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Evaluating the potential of thermal read-out techniques combined with Molecularly Imprinted Polymers (MIPs) for the sensing of low-weight organic molecules

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In recent years, there has been a tremendous increase in the papers published on synthetic recognition elements. Molecularly Imprinted Polymers (MIPs), also referred to as ‘man-made mimics’ of antibodies, are able to rebind their template molecules with high affinity. Advantages compared to natural receptors include their excellent thermal and chemical stability, low-cost, and ease of the production process. However, their use in commercial biosensors is limited due to the difficulty to incorporate MIPs into suitable sensing platforms and traditional detection techniques, such as chromatography, that require bulky and sophisticated equipment. In this review, we evaluate the potential to use MIPs combined with thermal read-out for the detection of low-weight organic molecules. We discuss thermal methods to study MIP-template complexation and to determine neurotransmitters concentrations. In particular, we highlight the heat-transfer method (HTM), a recent technique that is straightforward, low-cost, and requires minimal instrumentation. Until now, sample preparation involves a two-step process, making it time-consuming, and measuring biological samples is difficult due to the noise in the signal. Different sample preparation methods are discussed and it will be demonstrated how this affects the thermal response. An outlook is given in novel methods that can simplify and speed up sample preparation. Finally, we show a novel thermal technique, which is based on the analysis of transport of thermal waves rather than evaluating the fixed heat-transfer resistance. Through applying the concept of thermal waves, signal-to-noise ratio is significantly increased, which will result in lower detection limits and has a high potential for the study of biological samples.

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1. Introduction

1.1 General Introduction on MIPs

Molecularly Imprinted Polymers are polymers containing nanocavities with a pre-determined selectivity towards their template molecules [1],[2]. They are sometimes referred to as ‘man-made mimics of antibodies’, but compared to natural antibodies their advantages include: low-cost [3], robustness [4], and stability under extremes of pH and temperature [5]. The molecular imprinting technique dates back to the 1930’s, when Polyakov concluded that the selectivity of silica matrices for a certain template depended on the chemical nature of the additive that was introduced during the synthesis [6]. Even though several papers were published on this topic in the 1930’s, the start of the current molecular imprinting technique technology did not emerge until the 70’s when the groups of Wulff [7] and Klotz [8] independently reported on organic polymers with pre-determined ligand specificity. When the Mosbach group first introduced the concept of non-covalent binding [9], it was a real breakthrough in the field as this allowed easy extraction of the template from the matrix. As a result, while from the first paper of Polyakov in 1931 until 2006 a number of 1450 MIP-based articles was documented [10], in the period from 2004 to 2011 alone an exponential increase was seen with 3779 references MIP-related articles [10, 11]. Over the past years this number is steadily increasing, with the focus shifting from development of solid materials and monolayers for chromatography towards biosensor based applications [12],[13]. The first chemical sensor arrays were developed by groups of Dickert [14],[15], Shimizu [16],[17] and Takeuchi [18],[19]. The use of MIPs for sensing applications remains challenging, current problems include the development of a suitable interface layer [1, 20], leaching of trapped template [21, 22], and the lack of straight-forward and low-cost read-out techniques[23, 24]. However, with current methods it is possible to compete with enzymes, as was demonstrated by Chianella *et al.*[25]. The group of Piletsky has developed a straightforward approach to coat microplate wells with nanosized MIPs (Figure 1).

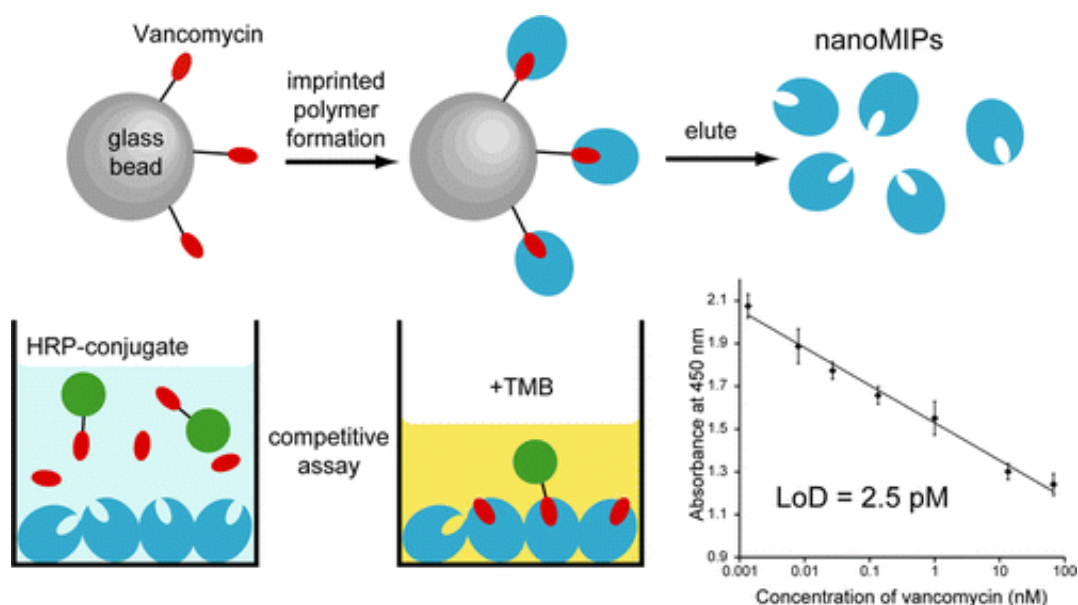


Figure 1. NanoMIPs were synthesized with a solid approach with vancomycin as the template. With a horseradish peroxidase-vancomycin conjugate, detection limits in the order of 2.5 pM were achieved in buffer and in blood plasma[25]. Reproduced with permission from ref 25. Copyright 2013, American Chemical Society.

The specificity of the nanoMIPs towards vancomycin was tested with a competitive assay as follows. First, well plates were coated by addition of nanoMIPs solutions, which were evaporated overnight. To prevent non-specific binding, the wells were blocked by incubating them with bovine serum albumin (BSA) solutions in PBS. Finally, solution of horseradish peroxidase (HRP) and free vancomycin were added. The concentration was then calculated by using the absorbance values at 450 nm and comparing this to the calibration curve. To test the cross-reactivity, the same competitive assay was performed with three other commonly used antibiotics, respectively, gentamicin, bleomycin, and amoxicillin.

This approach is a promising alternative to traditional ELISA assays as the sensitivity of the described assay is a factor of three orders of magnitude better. In addition, MIPs can be stored for a long period of time without adverse effect to the binding properties and non-specific binding in biological samples is minimal [26],[27]. In the case of the nanoMIP assay for vancomycin, well plates MIPs could be stored 1 month at room temperature before adverse effects on the detection of the template were observed [25]. The approach is not just limited to vancomycin, in the past the same assay has been reported for beta-agonists such as norepinephrine and epinephrine. With the generic nature of the nanoMIP preparation in mind, this suggests the assays for more analytes, such as pollutants, peptides, proteins and disease markers, can be developed in a relatively short time. In 2015, MIP Diagnostics Ltd. was founded, a spin-off from the University of Leicester, who have developed an automated device for synthesizing MIPs within several weeks of receiving the target.

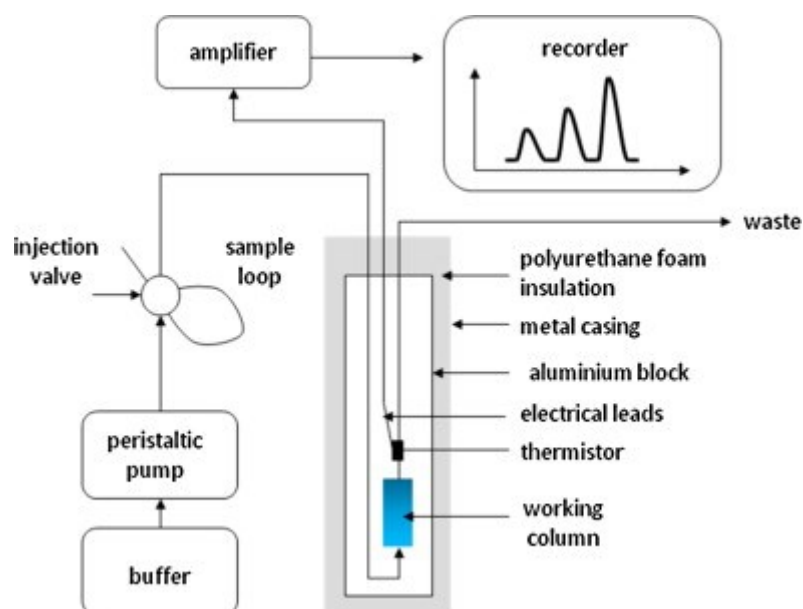
For biosensing purposes, most MIP-based biosensing platforms are focused around optical, fluorescent or electrochemical detection. In this review, we focus on the application of thermal read-out techniques for the detection of small organic molecules with MIP-based sensor platforms. First, we discuss the use of ultrasensitive isothermal calorimetry, which generates thermodynamic data and measures template-MIP binding in solution by evaluating the binding enthalpy. In the next section, thermal techniques for binding of organic small molecules to immobilized MIP-layers are reported; including thermistors and the heat-transfer method. Finally, an outlook is provided on a new thermal technique that relies on the thermal wave transport analysis.

1.2. Comparison of thermal detection techniques

We will briefly introduce four different thermal techniques here and describe their similarities and differences. Examples of small molecule detection with the methods will be discussed in more detail in section 2, 3, 4 and 5.

Isothermal titration calorimetry is the only method in which the MIPs do not need to be immobilized on the surface. To perform measurements, the template is dissolved in a buffer solution into which aliquots of either the monomer or the polymer microgel are titrated [28]. Changes in the heat (W) are recorded and can be correlated to the fraction of bound ligand. It is used to determine binding affinities (K_a) and study thermodynamic parameters, such as the binding enthalpy (ΔH), entropy changes (ΔS) and binding stoichiometry or complexation ratio [29]. This makes this technique perfectly suitable for high-throughput screening of monomers, but it is not commonly used for detection.

The second method is based on thermistors; a type of resistor, in general a polymer or a ceramic, whose resistance is highly dependent on the temperature. A typical sensor would involve MIP particles packed into columns (Scheme 1) and subsequently placed into insulated thermistor blocks. To perform measurements, analyte solutions are introduced into the system and temperature differences are monitored [30].



Scheme 1. At the top of the column packed with MIP particles, a thermistor is fixated that is connected to a Wheatstone bridge to determine temperature changes of 0.001°C . Reproduced with permission from ref 30, copyright 2013 from Elsevier.

A temperature profile is measured over time and binding of substrates to the MIP layer results in changes in the mK range. The temperature peak height, in addition to area under the curve, is proportional to the enthalpy and corresponds to a specific substrate concentration. For most reactions, concentrations can be determined in the 0.01-100 mM range [31].

The heat-transfer method is significantly different to these other methods; instead of measuring a temperature, a temperature gradient is obtained by applying a heat-flow through the MIP particles. The thermal resistance is then determined by dividing the gradient over the power into account, which is then correlated to the concentration of target molecules bound to the MIP layer [32]. Measurements are performed with a low-cost and home-made set up, while the other two thermal detection methods require expensive equipment. The main advantage is the limit of detection, with HTM this is in the nM range compared to mM concentrations measured by thermistors. However, this method is not suitable to perform kinetic measurements and no additional information about the thermodynamics parameters ΔH or ΔS is obtained [32].

Finally, thermal wave transport analysis is described, a novel method that has only been used for the determination of dopamine concentrations [33]. Experiments were performed with the same set up as for HTM. Instead of keeping electrodes functionalized with MIPs at a fixed temperature, a thermal wave is sent through the MIP sample by first heating it up and subsequently cooling it down. The element that is measured is not the thermal resistance but the phase shift, as this is associated with how fast or how slow a system responds when it is subjected to temperature changes. It has to be noted that increases in the thermal resistance, which occur due to binding of small organic molecules

to the MIP layer, will also result in a delay in the phase shift. Advantages include that this two methods can be applied simultaneously, but advantages of TWTA are a shorter measurement time and higher effect size due to decrease of the noise on the signal.

This information is summarized into Table 1 to demonstrate the strength and weaknesses of each technique.

1.3 Advantages thermal detection for biosensing of small organic molecules

Biosensors with MIPs as recognition element instead of natural receptors offer several advantages, including; long-term storage, excellent stability, re-usability, possibility to tailor the MIP to its target, and animal use is not required [3, 11]. The area of MIPs for separation and extraction is well-established and chromatography is commonly used to determine target concentrations. MIP particles are directly packed into columns and can distinguish between targets and their corresponding enantiomers. The main drawbacks are the cost of the equipment and the fact that it is not possible to develop a portable sensor, which can measure concentrations on the spot. Miniaturization is important when designing a point-of-care sensor, and optical and gravimetric techniques do not offer a viable alternative. Electrochemical sensors can be operated as handheld devices, are suitable to determine traces of molecules, and are relatively low-cost[20]. However, issues can arise with the selectivity, interpretation of the data, and it is often limited to the use of monomers or a template that possesses electroactive properties.

All discussed thermal detection techniques have the advantages that they are simple, easy to interpret, and can be applied to all templates and versatile MIP polymer structures. ITC and thermistors provide valuable information for the screening of monomers and thermodynamic parameters that are difficult to obtain with other methods. The other two methods, respectively, HTM and TWTA, have the possibility of integration into portable on-site sensors. The acquired limits of detection are suitable for detection in buffer solutions, but more research needs to be performed to demonstrate their proof-of-application with relevant biological samples.

2. The use of calorimetry for MIP template complexation and recognition

In order to use molecular imprinting technology for commercial applications, there needs to be a high affinity between the developed MIP and its template. In previous experimental studies, techniques that were used to study the template/functional monomer interaction include Nuclear Magnetic Resonance (NMR) studies [34],[35], FTIR spectroscopy [36], [37], and to a lesser extent, calorimetry [38].

With calorimetry, the release and absorbance of reaction heat is measured during physical state changes of a material by heating [39, 40]. It offers the advantage that no immobilization of the MIPs or any modification to starting reactants is required, but the equipment is not generally available in labs in contrast to FTIR and NMR, thereby limiting its application. Studies on the pre-polymerization complex have been performed with two calorimetric methods; respectively, isothermal batch and titration calorimetry. The latter is preferred, since in batch calorimetric experiments unstirred samples are affected by strong solvent-polymer interactions, such as swelling of the material over time.

Nicholls has determined that the degree of template complexation is associated with the change in Gibbs free energy of formation of each mode of template-functional monomer interaction [29]. It is assumed that MIPs do not undergo conformation changes during recognition of the template and hydrophobic interactions are negligible since rebinding experiments are in general performed in lipophilic solvents. This means that the energetic contributions of template-functional monomer interactions, according to Nicholls, can be simplified to [41] :

Equation 1:

$$\Delta G_{bind} = \Delta G_{t+r} + \Delta G_r + \Delta G_{vib} + \Sigma \Delta G_p$$

where the Gibbs free energy changes are: ΔG_{bind} , complex formation; ΔG_{t+r} , translational and rotational; ΔG_r , restriction of rotors upon complexation; ΔG_{vib} , residual soft vibrational modes; $\Sigma \Delta G_p$, the sum of interacting polar group contributions.

There are several examples in literature of the use of ultrasensitive isothermal titration calorimetry to evaluate the binding properties of MIPs towards its template. Weber et al., [28] studied the response of microgels imprinted with L-Boc-phenylalanine anilide (L-BFA) to a range of probe molecules, observing significant difference between the binding enthalpy of the original template compared to the other competitors. Hsu et al. [42] determined that insertion of a styrene monomer into a MIP composed of polyethylene glycol dimethacrylate enhanced the extraction of the enzyme Ribonuclease A. This was determined via two different detection methods, respectively, with Enzyme-Linked ImmunoSorbent Assay (ELISA) tests and with microcalorimetry. The results are summarized in Table 2, where the number after SM indicates the percentage of styrene incorporated into the monomer mixture.

From this table, it can be seen that the imprint factor (IF) as calculated by ELISA assays and microcalorimetry follows roughly the same trend. The results for SM-40 are significantly different however, where ELISA assays predict a lower IF (1.53) with microcalorimetry a significant increase

(4.96) is determined. This could be because in the case of ELISA tests MIPs are functionalized onto a surface and non-specific binding becomes a more important factor.

Overall, isothermal calorimetry allows fast and low-cost screening of template-monomer interactions and is used for the design and optimization of MIPs for a variety of targets. Selectivity can be determined up to a level of chiral recognition, as was demonstrated with a MIP for phenyl- α -mannopyranoside [43]. At a low degree of occupation of the binding sites, significant differences were observed in the endothermic heat effect for the D and L enantiomers, indicating enantioselective rebinding of the template. Besides detection, microcalorimetry is also used to evaluate binding mechanisms, complexation ratios and material properties of MIPs. The binding mechanism of 2-4-dichlorophenoxyacetic acid to a vinylpyridine MIP was elucidated by microcalorimetry [44]. It occurs in several distinct steps, starting with the dehydration of the pores, followed by binding of the template, and finally, rearrangement of water molecules. The process was pH dependent, as shown in Table 3 where the thermodynamic parameters for the MIP and NIP are listed at different pH values. The values obtained in this study are comparable to previous literature [45]. It shows that at acidic pH values, binding to the MIP is driven by entropy while for the NIP enthalpy is the dominating force. This is line with the proposed binding mechanism, which involves steric complementary. At basic pH values the electrostatic repulsion forces control the binding and a higher positive enthalpy is obtained [44]. The use of isothermal titration calorimetry to monitor the energetics of formation of the MIP pre-polymerization complex was demonstrated by Zhao *et al.* [46]. A MIP for patulin, a contaminant, was prepared by grafting polymer brushes onto a silica support. The formation of the pre-polymerization complex was determined by titrating solutions of the functional monomer, acrylamide, to template solutions in the calorimetric cell. By measuring the heat changes after each addition the binding constant (K) and enthalpy change (ΔH) were calculated.

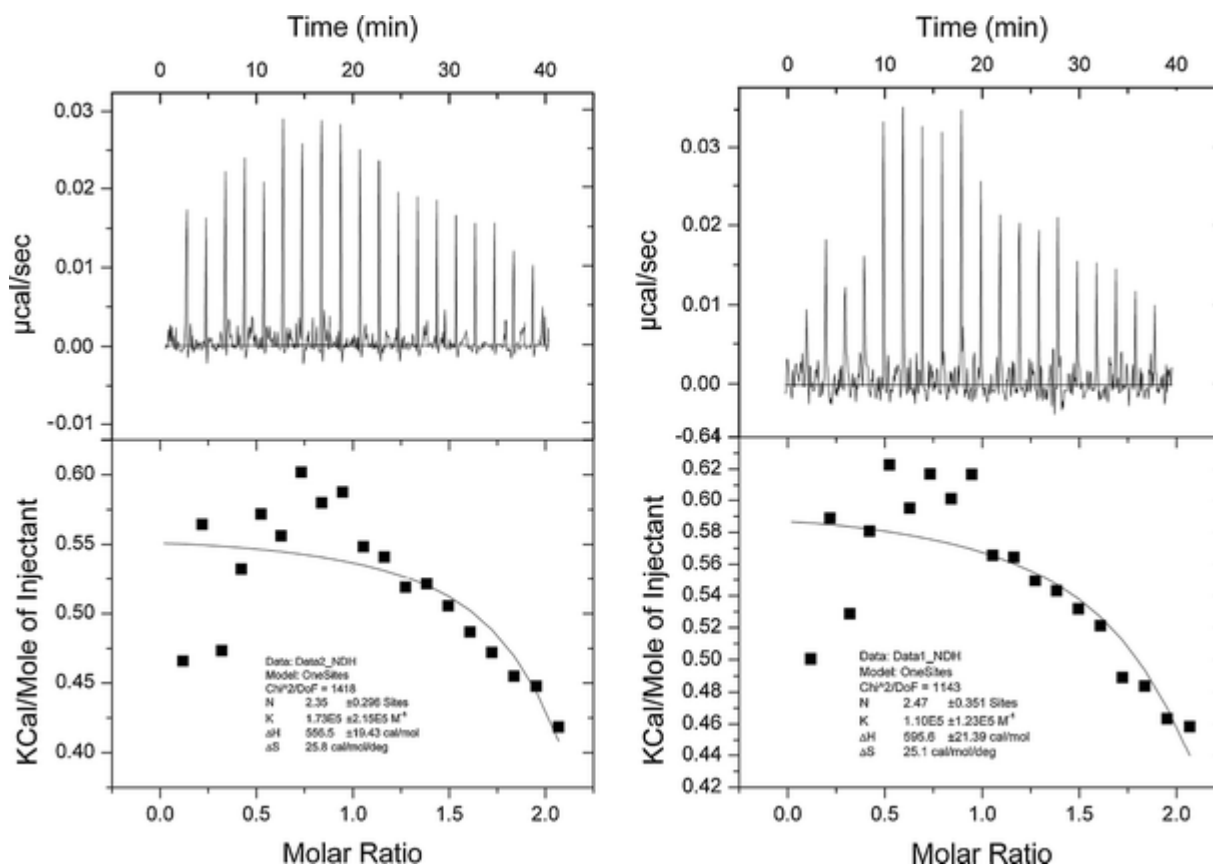


Figure 2. Isothermal titration calorimetry data for the template binding to the functional monomer acrylamide. The profiles are given of titration of acrylamide with patulin (left) and 6-hydroxynicotinic acid (right) in aqueous solutions. In the upper panel the raw data of the titration is provided, with in the lower panels the normalized data is provided[46]. Reproduced with permission from ref 46, copyright 2011 from Springer.

From the microcalorimetry graphs, it was determined that there is a strong donor-acceptor hydrogen bond formed between the monomer and both templates. The plot of the kcal/mole of the injectant vs the molar ratio demonstrates the affinity distribution and the maximum peak height at a molar ratio of 1 indicates that there is a 1:1 ratio of binding for the monomer to the template. This was confirmed by molecular modeling, and by performing batch rebinding experiments and analyzing them with Scatchard analysis. While for these two templates a similar pattern in titration experiments is observed, for a similar molecule, 5-hydroxymethyl-2-furaldehyde, a distinctly different result is obtained. As a consequence, the synthesis needs to be adapted and after identifying the groups participating in pre-polymerization complex formation need to be matched with the functional monomer that is employed in the MIP synthesis.

Fish et al.[47] used isothermal titration calorimetry to study complexation ratios between the functional monomer, methacrylic acid, and the template, the drug cinchonidine. Calorimetric titrations elucidated that the minimum energy of the complex was at a ratio of 4:1 mol/mol for the

monomer relative to the template. This demonstrates that isothermal calorimetry is a useful tool for the synthesis of MIPs, as it speeds up the optimization process and allows for screening of different monomers. It can also be used to model the presence of heterogeneous binding sites in MIPs [48, 49], which supports in the design process[50].

3. Thermistors as read-out technique for biosensing with MIPs

Thermistors are resistors that exhibit a large and precise change in electrical resistance when subjected to a temperature change [31]. Biochemical processes involve the evolution of heat, with the total heat that is produced depending on the molar enthalpy change (ΔH) and the total number of moles of product (n) used in the reaction [30], [51]. The relative temperature change associated with the reaction is dependent on the total heat capacity (C_s) of the system. This difference ΔT , as is measured with the read-out of the sensor, is then defined by Equation 2 [30].

Equation 2:

$$\Delta T = -(n \Delta H / C_s)$$

Enthalpy changes for enzyme catalysis are associated with values up to 200 kJ/mol, which is sufficient to determine metabolites concentrations up to the low micromolar regime [52, 53]. In molecular imprinting, as described previously, binding of the template to the nanocavities results in an enthalpy change. The benefit of this technique is that it is label-free and the MIPs used in analysis do not need to be integrated on the sensor, eliminating the need of an immobilization step.

Nowadays, they are used for several sensing applications, but examples of MIP-based platforms with thermistors as read-out are sparse. The first combination of a thermistor transducer for the recognition of small organic molecules with catalytically active MIPs was described by Lettau et al. [54]. In this work, MIPs were prepared that possessed catalytic activity towards the substrates phenylacetate and 4-nitrophenylacetate. For functionalization of the particles, 150 mg of MIP or NIP were packed in a column in a flow reactor. Phenylacetate concentrations in aqueous solutions (0.5 – 5 mM) were introduced to the system, leading to a peak in the temperature due to conversion of the substrate. After flowing the system with buffer, a negative peak was observed that returned to the baseline, which was because the substrate was removed from the reactor. Saturation occurred from concentrations higher than 0.5 mM, with the operating regime of the sensor from 0.05 to 0.5 mM. The specificity of the MIPs was demonstrated by the addition of the reaction products phenol and acetic acid, which gave a significantly different response in the calorimetric signal. The data was then used to determine kinetic parameters of the MIPs and a Michaelis Menten constant of 2.2 mM was calculated, which is similar to what was previously determined for esterolytic MIPs.

In a follow up on this work, a MIP for nitrofurantoin was optimized by combining diaminopyridine derivative (BMP) as the first monomer with a urea based derivative (VFU) as the second monomer. For the first time, detection with a MIP-based thermistor was achieved in an organic solvent[55] [56]. The binding of the template to the MIP is observed as an exothermic peak signal that appears after injection of the analyte, which is subsequently followed by endothermic desorption after washing with the solvent. This exothermic peak correlates to enthalpy changes associated with non-covalent complex formation and changes of solvation of the template at the imprinted polymer[43, 57]. By monitoring the exothermic peaks, it was possible to measure nitrofurantoin in the micromolar regime in a combination of acetonitrile and dimethylsulfoxide (DMSO). In Figure 3, the binding of the template nitrofurantoin is given at the exothermic peak for both the MIP and the reference NIP [56].

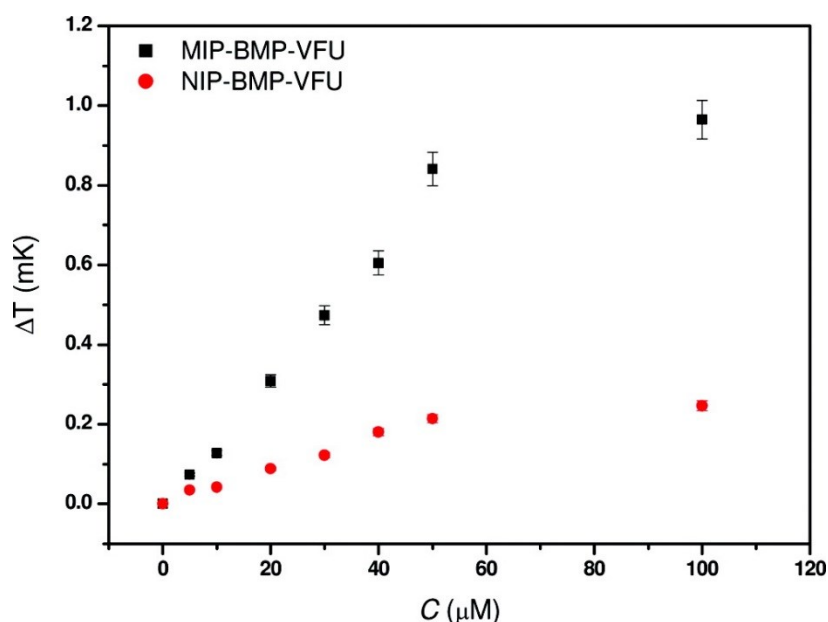


Figure 3. Concentration dependence of nitrofurantoin binding to the imprinted polymer based on two functional monomers MIP-BMP-VFU and the control polymer NIP-BMP-VFU investigated by thermometric sensing in acetonitrile + 0.2% DMSO. (Data represent mean (s.d., n = 3).

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The imprint factor that was achieved with thermal detection was similar compared to traditional batch rebinding experiments. There are examples in literature which show significant improvement in the imprint factor such as MIPs containing phenyl boronic acid residues for the rebinding of fructosyl valine[58]. In contrast to previous experiments, here a flow through setup was used to enhance the sensor performance. This concept was extended to fructose and pinacol and showed that the choice of the cross-linkers has a significant impact on the non-specific binding to the control polymer [59].

With this method, label-free detection of fructose was achieved in the range of 0.5-10 mM in buffer solutions and the imprint factor was increased with a factor or nearly tenfold compared to traditional batch rebinding experiments. An additional benefit of combining MIPs with thermistors is that it can be used for characterization of the material since differences in heat generation reflect different recognition sites present in the MIPs [54].

4. The Heat-Transfer Method (HTM)

In 2012, van Grinsven et al. [32] discovered an anomaly in the thermal behavior of DNA at its melting temperature. At this temperature, DNA undergoes a structural conformation from a well-defined elongated brush to a spaghetti-coiled structure, increasing the surface area by nearly 150%. This effect is known to result in an increase of the electrical resistance due to the formation of an additional insulating layer [60], but the increase in the thermal resistance (R_{th}) had not been reported in literature before. The thermal resistance is analogous to the electrical resistance and is defined according to Equation 3 [61].

$$\text{Equation 3: } R_{th} = \frac{\Delta T}{P}$$

In this formula, ΔT ($^{\circ}\text{C}$) corresponds to the temperature difference over the sample and P (W) to the heating power of the heat sink. The schematic design of this heat-transfer device is shown in Figure 4.

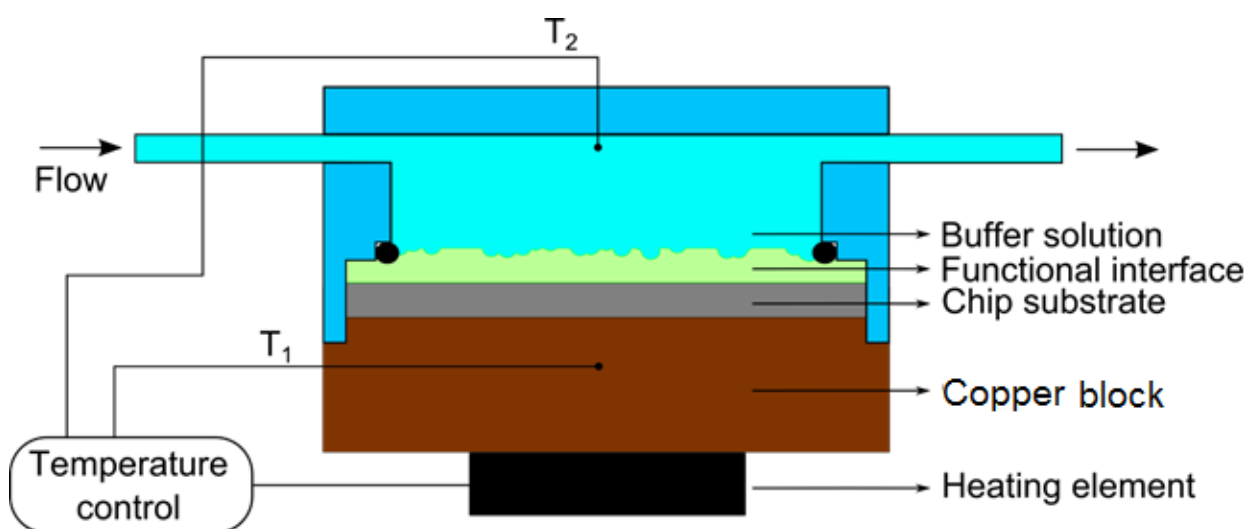


Figure 4. Schematic layout of the HTM setup, in which the sensor chip is connected to a copper block with the functional interface facing the liquid flow cell. The temperature T_1 is actively steered, while temperature T_2 in the fluid is recorded with a second thermocouple. With the heat-

transfer related parameters T_1 - T_2 and the input power P , the thermal resistance in time at the solid liquid-interface is calculated. Reproduced with permission from ref 61. Copyright 2012, American Chemical Society.

This technique, referred to as the heat-transfer method (HTM), has been extended to a variety of biosensing applications since it is label-free, low-cost, fast, sensitive, and user-friendly due to the nature of the proposed read-out technology. At the moment, current use is for determining phase transitions in lipid vesicles [62], single nucleotide polymorphisms in DNA[32], measuring proteins concentrations by aptamers[63] [64], and molecular imprinting technology [65, 66]. In the latter, we can distinguish between the surface imprinting technique that is used for larger templates such as cells and proteins[67, 68] , and the bulk imprinting technique, which is more common for small organic molecules [2, 69]. For surface imprinting, cells are pressed onto a semi-cured polyurethane layer and subsequently removed after cross-linking [66]. This removal results into the presence of micron-sized cavities on the surface and when template cells rebind, heat-transfer is blocked into that direction. With HTM as read-out technique, specific detection of cells with SIPs with a limit of up to 10^4 cells/ml was achieved. In follow up of this research the detection limit was improved by a factor of 30% by using polished alumina substrates, eliminating the use of conductive silver paste and thereby lowering the resistance at the interface [70]. This principle is not just limited to detection, but has also been used for screening of cell quality assays and demonstrated that after culturing cells for a prolonged period of time, the bond to the imprint is weaker due to the difference in functional groups on the membrane [71]. Selectivity problems can be overcome by coupling the system to a peristaltic pump in order to control the flow-rate during sample addition. The competitor molecules bind with a lower affinity to the MIP cavities on the surface and were easily removed after flushing at a high rate with a buffer solution. This shows that non-specific binding to the imprints is completely overcome by shear forces imposed by the liquid flow. In past work, it was demonstrated this is sufficient to prevent binding of different types of breast cancer cell lines.

In the case of bulk imprinting, the cavities are not present on the surface but inside the material and rebinding of the template results into partial blocking of the heat-transfer inside the pores. The underlying principle detection principle by HTM is referred to as the “pore-blocking model” [65], shown in Figure 5.

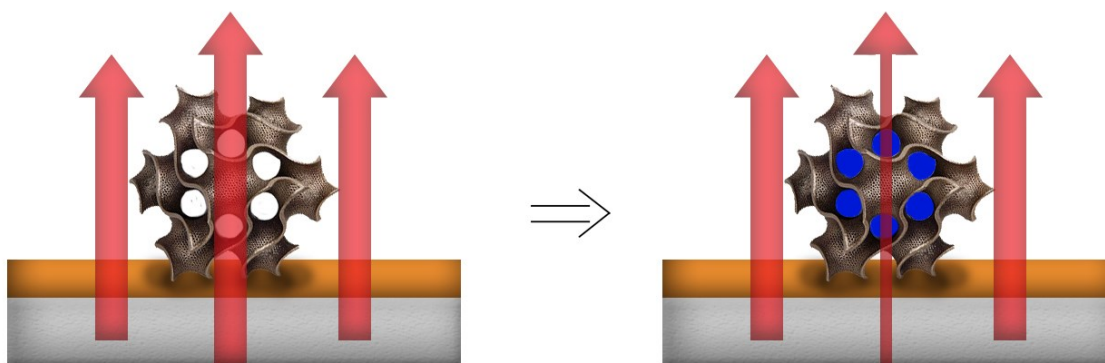


Figure 5. Artistic impression of the “pore-blocking model”, with the MIP particle embedded in the surface with various pores or binding sites of the template molecule. When the channels are filled by the target molecule (indicated by blue dots), heat-flux through the MIP layer is strongly reduced and the overall heat-transfer resistance is increased. Reprinted with permission from ref 65.

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To functionalize the MIP particles on the surface, the particles are applied onto an adhesive layer that was spin-coated prior on the electrode. Several polymers were tested as adhesive layers, including polyvinylchloride (PVC) and modified MDMO-PolyPhenyleneVinylene (MDMO-PPV). Both materials have a low glass transition temperature and by slight heating of the substrate, the polymer coating will exhibit liquid-like properties and MIP particles are allowed to sink into the adhesive layer. The main difference is that PVC is insulating compared to MDMO-PPV, and for performing heat-transfer measurement it was preferred to have a conductive adhesive layer on the substrate in order for heat to freely pass through the electrode surface. After functionalizing the MIPs onto the electrodes, attachment was studied with optical microscopy and it was determined that ~ 30% of the surface was occupied with MIP particles [65].

This process has been performed for MIPs designed for neurotransmitters histamine, L-nicotine, and serotonin, and detection limits between 50 to 100 nM were achieved in buffered saline solutions using HTM as read-out technique. As references, the NIPs were used and the response of the MIPs was tested against competitor molecules. For L-nicotine its metabolite cotinine was studied, for the template serotonin dopamine was employed, and histamine was tested against histidine. As a first proof-of-application, saliva samples spiked with L-nicotine were measured. This was proven to be difficult, since high noise levels were obtained that originate from the viscosity of the sample and from the noise generated by the power supply [65]. By fine tuning the settings of the feedback loop,

the detection limit of the neurotransmitter L-nicotine was brought down to 33 nM in buffer solutions [72].

A first array sensor set up of the HTM equipment was developed by Wackers et al., [73], who segmented a flow-through sensor into four quadrants with a sample volume of $\sim 20 \mu\text{L}$. With this design, it was possible to discriminate between histamine, serotonin and L-nicotine without significant cross-selectivity. To enhance the detection limit with HTM as read-out, improvements can be done on the heat-transfer of the whole system, which includes both the MIPs and the supporting electrode, and on the functionalization of the synthetic receptors on the surface. The MIPs itself are insulating, therefore thinner layers rather than micron-sized particles would lower the overall thermal resistance and increase the relative effect size. There are other supporting materials available rather than electrode bare alumina, which could also stimulate the heat-flux for the sample. Grafting MIPs onto graphene fulfills both these criteria since it allows a small dimension, high surface-to-volume-ratio and graphene possesses superior thermal properties[74]. While there are some examples in literature [75] [76], it is not possible to directly graft MIP layers onto graphene and therefore graphene oxide is a more viable alternative due to the presence of oxygen functionalities. Peeters et al. [77] described a method for developing a MIP-graphene oxide (GO) hybrid by employing reversible addition-fragmentation chain transfer (RAFT). The nanosized layers were capable of detecting histamine with HTM in the nanomolar regime with a similar detection limit as what was obtained with micron-sized particles. However, the drawback was that the MIP-GO hybrids still needed to be physically absorbed onto the electrodes, and directed grafting strategies from literature might further improve the detection limit [78, 79].

The main drawback in terms of sample preparation is the long preparation time and there is no direct control of the deposition of particles on the surface. Electro polymerization is an alternative that could be explored, allowing fast synthesis and monolayer coverage of the surface [20, 80, 81]. Control over the synthesis remains complicated however, which could be optimized by using controlled polymerization techniques [82-84]. Controlled grafting of MIPs from electrode surfaces or supports, such as silica beads, resulted in thin imprinted polymer films with a thickness in the range of 1-2 nm. The obtained structures were more homogeneous and the higher surface area lead to superior chromatographic performance compared to MIPs prepared by bulk polymerization. In the case of thermal detection, these controlled methods were not explored yet. This is particularly interesting in the case of graphene as support material since it exhibits superior thermal properties compared to the metals that are as electrodes now, such as alumina and silicon.

5. Thermal wave transport analysis

In the case of HTM, the sample is kept at a fixed temperature with a constant thermal flux and stringent control is required through the feedback system. While nanomolar concentrations are measured with this technique, drawbacks include the noise originating from the temperature control and the long measurement time (15 min), which does not allow kinetic experiments. Recently, a new method was developed which focuses on a thermal wave (amplitude = 0.1°C) that is transmitted through the functional interface and its output measured in the liquid (Figure 4) [33]. Thermal waves through polymers have been used in past work to study the charge distribution and transport phenomena in polymers with resolutions down to 1 micron [85]. In addition, glass transition temperatures of nano-sized polymer films were accurately determined [86]. The applications in literature for polymers are only limited and no attempt has been recorded on combining thermal waves with MIPs.

From previous research, it is known that when binding occurs onto the MIP layer, the heat-transfer resistance increases[65]. As a result, the overall response of the system to temperature fluctuations is delayed and the phase shift can be related to the amount of neurotransmitter bound on the surface of the polymer.

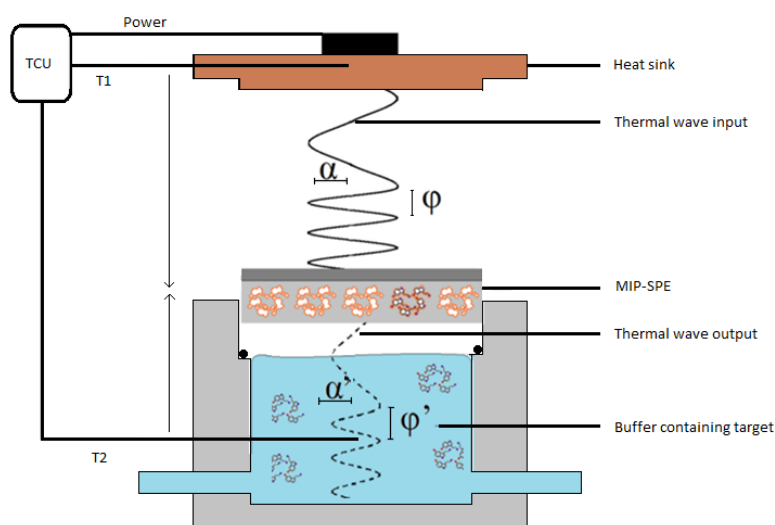


Figure 6. Schematic representation of the thermal wave analysis transport set up. A thermal wave was applied from the heat sink to the MIP-layer and the output was measured in the liquid. When targets binds to the MIP, it induces a delay in the phase ($\varphi \neq \varphi'$) and a reduction in amplitude ($\alpha \neq \alpha'$) of the signal[33].

This technique is measured with the same equipment as for HTM and since both methods are performed simultaneously, it provides the option to directly validate results. In first proof-of-principle tests, a different approach for sample preparation was used involving the use of screen-printed

electrodes (SPEs). SPEs were selected since they are cost-effective, highly reproducible, and suitable for incorporation into portable devices [87-89]. There are a few examples of MIP-based sensors that are combined with SPEs, but literature examples are sparse [89-91]. Peeters et al. have presented a new concept for the design of functionalized SPEs, which was based on direct mixing of the MIP particles with the bulk of the ink used to fabricate the SPEs. Various particle to ink ratios were tested, with 30% of particles vs ink giving the optimal result. Lower particle ratio, such as 5%, resulted in significantly lowering the effect size. A ratio of higher than 30% was not achievable; above a certain threshold the ink loses its printability because there is not enough graphene ink present.

This is the first time MIP functionalization on SPEs is shown in a single step, allowing fast preparation and high-throughput printing of the electrodes. As proof-of-principle, MIP particles for dopamine were incorporated onto SPEs. Dopamine is a neurotransmitter that is indispensable for the efficient functioning of a variety of physiological functions and abnormalities in its concentration are associated with psychiatric disorders such as schizophrenia and depression [92, 93]. Subsequently, MIP-SPEs were mounted into the heat-transfer set up and measurements were performed with aqueous solutions that were spiked with dopamine. Detection of dopamine was proven to be specific, as no response was observed of the NIP to dopamine. To demonstrate proof-of-application of the technique, one spiked food sample was studied. In Table 4, the detection limits for SPE-MIPs are provided with three different techniques, respectively, cyclic voltammetry, HTM and TWTA. From this table, it is directly clear that TWTA has the benefit of significantly reducing measurement time and lowering detection limits in complex samples compared to HTM. To demonstrate this technique is generic, the system has to be extended to other neurotransmitters or proteins by adapting the MIP interface layer. In addition, several parameters can be varied to optimize measurements, such as the composition of the screen-printing ink, use of different SPE substrates and the thickness of the substrates.

6. Summary

MIP-based biosensor platforms provide a viable alternative towards traditional ELISA assays but the potential of thermal detection technique remains relatively unexplored. Therefore, the potential of thermal methods for the detection of neurotransmitters with MIPs has been evaluated. It is focused on four detection methods, respectively: calorimetry, thermistors, the heat-transfer method, and a new technique, thermal wave transport analysis. Calorimetry allows fast and low-cost screening of template-monomer interactions and its main application is for the design and optimization of MIPs, as it enables determining heterogeneous binding sites in the synthetic receptors. The examples of MIP-based thermistors in literature are sparse and detection limits are not sufficient to measure biological samples, but it provides useful insight into the distribution of binding sites into MIP

particles. The heat-transfer method (HTM) is an exciting and emerging technique based upon heat-transfer, which can measure neurotransmitter concentrations in buffer solutions in a fast and low-cost manner. Through adapting the thermal resistance from measuring a fixed heat-transfer resistance to the application of thermal waves, the new concept thermal wave transport analysis (TWTA) was presented. Preliminary results indicate an enhanced effect size compared to HTM by reducing the noise on the signal, which provides the opportunity to measure in complex samples. In summary, thermal techniques provide a low-cost and straightforward alternative for detection of low-weight organic molecules with MIPs, which will require more study in the future to transform this platform into array formats.

Abbreviations

BMP – Diaminopyridine derivative

BSA – Bovine Serum Albumin

DMSO – DiMethylSulfOxide

ELISA – Enzyme-Linked ImmunoSorbent Assay

FTIR- Fourier Transformed Infrared Spectroscopy

GO- Graphene Oxide

HRP – HorseRadish Peroxidase

HTM – Heat-Transfer Method

L-BFA - L-Boc-Phenylalanine Anilide

MIP – Molecularly Imprinted Polymer

NMR – Nuclear Magnetic Resonance

PPV - PolyPhenyleneVinylene

PVC – PolyVinyl Chloride

RAFT - Reversible Addition-Fragmentation chain Transfer

R_{th} – Thermal Resistance

SPE – Screen-Printed Electrode

TWTA – Thermal Wave Transport Analysis

VFU – Vinylphenyl Urea based derivative

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Tables

Table 1. The different properties of the detection methods ITC, thermistors, HTM, and TWTA are compared, demonstrating the weaknesses and the strength of each technique.

	ITC	Thermistor	HTM	TWTA
LOD	-	-	+	+
Measurement time	++	+	-	+
Costs set up	-	+	++	++
Operating costs	++	++	++	++
Selectivity	++	+	+	+
General applicability	++	++	++	?
Stability	++	+	+	+
Sample preparation	++	+	-	+
Portability system	-	-	+	+

Table 2. Rebinding efficiency of MIPs and exothermal rebinding of RNase A (0.05 wt% in PBS) to various styrene containing MIPs and NIPs, as measured by ELISA and isothermal calorimetry.[42]

Polymer compositions	Imprint factor by ELISA	Imprint factor by calorimetry
SM-00	2.27	2.78
SM-10	8.56	2.75
SM-20	10.43	6.15
SM-40	1.53	4.96

Table 3. The thermodynamic parameters of 2,4-D binding to MIP and NIP at different pH values[44]

Thermodynamic Parameter	pH 3		pH 6		pH 9.5	
	MIP	NIP	MIP	NIP	MIP	NIP
ΔG_{ads} (kJ/mole)	-4.35	-2.78	-3.11	-5.38	-3.27	-6.26
ΔH_{ads} (kJ/mole)	3.25	3.25	0.85	-9.52	8.14	10.25
$\Delta T S_{ads}$ (kJ/mole)	7.6	5.63	3.96	-4.14	11.41	16.51

Table 4. Detection limits for MIP-modified SPEs of dopamine in buffer solutions and in a food sample for cyclic voltammetry, and the thermal methods HTM, and TWTA. The sample preparation time is associated with the preparation time per sample.

Detection method	LOD buffer solutions (nM)	LOD food sample spiked with dopamine (nM)	Sample preparation time (min)	Sample measurement time (min)
Cyclic voltammetry	4700 ± 50	-	1	2
Heat-transfer method (HTM)	350 ± 30	500 ± 50 nM	45	15-20
Thermal wave transport analysis	260 ± 35	150 ± 40 nM	1	3-5