

Chapter 9

Theoretical Considerations for the Application of Molecular Imprints for Chemical Analysis[#]

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Abstract

Development of new selective analytical methods and sample enrichment techniques remains of interest. The implementation of molecular imprints in chemical analysis may offer objective advantages over existing methodologies. Criteria for the applicability of molecular imprints (MIPs) in separation methods, ligand-binding assays, sample pretreatment and sensors are defined. It should be noted that each analytical problem asks for imprints with specific characteristics. Affinities can be comparable with those observed for antigen-antibody interactions, which enables application in ligand-binding assays and for sensors. However for application in separation methods, moderate affinities, allowing fast mass transfer between mobile and stationary phase are essential to obtain highly efficient separations. For sample pretreatment procedures a large range of affinities can be used, however large sample volumes can only be processed with high affinity materials. Considering the state-of-the-art of molecular imprinting, the application for sample pretreatment directed to pre-concentration looks the most promising. It is anticipated that molecular imprints can find a prominent place in pharmaceutical research not only as a tool to improve selectivity and sensitivity in analytical methods, but also for selective extraction of drugs or intermediates from reaction media. In an environmental context, these imprints may be used for the analysis and/or removal of pollutants.

9.1 Introduction

Trace analytical methods for complex matrices rely on efficient sample enrichment techniques and specific assays. A low selectivity and/or a low capacity for the target analyte often limit current enrichment techniques.

Development of new selective analytical methods and sample enrichment techniques are therefore of broad analytical interest. One technique that may find general use is molecular imprinting, which is used for the preparation of phases with tailor-made binding sites [1-7]. The phases can be prepared by the synthesis of (highly cross-linked) polymers in the presence of a target template or print molecule followed by removal of the

template. The remaining polymer will contain binding sites, which are complementary to the template molecule. The selection of monomers and the polymerization conditions will yield materials with different binding properties, which can be explained by the interactions between functionalities of the polymer and the template molecule.

The implementation of molecular imprints in chemical analysis should offer objective advantages over existing methodologies. This implies that production of a range of imprints and looking for a possible application is not the way to go.

At this point the properties of any imprinted materials cannot be fully programmed or even forecasted. However, for any application, criteria can be defined which should be met, to end up with superior materials and methods.

Table 9.1. Key references to the four major applications of molecular imprinting.

Applicability of molecular Imprinting	Key references, Reviews (<u>underlined</u>)
Separation methods	[<u>3,5,8,9</u>]
- chiral	[<u>10,11</u>]
- electrophoresis	[12,13]
Ligand-binding assays	[<u>3,5,8,14,15</u>]
Sample pretreatment	[<u>5,8,16-18</u>]
Sensors	[<u>3,8,19,20</u>]

In the present article we attempt to define criteria for the applicability of molecular imprints (MIPs) in different analytical techniques. Where appropriate, additional criteria indicated by the application area are given and discussed.

We consider four major analytical techniques, operating in a liquid environment: Analytical separation methods (liquid chromatography and capillary electrophoresis); sample pretreatment; ligand binding assays; sensors. The key references to the analytical techniques are given in table 9.1. Special attention is paid to the identification of approaches to control the binding characteristics within the physical boundaries of the analytical technique and the application area: Preparative separations; bioanalytical separations and trace analysis.

9.2 Theory

The most common procedures for the synthesis of molecular imprints have been reviewed in detail [5,7,21]. Single or multiple interactions of functional groups of both compounds can describe the interaction between analytes and/or template molecules and the monomers. The types of interaction can be hydrogen bonds, ion pairing, π - π interaction or driven by the hydrophobic effect. The selection of the monomer(s) is based on the generation of these particular interactions. This implies that polymers generated from these monomers will always interact with the analyte or template molecule as well as with compounds that contain functional groups similar to those of the template.

The affinity increases with the number of interacting groups, however, each individual interaction can be strongly dependent on the properties of the solvent, e.g. protic or aprotic, polarity, dielectric constants, presence of complex forming agents, etc. [22-24]. These effects can occur through interaction with the polymer, the analyte or both. This implies that affinities can vary from zero to nanomolar equilibrium dissociation constants. Therefore, application of molecular imprints appears to have potential for a range of analytical applications.

It is assumed that a change in selectivity of molecular imprinted polymers in comparison to non-imprinted polymers can only occur when the analyte and/or the matrix components of the sample will have an increased number of interaction points [25]. So, single and part of the dual point interactions that take place between the analyte and the polymer will affect the selectivity. Note that non-imprinted polymers are successfully used for analytical applications [26]. In other cases, the interaction of analytes and matrix components with non-imprinted parts of a polymer may overshadow the interactions of the analyte with the true imprint so that the selectivity is not as good as required for the application, irrespective of the imprinting efficacy. The number of binding sites present in the polymer that play a role in the selective interaction of the analyte with the imprint (cavity) is typically less than 1% but can mount to 35% of the theoretical maximum amount of binding sites [27].

For that reason the impact of both the selective (imprinted part of polymer) and the non-selective (non-imprinted part of the polymer)

interactions on the applicability of imprinted polymers in different analytical techniques is discussed here. A schematic representation of the selective and the non-selective interactions is given in Fig. 9.1.

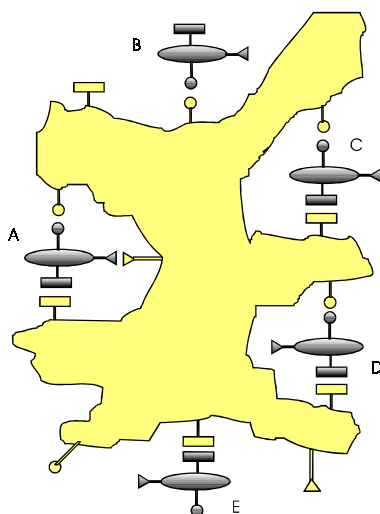


Figure 9.1. Schematic representation of the interaction of the analyte and the synthesized polymer. The polymer contains several binding sites. The most favorable energetic interaction is shown at location A, where three binding sites result in the most selective (specific) interaction of the analyte with the polymer. Two-point interactions, which are energetically less favorable, are depicted at locations C and D, where the analyte is still allowed to approach the binding sites at more than one way, and therefore is less selective in its interaction with the polymer. Locations B and E show the least selective interaction possible and are therefore the least favorable.

9.3 Separation methods

In chromatographic methods the selectivity is dependent on differences in interaction (specific and non-specific) between analytes and imprint. The selectivity is generally defined as α , where α is the ratio of the capacity factors of two compounds. These are proportional to the interaction of the analytes with the stationary phase.

For separation systems based on modified silicas, α -values exceeding 1.05 are considered adequate to obtain resolutions between eluting compounds $R_{ij} > 1.5$ allowing quantitation of the individual compounds. An α -value of 1.05 implies that the last eluting compound has a 5% higher affinity towards the stationary phase in comparison to the first eluting compound.

The resolution is not only dependent on the α -value, but also on the efficiency of the system. The efficiency is an indicator for peak broadening during the separation process. This implies that a high α -value is required in systems with severe peak broadening, whereas in highly efficient separation systems low α -values are acceptable. Fast and efficient separation systems are also important to warrant the detectability of the analytes.

Chromatographic systems using molecular imprints described by various authors show that extreme α -values can be obtained, but that hardly adequate resolutions were reported [10,28-30]. The processes responsible for peak broadening are: (1) Eddy-diffusion dependent on particle-size, particle-shape, porosity and flow rate. (2) Longitudinal diffusion dependent on the viscosity of the mobile phase and the diffusion constants of the analytes. (3) Mass transfer in/between the phases dependent on intraparticle diffusion, association with binding sites, dissociation from the binding sites, reassociation (number of binding sites).

The production process of imprinted polymers is not very elegant. Polymerization often takes place in glass tubes, which are crushed afterwards. The polymer materials are milled in a mortar and sieved. Most researchers used the fraction between 25 and 40 μm particles with irregular shapes. This explains a substantial contribution from the Eddy-diffusion to the peak broadening process. Smaller, spherical particles are much more efficient. Using polystyrene seeding microspheres with a starting diameter of 1 μm and dispersion or precipitation polymerization might be a major improvement, provided that neither the template nor the other ingredients of the polymerization mixture diffuse into the medium.

However, mass transfer plays a large role in the peak broadening process. This can be explained by the fact that retention of a compound means that molecules migrate from the mobile phase into the stationary phase independent of each other. If one molecule is captured in the stationary phase, the other molecules in the mobile phase are moving

forward. After a while this molecule will be released by the stationary phase and move forward by the mobile phase. The mean residence time of a molecule can be calculated by subtraction of the retention time of the analyte (t_r) and the time for the mobile phase to pass the column (t_{r0}). The mean residence time is composed of the sum of numerous short residences of the molecule in the stationary phase during the separation process. Because this is a random variable it means that the standard deviation of the mean residence time, *i.e.* peak broadening, is dependent on the number of phase-changes. Fast and frequent exchange therefore, implies reduced peak broadening. The situation with molecular imprints is rather complex, because the analyte molecule can be in the stationary phase in three different conditions, dissolved in the solvent in the inner pores of the particles, non-specifically bound to one or two functional groups of the polymer, or specifically bound in the cavities of the imprint. The condition that determines to a major extent the peak broadening is the binding kinetics of the latter interaction. A comparable situation exists with immunoaffinity columns. To make them suitable for chromatographic separations and not as a preconcentration device, so called soft-antibodies with affinities in the micromolar range are to be developed [31]. For both imprints and antibodies this is not so simple because reduction in affinity will often imply a reduction and a change in selectivity.

In capillary electrophoretic systems, molecular imprints can be used as well [32-34]. Capillaries can be filled with particles of the imprinted polymer [32,35,36], or alternatively polymerization can take place inside the capillary yielding a coated capillary or a continuous bed [13,33,34,37]. The separation process is dependent on differences in electromigration and on differences in interaction with the imprinted polymer. This implies that a different type of selectivity will be obtained in comparison with chromatography. In capillary electrophoretic separations selectivity values $\alpha > 1.01$ are often adequate to obtain resolutions $R_{ij} > 1.5$ between eluting peaks, allowing quantitation of the individual compounds. The low selectivity requirement can be explained by the efficiency of the separation process due to the absence of Eddy-diffusion. What remains is that, also in this separation technique using molecular imprints, the efficiency is strongly dependent on the binding kinetics of the analyte and the imprint.

For separation processes it can be concluded that molecular imprints can add different types of selectivity. It should be noted, however, that both the production process of polymers and the selection of mobile phase or run buffers should be focussed on improvement of mass transfer processes. Low affinity binding sites are essential.

The selection of monomers and cross-linkers and the extent of cross-linking are important parameters. The selection of mobile phases or run buffers should be directed to a reduction of strength of interaction between functional groups of the analytes and of the polymer and not to the elimination of a particular type of interaction, because that may lead to the situation where "recognition" by an imprinted polymer is comparable to the recognition by non-imprinted polymers.

9.4 Ligand-binding assays

For analytes that lack physicochemical properties that allow selective and sensitive quantitation in a given matrix, ligand-binding assays can be used. Ligand-binding assays are based on the competition between an analyte and a labeled ligand for binding to a macromolecule. The change in the free or bound fraction of the labeled ligand can be related to the concentration of the analyte present in the sample. So far, mainly proteins, *e.g.* antibodies or receptors have been used as binding macromolecule. Generally, binding affinities of the analyte and the label for the macromolecule are in the range from low picomolar to nanomolar concentrations. The selection of the label, *e.g.* radioactive, fluorescent or enzymatic, allows its quantitation at the same concentration level.

Receptors and antibodies do have a different type of selectivity. Only pharmacologically active compounds, eutomers and active metabolites, can be measured with receptor assays. If these compounds are present in combination, the sum of compounds will be determined providing a therapeutic relevant parameter. Immunoassays will recognize compounds dependent on the way the antibodies have been raised. Because most small molecular compounds are not immunogenic, they have to be linked via a chemical spacer to a larger protein to induce antibody formation. The antibody will discriminate between substances with

differences in chemical structure with the exception of the site of the analyte where the spacer was linked. This implies that selectivities can be modulated to a large extent. If more compounds with affinity for the antibody are present in a sample they will be codetermined. However, the outcome of such an assay is often meaningless [38,39]. The use of molecular imprints that recognize a particular analyte is principally interesting because, in contrast to antibodies, all functional groups in the analyte molecule can contribute to its recognition by the imprinted polymer. The imprint should selectively recognize both the analyte and labeled ligand. Dependent on the type of label this might imply some difficulties. Radioactive tracers do hardly affect the structure of the molecule, but fluorescent labels are rather bulky and are coupled directly or via a spacer to a functional group of the analyte molecule. This can have a large impact on the interaction with the imprint although some successful examples have been shown [19,40]. Recently, the preparation of a fluorescent molecularly imprinted polymer has been reported [15].

The selectivity of a ligand-binding assay is dependent on differences in interaction between analytes and sorbent. However, the differences needed to obtain accurate measurements are magnitudes higher than for separation processes. A sorbent used in chromatography showing an α -value of 1.05, that is considered to be adequate to obtain baseline separation, implies a cross-reactivity of approximately 95% in a ligand binding assay. Cross-reactivities of other compounds should be smaller than 1% to allow quantitation of the compound with the highest affinity.

The sensitivity of ligand-binding assays is dependent on the affinity of the analyte and the labeled ligand towards the imprint. A small number of binding sites relative to the number of analyte and labeled ligand molecules is a prerequisite to obtain competition. It would be preferable if there is a single population binding sites. However, it seems that there is a gradient of affinities. It is claimed that production processes of molecular imprints are highly reproducible which might be favorable over the production of mono- and polyclonal antibodies. Nevertheless, experimental data disprove the idea that small variations in the polymerization process do not affect the gradient of affinities for a given imprinted polymer. It remains to be seen whether or not affinities can be obtained that can compete with those of antibodies. Association and dissociation kinetics are

less critical in comparison with separation methods, although dependent on the method to separate bound and free fraction of the labeled ligand. A slow dissociation rate is favorable for the sensitivity and for the separation in that equilibria are maintained.

To facilitate this separation step, imprints can be bound to the surface of test tubes or microtiter plates, enabling automation of assays. The required number of binding sites depends on the detectability of the label and its affinity towards the binding sites. The number of binding sites should be constant for each test. It should be noted that the imprint might not interfere with the detection of the labeled ligand.

For ligand-binding assays it can be concluded that molecular imprints do have potential advantages. Provided that, the analyte or template molecule is readily available at reasonable prices, imprints can be made available at higher quantities than biological macromolecules like antibodies and receptors. Moreover, it is still believed that the imprints can be made in a reproducible fashion. Compatibility with biological, aqueous samples is highly commendable.

It is unclear whether or not ligand-binding assays based on imprints can compete with the sensitivity and selectivity of immuno- and receptor assays. More complex monomer and cross-linking mixtures and step-by-step polymerization procedures might attribute to obtain imprinted polymers with the desired binding characteristics.

9.5 Sample pretreatment

For most bioanalytical assays, sample pretreatment steps are mandatory. The objective of a sample pretreatment procedure is to achieve preconcentration of the analytes of interest and/or clean-up of the sample and/or making the sample compatible with the analytical technique.

Preconcentration of analytes is required when the concentration of analytes in the sample is low, or when the sample volume that can be introduced in the assay is small, or for the isolation of compounds.

Clean-up of the sample is directed to removal of compounds interfering with the detection of the analyte of interest (e.g. co-eluting and detected at the same wavelength in an UV-detector) or removal of compounds that

affect the performance of the instrumentation (e.g. co-eluting and affecting the ionization efficiency of the analyte in the interface of a mass spectrometric detector).

Furthermore, the analytes of interest should be present in a solvent that can be introduced into the analytical system. For gas chromatographic methods the solvent should be volatile whereas for liquid chromatographic analysis, the sample should be miscible with the mobile phase. Generally, this implies a solvent exchange.

Molecular imprints may serve as a stationary phase in solid phase extractions [16-18,41,42]. The properties of the imprinted polymers and their treatment are similar to those described in the section on separation methods when used in on-line extraction procedures. However, not to the same extent. Essential is that the elution volume containing the analyte of interest is small enough not to have a detrimental effect on the performance of the coupled analytical technique. During sample application and washing steps the analyte should be strongly bound to the sorbent so that when larger volumes of sample and/or washing solvent are applied, there is no break-through of the analyte. The elution medium should enable a fast dissociation of the analyte from the sorbent. This can be achieved by choosing conditions that completely destroy the interaction between functional groups of the analyte and of the sorbent (imprint). This will induce fronting of the analyte but also of most of the bound matrix components. In on-line procedures using small particles of modified silica (<10 μm) the elution step can be considered as a true chromatographic separation implying that retention of the analyte takes place. This means that fast dissociation and association kinetics of the analyte are mandatory. Considering the remarks made in the section on separation methods, the latter approach is not applicable yet, but stronger solvents can be applied.

Thus, on-line SPE means elution under rather harsh conditions, forcing an instant dissociation of the analyte from the sorbent. A limiting factor is that the eluting solvent should be compatible with the analytical technique used for quantitation of the analyte.

Off-line solid phase extraction procedures can handle a larger variation of solvents. In contrast to on-line procedures, miscible and immiscible solvents can be used sequentially when the sorbents are dried in

between. An other aspect is that a solvent used for elution can be evaporated and the analyte containing residue can theoretically be dissolved in the solvent of choice. Because off-line procedures are never used as a mini-chromatographic separation, association and dissociation kinetics are less critical albeit that small elution volumes do have practical advantages and are crucial when preconcentration of the sample is an objective of the sample pretreatment procedure.

Any degree of selectivity of the imprinted polymer can be used. The higher the selectivity of the sample pretreatment procedure, the lower the requirements for the selectivity of the analytical technique used for the quantitation of the analyte of interest. If methods are to be used for the extraction of a group of analytes, imprints should not be selective towards individual compounds. Imprints can be made with a single analyte or template molecule and subsequently these imprints can be mixed, otherwise a mixture of analytes can be imprinted in one polymerization process.

It can be concluded that molecular imprints do have a high potential for application in solid phase extraction procedures. In off-line procedures, selectivities and clean-up steps can be better due to the larger variation of solvents that can be used. It should be noted that on-line procedures are preferred because these are amenable to automation whereas no analyte is lost in transfer steps.

Efficiency of extraction columns is not important if elution takes place with solvent mixtures with very high elution strength. Fronting of the analyte will take place yielding a higher concentration in the eluate at the expense of clean-up.

9.6 Sensors

The use of sensors in analytical chemistry can be divided into application for continuous measurements or for measurement of separate samples [19,20,43]. Imprints are attached to a surface, forming a thin layer. Binding of analytes to the imprint will change a number of physicochemical properties of that layer, which can be monitored [43]. Only for the

measurement of separate samples, indirect detection protocols are possible. Essentially it can be regarded as a competitive ligand binding assay in which a unique property e.g. fluorescence of a labeled ligand, which also binds to the imprint, is monitored. This implies that a fixed concentration of a labeled ligand is added to a sample, which needs incubation with the sensor until equilibrium is reached. The binding molecule, the imprinted polymer, is re-used for many assays. Generally, this is done to monitor ultra-trace concentrations of analytes with physicochemical properties that do not allow direct detection.

In all other cases, the interaction of the analyte with the imprinted polymer changes the properties of the outer layer of the sensor, e.g. inducing capacitance or optical effects. It is essential to realize that also binding of the analyte to non-imprinted parts of the polymer will change the signal, although possibly not to the same extent. Actually, any matrix component that can interact with the polymeric layer in a selective or non-selective manner will change the signal of the sensor. This implies that the interaction of imprinted polymers with matrix components should be minimal and constant. The latter explains why sensors are frequently used in monitoring of continuous chemical or biotechnological processes. The time-lag between a change in concentration of analyte and change in sensor signal is dependent on the kinetics of the association and dissociation of the analyte and the imprint. As mentioned previously, high affinities of an imprint for a particular analyte, required for the detection of low concentrations thereof, can generally be attributed to a slow off-rate. This implies that there is a trade-off between the sensitivity and the time-lag of the sensor.

With the indirect sensors using a labeled ligand, interactions of analyte and matrix components with the non-imprinted parts of the polymer do not change the signal of the sensor.

The selectivity of a sensor is dependent on differences in interaction between analytes and sorbent to the same extent as in ligand-binding assays. The sensitivity of a sensor is dependent on the affinity of analyte or of the labeled ligand and on the detection principle.

In conclusion, due to their stability, molecular imprints can be readily used as the sensing surface of a chemical sensor. A thin layer with a high density of imprints is a prerequisite for a sensitive sensor. The sensitivity is strongly dependent on the interaction of the analyte and in some cases the interaction of a labeled ligand with the imprint. In cases, where the detection principle is based on properties of the analyte itself, interaction of the analyte and other matrix components with non-imprinted parts of the polymer affect the sensor signal. Particularly in cases where the matrix composition is constant and the analyte is present in variable concentrations, sensors can have an application.

9.7 Summary

It is clear that each technique has its own requirements with regard to the binding properties of the imprinted polymer. Also for every technique each analytical problem requires imprints with specific characteristics. Nevertheless, a number of recommendations can be given.

A high density of imprints in the polymer is advantageous for any technique because it reduces interactions of analytes and matrix components with the non-imprinted part of the polymer. Generally, due to the production process, the population of binding sites is heterogeneous. This is not a problem as long as the imprinted polymer can be produced in a reproducible fashion and that the range of affinities is small and substantially (>100-fold) higher than the affinity of analytes and of matrix components for the non-imprinted parts of the polymer. The latter is important because the number of binding sites on the non-imprinted part is considerably higher than the number of imprints present in the polymer. If this is not the case, it should be considered using non-imprinted polymers instead of imprinted polymers.

Affinities can be comparable with those observed for antigen-antibody interactions, which enables application in ligand-binding assays and for sensors. Preparation of molecular imprints is much faster and probably more reproducible than the preparation of antibodies.

The selectivity we are looking for can differ quite dramatically. If we are only interested in the analysis of a single compound, e.g. an

enantiomer, it is advisable to use that particular compound or the other enantiomer for imprinting. If we are interested in one class of compounds, we can look for a structural analog with the same functional groups, required for interaction with functional groups of the monomers (during selfassembly) or for a polymer having slightly bigger substituents, so that all target analytes will fit in the cavities of the imprinted polymer. Alternatively, imprints can be generated against a mixture of template molecules, or imprints of different templates can be mixed afterwards.

The use of the analyte molecule itself as template molecule for imprinting can be problematic in a number of cases. When the compound is not available in pure form in reasonable amounts and at reasonable costs, analog compounds that meet the criteria should be considered. The latter is also the case for highly toxic compounds, chemically unstable compounds, or poorly soluble compounds under the applied polymerization conditions. However, there are also some analytical arguments that may exclude the use of the analyte as template molecule. During polymerization a small percentage of the analyte will remain in the polymer and can leak during application [33]. In separation methods this may lead to drifting baselines or extra peaks induced by the solvent composition of the injected samples. In ligand-binding assays, sample pretreatment and sensors, it will affect the accuracy and precision particularly at lower concentrations. Because these analytical methods are particularly valuable for trace analysis the use of the analyte as template molecule should preferably be avoided. Another element is that using the analyte as template might lead to high affinities of the polymer with slow dissociation kinetics. For application in chromatography or capillary electrophoresis this will lead to extensive peak broadening.

Considering the state-of-the-art of molecular imprinting the application for sample pretreatment directed to preconcentration looks the most promising. For sample enrichment, rather high binding affinities are favorable to extract large sample volumes and allow the use of rather harsh washing steps, which can boost the selectivity.

Preparation of thin coatings containing imprints can contribute to the development of substance selective sensors.

It becomes clear that the suitability of a given imprint is dependent on its application. This implies that extensive interaction between organic

chemists and analytical chemists, is required to produce materials and procedures that pay off.

It is anticipated, that molecular imprints can find a prominent place in pharmaceutical research not only as a tool to improve selectivity and sensitivity in analytical methods, but also for selective extraction of drugs or intermediates from reaction media independent of their generation (chemical or biotechnological). In an environmental context, these imprints may be used for the analysis and/or removal of pollutants.

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