Small-Molecule Dengue Virus Co-imprinting and Its Application as an Electrochemical Sensor

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Polymers can be synthesized to recognize small molecules. This is achieved by introducing the target molecule during monomer self-assembly, where they can be incorporated during cross-linking polymerization. Following additional pre-processing, the material obtained can then be applied as a sensing layer for these molecules in many applications. The sensitivity of the polymers depends on the “active sites” imprinted on the surface. Increasing the number of active sites on the polymer surface can be achieved by using nanoparticles as a platform to support and concentrate the molecules for imprinting. In this work, we report the first use of dengue virus as a supporting nanoparticle to make for a more effective polymer composite sensor for the detection of bisphenol A (BPA), which is an environmental contaminant. The dengue virus has a nanoparticle size of around 100 nm and its surface provides regions where lipids and hydrophobic compounds can bind, making it an ideal support. The mixing of BPA with dengue prior to monomer self-assembly led to imprinted polymer surfaces with much higher density BPA binding sites and a limit of detection of 0.1 pM. We demonstrate that a BPA–dengue co-imprinting polymer composite sensor shows a very high sensitivity for BPA, but with lower production costs and technical requirements than other comparable methods.

A material surface can be prepared with molecular recognition properties by using several techniques.[1–4] One of these methods is the self-assembled monolayer (SAM) polymerization process in the presence of target structures. In this method, molecular recognition capability is established from active sites created through the self-assembly of monomers around the target structures to form shape and/or charge complementarity before polymerization.[5] These materials are sometimes referred to as molecularly imprinted polymers (MIPs).[6] The molecular recognition property makes MIPs suitable for sensor fabrication, as reported in literature over many years.[7–11] MIP-based sensors can identify structures ranging from small to large molecules, proteins, or even whole microorganisms such as bacteria or viruses.[12,13] In case of virus MIP biosensors, the main applications are virus detection, classification, or virus binding assays.[14,15] However, applications of virus imprinting beyond these types have not been reported.

It is known that a number of active compounds against dengue contain hydrophobic moieties that allows them to bind to hydrophobic areas on the dengue envelope.[16] This feature, and our previous success in imprinting whole virus particles,[17] led us to consider using the large surface area of the dengue virus as a means to more effectively imprint hydrophobic molecules by exploiting their binding to the dengue virus surface. In this case, the MIPs should have recognition sites for dengue, but, more importantly in our case, binding sites for the molecules that are known to bind to the virus surface. The main advantage of co-imprinting is that the virus nanostructure has a high surface area, which means a potentially higher number of binding molecules on the virus surface for SAM formation. The utility of this type of approach has been demonstrated for other types of 3D nanostructured MIPs including nickel nanospheres and doped graphene sheets.[17–19] These modifications to the traditional imprinting approach (i.e. template + polymer alone), termed molecularly co-imprinted polymers (MCIPs), have a higher number of active sites for the templates in question, and display much improved sensitivity and specificity.

In this study, we investigate the use of template binding to virus particles for the co-imprinting of BPA for the preparation of SAM polymer–graphene oxide composites. BPA was chosen in this study because it is a commonly used reagent in the polymer industry and a widespread environmental contaminant. BPA poses a significant risk to humans, as it can modulate estrogen receptor signaling.[20] It has been shown to play a role in several human disorders including infertility, early onset of puberty, hormone-dependent tumors such as breast and prostate cancers, and a number of metabolic disorders including polycystic ovary syndrome. The similarity of BPA to known aromatic dengue virus entry inhibitors of the molecule...
meant it was a suitable candidate for assessment in our co-imprinting studies with the dengue virus. Several methods for BPA detection have been investigated to obtain a high-performance technique that can detect BPA at low concentrations. BPA-contaminated samples including drinking water, food, serum, and urine were examined by using methods such as gas chromatography (GC), high-performance liquid chromatography (HPLC), immunoassays, and electrochemical techniques with limits of detection (LODs) ranging from $10^{-15}$ to $10^{-9}$ mol L$^{-1}$.[21–23] A recent study reported an MIP application involving silica mesopores in conjunction with spectroscopic detection, which offered a cheap and reliable means to determine BPA contamination with an LOD of 0.5 ppm (2.2 μM).[24] Another notable study reported the use of aptasensor-based Au nanoparticles with an LOD of 7.2 fm.[25] In fact, a method using an aptamer-based sensor can access low LODs, but the sensor detects BPA by using short-chained synthetic RNAs, whereas our method requires no other specific reagents to functionalize the electrode surface.

The efficiencies of MIP composites sensors prepared using dengue virus with BPA and without will be investigated in terms of substrate recognition. Available data on the use of MIPs based sensors to detect BPA has been used to compare to the results from this work.[26–28] LOD by a virus assisted MCIP sensor determined here to detect BPA was lower than LOD from other MIPs based methods.

Prior to the MCIP production, the target template was theoretically assessed for its ability to bind to the virus surface. Therefore, the binding capability of BPA to dengue virus had to be justified by using docking simulations. The experiment was carried out with the molecular docking program GOLD[29] and a known drug targeting the dengue envelope protein as a reference. It was found that BPA was predicted to bind in the same areas as a drug on the virus surface (Figure S1 in the Supporting Information), owing to the obvious hydrophobic structure of dengue virus inhibitors.

SEM images reveal a change in MCIPs surface produced with dengue, as presented in Figure 1a. Further assessment of the BPA recognition by BPA–dengue MCIPs was carried out by using electrochemical impedance spectroscopy (EIS) experiments. The circuit analog used to fit the EIS data was the Randles RC circuit type, where $R_s$ is the charge-transfer resistance, $C$ is the double-layer capacitance, $R_i$ is the solution-phase resistance, and $W$ is the Warburg impedance. The obtained data showed that BPA absorption reduced the $R_{ct}$ of both MCIP- and MIP-modified electrodes. The plots of $R_i/R_s$ values calculated from simulated circuit with log BPA concentration showed linearity across a 4-log-unit range. However, the MCIP-based electrode with virus-assisted imprinting showed a higher signal response compared to the MIP-based electrode prepared without the virus at the same BPA concentration, and the lowest LOD down to 0.09 pM (Figure 2). The MCIP ele-

![Figure 1. All SEM images of MCIP, MIP, and non-imprinted polymer (NIP) surfaces show large cavities with sizes between 100 and 2000 nm. Small holes of 50–70 nm are found across the areas in MCIP surfaces (a) and at some areas with lower density in the MIP surface (b). These small holes are not seen in NIPs (c), which is a blank co-polymer surface.](Image)

![Figure 2. Data from the EIS spectrum (inset) were used to calculated $R_{ct}$ values. The plot of $R_i/R_s$ with log[BPA] shows that the sensitivity of MCIPs (a) to BPA (according to the EIS signals) was highest followed by MIPs (b) and NIPs (c).](Image)
molecules to be left on another surface, where an external force is applied. However, it is not widely used in small molecule imprinting because it is hard to control the force applied, as quite often the polymer film used would stick to the platform. In our case, the microorganism itself is a nano-stamping device without the need for the applications of an external force. In brief, the dengue virus represents a medium for re-staining BPA during the self-assembly process of monomers to form both BPA and dengue recognition sites on MCIPs. The BPA molecule is not a virus inhibitor and does not resemble lipids, which bind to the dengue surface. However, it contains hydrophobic parts similarly to structures reported as compounds that bind to dengue virus.

Previously, we have found that the number of species pre-mixing with influenza A virus reduces the absorption of the virus particles on the virus MIPs. When microorganisms are presented in the same medium, the template molecules can interact on either surface and may not be available for imprinting. Therefore, the surface property of the organism will play a role, which will result in less absorption on the corresponding MIPs. In that work, we designed an MIP-based binding assay for small-molecule virus binding assessment. Here, further studies have led to a new application of molecular absorption on a virus surface, as presented by using the dengue virus to assist BPA MCIP production. Such materials could be applied for virus subtype classification as well as virus or virus-binding molecule identifications. The BPA–dengue MCIPs reported here increased the sensitivity to BPA, as compared to conventional MIPs. Although there are active compounds against dengue targeting the virus surface, we have not used these molecules in this experiment. We would like to exploit the dengue binding property only to produce MCIPs, regardless of their antivirus activity. The target structures can be any molecules of interest and the binding affinity can be assessed at least by means of computational simulations.

The sensitivity of an MIP-based sensor depends on number of active sites presented on a polymer surface, however, it is hard to consistently produce MIPs with high numbers of these sites. Several methods aimed at increasing the number of MIP active sites have been reported, including surface-imprint ed nanoparticles and MIP membranes. Here, we have proposed another alternative method to increase number of active sites, which has been confirmed experimentally. Binding of BPA to dengue virus before imprinting can increase the number of BPA molecules imprinted on polymer–graphene oxide composites film. Therefore, the BPA electrochemical sensor based on MIPs associated with dengue virus shows higher sensitivity as compared with other MIP-based methods (Table 1).

| Electrochemical sensor LOD [mol L⁻¹] Ref. |
|----------------------------------------|-------------------|
| MIPsbased methods                      |                   |
| MIP–AB/GCE                             | 2 x 10⁻⁸ [30]     |
| MIP–NG/GCE                             | 1.38 x 10⁻⁷ [31]  |
| MIP–MWNPE                              | 2.2 x 10⁻⁸ [32]   |
| MIP–sol-gel/MWCNTs                     | 3.6 x 10⁻⁹ [33]   |
| MIPs                                   | 9 x 10⁻¹⁰ [34]     |
| Other methods                          |                   |
| MCH/aptamers/Au-NPs/BDD                | 7.2 x 10⁻¹⁵ [25]   |
| Na-doped WOₓ/CPE                       | 2.8 x 10⁻⁸ [42]    |
| porous polymerized ionic liquid film GCE| 8 x 10⁻⁸ [43]      |
| AuNPs/GNs–GCE                          | 8 x 10⁻⁸ [44]      |
| protein-immobilized graphene electrode | 5 x 10⁻¹⁵ [45]     |

Type 4 dengue virus (DENV-4) (strain H241) was prepared in a biosafety level 2 (BSL-2) laboratory. The mosquito cells (C6/36) were used for propagation of DENV-4 and incubate for 5 days at 25°C with 5% CO₂ atmosphere prior to being kept at −70°C until used.

**Experimental Section**

**Dengue Virus Preparation**

The most common method for synthesizing GO is Hummer’s method. Starting with graphite (5 g) and NaNO₃ (2.5 g), they were mixed into H₂SO₄ (115 mL) whilst stirring in an ice bath. Then, KMnO₄ was added and the temperature was kept around 0°C. Next, the reaction was heated at 35°C for 2 h. Then, deionized water (230 mL) was added dropwise into the solution and heated to 98°C for 15 min. An additional 700 mL of deionized water was slowly added and the reaction was treated with 30% H₂O₂ (50 mL). The color of the mixture changed from dark brown to bright yellow. The metal ions in the mixture were removed by using HCl (100 mL). Lastly, GO was purified by using a dialysis membrane for 1 week with the deionized water changed every day until neutralization, which was tested by using pH indicator. The GO powder was obtained after drying under vacuum at 60°C for 12 h. The GO ready to use after sonication for 30 min to exfoliate.

**Graphene Oxide (GO) Synthesis**

The composites were prepared by using a previously reported method. Acrylamide (AAM), methyl methacrylate (MMA), methacrylic acid (MAA), and n-vinylpyrrolidone (NVP) were mixed with graphene oxide sheets. In the composites, graphene oxide was used to enhance the polymer conductivity, making it suitable for EIS measurements. The monomers served as sensitive layer building blocks with side-chain groups covering a range of interac-

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**Table 1. Comparison of electrochemical sensors for BPA detection.**
tions, which were present on the virus surface and BPA. The EIS electrode was prepared as follows: the monomers were polymerized through the radical polymerization reaction at 70 °C until a gel form was obtained. A working electrode was coated with polymer–graphene oxide composites. The electrode fabrication was as follows: the polymer mixture was dropped onto a gold electrode (1 x 1 cm²) and then spun at 1000 rpm for 10 s until a thin film polymer was obtained on the gold surface. The mixture of dengue virus and BPA template was incubated for 30 min in a refrigerator before being dropped onto thin film polymer. Leaving the prepared electrodes under UV light overnight and subsequently at 55 °C for 2 h allowed the polymerization process to complete. The scheme for the MCIP biosensor fabrication on a gold sheet is shown in Figure 3. The NIP gold electrode was prepared by using the same procedure, but without the template molecule. The MCIP biosensor was fabricated in laminar flow cabinet (BSL-2) to prevent contamination of the environment with the dengue virus.

**EIS Measurements**

EIS was carried out by using FRA mode over the frequency range 0.01–10000 Hz with 5 mM potassium ferricyanide (K₃[Fe(CN)₆]) in 0.01 M phosphate-buffered saline (PBS). This experiment was operated with three electrodes, which were the MCIP biosensor, Ag/AgCl, and a platinum sheet as the working, reference, and counter electrodes, respectively. BPA was dissolved in deionized water and added to Fe³⁺ electrolyte solution (20 mL) to obtain 0.01, 0.1, 0.5, 1, 5, 10, 50, and 100 ppm as the final concentrations. Nyquist plots were used to represent the impedance data and fitted to an equivalent circuit with NOVA 1.6 software.

**SEM Surface Morphology**

The modified MIP surface on the gold electrode was investigated by using a Quanta 450 FEI scanning electron microscope. Secondary electrons were generated at 25 keV and operated in samples with high vacuum mode.

**Molecular Docking**

Molecular docking was performed by using the GOLD V5.2.2 program with binding mode for BPA on the dengue virus structure (1OKE.pdb). The reference and template molecules were optimized by the HF/6-31G* program. The active-site radius was set to 12 Å for the dengue protein and the number of GA runs was set to 100. There are three active sites in this protein. After docked, BPA influenced amino acids similarly to the reference inhibitor does. The orientation and the interaction between the ligand and receptor were evaluated and ranked by the GOLD score.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Keywords:** bisphenol A • dengue virus • electrochemical sensing • molecularly imprinted polymers • self-assembly

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