Detection of Aspartame via **Micro**sphere-Patterned and Molecularly Imprinted Polymer Arrays

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ABSTRACT

A colloidal sphere-patterned polyterthiophene thin film sensor with high binding affinity and selectivity towards aspartame was fabricated using a technique combining molecular imprinting and colloidal sphere lithography (CSL). The successful imprinting of aspartame into electropolymerized molecularly imprinted polymer generated artificial recognition sites capable of rebinding aspartame into the microporous film, which was sensitively detected using quartz crystal microbalance measurements. The resulting sensor exhibited a good linear response after exposure to aspartame concentrations ranging from 12.5 μM to 200 μM and limit of detection of ~ 31 μM . It also demonstrated a high selectivity towards aspartame as compared to other peptide-based analogs including alanine-phenylalanine (Ala-Phe), alanine-glutamine (Ala-Gln), glycylglycine (Gly-Gly), and arginylglycylaspartic acid (RGD). The formation of the highly ordered micro-patterned surface was induced and monitored *in situ* by electrochemical quartz crystal microbalance and atomic force microscopy. Analyte imprinting and removal were characterized using X-ray photoelectron spectroscopy. Based on molecular modeling (semi-empirical AM1 quantum calculations), the formation of a stable pre-polymerization complex due to the strong hydrogen bonding interactions between the terthiophene monomer and aspartame played a key role in the effective aspartame imprinting and detection.

KEYWORDS: aspartame, molecular imprinting, conducting polymer, electropolymerization, colloidal sphere lithography, polythiophene, quartz crystal microbalance, electrochemical quartz crystal microbalance

INTRODUCTION

Despite widespread consumption, the controversy surrounding aspartame persists due to adverse health risk reports [1]. Concern over this issue continues to heighten as it led *PepsiCo Inc.*, one of the two biggest beverage companies in the world, to succumb under consumer pressure and completely exclude aspartame in its diet soda recipe beginning August 2015 [2]. Since its accidental discovery in 1969 [3], the non-carbohydrate-based aspartame (APM) gained massive attention because of its highly potent sweetness that is 200 times more than sucrose [4]. Consequently, fewer intakes are required to achieve the same effect, which is particularly attractive for weight control, body sugar management and even dental cavity prevention [5,6]. For most artificial sweeteners, the disparity in taste is inevitable but aspartame's sweetness closely resembles that of sucrose but longer lasting, thus making aspartame a leading option [7]. However, upon ingestion, aspartame undergoes hydrolysis to form methanol after absorption in the intestinal lumen [1]; the remaining dipeptide is completely metabolized to form amino acid isolates L-aspartate and L-phenylalanine at the mucosal surface and absorbed by the body [8]. According to previous reports, the amount of produced methanol, which eventually converts to formaldehyde and then to formic acid for both human and rat experiments [9], is related to increased carcinogenicity of aspartame [4,10,11]. On the other hand, phenylalanine are reported to be neurotoxic and is capable of severely altering the concentrations of important inhibitory catecholamine neurotransmitters including norepinephrine, epinephrine, and dopamine within certain regions in the brain [1,12,13]. In addition, cases of memory loss, headaches, insomnia and even mental disorders have been macroscopically related to aspartame intake [12,14,15]. Hence, these findings provide the strong impetus in designing novel sensitive and selective aspartame detection systems for consumer protection.

Most widely-used detection protocols for aspartame employ capillary electrophoreses [16–18] and high performance liquid chromatography (HPLC) techniques [19]; however, these techniques require highly sophisticated equipment and time-consuming preparatory and pre-treatment steps [20]. Other reports involve the co-immobilization of enzymes such as alcohol oxidase (AOX) and carboxyl esterase (CaE) on screen printed electrodes using various crosslinking chemistries [20–22]. When the analyte reaches the functionalized electrode, the CaE hydrolyzes the methyl ester group to produce methanol leaving the dipeptide group. AOX oxidizes the methanol to produce hydrogen peroxide, which can be quantified electrochemically. Due to high cost and difficulty in maintaining the active ingredient, immobilizing enzymes may be effective but very limited and impractical especially for industrial applications [23]. In this regard, utilizing molecularly imprinted polymers offers a faster, longer-lasting, and more sensitive approach in detecting a wide range of analytes. Molecular imprinted polymers (MIPs) can be referred to as synthetic antibodies containing cavities with complementary shapes, sizes and strategically-situated recognition sites that possess a highly specific binding affinity towards the “imprinted” analyte [24]. The concept of molecular imprinting was first demonstrated by Polyakov from Kiev using silica particles that exhibited unusually specific adsorption towards the additives and solvents incorporated during its fabrication [25]. MIPs are typically prepared by co-polymerizing functional monomers and cross-linkers with the target analyte to form a composite wherein the analyte molecules are embedded in surrounding polymeric material [26]. Prior to co-polymerization, strong interactions either through covalent or non-covalent interactions should be induced to form the pre-polymerization monomer-template complex to hold the functional groups in the most thermodynamically stable position before the MIP is formed. Typically, cross-linkable monomers are added to help maintain the orientation of the

pre-polymerization complex. Using solvent extraction and possible stimuli-triggered swelling techniques, the target analyte is removed from the matrix thus leaving “imprints” capable of re-capturing the analyte. Similarly, MIPs possess a lock-and-key mechanism; an artificial MIP recognition site is the lock while the analyte is the key. Due to this strong and selective binding affinity, MIPs have been employed for various chromatographic separation systems, binding assays and sensing devices [26]. So far, apart from the aspartame-imprinted zwitterionic polymer grafted onto a silica surface [1], the application of molecular imprinting in aspartame detection is very limited thus presents a huge opportunity area for development.

Synthetic schemes for producing MIPs are mostly based on bulk free radical polymerization of functional vinyl monomers [27]. However, extracting the imprinted analyte from these bulk MIP monoliths is a major concern that results to poor sensor performance. Based on recent reports, surface imprinting in thin film formats provide a more appealing strategy since the artificial recognition sites are easily accessible and more closely attached to transducer surfaces and the signal due to analyte rebinding is amplified [28,29]. In the past few years, our group has been using thin films of electrochemically polymerized conducting polymers in developing molecularly imprinted polymer sensors for various analytes including drug molecules [30] and several toxic chemicals [31,32]. Through this technique particularly with cyclic voltammetry, modifying electropolymerization variables such as the potential range, scan rate, and the number of scans provides direct control over the resulting thickness and the oxidation-reduction (redox) state of the polymeric film, which can potentially affect sensor performance [33]. In this study, we employed a terthiophene-based monomer with an acetic acid moiety (3-TAA) in synthesizing the MIP via anodic electrochemical polymerization. Aside from its chemical stability, 3-TAA

has carboxylic acid units capable of forming hydrogen-bonding interactions with aspartame. Moreover, it does not require the use of a cross-linkable monomer to form a robust MIP film.

Meanwhile, recent reports have demonstrated the improved sensitivity of **colloidally templated and** microporous MIP sensors over planar formats by increasing the exposed surface area/volume ratio and providing more access to recognition sites that are obscured within the film [34,35]. Hence, in this investigation, we have fabricated a sensitive and highly specific aspartame-imprinted MIP sensor as synthesized via colloidal template-assisted electrochemical polymerization. As pioneered by Van duyne's research group [36], **colloidal sphere** lithography employs hierarchically assembled colloidal spheres usually made of silica or polystyrene as sacrificial masks for subsequent deposition of other materials. The monolayer colloidal crystal (MCC), a hexagonally close-packed assembly that closely resembles a honeycomb structure, is the most common formation used in **colloidal sphere** lithography. Recently, our group has been widely employing the sacrificial polystyrene (PS) MCC template in co-patterning conducting polymers with various inorganic materials such as gold nanoparticles [37], carbon nanotubes [38], and even graphene [39]. Since the interstitial voids of the MCC still expose electrochemically accessible areas, the pre-polymerization complex composed of the terthiophene-acetic acid monomer and aspartame can be simultaneously electropolymerized and deposited within these tight spaces. An inverse opaline poly(3-TAA)/APM composite pattern is then revealed after dissolving the colloidal sphere templates. Moreover, the embedded aspartame molecules are extracted forming artificial recognition sites capable of rebinding the analyte to the MIP array. Mass adsorptions as monitored by quartz crystal microbalance (QCM) were mathematically correlated to the concentration of the analyte solution to which the MIP was

exposed. The simple and robust protocol was able to produce a highly sensitive and aspartame specific detection system.

MATERIALS AND METHODS

Materials

Without prior purification, Polybead® polystyrene (PS) microspheres (500 nm in diameter, 2.59 wt % latex in water) from Polysciences, Inc. (Warrington, PA) have been employed as the sacrificial template for **colloidal sphere** lithography. The anionic surfactant sodium N-dodecyl sulfate (SDS) has been purchased from Alfa Aesar (Ward Hill, MA). The monomer (2-(2,5-di(thiophen-2-yl)thiophen-3-yl)acetic acid), an acetic acid functionalized-terthiophene molecule referred to as 3-TAA, has been synthesized in our group using a previously reported procedure [34]. Meanwhile, the sensing analyte aspartame or L-Aspartyl- L -phenylalanine methyl ester (98%, 22839-47-0) was obtained from Acros Organics (Geel, Belgium). The supporting electrolyte tetrabutylammonium hexafluorophosphate (TBAH) and phosphate buffer saline tablets were obtained from Sigma-Aldrich (St. Louis, MO). Most solvents including acetonitrile (ACN), tetrahydrofuran (THF), and methanol have been purchased from Fisher Scientific.

Formation of 2D PS monolayer colloidal crystals (MCC)

The sensor substrate selected is a standard AT-cut polished Au QCM crystal with a 5 MHz fundamental resonant frequency and 1 in (25.4 mm) diameter. **The use of an electrically conductive substrate for the deposition is vital in nucleating the potential-induced oxidation coupling polymerization and the subsequent deposition of the newly formed polymer.** Before any deposition, it was washed using the Piranha solution (70% H₂SO₄ and 30% H₂O₂) then

repeatedly rinsed with de-ionized H₂O. *Caution! Using the Piranha solution requires extreme care because it is highly reactive and corrosive particularly to organic materials.* After drying with nitrogen, the crystal was subjected to plasma treatment in an oxygen plasma etcher (Plasmod, March) for 30 sec. Prior to MIP synthesis, a sacrificial template made up of hexagonally packed array of PS monolayer colloidal crystals (MCC) has been assembled on the QCM crystal surface using the Langmuir-Blodgett (LB)-like deposition method [40]. In brief, a pre-cleaned Au QCM crystal was clipped in one end to achieve a vertical orientation and immersed an aqueous dispersion containing 1 wt% PS microspheres and 34.7 mM SDS. The colloidal dispersion should be pre-sonicated for at least an hour to ensure uniform dispersion and minimize aggregation in the resulting array. Using a motorized dip coater, the substrate is slowly raised at a controlled rate of 0.1-0.3 mm/min from the colloidal dispersion. Finally, the substrate was dried in vacuum for at least an hour before use.

Molecular modeling for optimized monomer-template complex formation

Favorable formation of monomer-template pre-complex can be quantified as energy of formation ($\Delta E_{complex}$) and optimized by varying the concentration ratios of the components through molecular modeling studies. The modeling software *Spartan '08* Version 1.2.0 (Wavefunction, Inc.) was used to perform semi-empirical Austin Model 1 or AM1 calculations that considers the geometry, dipole moments and relevant interactions. These include hydrogen bonding, which is known to play a significant role in designing molecular imprinting sensors. $\Delta E_{complex}$ was calculated using Eq. 1. Based from *Spartan '08* modeling results, a 2:1 ratio of 3-TAA and aspartame has been used to prepare the MIP solution. Specifically, an acetonitrile solution containing 400 μ M 3-TAA, 200 μ M aspartame and 0.1 M TBAH was used to fabricate

the MIP film. The resulting solution was stored in refrigerated condition after sonication to allow better complexation between the monomer and template. On the other hand, the non-imprinted polymer (NIP) film was also synthesized using an ACN solution with 400 μM 3-TAA and 0.1 M TBAH.

Fabrication of patterned and molecularly imprinted poly(3-TAA) films

The MIP film has been electrochemically polymerized and simultaneously deposited by cyclic voltammetry method onto the PS-templated Au-coated QCM crystal. The set-up used was a standard three-electrode electrochemical cell that consists of an Ag/AgCl reference electrode, platinum counter electrode, and the Au-coated QCM crystal (working electrode) pre-patterned with PS MCCs as the working electrode. The electropolymerization was performed by gradually sweeping the potential between 0 V and 1.1 V at a scan rate of 100 mV/sec for 15 cycles. The resulting film was thoroughly rinsed with ACN to wash away the undeposited monomers and oligomers. A second cyclic voltammetry experiment was done using only an ACN solution with 0.1 M TBAH to confirm the stable formation of the polyterthiophene. Since the polystyrene particles are electrically insulating, only the areas that are not being covered by the colloidal sphere template are electrochemically accessible and capable of nucleating polymer formation. Consequently, immersing the PS-MIP film in THF for 30 min twice to selectively dissolve the PS sacrificial template unveils the resulting inverse opal pattern. Briefly, similar above procedure was done for the preparation of the NIP film (control) by electropolymerization of the same monomer without exposure to the analyte. The aspartame analyte was extracted from the MIP film matrix by thorough washing with methanol. The sample was dried in vacuum for at least an hour before sensing.

Quartz Crystal Microbalance for Electrosynthesis of MIP film and Aspartame detection

The RQCM – quartz crystal microbalance research system (Inficon, Inc.) which includes the main apparatus, probe and crystal was used in monitoring mass changes during the electropolymerization of the MIP film and the rebinding process for aspartame. The apparatus consists of a built-in phase lock oscillator with a measurement resolution of $<0.4 \text{ ng/cm}^2$ and frequency range of 3.8 – 6 MHz and is capable of crystal capacitance cancellation to remove unwanted capacitance effects of the crystal. QCM measurements were logged and graphically monitored using the RQCM data-log software. For the electrochemical polymerization, the Amel 2049 potentiostat and power lab system (Milano, Italy) were connected to the RQCM to perform an electrochemical-quartz crystal microbalance (EC-QCM) measurement, wherein any film depositions induced by electrochemistry is monitored gravimetrically. The voltage bias applied by the potentiostat is conducted through the QCM holder, POGO electrodes and to the main QCM surface hence frequency changes and current measurements can be logged in real time while the electrosynthesis is on going. On the other hand, for the aspartame detection, changes to the resonant frequency after exposure to varying analyte concentrations are then used as the main transduction signal for the MIP sensor.

Characterization of Colloidally-templated Poly (3-TAA) Films

An atomic force microscope (PicoScan 2500, Agilent Technologies) was used to study the surface morphology of the substrates in tapping mode. AFM is equipped with a piezo scanner that was set to run at 1-1.5 lines/s using commercially available tapping mode tips (Tap 300-10,

silicon AFM probes, Tap 300, Ted Pella Inc). The software Gwyddion 2.19 was used to analyze and enhance the AFM images.

X-Ray photoelectron spectra for monitoring the presence of key elements were performed using an X-ray photoelectron spectrophotometer (PHI Model 5700), which was equipped with a monochromatic Al K α X-Ray source ($h\nu = 1486.7\text{eV}$). The X-ray source is positioned at 90° relative to the axis of a hemispherical energy analyzer. The spectrophotometer was operated both at high and low resolutions with pass energies of 23.5 and 187.85 eV, respectively, a photoelectron take off angle of 45° from the surface. All XPS measurements were performed at a base pressure of 1×10^{-8} Torr.

RESULTS AND DISCUSSION

Scheme 1 illustrates the step-by-step strategy in fabricating the molecularly imprinted polyterthiophene film for aspartame detection applications. First, a hexagonally close-packed formation of 500 nm PS latex microbeads, also referred to as a monolayer colloidal crystal (MCC) is assembled on a Au QCM substrate via the *Langmuir-Blodgett*-like deposition method. Employing the colloidally-templated substrate as the working electrode, the terthiophene-carboxylic acid/aspartame complex was electrochemically polymerized via cyclic voltammetry. The polyterthiophene film subsequently formed in the electrochemically accessible interstitial voids of the colloidal pattern. An inverse opal structure is then revealed after dissolving the latex PS spheres. Lastly, aspartame is removed from the matrix by thorough washing in methanol to expose molecular imprints capable of capturing the analyte.

Molecular Modeling and Simulation of Monomer-Template Prepolymerization Complex

In designing molecularly imprinted polymer sensors, inducing strong non-covalent interactions between the monomer and analyte molecules is vital in ensuring that the resulting film will have cavities with strategically placed chemical handles capable of capturing the analyte back to the film. Molecular modeling and simulating the interactions between the components is an effective approach to address this concern. Based from its chemical structure, 3-TAA has a carboxylic acid moiety that can potentially form hydrogen-bonding and electrostatic interactions with the amine and carboxyl groups of aspartame. The interaction energy of the resulting monomer-analyte pre-complex can be estimated using Eq. 1.

$$\Delta E_{interaction} = (\Delta H_{f,3-TAA/Aspartame}) - (\Delta H_{f,3-TAA} + \Delta H_{f,Aspartame}) \quad (1)$$

It accounts for the individual enthalpies of formation of the complex, monomer and analyte, which have been determined using semi-empirical AM1 force field calculations as performed in *Spartan '08* (V1.2.0; Wavefunction, Irvine, CA). By changing the composition ratios, the most thermodynamically stable complex formation can be achieved using 1:2 analyte-monomer ratio which accounted for an interaction energy of -25.1 kJ/mol. In comparison, a 1:1 system corresponds to 10.7 kJ/mol while a 3:1 monomer/analyte system is calculated to have 15.2 kJ/mol. The negative interaction energy value suggests a highly spontaneous complex formation, which is key in forming stable imprinting of the analyte within the polymeric system. **Fig. 1** shows the theoretical model of the pre-polymerization complex, which also suggests a potential arrangement of 3-TAA and aspartame in the matrix. Space-filling and tube models are shown in **Fig. S1**. Based on the space-filling simulation, the aspartame analyte can be found squeezed in the middle of two 3-TAA units. During polyterthiophene formation, interaction between the monomer and aspartame should be stable enough in such a way that a robust conducting polymer/analyte composite can form on the electrode surface.

Aspartame-Imprinted Poly(tertiophene-acetic acid) Inverse Opal Film Formation

The simulated optimum monomer:analyte ratio is then translated in preparing the electropolymerizing solution for synthesizing the MIP film. However, according to previous reports [34,35,41], incorporating nano/microstructures throughout the film improves the performance of the sensor by increasing the contacting surface area to analyte. Hence, a sacrificial micropatterning template composed of hexagonally close-packed assembly of PS latex beads was first arranged on the QCM surface using a controlled vertical deposition technique. Covered areas within the **insulating** honeycomb structure also referred to as a monolayer colloidal crystal (MCC) will mask subsequent depositions, however, the interstitial spaces between the spheres still expose electrochemically-accessible areas capable accepting electrochemically polymerized materials. **Fig. 2A and 2B** are 2D and 3D topographical tapping mode AFM images that demonstrate successful ordering of PS microspheres. According to the x-axis of the cross-sectional profile in **Fig. 2C**, the average diameter of the colloidal PS is **463.0 nm $\pm$ 4 nm**, which is close to the actual 500 nm PS diameter. Meanwhile, the y-axis of the cross-sectional profile only accounts for **162.4 nm $\pm$ 8 nm** for PS diameter since the tight spaces between beads are difficult to reach and image accurately. The highly ordered hexagonally close-packed surface was prepared using the *Langmuir-Blodgett*-like deposition method, wherein the QCM crystal is immersed in an aqueous dispersion of latex particles and anionic surfactant and then slowly raised at a steady pace. The anionic surfactant SDS assumes a vital role in inducing the well-ordered arrangement of the colloidal particles as it helps meet the required ionic strength to force the particles to the air-liquid interface [40]. At this point, the colloidal materials are driven to a more thermodynamically stable honeycomb orientation that is also capable of seeding

wider range ordering. Moreover, the surfactant also helps in inducing electrostatic repulsion within the particles, which protects the pattern from colloidal aggregation. As the substrate is slowly lifted, the close-packed formation is transferred onto and maintained at the surface.

The fabricated PS MCC was then used as an electrochemical mask for **colloidal** patterning the molecularly imprinted polyterthiophene film. The PS-patterned Au-coated QCM crystal was immersed in an acetonitrile solution containing 3-TAA (400 μM), aspartame (200 μM), and tertbutylammonium hexafluorophosphate (0.1 M). Using a standard three-electrode cell with the Au-coated QCM crystal as the working electrode, the potential was swept back and forth between 0 and 1.1 V at 100 mV/sec for 15 times. The ease in dissolving the sacrificial latex template is one of the most attractive features of **colloidal** sphere lithography but it also limits the range of solvents that can be employed in depositing a material through it. Based on **Fig. S2**, it can be seen that the AFM image is similar to **Fig. 2A** and that the electropolymerization conditions did not disturb the highly ordered system. After thorough washing the QCM crystal using tetrahydrofuran to remove the PS microbeads and then aspartame extraction via methanol dissolution, the successful fabrication of the inverse opaline polymer film, as presented in **Fig. 2D and 2E**, is unveiled. **Fig. 2F** shows the AFM cross-sectional profile of the inverse honeycomb pattern. The macropore diameter or the peak-to-peak distance of the macropore is determined to average at **473.6 nm $\pm$ 31 nm**. Moreover, the center-to-center distance equals **464.0 nm $\pm$ 12 nm**. Both values closely resemble the geometrical features of the original PS pattern. On the other hand, the height of the cavities averages at **95.9 nm $\pm$ 7 nm**. As summarized by the chemical reaction in Fig. 3A, the real-time formation of the poly(3-TAA)/aspartame complex was simultaneously facilitated and monitored using electrochemical quartz crystal microbalance (EC-QCM), a technique that measures current changes, resonant quartz

frequencies, and mass depositions. Cyclic voltammograms of **Fig. 3B and 3C**, which correspond to the molecularly-imprinted and non-imprinted polymer films, depict the current responses due to the electropolymerization. The mechanism of polythiophene electropolymerization has been previously reported [42]. Typical to the oxidation-reduction behavior of polythiophenes, an onset potential was observed at 0.4 V. Already evident in the first anodic cycle (forward scan from 0V to 1.1V), an oxidation peak emerged after 1.0 V that corresponds to the release of electrons from the monomer and subsequent formation of radical cation species also known as polarons. As the potential sweeps back from 1.1V to 0 V, the polarons attach to each other through a 2,5 linkage and undergoes reduction as signified by the wide cathodic peak between 0.4 V – 1.1V. For succeeding cycles, another anodic peak becomes evident at 0.6 V, which is attributed to the oxidation of the radical dications or bipolarons. The anodic potential is much lower than that of the polarons since the dications readily oxidize due to extended π -conjugation as compared to the radical cation species. Similar behavior is observed for the electropolymerization of both the aspartame-imprinted and non-imprinted polymer films. However, the total cyclic voltammogram area for the non-imprinted polymer film is much more wider as compared to the imprinted film, which is intuitive because incorporating aspartame in the matrix resulted to a slightly decreased CV area as compared to the electropolymerization of the monomer alone. On the other hand, the current responses observed in the cyclic voltammograms reflect corresponding frequency shifts ΔF in the QCM data as shown in **Fig. 4A**. For both MIP and NIP films, a decreasing staircase behavior is observed wherein the number of “stairs” corresponds to the number of electrochemical polymerization cycles. In a typical electrochemical oxidation-reduction cycle, a sudden decrease in ΔF is observed as the potential is swept from 0 V to 1.1 V (oxidation), which is followed by a slight increase in ΔF when the potential cycles back from 1.1 V to 0 V

(reduction). In addition to the stepwise polymeric depositions per cycle, the oscillating behavior is also due to the cyclic capture and release of supporting electrolyte PF_6^- ions. During the anodic cycle, the monomer releases an electron forming the positively-charged polaron, capable of accepting negatively-charged supporting electrolytic PF_6^- ions. This behavior as combined with polymeric deposition is signified by a sudden decrease in ΔF . Meanwhile, during the reduction half-cycle, the cationic nature of the polymeric backbone neutralizes thus releasing the supporting electrolyte ions. Comparing the MIP and NIP EC-QCM data, it can be observed that the increased ΔF shifts due to reduction is much higher for the non-imprinted polymer film than the MIP. It is possible than during the polymerization of 3-TAA/aspartame complex, aspartame hinders the diffusion of the supporting electrolyte ions in and out the resulting polymeric matrix, which is not the case for the electropolymerization of the pure monomer. After 15 cycles, the total ΔF shift amounted to -823 Hz for the 3-TAA/aspartame complex, which is much higher than that of the pure case which only achieved a total of -406 Hz. Based on the measured mass shifts presented in **Fig. 4B**, a total of $14.6 \mu\text{g}/\text{cm}^2$ deposited due to the electropolymerization of 3-TAA/aspartame while for the 3-TAA only case, $7.3 \mu\text{g}/\text{cm}^2$ of material was formed. These findings complement the claim made from the CV data that the interaction between the monomer and the analyte is strong enough to co-deposit the electrochemically inactive aspartame with the polyterthiophene on the QCM surface, which is vital in fabricating the analyte imprints of the MIP film.

The high resolution X-ray photoelectron spectroscopy (XPS) data presented in **Fig. 5 and 6** confirm the electrodeposition of a robust poly(3-TAA)/aspartame composite array (after the removal of PS latex beads) on the Au QCM crystal. The emergence of the S 2s singlet peak at 228 eV and 2p doublet peaks at 163 eV and 166 eV, as shown in **Fig. 5A and 5B**, confirm the

robust deposition of the patterned film [43]. Moreover, detected C 1s and O 1s intensities, shown in **Fig. 5C and 5D** respectively, are also characteristic peaks for polythiophene films. Two peaks can be observed from the C 1s spectra. The more intense C 1s peak centered at 284.6 eV is attributed to the –C-C- and –C-H- functional groups of the polymer; the less prominent peak at 288.9 eV correspond to the –O-C=O- moieties [43]. On the other hand, O 1s peak which begins at 529.01 eV and ends at 535.42 eV is due to the –OH and –O-C=O- units of poly(3-TAA) [43]. Aside from confirming the synthesis of polymeric film, XPS is also an important tool in determining the presence of aspartame in the system by monitoring the elemental marker N 1s. Based on **Fig. 6**, the electrodeposited poly(3-TAA)/aspartame indeed demonstrated an intense N 1s peak from 399 eV-402 eV and centered at 400 eV [43], which is, as expected, not present in the spectra for the non-imprinted film. In order to finally generate the imprinted cavities, the composite film was repeatedly washed in methanol for 1 hr. As a result, the N 1s spectra significantly decreased suggesting complete removal of most amounts of analyte in the system.

Aspartame QCM Detection Using Molecularly-imprinted and Microporous Poly(3-TAA) Inverse Opaline Films

The capability of the aspartame-imprinted and colloidal-patterned poly(3-TAA) arrays to capture the analyte is measured using *in situ* quartz crystal microbalance adsorption studies. **Fig. 7A** presents time-dependent ΔF measurements after the injection of 50 μM aspartame in PBS buffer solution (0.1 M, pH 7) onto MIP and NIP films. Adsorption onto the micropatterned MIP film had a much greater ΔF decrease equal to **-55 Hz**, which is more than twice as compared to the measured rebinding of **-21 Hz** on a non-imprinted film. Based from this concentration, the imprinting factor, which is defined as the ratio of the transduction response of the imprinted film

over the NIP, is determined to be equal to 2.64. **Fig. 7B** compares ΔF signals due to aspartame adsorption on both MIP and NIP using various concentrations. Clearly, the adsorption data confirms the successful imprinting of aspartame within the MIP film and its ability to capture the analyte with higher sensitivity as compared to a non-imprinted polymer film. The sensing performance of the colloidal sphere-patterned, microporous MIP film was further evaluated by measuring the response over a range of concentrations from 12.5 μM to 200 μM aspartame. *In situ* QCM adsorption measurements are collated in **Fig. 7C** from which a relatively good linear calibration plot was constructed relating the final frequency shift as a function of aspartame concentration and presented in **Fig. 7D**. The determined Pearson's coefficient R^2 is 0.9845 while the slope of the calibration plot is 1.2517 Hz/ μM . Moreover, the limit of detection (LOD), which is the minimum analyte concentration that can be detected at a certain confidence level, was also determined based on **Eq. 2**, wherein σ is the standard deviation of the y-intercept and m is the slope of fitted line [31]. Assuming a confidence level of 95%, the LOD is calculated to be 31.75 μM (9.34 mg/L)8/4/2016 11:01:00 AM.

$$\textbf{Limit of Detection (LOD)} = \frac{3\sigma}{m} \quad (2)$$

On the other hand, the limit of quantification is calculated to be 105.84 μM (31.15 mg/L) according to **Eq. 3** [44].

$$\textbf{Limit of Quantification (LOQ)} = \frac{10\sigma}{m} \quad (3)$$

In 2013, the European Food Safety Authority (EFSA) has stated that the acceptable safety limit for aspartame is 40 mg per kg of body weight while the US Food and Drug Administration (FDA) recommends 50 mg per kg of body of weight [45,46]. Moreover, the maximum permitted level (MPL) for aspartame content in soft drinks is set to 600 mg/L by the EFSA [45]. Based on

these values, the detection and quantification limits of the proposed MIP sensor is suitable for aspartame detection within the legally set safety limits.

Aside from the sensitivity of the detection mechanism, the selectivity of the micropatterned aspartame-imprinted poly(3-TAA) sensor was also investigated by measuring the ΔF response after exposure to aqueous solutions containing 200 μM of various analogs with chemical structures that closely resemble that of aspartame, the imprinted analyte. **Fig. 8A** shows the chemical structures of the selected analogs, which include peptides alanine-phenylalanine (Ala-Phe), alanine-glutamine (Ala-Gln), glycylglycine (Gly-Gly), and arginylglycylaspartic acid (RGD). As presented in **Fig. 8B**, the corresponding time-dependent QCM ΔF shifts reveal that aspartame, indeed, exhibited the highest amount of adsorption towards the aspartame-imprinted poly(3-TAA) array. This behavior can be attributed to the successful formation of cavities with same shape and size as aspartame within the microporous MIP film. The selectivity of the sensor can also be expressed in terms of the cross-reactivity ratio, which is defined the ratio of the ΔF shift due to the capture of the similarly structured analogs over the ΔF response when the micropatterned MIP film is exposed to the aspartame solution. Based on **Fig. 8C**, the cross-selectivity ratios arranged in a decreasing order are as follows: Ala-Gln (70.8%), Gly-Gly (59.2%), Ala-Phe (44.3%) and RGD (18.2%). The high interference ratio of Ala-Gln can be attributed to the three groups of amine moieties, which are capable of forming strong hydrogen bonding interactions to the $-\text{COOH}$ units of the polymeric film. On the other hand, it is possible that the smaller size of the molecular structure of Gly-gly caused the analog to fit into and be captured by the molecular imprinted cavities. Unlike the other molecules, the poor binding affinity of Ala-Phe and the RGD peptide might be attributed to the relatively larger sizes of the structures, which hindered the rebinding of the analogs onto the MIP sensor. Despite these

issues, the aspartame-imprinted poly(3-TAA) film still exhibited high selectivity towards aspartame as compared to other structurally related analog molecules.

CONCLUSION

We have fabricated a micropatterned thin film sensor for aspartame based on a surface-bound molecular imprinting technology. Inspired by previous reports [34,41], colloidal sphere lithography was employed in order to introduce periodically-spaced macropores that exposed hidden artificial recognition sites and increased the surface area/volume ratio of the MIP sensor. Hexagonally close-packed PS MCC sacrificial masks were successfully prepared using the *Langmuir-Blodgett*-like deposition method. Prior to electropolymerization, the optimum 2:1 monomer/aspartame composition ratio was calculated from simulated semi-empirical AM1 calculations to maximize non-covalent interactions between the components. Using the PS-patterned gold substrate QCM crystal as a working electrode, the pre-polymerization monomer/aspartame complex was simultaneously electropolymerized and deposited within the interstitial voids of the PS MCC template. Dissolving the PS mask revealed a highly ordered inverse opal pattern and array fidelity. Monitoring the N 1s peak obtained by XPS as well as the current responses and ΔF shifts from the electrochemical quartz crystal microbalance technique supported the claim the aspartame was embedded within the electropolymerized poly(3-TAA) due to the strong interactions between the components. By tracking the elemental N 1s XPS marker, aspartame was successfully extracted from the composite array by repeated washing in methanol. The micropatterned, aspartame-imprinted polyterthiophene array exhibited sensitivity towards analyte concentrations from 12.5 μM to 200 μM , as characterized by a Pearson's coefficient R^2 of 0.9845. The nanostructured thin film sensor also showed specificity towards

similarly structured analog molecules which includes peptides such as Ala-Gln, Gly-Gly, Ala-Phe and RGD, as arranged in a decreasing cross-selectivity ratio. Based on these results, it was confirmed that the molecular imprinting technology using a colloidally-templated and electrochemically polymerized conducting polyterthiophene film was able to build synthetic antibody-type recognition sites for rebinding aspartame which was tracked using the QCM technique. To our knowledge, this is the first account wherein an artificial nanostructured conducting polymer film is used to detect aspartame. As compared to a previous report on aspartame-imprinted zwitterionic polymer grafted silica [1], the performance of our proposed protocol offers a much lower detection range, thus better sensitivity. Due to its promising sensitive and selective binding affinity towards aspartame, the technology can easily be translated to various separation systems. In particular, molecularly imprinted polyterthiophenes can be electrodeposited on commercially available steel meshes and be used to filter out the imprinted molecule from a given mixture, which can be manufactured on a larger scale. In terms of performance, other stimuli-responsive functional groups can be added to the polymer matrix to help induce swelling, which can be turned on and off upon trigger for developing smart functional detection systems.

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ARTWORK

Scheme 1. Schematic diagram of the stepwise fabrication of the nanopatterned aspartame imprinted poly(3-TAA) sensor. (A) Assembly of PS microbeads via *Langmuir-Blodgett*-like deposition (B) Electrochemical polymerization of 3-TAA with Aspartame (C) Dissolution of PS microspheres (D) Aspartame extraction

**Scheme 1 is a double column-fitting image.*

Figure 1. Molecular model ball-and-spoke representation of 3-TAA/aspartame pre-polymerization complex (2:1 ratio) based on semi-empirical AM1 calculations

*Figure 1 is a 1.5 column-fitting image.

Figure 2. Topographical tapping mode AFM images of (A) monolayer colloidal PS microsphere assembly and (B) inverse opal poly(3-TAA) MIP film; (C) and (D) Corresponding 3D AFM images. (E) and (F) are the cross-sectional profiles of the PS MCC and the inverse opaline film

*Figure 2 is a double column-fitting image.

Figure 3. (A) Chemical equation of the electropolymerization of 3-TAA. Cyclic voltammograms of (B) molecularly imprinted (with the monomer free scan in the inset) and non-imprinted (C) poly(3-TAA) films

* Figure 3 is a 1.5 column-fitting image.

Figure 4. Progress of electrochemical polymerization as monitored from the (A) frequency change and (B) mass data versus time recorded using an electrochemical quartz crystal microbalance (EC-QCM)

* Figure 4 is a 1.5 column-fitting image.

Figure 5. High resolution XPS (A) S 2s, (B) S 2p, (C) C 1s, and (D) O 1s spectra of aspartame-imprinted poly(3-TAA) film

* Figure 5 is a 1.5 column-fitting image.

Figure 6. High resolution N 1S XPS spectra of non-imprinted and molecularly imprinted polymer film, before and after analyte removal

* Figure 6 is a 1.5 column-fitting image.

Figure 7. (A) Comparison of the *in situ* rebinding of 50 μM aspartame onto MIP and NIP film (B) QCM responses of MIP and NIP film toward various concentrations of aspartame (C) ΔF response shifts of aspartame-imprinted poly(3-TAA) toward various analyte concentrations (D) Corresponding aspartame sensing calibration curve of the MIP film

* Figure 7 is a 1.5 column fitting image.

Figure 8. (A) Chemical structures of analog molecules that closely resembles aspartame for selectivity analysis (B) Time-dependent QCM ΔF responses using a constant 200 μM aspartame in PBS solution (C) Selectivity ratios of QCM ΔF shifts corresponding to the rebinding of various similarly structured peptides

**Figure 8 is a double column fitting image.*