separations

Editorial

Is Chromatographic Efficiency Possible Using Molecularly Imprinted Stationary Phases?

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Abstract: Molecularly imprinted polymers are prepared in the presence of a template. They have been shown to resolve enantiomers when used as stationary phases for liquid chromatography. However, the separation efficiency is not as good as that observed with silica stationary phases. This manuscript identifies the main problems as the slow transfer kinetics between the mobile and stationary phase and the heterogeneity of the stationary phase binding sites. It suggests that preparing templated polymers that have predominantly or exclusively noncovalent crosslinks is the most promising approach for improving efficiency.

1. Molecularly Imprinted Polymers

Molecularly imprinted polymers (MIPs) are prepared in the presence of a template. When the template is removed, the polymer is left with a binding site that has the correct shape to rebind the template. The original method for making these materials involved a template covalently bonded to a monomer that would be incorporated into the polymer [1]. Removing the template from the polymer required this bond to be broken. While this method is viable, it is more convenient to use noncovalent interactions such as hydrogen bonding for the interaction between the template and a recognition monomer that is designed to interact with the template. Polymerization is performed in a solvent that does not form hydrogen bonds so that the only hydrogen bonds are between the template and the recognition monomer.

MIPs were originally touted as replacements for antibodies in the many analytical applications that involve antibodies as the recognition element that selectively binds to analyte. This has not come to pass. The advantages of MIPS are that they are easy to prepare and are more robust than antibodies. These advantages are real. However, the MIPs prepared to date do not come close to matching the selectivity and sensitivity of antibodies, with the result that they are now viewed as a technology that did not live up to its hype. Furthermore, the rate of binding by MIPs is much slower than with antibodies. This author believes that the reason for this is that most MIPs involve a high degree of covalent crosslinking to maintain binding site integrity. The result is a rigid material that does bind the template selectively but only relatively slowly. MIPs with a high degree of crosslinking are necessarily separate solid phases. Slow mass transfer through this phase is thought to be a major component of the slow response. Current efforts on improving response times are usually focused on making the molecularly imprinted polymer thinner so that the mass transfer distance is shorter.

Naturally occurring compounds that are used for analyte recognition, i.e., both antibodies and aptamers, involve noncovalent bonds to produce a shape that selectively binds the template. These bind to the analyte much more rapidly and with higher affinity than MIPs. This observation suggests that MIPs with noncovalent crosslinks might have a faster response and be more competitive with naturally occurring recognition molecules with respect to the rate of response.

The "imprinting factor" has been defined as the affinity of an MIP for a template divided by the affinity of a nonimprinted polymer for the same template [2]. This citation,

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while not the original citation in which the imprinting factor was defined, provides an excellent description of the imprinting process and the properties of molecularly imprinted polymers. The nonimprinted polymer, often designated the NIP (for nonimprinted polymer), is prepared using the same conditions as the MIP, except without any template. An imprinting factor of two to five is strong evidence that there is a templating effect when preparing polymers in the presence of the template. However, the values that are typically observed, while they confirm the basic hypothesis that templating occurs, are not nearly large enough to lead to a polymer that is suitable for sensing applications. However, only a relatively small factor, let us say a modest value of two, is sufficient to lead to a large change in template retention if the MIPs are used as the stationary phase for a chromatographic separation. Therefore, one line of research has explored the utility of MIPs as stationary phases for chromatography.

An inevitable consequence of the methods used to prepare MIPs is that not all binding sites are the same. The heterogeneity of binding sites has not normally been a subject of study. One exception is a study where a three-site model was shown to accurately predict the shape of chromatographic peaks separated using a molecularly imprinted stationary phase [3].

Although the heterogeneity of binding sites has not been a common object of study, one can infer heterogeneity from the dynamic range of an MIP. If there were a binding site with a single binding constant, one would expect a dynamic range of approximately two orders of magnitude. One can show with an easy calculation that 91% of all binding sites are occupied if the concentration is ten times larger than the binding constant and 9% of all binding sites are occupied if the concentration is ten times smaller than the binding constant. This means that 82% of all binding occurs over a dynamic range of 2. If the dynamic range is much greater than 2, this signifies binding site heterogeneity.

There are several studies of MIPs that report impressively low detection limits and very wide dynamic ranges. A recent example reports an impressive detection limit for creatinine, 0.1 pg/mL, and a dynamic range of 10 orders of magnitude, a sure sign that there is a wide variation in the nature of binding sites and their affinity for the template [4]. This particular molecularly imprinted polymer will not work well as a stationary phase for chromatography.

The heterogeneity of molecularly imprinted polymers has other consequences. The imprinting factor, commonly used as a measure of templating effectiveness, will depend on the template concentration used for the measurement. At low template concentrations, the stronger sites will bind all of the template and the imprinting factor will be larger. At high template concentrations, the stronger sites will be overloaded and more weakly binding sites will be occupied, with the result that the imprinting factor will be smaller.

The literature suggests that the dynamic range is not the same from study to study. This means that the binding site heterogeneity will vary from preparation to preparation. For the purposes of chromatography, it is important to minimize heterogeneity. For sensing purposes, it is important to maximize heterogeneity if a wide dynamic range is desired.

Of particular interest is the possibility of chiral separations using polymers that are imprinted with one enantiomer. That enantiomer will be retained more than the other enantiomer, leading to a separation of enantiomers that differ only in configuration. The reason this is particularly attractive as an application of MIPs is that chiral separations require a chiral stationary phase. Most stationary phases are not chiral. However, chiral phases are easily prepared as MIPs provided one enantiomer is available as a template. Otherwise, the requirement of a chiral stationary phase limits the number of chromatographic phases available for a separation. That chiral separations are the most likely application of molecularly imprinted stationary phases is widely recognized. Most separation studies involve chiral separations. It has been shown in many studies that enantiomers can be separated using molecularly imprinted polymers. This is because the separation factor is very large, rather than reflecting efficient chromatography.

The purpose of this polemic is to address the question of whether MIPs can be developed to be practical chromatographic phases. It is believed that the most likely application of MIPs as stationary phases will be chiral phases. This manuscript will not attempt to be comprehensive. Instead, the reader is referred to recent reviews [5–7]. Two of them cover the literature on the use of MIPs as stationary phases for high-performance liquid chromatography [5,6]. The most recent review [5] acknowledges that molecularly imprinted stationary phases have yet to make a practical impact, stating "Due to the low column efficiencies (several thousand plagtes/m) of the MIPs based sgationary phase, they have only been studied in the laboratory and are still far from mass production and commercialization". The third review cited [7] focuses on the application of MIPs for the solid-phase extraction of an analyte [7]. Solid-phase extraction is widely used as a method for removing the analyte from the sample before a separation, thus cleaning up the sample and prolonging the life of a stationary phase. It is a potential application of MIPs but will not be considered further here. Readers interested in the topic of molecularly imprinted polymers are referred to reviews of this subject, including less recent reviews cited in the reviews that are cited.

2. Chromatographic Efficiency Using Liquid Mobile Phases

Chromatography using gases as the mobile phase, known as gas chromatography, was developed many years before high-performance liquid chromatography. It was found that column efficiency, measured as the height of a theoretical plate, went through a maximum (the maximum efficiency corresponds to the minimum plate height) as a function of flow rate. The experimental curve could be fitted by the following equation:

$$\text{Height of a theoretical plate} = A + B/v + Cv \quad (1)$$

The first term is the eddy dispersion term. It arises because the mobile phase in the center of the column moves faster than the mobile phase near the walls of the column. The second term is due to the longitudinal diffusion of the solute. The third term is due to slow equilibration between the mobile and stationary phase.

When a liquid is used as the mobile phase, the diffusion constants of the solutes being separated decrease by about four orders of magnitude. Because of this, longitudinal diffusion is no longer a significant source of chromatographic inefficiency, except at impractically slow flow rates. Instead, the slow equilibration term is the dominant source of chromatographic inefficiency under normal operating conditions using a liquid mobile phase.

The slow equilibration term arises because the solute in the mobile phase moves as the mobile phase percolates through the column. The solute in the stationary phase does not move. This gives rise to a situation where the concentration of the solute in the mobile phase is greater than the equilibrium concentration at the downstream end of the solute band. Conversely, the concentration of the solute in the stationary phase is greater than the equilibrium concentration at the upstream end of the solute band.

The rate at which equilibrium is established determines chromatographic efficiency. Two factors contribute to this. One is the rate of mass transfer. The solute has to diffuse to the phase boundary before it can enter the other phase. Since the rate of diffusion is much slower in a liquid mobile phase, mass transfer is slow. The only way to overcome this is to minimize the mass transfer distance. This is achieved by reducing the size of the stationary phase particles. This in turn increases the backpressure so that a pump is required to propel the mobile phase through the column. The recent trend in HPLC has been towards decreasing the particle size in order to increase chromatographic efficiency, i.e., reduce the height of a single plate. This is performed at the expense of increased backpressure, but means that less mobile phase solvent is required to complete a separation with a given resolution.

The other factor determining how long it takes for the solute to equilibrate between mobile and stationary phases is the kinetics of this transfer. In many contexts, the kinetics

of phase transfer are so rapid that they may be ignored relative to the rate of solute mass transfer. When this is carried out, there is an assumption in the so-called van Deemter equation that describes the relationship between chromatographic efficiency, measured as the plate height, as a function of flow rate. This assumption is often unstated. For example, a recent review of chromatography using molecularly imprinted stationary phases [5] states a form of this equation that makes this assumption, even though slow kinetics are almost certainly the cause of band broadening in most, if not all, systems involving molecularly imprinted phases. This suggests to the author that some members of the molecularly imprinted polymer community may not be aware of the importance of slow phase transfer kinetics as a source of chromatographic inefficiency. On the other hand, the other cited review [6] is aware of the importance of the effect of binding kinetics on chromatographic efficiency.

Binding site heterogeneity is another widely unrecognized problem using molecularly imprinted stationary phases. This is not a problem with the van Deemter equation. Instead, this involves an overloading problem. Overloading occurs when the amount of solute in the mobile phase exceeds the capacity of the stationary phase. When this happens, the partition coefficient (=amount in mobile phase/amount in stationary phase) increases because the stationary phase cannot accept all of the solute. An increased partition coefficient means that the center of the band where the solute concentration is highest moves more rapidly than the wings of the band where the solute concentration is lower and the partition coefficient is smaller. The result is a tailed peak because the center of the band catches up with the downstream wing of the band and leaves the upstream end of the band behind. In an extreme case, the retention time will be shorter when the solute concentration is higher. Fortunately, in most circumstances, overloading can be avoided by working with low enough solute concentrations such that there are enough sites on the stationary phase to bind with the solute, such that the partition coefficient is not a function of the solute concentration.

To understand why binding site heterogeneity is an issue for imprinted stationary phases, let us first envision a stationary phase that has two binding sites, one strong and one weak, in equal amounts. In this case, overloading of the strong site will occur at half the concentration at which it would occur if all the binding sites were equal in affinity for the solute. This may not seem like a major problem, but it becomes one if there is a large distribution of binding sites. One result will be that the retention time will decrease at higher solute concentrations because this will overload the higher affinity sites [3]. A second result will be that peaks will be severely tailed [3]. Both results are unacceptable for modern chromatography. One of the problems that will need to be addressed to make MIPs practical stationary phases for modern HPLC is preparing polymers that are close to being uniform.

3. How to Prepare MIPs That Are Efficient HPLC Stationary Phases

This discussion leads to the conclusion that MIP stationary phases need to address three issues if they are to have the chromatographic efficiency required to be practical. Even if these challenges are effectively addressed, users may already be comfortable using phases they know rather than trying out new phases. As a result, it is not a given that MIP stationary phases will ever be useful for high-performance chromatography.

The three issues that need to be addressed in MIP phase preparation are: (1) mass transfer distance, (2) the kinetics of phase transfer, and (3) binding site heterogeneity.

Mass transfer distance can be addressed using the technique of surface imprinting [8]. This involves immobilizing the template on silica before preparing the templated polymer. These nanoparticles can then be attached to micrometer-sized silica particles to make phases that have only surface sites. This effectively results in phases where the mass transfer distance depends only on the size of the silica particles. It can be performed using silica particles that are known to be the appropriate size for efficient chromatography. This has been carried out but has not resulted in highly efficient chromatography [9]. While

enantiometric separation is excellent, the peaks are severely tailed. This suggests to this author that reducing the mass transfer distance is not sufficient for efficient chromatography. This otherwise-elegant approach still results in solid-phase materials that are likely to have slow binding kinetics and a high degree of heterogeneity.

Heterogeneity is not normally investigated as a property of MIPs. However, there was a study comparing a phase prepared using hydrogen bonding between the functional monomer and the template to a phase prepared with a covalent bond between the functional monomer and the template [10]. This study found more efficient chromatography using the phase that had the covalent bond. The covalent bond would require a fixed spatial relationship between the template and the functional monomer. This is likely to reduce the heterogeneity in the resulting material. This result suggests to the author that phase heterogeneity is a problem affecting separation efficiency.

The problem of slow phase transfer kinetics is most easily addressed by increasing temperature. This does lead to improved separation efficiency but at the expense of enantiomeric resolution [3]. Superfluid chromatography has been suggested as a possible method that would yield improved separation efficiency [6]. This is effectively an increase in temperature because higher temperatures are required to produce supercritical fluid mobile phases. It is not clear whether or not this will lead to high separation efficiencies.

This author believes that the most effective approach will be to use molecularly imprinted polymers prepared using completely or predominantly noncovalent crosslinks. We have already shown that this leads to much faster binding [11]. Template binding at room temperature was complete in less than two seconds. This greatly improves the kinetics of phase transfer, a problem this author believes is a large factor in the relatively poor efficiency observed so far. I believe the main reason for the greatly improved kinetics is that this polymer is soluble in water rather than forming a separate phase. Furthermore, it is much more flexible than MIPs prepared with a high level of covalent crosslinker. These materials are closer to naturally occurring antibodies and synthetically prepared aptamers, both of which achieve molecular recognition with noncovalent crosslinks.

The polymer used to prepare an MIP with predominantly noncovalent crosslinks was synthesized via reverse addition fragmentation transfer [12], a form of living polymerization based on chain transfer. It produces polymers of controlled length that have a terminal chain transfer agent. One of the commercially available RAFT agents is 4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid N-succinimidyl ester. This reacts with aminated silica. There is also a RAFT agent, cyanomethyl [3-(trimethoxysilyl)propyl] trithiocarbonate, that reacts directly with silica. While this material has not been used for separations yet, it has been attached to silica, suggesting that it could easily be grafted onto silica beads known to produce highly efficient separations.

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