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A NOBLE MOLECULAR IMPRINT POLYMER BIOSENSOR FOR CAFFEIC ACID DETECTION IN *ORTHOSIPHON STAMINEUS* EXTRACTS

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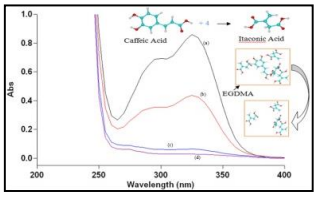
Graphical abstract



Abstract

A molecular imprint polymer (MIP) biosensor has been developed to determine caffeic acid in misai kucing (*Orthosiphon stamineus*) samples. The simulation of HyperChem 8.0 software gave a suitable template and functional monomer ratio for the MIP preparation. The MIPs were prepared by non-covalent bulk polymer approach. The analytical performance of MIP and NIP studies were based on the frequency change of mass sensitive quartz crystal microbalance sensor. The MIP biosensor showed good sensitivity to caffeic acid from 1.5 ng/ml - 12.5 ng/ml with a R^2 value of 0.98 whereas NIP sensor showed very low response. The caffeic acid in *O. stamineus* extract and two commercial products were quantified using the MIP biosensor.

Keywords: Molecular Imprint Polymer (MIP); Quartz Crystal Microbalance (QCM), caffeic acid

Abstrak

Satu salinan polimer molekul (MIP) biosensor telah dibangunkan untuk menentukan asid caffeic dalam misai kucing (Orthosiphon stamineus) sampel. Simulasi perisian HyperChem 8.0 memberikan template yang sesuai dan nisbah monomer berfungsi untuk penyediaan MIP itu. MIPS yang telah disediakan oleh pendekatan polimer pukal bukan kovalen. Prestasi analisis MIP dan kajian NIP adalah berdasarkan perubahan frekuensi jisim sensitif kristal kuarza microbalance sensor. The biosensor MIP menunjukkan sensitiviti yang baik untuk asid caffeic daripada 1.5 ng / ml - 12.5 ng / ml dengan nilai R^2 0.98 manakala NIP sensor menunjukkan sambutan yang amat rendah. Asid caffeic dalam ekstrak *O. stamineus* dan dua produk komersial dikuantifikasikan menggunakan biosensor MIP itu.

Kata kunci: Molekul Imprint Polimer (MIP); Quartz Crystal Microbalance (QCM), asid kafeik

1.0 INTRODUCTION

The use of herbal products is wide spread at the present time. They have vast therapeutic properties to maintain good health and their consumption have minimal side effects. *O. stamineus* or Java Tea (*Orthosiphon stamineus* Benth) is a medicinal herb found in the shrub of Southeast Asia. It contains more than 20 phenolic compounds, together with nine caffeic acid derivatives, such as rosmarinic acid (RA) and 2,3-dicaffeoyltartaric acid, two flavonol glycosides and nine lipophilic flavones[1]. The caffeic

acid derivatives are recognized as one of the major contributors in *O. stamineus*[2]. It has broad spectrum of biological and pharmacological activities in human health including anti-inflammatory, antioxidant and immune-modulatory effects[3,4].

A suitable quality control method is required to check the authenticity, quality and purity of herbal products to use their full potential[5]. Therefore, an analytical device is essential to detect and quantify bioactive compounds in the medicinal plants and their products.

Molecularly Imprinted Polymer (MIP) is an affinity material for biosensors, antibodies, absorbents and solid phase extraction[6]. MIP is a design of polymeric matrices with a predetermined selectivity and specificity for a given template, which can be used as a complimentary to biosensor[7]. It is a broad technology, which set up detection properties into synthetic polymers as a biosensor[8]. MIP forms a complex of functional monomers with template by covalent or non-covalent interactions and are then bonded by cross linker. Artificial binding sites are exposed which is similar in shape, size and position of the template molecule.

The molecular sensing technology is based on the frequency changes of the quartz crystal vibration when template molecules interact on the sensor surface. MIP-QCM instrument feature a sensing film immobilized on the surface of quartz crystal which develop the sensitivity of the fluctuating frequency of the crystal up to nanogram level of mass change[9]. The MIP-QCM biosensor possesses several advantages over the conventional equipments such as fluorescence polarization immunoassays (FPIA) and infrared spectroscopy (FT-NIR).

The popular medicinal plant, *O. stamineus* contains various medicinal compounds including caffeic acid. The present research MIP biosensor would be a promising analytical device to detect the caffeic acid in the target samples.

2.0 EXPERIMENTAL

2.1 Chemicals and Materials

Caffeic acid, azobisisobutyronitrile (AIBN), ethanol (absolute) and allyl mercaptan were purchased from Aldrich Chem. Co. (Milwaukee, WI, USA). EGDMA was obtained from Fluka A.G. (Buchs, Switzerland). All other chemicals were of analytical reagent grade and purchased from MerckAG (Darmstadt, Germany). Water used in experiments was obtained from Zeneer PowerII water purification system.

2.2 Instruments

An AT-cut gold/Cr polished 5 MHz quartz crystals and a quartz crystal analyzer (QCM 200) purchased from Standford Research Systems were used to perform micro gravimetric measurements. Saurbrey's equation relates the shear mode of QCM and mass change

$$\Delta f = -\frac{2f_0^2}{A\sqrt{\rho_q \mu_q}}\Delta m \quad (1)$$

where Δf is the measured frequency shift due to the added mass in Hertz, f_0 is the fundamental oscillation frequency of the dry crystal, Δm is the mass loading on crystal surface in grams, p_q is the density of quartz (2.648 g/cm^3), μ_q is the shear modulus (2.947x10^{11} g/cm.s^2), and A is the electrode area (0.197 cm^2).

2.3 Optimization of Geometries and Binding Energies

The structures of carboxyl groups in methacrylic acid (MAA), itaconic (IA) and caffeic acid (CA) were optimized based on molecular mechanics and semi-empirical calculations using the HyperChem 8.0 molecular visualization and simulation program. Initial molecular geometries were optimized with the PM3 semi-empirical calculations. Single point calculations were used to get lowest energy and to determine the total energies. The optimized structures of MAA and CA were placed with a starting inter-planar distance of 2.3 Å and the angle made by hydrogen bonds. Further, the geometries were optimized based on PM3 semi-empirical calculations with Polak-Ribiere routine and root mean square (RMS) gradient of 0.01 Kcal/Å.mol as the termination condition[10,11].

2.4 Preparation of MIP and NIP

Based on the optimization data, the MIP has been synthesized in order of two stages.

(i) Polymerization: Caffeic acid and itaconic acid (IA) was mixed for the complex formation between a template and a functional monomer. Azobisisobutyronitrile (AIBN) (10 mg) was added as initiator. EGDMA was added to the mixture and stirred for 5 min at 800 rpm[7]. The mixture was then purged with nitrogen gas and sonicated for 3 min to remove any oxygen content. Non-imprinted polymer (NIP), for control purpose, was also prepared and treated in the similar manner as MIP without caffeic acid. The MIPs are grounded into small particles in a ball mill and entered into the sieving machine to get microsized particles below 38 µm diameter.

(ii) Template Removal: Before the MIP was coated on QCM surface, the template molecules were removed from the polymer. Soxhlet extractor has been used to remove template from MIP with methanol and acetic acid (9:1 v/v) solvent. Acetic acid act as modifier as it can enhance the elution strength of solvents during desorption studies[12].

2.5 Pretreatment of Quartz Crystal and Immobilization of MIPs

Figure 1. shows the binding of carboxyl group and alcohols to the gold through 2-aminoethanethiol and glutaraldehyde, leaving the functionality exposed at the upper surface of the monolayer. The 2-

aminoethanethiol coordinates as self-assembled monolayer on gold surface in the form of two dimensional crystalline lattices. Dip coating was found suitable and easier compared to other methods. 5 mg of MIP powder mixed in 50 ml THF solvent. The pre-treated gold quartz crystal was dipped slowly into the MIP solution until no bubbles were observed. After 30 sec, the crystals taken out and dried in room temperature. A thin film was seen on the surface of the crystal. The crystal was washed with deionized distilled water.

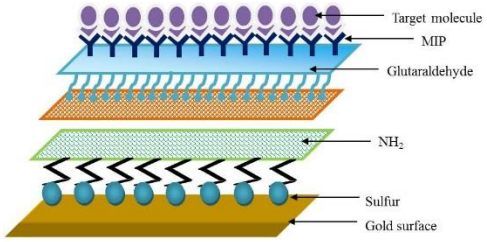


Figure 1 Self-assemble monolayer of glutaraldehyde and thiol on gold surface

2.6 MIP-QCM Measurement

Methanol:acetic acid (9:1) were used as washing solution for each repetition. The pre-treated gold quartz crystal was connected to the QCM device. One ml of caffeic acid solution was dropped on the crystal in each case. The crystals subjected to several washing with methanol:acetic acid (9:1) solution in between each measurements. The QCM frequency recorded for 25 mins.

3.0 RESULTS AND DISCUSSION

3.1 Optimization of MIP

According to Chen *et. al.*[13], H-bonds forms when a hydrogen atom is positioned between two electronegative atoms. The formation of an H-bond may polarize a donor to enhance its stability. Strong H-bonds have significant covalent characteristics. Therefore, it is favourable to have normal binding energy between 3-5 kcal/mol. From the Figure 2, IA was chosen as the suitable monomer for caffeic acid MIP preparation at the ratio of 4:1.

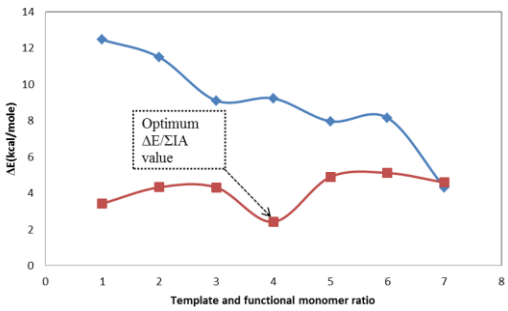


Figure 2 Binding interaction energies for methacrylic acid (◆) and itaconic acid (■)

The highest complex energy ($E_{complex}$) was chosen to estimate and to calculate the binding energy difference between template and functional monomer. However, the average hydrogen bonds energy required to get the stable complex since the hydrogen bond gives high stability and selectivity with MAA and IA[14].

According to Mulliken charges calculation[15] which optimizes using PM3 semi-empirical method the interaction energies (ΔE) was calculated from following equation:

$$\Delta E = E_{(template–monomer\ complex)} - [E_{(template)} + nE_{(monomer)}] \quad (2)$$

Where,

$E_{(binding)}$ = Energy of MAA and IA to bind with Caffeic Acid

$E_{(complex)}$ = Energy after optimization

$\Sigma E_{(monomer)}$ = Energy of monomer ration (1,2,3,4,5)

3.2 Extraction Efficiency

Figure 3 shows the conjugation process of template removal using UV-vis spectrophotometic analysis. The sample solution (pure caffeic acid, MIP before template removal, MIP after template removal and NIP) were prepared at 0.001 mg/ml. An adsorption peak at 328 nm was observed for pure caffeic acid sample (Figure 3. (a)). When the pure caffeic acid was interacted with itaconic acid, the peak did not display the absorption peak of caffeic acid (Figure 3. (c)). The result indicates that the free caffeic acid bonded to the monomers. However, the shape of the peak shows the presence of caffeic acid in MIP (before extraction). On the contrary, an obvious peak was obtained after the MIP was extracted with methanol-acetic acid solution (Figure 3. (b)). The reduction of absorption peak of MIP (after extraction), concludes that almost 99% of the template removed from MIP polymer. Another strategy is the peak of MIP (after extrcation) shows similar absorbance to NIP (Figure 3. (d)), which do not contains caffeic acid.

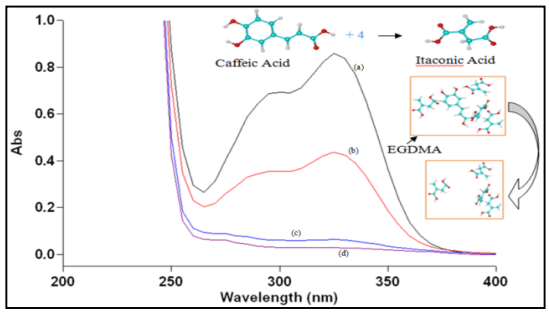


Figure 3 UV–vis absorption spectroscopy of (a) NIP (b) MIP after extraction (c) MIP before extraction, and (d) pure caffeic acid

3.3 Evaluation of Caffeic Acid MIP Biosensor

The caffeic acid imprinted biosensor was washed with 0.01% methanol:acetic acid (9:1) until the frequency reaches consistent value F_0. The response frequencies for different concentrations caffeic acid were measured using the biosensor. The frequency shift for each concentration calculated using equation $\Delta f = F_0 - F$. The frequency changes due to the adsorption of caffeic acid on MIP and NIP sensors are shown in Figure 4. The NIP sensor response is low because caffeic acid does not bind on NIP surface.

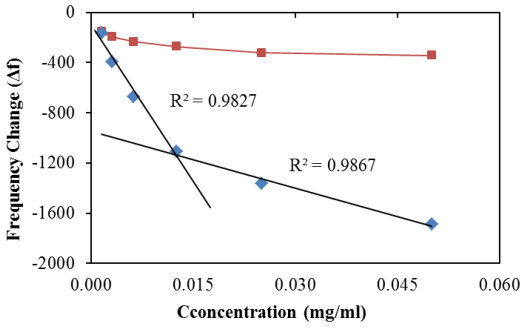


Figure 4 QCM response of the caffeic acid imprinted polymer (MIP) (◆) and non-imprinted (NIP) (■) biosensor

The MIP line shows the frequency decrease as the concentration increase due to the increase in mass on quartz crystal. When the cavities of MIP on quartz crystal filled with caffeic acid, the mass on the quarts surface does not increase. In this case the frequency change rate is reduced. The rebinding assay showed a convergence due to saturation of empty cavity of MIP with caffeic acid. The existence of convergence line in MIP response is due to this phenomenon. The R^2 values before and after the convergence are 0.9827 and 0.9867, respectively. The mass changes calculated based on Sauerbrey equation. The detection limit of caffeic acid was found 585ng/cm^2.

3.4 Sensor Response on Natural and Commercial Products

The content of caffeic acid in different concentrations of *O. stamineus* extract and commercial tea samples were measured by the sensor. The binding of caffeic acid on imprinted MIP
causes a mass change that is reflected in frequency change in Figure 5. The similar plot of the frequency changes proves that caffeic acid imprinted MIP biosensor is able to detect caffeic acid in *O. stamineus*.

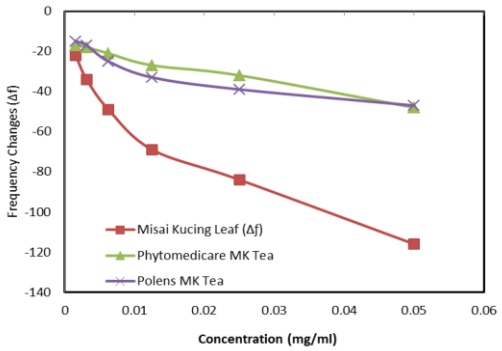


Figure 5 Caffeic acid detection in various samples

4.0 CONCLUSION

A bio-mimicking sensor has been developed to detect caffeic acid in *O. stamineus* and its samples. A computational approach was used to predict a suitable monomer and template-monomer ratio using HyperChem 8.0 software. Itaconic acid was chosen as the suitable monomer at ratio 1:4 with maximum binding energy. The caffeic acid MIP-QCM exhibited higher sensitivity to pure caffeic acid, with R^2 value 0.9827, compared to NIP-QCM. The frequency changes in MIP and NIP clarified that caffeic acid MIP detects only the target molecule (caffeic acid).

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