imprinting. This system may be helpful in understanding the modes of peptide recognition processes.[32] They may also find use as artificial sensors for screening of peptides and peptidomimetics.

Experimental protocol

(Boc-L-Cys)$_2$, acrylic acid, acrylamide, oxytocin, angiotensin II, bradykinin and vasopressin, were obtained from Sigma-Aldrich (St. Louis, MO). *N*-Benzylacrylamide was purchased from Lancaster (Lancashire, UK). The 15-mer peptide derived from nonstructural protein 1 of Japanese encephalitis virus[29] was synthesized by a peptide synthesizer. The buffer used for all experiments was 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 9 MHz quartz crystal with a gold electrodes on both sides (diameter: 4.5 mm, area: 15.9 mm^2) of the crystal.

Preparation of imprinted polymer-coated QCM: The QCM disks were immersed in a 10 μM solution of (*N*-Acr-L-Cys-NHBn)$_2$ in HPLC-grade

acetonitrile for 16 h; they were then rinsed exhaustively with acetonitrile and dried under vacuum. A solution of acrylic acid (55 μmol), acrylamide (55 μmol), *N*-benzylacrylamide (110 μmol), and template (3 μmol, oxytocin or vasopressin) were mixed in mixture of acetonitrile/water (1:1; 0.3 mL). After depositing a 4 μL 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 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 phosphate buffer (20 mM, pH 3–4) to remove the template. This was followed by washing with methanol and drying. The thickness of the films were measured using a surface profiler from Veeco Inc. (Dekatak^3 ST).

Biosensor system: The flow injection system was compose of an HPLC pump (Model L7110, Hitachi, flow rate = 0.1 mL min^{-1}), home-built flow cell, a sample injection valve (Model 1106, OMNIFIT), a home-built oscillation circuit (including oscillator and frequency counter), 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. PBS was used for circulating, washing and testing. The specificity of the MIP-grafted QCM was evaluated by injecting 100 μL of oxytocin or vasopressin solutions at different concentrations.

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