Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 804 (2004) 141–149
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Review

Characterization of the heterogeneous binding site affinity distributions in molecularly imprinted polymers

Robert J. Umpleby II[a,b], Sarah C. Baxter[a,b], Andrew M. Rampey[a,b], Gregory T. Rushton[a,b], Yizhao Chen[a,b], Ken D. Shimizu[a,b,*]

[a] *Department of Chemistry and Biochemistry, University of South Carolina, Columbia, SC 29208, USA*
[b] *Department of Mechanical Engineering, University of South Carolina, Columbia, SC 29208, USA*

Abstract

Molecularly imprinted polymers (MIPs) are polymers that can be tailored with affinity and selectivity for a molecule of interest. Offsetting the low cost and ease of preparation of MIPs is the presence of binding sites that vary widely in affinity and selectivity. Presented is a review of methods that take into account binding site heterogeneity when calculating the binding properties of MIPs. These include the bi-Langmuir, Freundlich, and Langmuir–Freundlich binding models. These methods yield a measure of heterogeneity in the form of binding site affinity distributions and the heterogeneity index. Recent developments have made these methods surprisingly easy to use while also yielding more accurate measures of the binding properties of MIPs. These have allowed for easier comparison and optimization of MIPs. Heterogeneous binding models have also led to a better understanding of the imprinting process and of the advantages and limitations of MIPs in chromatographic and sensor applications.

Keywords: Reviews; Binding site heterogeneity; Affinity distribution; Molecularly imprinted polymers

Contents

1. Introduction

Molecularly imprinted polymers (MIPs) are highly cross-linked polymers that are formed in the presence of a template molecule [1–3]. Removal of the template leaves binding cavities with affinity and selectivity for the original template. The ease in preparation of these synthetic

* Corresponding author. Tel.: +1-803-777-6523; fax: +1-803-777-9521.
E-mail address: shimizu@psc.sc.edu (K.D. Shimizu).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.01.064

polymeric receptors is offset by the low fidelity of the imprinting process. MIPs typically contain binding sites that possess a wide range of affinities and selectivities. This binding site heterogeneity strongly influences the binding properties of MIPs. For example, heterogeneity has been cited as a major source of peak broadening and asymmetry in HPLC applications using MIP stationary phases [4]. It has also been cited as a contributor to the low selectivities seen when using MIPs as catalysts as enzyme analogs [5]. Binding site heterogeneity also severely complicates the measurement of the binding properties of MIPs because it leads to binding properties that are highly dependent on the concentration range in which they were measured. Despite the consequence of heterogeneity in MIPs, only recently have binding models been applied that take into account the full breath of the heterogeneity in MIPs. The goal of this review will be to introduce some of these methods and to discuss their advantages and disadvantages in characterizing MIPs.

2. Homogeneity and heterogeneity

Surfaces with recognition abilities can be divided into two general categories: *homogeneous* and *heterogeneous*. These are shown schematically in Fig. 1. In homogeneous system, all the binding sites have the same affinity and selectivity as depicted by the identical depths and shapes of the binding cavities. In contrast, heterogeneous system contain binding sites of varying affinity and selectivity as denoted by the varying depths and shapes. Binding site heterogeneity in MIPs has often been compared to polyclonal antibodies. A key difference, however, is that the individual antibodies in a polyclonal mixture can be separated to yield homogeneous monoclonal systems. The analogous purification strategy cannot be applied to MIPs as all the binding sites are physically bound together in the polymer matrix.

Various methods have been applied to reduce the heterogeneity in MIPs such as the optimization of the imprinting process or selective chemical modification of the surface [3]. The most successful has been to use stoichiometric or covalent imprinting mechanisms [6]. This reduces the structural and statistical variability of the key prepolymerization complex. However, despite the rapid improvement of MIPs, binding site heterogeneity remains as an almost inherent property of MIPs. This is particularly true for the non-covalently imprinted polymers, which remain the most common class of MIPs because of their ease of preparation from commercially available monomers.

Fig. 1. Schematic illustration of binding site homogeneity and heterogeneity in MIPs.

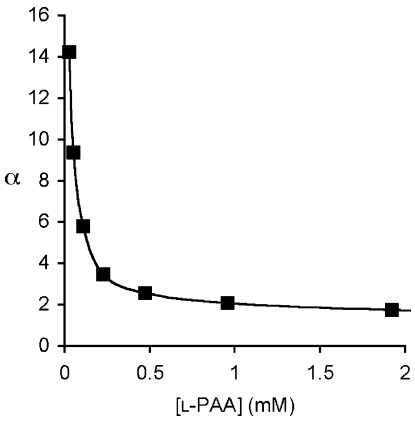

Fig. 2. The measured concentration dependence of the separation factor (α) as measured for an MIP imprinted for L-phenylalanine anilide (L-PAA). The separation factor was measured in batch rebinding studies and was calculated as: α = ([bound L-PAA]/[free L-PAA])/([bound D-PAA]/[free D-PAA]) [31].

2.1. Influence of heterogeneity

It should be pointed out that heterogeneity is not always detrimental to the binding properties and applicability of MIPs. In spite of their heterogeneity, non-covalent MIPs have been and will continue to be utilized in a wide range of applications including separations, sensing, and catalysis. In addition, it will be argued later that heterogeneity is a fundamental feature of the imprinting process. Heterogeneity, however, does usually temper and limit the capacity and selectivity of MIPs. Perhaps more problematic is that binding site heterogeneity severely complicates the characterization of MIPs. It makes the binding properties of MIPs highly concentration dependent, which obfuscates the comparison and optimization of MIPs. A striking example is the effect of heterogeneity on the chromatographic separation factor (α), which is a widely used figure of merit in comparing MIPs. At high analyte concentrations, α is a relatively stable but low value (Fig. 2). In contrast, α increases rapidly to very high values at low analyte concentrations. This behavior arises from the low affinity, low selectivity site being sampled at high polymer loadings, and the high affinity, high selectivity sites being sampled at low polymer loadings. This concentration dependence of α can yield very high separations factors but also complicates the comparison of MIPs using α. This highlights the importance of understanding the underlying assumptions behind the different binding parameters that are used to compare MIPs. It also demonstrates the need for methods for characterizing MIPs that take into account heterogeneity in MIPs.

2.2. Shape of the distribution in MIPs

Once it was been established that MIPs contained a heterogeneous distribution of binding sites, the question then arose: what is the shape of the distribution? We have

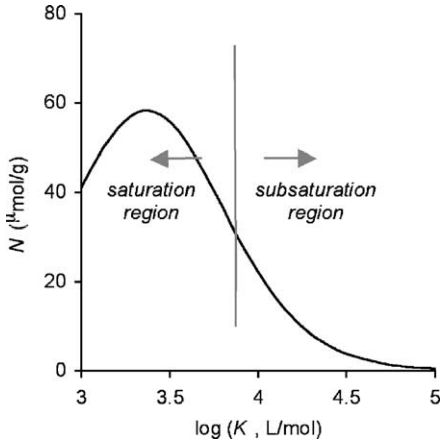

Fig. 3. The broad unimodal heterogeneous affinity distribution proposed to be present in molecularly imprinted polymers.

proposed that a broad unimodal distribution is most consistent with the observed binding properties and with the many sources of heterogeneity in MIPs (Fig. 3) [7,8]. These distributions are commonly represented as affinity distributions (AD), which plots the number of sites (N) that have association constant (K). The x-axis is commonly plotted in units of $\log K$ in order to make it proportional to the binding energy (ΔG), and therefore this plot is also called a site–energy distribution. This distribution is remarkably similar in shape to that originally estimated by Wulff et al. over 20 years earlier for the first imprinted polymers [9].

Initially, two distinct ADs were measured for MIPs: an unimodal peak and an exponentially tailing distribution [7]. Later, these were proposed to be features of the same broad unimodal distribution [8]. These distinct regions of the AD of MIPs arise from measuring different portions of the experimental binding isotherm. The exponentially tailing portion corresponds to the lower concentration portion of the binding isotherm where the high affinity binding sites are preferentially sampled. In this region, the MIP is at low loadings and is far from saturation. For most non-covalent MIPs, this is typically the subset of sites that are measured and utilized in most applications. This is because it is very difficult to reach saturation in most non-covalently imprinted polymers due to their heterogeneity. The less common region of the AD to sample is the unimodal peak with a maximum at K_0. These binding sites represent those measured at high loadings as the polymer reaches saturation. These have been measured in MIPs having very high affinity or MIPs containing a more homogeneous and narrow distribution such as the stoichiometrically imprinted polymers. However, with improvements in MIPs this region of the AD will become increasing more important.

This broad heterogeneous distribution helps to explain some of the unusual features of MIPs. For example, an extraordinarily wide range of binding constants (K) have been reported for MIPs from 10^{-2} to $10^{-8}\,M^{-1}$ [10]. A heterogeneous model proposes that MIPs effectively contain binding sites that span this entire region from low to high affinity. Thus, a subset of binding sites of can be found having just about any K, depending upon the concentration range being measured. A closer examination of the literature reveals that this is the case. For example, a binding study using UV spectroscopy which covered a concentration range from 10^{-3} to $10^{-6}\,M$, measured binding sites with association constants from 10^4 to $10^5\,M^{-1}$ [11]. Another binding study using radio-ligand assays were able to measure even lower concentrations of $10^{-8}\,M$ and identified binding sites with even higher binding affinities of $10^7\,M^{-1}$ [12]. Therefore, the question in assessing MIPs should not be “are there sites with a certain binding affinity?” but rather “how many sites in the MIP have that binding affinity?”.

3. Characterization of heterogeneity

Reviewed, in this section, will be methods for characterizing the binding properties of MIPs with particular emphasis on those that can take into account and measure heterogeneity. All the methods outlined further rely on calculations based on the experimental binding isotherm. A binding isotherm measures binding efficiency of a polymer over a range of analyte concentrations and is usually plotted as the concentration of analyte bound to a polymer (B) versus the concentration of free analyte remaining in solution (F). Binding isotherms for MIPs can be obtained from batch rebinding studies [11,13,14] in which a constant weight of polymer is equilibrated with a known concentration of analyte. This is then measured over a range of analyte concentrations. The concentration of the analyte remaining free in solution (F) is measured by HPLC, UV–fluorescence spectroscopy, or radio-ligand assay. The corresponding concentration of bound analyte (B) is calculated as the difference between the total (T) and free concentrations ($B = T - F$). Conversely, the concentration of analyte can be held constant and the weight of polymer varied. Binding isotherms can also be measured using frontal chromatography studies [15–17]. Binding isotherms measured by batch rebinding studies and chromatography may differ as the chromatographically measured isotherms also contains kinetic components, corresponding to rates of adsorption and release. The chromatographically measured binding isotherms are also measured under conditions where there are much weaker interactions between analyte and MIP in order to allow for elution. Thus, the measured capacities and affinities tend to be much lower, and the overall shape and ordering of isotherms measured by each method appear to be very similar and have lead to very similar conclusions as to which parameters are important in the imprinting process.

The relative binding ability of two polymers can be assessed by overlaying their respective isotherms. A more quantitative analysis can be made by comparison of the binding parameters that can be calculated from the respective isotherms such as the number of binding sites (N) and

association constant (K). The calculation of binding parameters from an isotherm requires the application of a specific binding model. Among those that have been applied to MIPs are the Langmuir, bi-Langmuir, Freundlich, Toth, and Langmuir–Freundlich isotherms [8,12,18,19]. Each of these models specifies a certain mathematical relationship between the bound (B) and free (F) concentrations in the binding isotherm. In addition, each model makes certain assumptions regarding the distribution of binding sites.

Selection of a binding model is primarily based on its ability to accurately reproduce the experimental isotherm. The physical basis for the model should also reflect the distribution of sites found in the measured system in order to generate realistic binding parameters. Due to the complexities in calculating the distribution of sites in heterogeneous systems, various simplifications and approximations methods are utilized. The most common is to assume that the distribution of sites conforms to a certain general shape. A few of the more common that have been applied to MIPs are shown in Fig. 4. Each can approximate the actual distribution with increasing degrees of accuracy.

Binding models can be grouped into two general classes: discrete and continuous distribution models. The most commonly applied binding models, the Langmuir and bi-Langmuir isotherms, are both examples of discrete binding models. Discrete binding models simplify a distribution into a finite number of different classes of sites, with each class of site having a different binding affinity. The Langmuir model assumes there is only a single class of sites, and the bi-Langmuir assumes there are only two classes of sites. The Freundlich and Langmuir–Freundlich are both examples of continuous distribution models in which a continuous function containing an infinite number of different types of binding sites is used to model the distribution. These models provide increasingly more accurate approximations for the heterogeneity present in most MIPs and also provide quantitative measures of heterogeneity. Both classes of binding models with examined separately in the following sections.

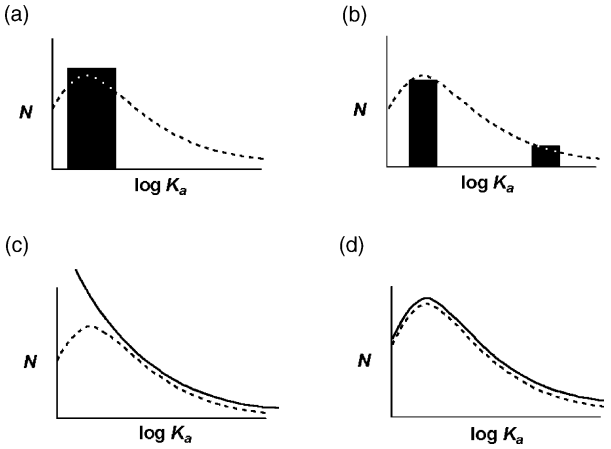

Fig. 4. Discrete (a and b) and continuous (c and d) binding models distributions, shown as bars and solid lines, respectively, which are overlaid on the broad heterogeneous distribution that is proposed for MIPs (broken lines). (a) Langmuir (narrow unimodal); (b) bi-Langmuir (bimodal); (c) Freundlich (exponential); (d) Langmuir–Freundlich (broad unimodal).

3.1. Discrete binding models

The discrete Langmuir and bi-Langmuir models are attractive because they are particularly easy to implement via Scatchard plots and readily generate the corresponding binding parameters: binding affinity (K) and number of binding sites (N). In the Scatchard analysis, the experimental binding isotherm is replotted in B/F versus B format. In homogeneous systems that contain only one type of binding site, the Scatchard plot falls on straight line (Eq. (1)) with a slope equal to the negative of the binding affinity ($-K$) and an x-intercept equal to the number of binding sites (N):

$$\frac{B}{F} = KN - KB \tag{1}$$

In contrast, the Scatchard plots for most MIPs are curved (Fig. 5). This curvature has been cited as evidence for binding site heterogeneity [14]. Heterogeneity can still be accommodated using the Scatchard analysis by modeling the curved isotherm as two separate straight lines, which is a graphical method for applying the bi-Langmuir isotherm. This limiting slopes method yields two separate sets of binding parameters (K_1, N_1 and K_2, N_2) for two classes of sites. The steeper line measures the high-affinity sites and the flatter line measures the low-affinity sites.

The graphical application of the bi-Langmuir model using the limiting slopes method is inconsistent as it depends upon individual decisions of which points to include in which subset. The bi-Langmuir isotherm can also be applied in a more systematic manner using curve fitting using the following expression (Eq. (2)). Higher order fits tri- and tetra-Langmuir models can, likewise be applied by adding

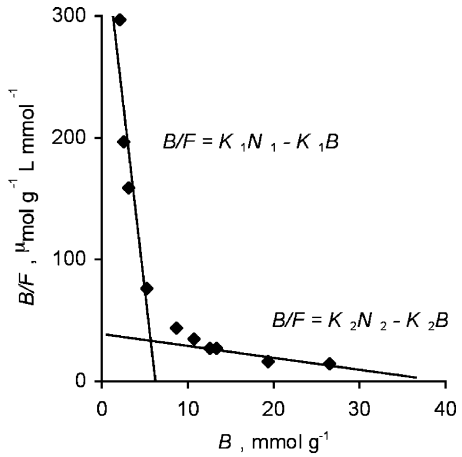

Fig. 5. Scatchard plot for an ethyl adenine-9-acetate imprinted polymer, with the limiting slopes estimated via regression.

additional terms to Eq. (2) [12]:

$$B = \frac{N_1 K_1 F}{1 + K_1 F} + \frac{N_2 K_2 F}{1 + K_2 F} \quad (2)$$

The bi-Langmuir binding model for curved Scatchard plots, however, are based on a series of approximations. Accurate estimates of the binding parameters using this method can only be found under the conditions when $K_2 N_2 \gg K_1 N_1$ [20].

The Langmuir and bi-Langmuir models are based on the assumption that MIPs are relatively homogeneous and contain only one or two distinct classes of binding sites. Measurement of the distributions in MIPs, on the other hand, have revealed a broad heterogeneous distribution [7]. This discrepancy between model and system leads to inconsistencies in the calculated binding parameters. For example, Scatchard plots yield highly concentration dependent binding parameters (N and K) for non-covalent MIPs, which, in theory, should be constants [7]. This variability in the calculated binding parameters for a single MIP becomes amplified when trying to compare two different polymers, often leading to ambiguous conclusions.

3.2. Continuous distribution models

Due to the shortcomings of discrete binding model in characterizing the heterogeneity, continuous distribution models, such as the Freundlich and Langmuir–Freundlich, have been more recently applied to MIPs [8,17]. These continuous distribution models are attractive as they can, in theory, more accurately approximate the broad unimodal distribution in MIPs. They should also yield more appropriate binding parameters. A second advantage is that the corresponding distribution and heterogeneity can be quantitatively measured.

3.3. Freundlich isotherm

The most easily applied continuous distribution model is the Freundlich isotherm (FI). This model is based on the Freundlich isotherm (Eq. (3)), which assumes a power function relationship between B and F. There are two fitting parameters a and m that both yield a measure of physical binding parameters. The preexponential factor a is a measure of the capacity (N_T) and average affinity (K_0). However, the individual contributions of N_T and K_0 to the preexponential factor cannot be directly extracted without additional experiments or assumptions. Therefore, this Freundlich fitting parameter is of lesser value. The second fitting parameter m is also known as the heterogeneity index. The value of which varies from zero to one, with one being homogeneous and values approaching zero being increasingly heterogeneous:

$$B = aF^m \quad (3)$$

The Freundlich model is most easily applied by replotting the experimental binding isotherm in $\log B$ versus $\log F$ format. In this form, systems that fit to the FI will fall on a straight line, having a slope of m and a y-intercept of $\log a$ (Eq. (4)). The Freundlich model has been shown to be generally applicable to most non-covalently imprinted polymers [19]. A literature survey of non-covalent MIPs, with widely varying templates, polymerization conditions, and binding affinities, found that 11 of 12 were in excellent agreement with the FI ($R^2 > 0.95$). Guiochon and co-workers [17] and Hwang and Lee [21] have also demonstrated the ability of the FI to accurately model the isotherms of MIPs:

$$\log B = m \log F + \log a \quad (4)$$

The ability to model the binding isotherm as a linear function has a number of practical advantages. First, it requires fewer experimental data points to accurately define than a curved function. Secondly, deviations from linearity in systems that follow the FI can be used as a diagnostic in identifying sources of error in the binding isotherm. Discontinuities of the experimental isotherm are diagnostic of systematic errors, whereas scatter of the data points from linearity is characteristic of random error. A third advantage of fitting the binding isotherm to specific function is that it simplifies the estimation of the corresponding AD. Systems that can be modeled by the FI are generally accepted to contain an exponentially decaying distribution of binding sites with respect to $\log K$ [22,23]. However, the FI by itself does not provide sufficient information for the exact solution of its AD. This calculation requires an accurate measure of the total number of binding sites (N_T) which is not possible for systems that strictly follow the FI [22,23]. Due to the prevalence of the FI, various approximations methods for estimating the exponentially decaying distribution of the FI have been developed. Most of these require additional physical measurements of the systems. Recently, Guiochon and co-workers have developed an analytically derived expression for the AD for a FI which requires only the experimentally derived Freundlich fitting parameters, a and m (Eq. (5)):

$$N(K) = a\frac{\sin(\pi m)}{\pi} K^{-m} \quad (5)$$

$$N(K) = 2.303am(1 - m^2)K^{-m} = 2.303am(1 - m^2)\mathrm{e}^{-2.303m \log K} \quad (6)$$

We have separately developed a similar expression for the AD of the FI (Eq. (6)).[1] The differences in these expressions (Eqs. (5) and (6)) arise because neither is an exact solution, rather both are approximations of the actual exponentially decaying FI distribution. Eqs. (5) and (6) have the same general form, with an identical decay factor of $-m$. The differences in the preexponential coefficient represent differences in approximation methods used in their derivations.

[1] The new AD function for the FI was derived by inserting the FI into the affinity spectrum approximation method for calculating affinity distributions. The equation was simplified by taking the limit as the 1/stepsize approaches unity. This was accomplished using the software package Mathcad, which automates symbolic limit taking.

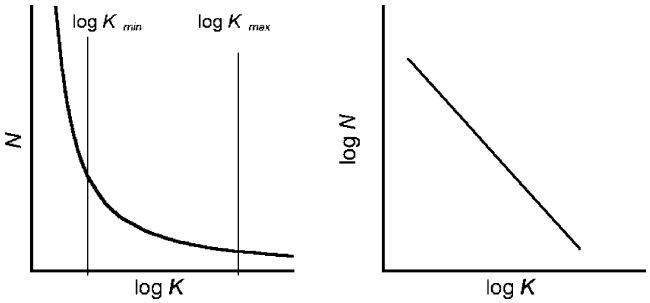

Fig. 6. Affinity distributions based on the Freundlich model plotted in semi-log (left) and log (right) formats.

The FI AD expressions make use of the FI for characterizing MIPs simpler than the more common limiting slopes Scatchard method. Both, however, are simple algebraic functions that can calculate the AD for a FI from the experimentally derived FI fitting parameters (a and m). This enables the comparison of MIPs via their ADs, which better takes into account the heterogeneity in MIPs. First, the experimental binding isotherm is measured and is plotted in $\log B$ versus $\log F$ format. The slope and the $10^{(y\text{-intercept})}$ of the linear fit of the isotherm yields the FI fitting parameters m and a. These are used to calculate a distribution exponentially decaying distribution using Eq. (6). While the AD expression (Eq. (6)), in theory allows the calculation of the AD over any range of binding affinities. It is in practice only valid within the limits ($K_{\min}$ and $K_{\max}$) set by the concentration ranges of the experimental binding isotherm:

$$K_{\max} = \frac{1}{F_{\min}} \quad \text{and} \quad K_{\min} = \frac{1}{F_{\max}} \tag{7}$$

ADs calculated using the FI can be presented in two formats (Fig. 6). The first is the common semi-log format (N versus $\log K$), which shows the exponentially decaying distribution. The x-axis is in units of $\log K$, which is proportional to the binding energy ΔG. Therefore, this plot is also called a site–energy distribution. The area under this curve is equal to the number of binding sites having binding affinities between the two limits. The second format for AD is in log format ($\log N$ versus $\log K$). This is useful because in this plot the exponentially decaying distribution becomes a straight line, which greatly facilitates the visual comparison of the AD of different polymers. ADs with similar heterogeneity are easily identified as parallel lines because the slope in the format is equal to the heterogeneity index m.

An important practical consideration in applying Eq. (6) is choosing the appropriate units throughout. The units of the binding isotherm ultimately determine the units of the AD and all other calculated binding parameters. In our studies, the binding isotherm ($\log B$ versus $\log F$) is in units of $\mu\text{mol g}^{-1}$ versus mol l^{-1}. This yields fitting parameters of m which is unitless and a, which has units of $\mu\text{mol g}^{-1}(\text{mol l}^{-1})^{-m}$. Note that m will be unitless regardless of the units of the binding isotherm and that a has units that contain the first FI fitting parameter m. The calculated AD then has N in units of $\mu\text{mol g}^{-1}$ and K in units of l mol^{-1}.

Expressions for two additional binding parameters can be derived from Eq. (5). These are the number of sites, $N_{K_1-K_2}$, and the weighted average affinity, $\bar{K}_{K_1-K_2}$ (Eqs. (8) and (9)).[2] As the subscripts on $N_{K_1-K_2}$ and $\bar{K}_{K_1-K_2}$ imply, these expressions yield values that represent only a subset of the entire distribution from K_1 to K_2. These limits are set by the concentration range over which the experimental binding isotherm was measured ($F_{\min}$ to $F_{\max}$). The binding parameters $N_{K_1-K_2}$ and $\bar{K}_{K_1-K_2}$ can be measured for any set for K_1 of K_2 values that are within the boundaries $K_{\min}$ and $K_{\max}$ as defined by Eq. (7). Further in comparing binding parameters $N_{K_1-K_2}$ and $\bar{K}_{K_1-K_2}$, it is important to calculate them over the same range of binding sites (K_1 and K_2):

$$N_{K_1-K_2} = a(1-m^2)(K_1^{-m} - K_2^{-m}) \tag{8}$$

$$\bar{K}_{K_1-K_2} = \frac{m}{m-1}\frac{K_1^{1-m} - K_2^{1-m}}{K_1^{-m} - K_2^{-m}} \tag{9}$$

An important limitation of the FI is that it is only accurate for a portion of the entire binding isotherm. Deviations at high concentration from the FI are expected because the FI is not able to model saturation behavior (Fig. 4). Deviations from the FI are also expected at very low concentrations where the binding isotherm approaches Henry's law where there is direct linear correlation between B and F [24]. Fortunately, the binding isotherms for most MIPs are constrained to the intermediate concentration range where the FI is applicable. The saturation portion of the isotherm is difficult to measure in MIPs because their low average binding affinities and binding site heterogeneity combine to keep the saturation point above the commonly measured concentration range (>mM). The low concentration extreme (<nM) is likewise difficult to measure in MIPs due to interference from the slow leaching of the template from the matrix.

3.4. Langmuir–Freundlich isotherm

The inability of the FI to model saturation behavior limits the types of binding parameters that can be calculated from it. Specifically, the FI cannot yield the global binding parameters such as the total number of binding sites (N_T) or the overall average affinity constant (K_0). Accurate measures of these values in heterogeneous systems as well as the heterogeneity index requires hybrid models that can accommodate both saturation and subsaturation regions of a binding isotherm such as the Jovanovic–Freundlich [24] or Langmuir–Freundlich [8]. We have explored the utility of the Langmuir–Freundlich isotherm (LFI) in characterizing MIPs. This binding model seems to be the most general as it can model both saturation and subsaturation behavior.

[2] Eq. (8) was derived by integrating Eq. (6) with respect to K over the limits K_1 to K_2. Eq. (9) was derived from the integral of the product of K and $N(K)$ (Eq. (6)) divided by Eq. (8).

The LFI is applied by curve fitting the binding isotherm to the LFI (Eq. (10)). There are three fitting parameters N_T, K_0, and m for the LFI. N_T and K_0 are the global binding parameters, total number of binding sites and median binding affinity, respectively. The fitting parameter m is identical to the heterogeneity index m from the FI. The difference between the LFI and FI are evident at higher concentrations where the LF can model saturation behavior. However, at lower subsaturation concentrations the binding models are virtually identical. In fact, the LF isotherm reduces to the FI at low concentrations:

$$B = \frac{N_T K_0^m F^m}{1 + K_0^m F^m} \tag{10}$$

We have also derived an analogous AD expression for the LFI (Eq. (11)). This more complex equation is still a simple algebraic expression and therefore, can be applied in similar fashion to the AD expression for the FI (Eq. (6)). Using the experimentally derived LFI fitting parameters, the corresponding distribution can easily be calculated. The limits over which the calculated AD is valid are set by the concentration range of the experimental binding isotherm using the same expressions used for the FI (Eq. (7)):

$$N(K) = 2.3 N_T m K_0^m K^{-m} \times \frac{(1 + 2K_0^m K^{-m} + K_0^{2m} K^{-2m} + 4K_0^m K^{-m} m^2 - K_0^{2m} K^{-2m} m^2 - m^2)}{(1 + K_0^m K^{-m})^4} \tag{11}$$

In general, the LFI is more universally applicable in characterizing MIPs because it can model saturation and subsaturation behavior together or individually. However, the LFI is usually not necessary. The binding isotherms for most MIPs have been measured in the substaturation region where the more easily applied FI is sufficient. The LFI is still equally capable of characterizing these systems since the LFI reduces to the FI at lower concentrations. A simple test to see if the LFI is necessary is to plot the binding isotherm in $\log B$ versus $\log F$ format. Isotherms that are linear over the entire concentration range are more easily characterized using the FI. Isotherms that are curved over the entire range are probably more easily characterized by the Langmuir isotherm. Only systems that show both the linear regions at low concentrations and the curvature at high concentrations would benefit from the LFI. An example of an experimental isotherm, which has these attributes is shown in Fig. 7.

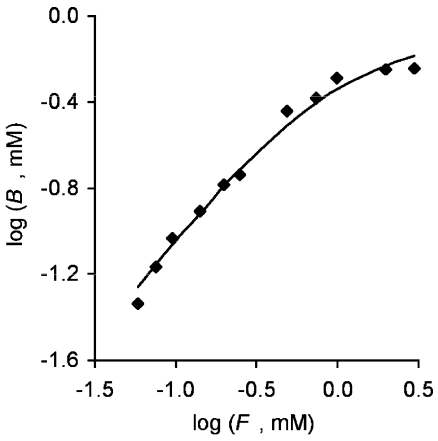

Fig. 7. Experimental isotherm and LFI fit for an atrazine imprinted polymer [32].

4. Limitations of AD methods

Two factors limit the accuracy of the AD methods in characterizing MIPs [25]. First are the practical limits in experimentally measuring the binding isotherm. Both the concentration range and the accuracy of the binding isotherm are usually constrained by the analytical methods used to measure analyte concentration. These in turn affect the accuracy of the calculated AD. The second factor limiting the accuracy of AD methods is more fundamental in nature and more vexing. The calculation of ADs from binding isotherms is a complex and "ill posed" mathematical problem. Only in the simplest homogeneous cases can the corresponding binding parameters (K and N) be exactly calculated. Even for an ideal heterogeneous binding isotherm, there are multiple ADs that could accurately reproduce the binding isotherm. The presence of experimental error and limitations in the concentration window only further obfuscates this problem. Thus, methods for calculating ADs from the binding isotherm of heterogeneous systems are all approximations of varying accuracy, and the selection of the appropriate binding model and AD are somewhat of a subjective exercise.

This inability to calculate the exact AD has contributed to the uncertainty in even assigning the general shape of the AD in MIPs. Our studies have suggested that MIPs contain a broad continuous unimodal distribution; whereas Guiochon and co-workers have recently suggested that a bimodal model may be more appropriate [26]. Based on the uncertainty in measuring binding isotherms and in calculating ADs, both binding models are probably valid mathematical solutions. Our choice of a continuous distribution over a discrete bimodal distribution is based on three factors. First, we followed the principle of Occam's razor. The simplest model with the fewest fitting parameters was chosen that accurately characterized MIPs. Both the bi-Langmuir and the Langmuir–Freundlich isotherms can accurately reproduce the experimental binding isotherm. However, the Langmuir–Freundlich does so using fewer fitting parameters, three versus four. Furthermore, comparison of the residuals from the two models also favors the Langmuir–Freundlich model as the experimental binding isotherms for MIPs typically show systematic variations

from bi-Langmuir isotherm. Second, if a bimodal distribution were present then application of the bi-Langmuir isotherm to different subsets of the same binding isotherm should consistently yield the same two classes of binding sites. However, this analysis yields a different set of association constants each time, which are more dependent on the concentration range being measured than the MIP being tested. This variability of binding parameters for a single polymer would seem to argue against a bimodal distribution and for a continuous heterogeneous distribution in MIPs. Finally, it is tempting to believe that the imprinting process will yield two distinct classes of sites: low affinity (background sites) and the high affinity (imprinted sites). However, given the many possible sources of heterogeneity including variability in the stoichiometry of the prepolymerization complexes, variability in the shape of the imprinted cavity [27], self-association of the template, and flexibility of the polymer matrix, it seems unlikely that a discrete bimodal distribution would form. More likely, the imprinting process yields a diversity of sites that is effectively a continuous distribution.

5. Advantages of AD methods

Due to the mathematical complexities, many of the methods for calculating ADs are computationally intensive and difficult to apply for the average user [26,28–30]. This is certainly the case for the initial methods such as the affinity spectrum and the expectation-maximization methods that were applied to MIPs. These methods were chosen for their ability to calculate ADs of varying shapes; however, this flexibility comes at the cost of computational complexity. Once the general shape of AD of MIPs has been specified, then regularized heterogeneous binding models such as the FI and LFI can be applied. These models significantly simplify the process and the corresponding ADs can be estimated using simple analytical expressions such as Eqs. (5), (6) and (11).

The advantage of using AD methods to characterize MIPs is that they take into account the heterogeneity in MIPs and therefore yield a more accurate description of their binding properties. This has the practical benefit of allowing for more accurate comparison and optimization of MIPs. The ADs express the concentration dependence of the binding properties as a graphical function. For example, differences in capacity and concentration window can shift the boundaries of the ADs (K_{min} and K_{max}), but will not change the general shape of the distribution. Therefore, ADs of two polymers can be compared for overlapping regions. In contrast, the binding parameters (N and K) calculated using Scatchard plots will vary widely with the concentration windows in which they were measured, leading to inconsistencies and ambiguities in comparing to evaluating the corresponding MIPs.

The LFI and FI also yield a quantitative measure of heterogeneity in the form of the heterogeneity index m. The ability to measure heterogeneity is important because heterogeneity strongly influences the binding properties and utility of MIPs. For example, heterogeneity is a major contributor to the peak asymmetry and tailing that inhibits the broader utility of MIPs in chromatographic applications [4]. It has also been cited as a source of the poor selectivity and cross-reactivity of MIPs in sensing and catalytic applications [5,12]. Thus, the ability to accurately measure heterogeneity in the form of the heterogeneity index is an essential tool for trying to reduce heterogeneity in MIPs.

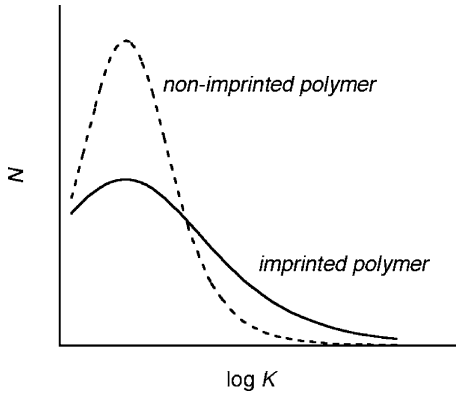

Fig. 8. Representative affinity distributions showing the greater heterogeneity of imprinted polymers (solid line) over non-imprinted polymers (broken line).

The application AD methods to MIPs have yielded a better understanding of the imprinting process and the imprinting effect. Perhaps the most surprising observation was that imprinted polymers are consistently more heterogeneous than their control non-imprinted polymers. This would seem counterintuitive as the formation of binding sites in the imprinting process would seem to go hand in hand with increasing homogeneity. This observation has changed our perceptions of the origins of the imprinting effect. The imprinted and non-imprinted polymers have very similar shaped broad unimodal distributions ADs. The primary difference appears to be that the imprinted polymer has a broader more heterogeneous distribution (Fig. 8). It is actually the greater heterogeneity of MIPs that shifts their distributions out into the high affinity region. For most applications, the MIPs are only tested in the exponentially decaying portion of the AD where MIPs shows higher capacity then non-imprinted polymers.

The above model suggests that heterogeneity is not only an intrinsic property of imprinted polymers but is characteristic of the imprinting effect. We propose that heterogeneity is actually a better figure of merit in comparing MIPs. The validity of using the heterogeneity index to compare MIPs was tested by optimizing the imprint effect by changing variables in the imprinting process. Consistent with previous studies: higher concentrations of template, lower temperatures and higher cross-linking percentages all improved the imprint effect as measured by increased capacity, affinity and also heterogeneity. In each case, the optimized polymer was more heterogeneous as measured by a lower heterogeneity index (m). The heterogeneity index has the advantage that it

remains constant for a particular polymer regardless of the concentration window in which it was measured. In contrast, previous figures of merits for MIPs such as binding affinity (K), capacity (N) or even selectivity (α) are all highly concentration dependent, which complicates their use in comparing MIPs.

6. Conclusions

Previous methods for characterizing MIPs have not directly addressed the issue of binding site heterogeneity despite the acknowledged importance of heterogeneity to the binding properties and utility of MIPs. Most likely, this has been due to the difficulties in applying heterogeneous models. However, recent developments have significantly simplified the use of continuous heterogeneous binding models such as the Freundlich and Langmuir–Freundlich isotherm in characterizing MIPs and also in the calculation of the corresponding binding parameters and affinity distributions. In addition to allowing for characterization of heterogeneity and for the more accurate measure of the binding properties in MIPs, the AD based methods also of value in the fundamental characterization of the imprinting process. Our studies have suggested that MIPs contain a broad unimodal distribution that exponentially tails into the high affinity region. This exponentially decaying region appears to be the most important with respect to the enhanced affinity and selectivity of MIPs.

References

[1] R.A. Bartsch, M. Maeda (Eds.), Molecular and Ionic Recognition with Imprinted Polymers, ACS Symposium Series, American Chemical Society, Washington, DC, 1998.
[2] B. Sellergren, Molecularly Imprinted Polymers. Man Made Mimics of Antibodies and Their Applications in Analytical Chemistry, Elsevier, Amsterdam, 2001.
[3] G. Wulff, Angew. Chem. Int. Ed. Engl. 34 (1995) 1812.
[4] B. Sellergren, K.J. Shea, J. Chromatogr. A 690 (1995) 29.
[5] G. Wulff, Chem. Rev. 102 (2002) 1.
[6] G. Wulff, K. Knorr, Bioseparation 10 (2001) 257.
[7] R. Umpleby II, M. Bode, K.D. Shimizu, Analyst 125 (2000) 1261.
[8] R.J. Umpleby II, S.C. Baxter, Y. Chen, R.N. Shah, K.D. Shimizu, Anal. Chem. 73 (2001) 4584.
[9] G. Wulff, W. Grobe-Einsler, W. Vesper, A. Sarhan, Makromol. Chem. 178 (1977) 2817.
[10] G. Vlatakis, L.I. Andersson, R. Müller, K. Mosbach, Nature 361 (1993) 645.
[11] K.J. Shea, D.A. Spivak, B. Sellergren, J. Am. Chem. Soc. 115 (1993) 3368.
[12] L.I. Andersson, R. Muller, G. Vlatakis, K. Mosbach, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 4788.
[13] J. Matsui, Y. Miyoshi, O. Doblhoffdier, T. Takeuchi, Anal. Chem. 67 (1995) 4404.
[14] C.J. Allender, K.R. Brain, C.M. Heard, Chirality 9 (1997) 233.
[15] B. Sellergren, Makromol. Chem. Macromol. Chem. Phys. 190 (1989) 2703.
[16] M. Kempe, K. Mosbach, Anal. Lett. 24 (1991) 1137.
[17] P. Sajonz, M. Kele, G.M. Zhong, B. Sellergren, G. Guiochon, J. Chromatogr. A 810 (1998) 1.
[18] Y.B. Chen, M. Kele, P. Sajonz, B. Sellergren, G. Guiochon, Anal. Chem. 71 (1999) 928.
[19] R.J. Umpleby, S.C. Baxter, M. Bode, J.K. Berch, R.N. Shah, K.D. Shimizu, Anal. Chim. Acta 435 (2001) 35.
[20] O.P. Norby, J.G. Jensen, J. Anal. Biochem. 102 (1980) 318.
[21] C.-C. Hwang, W.-C. Lee, J. Chromatogr. A 962 (2002) 69.
[22] R. Sips, J. Chem. Phys. 16 (1948) 490.
[23] R. Sips, J. Chem. Phys. 18 (1950) 1024.
[24] Y.B. Chen, M. Kele, I. Quinones, B. Sellergren, G. Guiochon, J. Chromatogr. A 927 (2001) 1.
[25] J.A. Schwarz, Colloids Surf. A 122 (1997) 265.
[26] B.J. Stanley, P. Szabelski, Y.B. Chen, B. Sellergren, G. Guiochon, Langmuir 19 (2003) 772.
[27] D.A. Spivak, J. Campbell, Analyst 126 (2001) 793.
[28] A.K. Thakur, P.J. Munson, D.L. Hunston, D. Rodbard, Anal. Biochem. 103 (1980) 240.
[29] A.M. Puziy, T. Matynia, B. Gawdzik, O.I. Poddubnaya, Langmuir 15 (1999) 6016.
[30] M. Cernik, M. Borkovec, J.C. Westall, Environ. Sci. Technol. 29 (1995) 413.
[31] G. Wulff, W. Vesper, W. Grobe-Einsler, A. Sarhan, Makromol. Chem. 178 (1977) 2799.
[32] T. Takeuchi, J. Matsui, J. Acta Polym. 47 (1996) 471.