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Measurement of the continuous distribution of binding sites in molecularly imprinted polymers†

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Reported is the first affinity spectrum (AS) [number of binding sites (*N*) *vs*. association constant (*K*)] for a non-covalently imprinted polymer. The AS method yields the distribution of sites over a continuous range of binding constants and characterizes the heterogeneity present in imprinted polymers better than current methodologies. To demonstrate the generality of the AS method, the distributions for three different imprinted polymers (two of which were taken from the literature) were calculated from their respective binding isotherms. The shapes of the distribution curves were different yet consistent with the respective covalent or non-covalent imprinting mechanisms. Finally, the binding parameters derived from the AS method were compared with those determined by the more common Scatchard analysis and were in general agreement.

Introduction

Molecularly imprinted polymers (MIPs) have become an increasingly active field of study for the construction of new materials capable of molecular recognition.[1] Imprinted polymers have been developed that compare favorably with synthetic and biological receptors with respect to thermal and chemical stabilities and binding affinities. A particularly attractive attribute of imprinted polymers is their ease and versatility of synthesis. MIPs are formed in a single step by a cross-linking reaction in the presence of a template molecule. Subsequent removal of the template leaves a cavity that retains specificity and affinity for the template. A wide variety of molecules, both organic and inorganic, have been imprinted for applications that require recognition, including enantiomeric separations, affinity chromatography, enzyme models and sensors.[2–5]

A property of MIPs that has limited their wider applicability is their heterogeneity. The imprinting process typically proceeds with poor fidelity, leading to a wide distribution of association constants.[6] We set out to improve the heterogeneity and shift the distribution toward the higher affinity sites; however, we were hampered by the limitations of current methods for characterizing MIPs. Most methods apply models having only one or two types of binding sites,[7] whereas MIPs contain a heterogeneous continuum of sites. Therefore, a procedure for calculating the continuous distribution of sites was developed based on a method originally described by Ninomiya and Ferry.[8] The analysis is general and easily applied, yielding the first quantitative measure of the continuous distribution of association constants in a non-covalently imprinted polymer.[9] The resulting binding parameters were compared with those determined by the popular Scatchard plot method. Finally, the analysis was applied to the binding isotherms of representative covalently and non-covalently imprinted polymers reported in the literature. The resulting distributions of binding sites were calculated and correlated with differences in the imprinting processes.

† Electronic Supplementary Information available. See http://www.rsc.org/suppdata/an/b0/b002354j/

Experimental

General procedure

Ethyl adenine-9-acetate (EA9A) was obtained from Aldrich Chemicals (Milwaukee, WI, USA). Ethylene glycol dimethacrylate (EGDMA) (Aldrich) was first washed twice with aqueous 1 M NaOH and once with aqueous saturated NaCl solution to remove the inhibitor. The monomer was further dried with anhydrous $MgSO_4$ and filtered from the solids. Methacrylic acid (MAA) (Aldrich) was distilled over $CaCl_2$ (10 mmHg, 80 °C). Azobis(isobutyronitrile) (AIBN) (Aldrich) was recrystallized from methanol. Acetonitrile was obtained from Fisher Scientific (Pittsburgh, PA, USA). Reaction mixtures were degassed using a Branson (Danbury, CT, USA) ultrasonic cleaner. Particles were sized with standard testing sieves (VWR Scientific, Media, PA, USA). UV measurements were taken on a Beckman (Fullerton, CA, USA) DU-7 spectrophotometer.

Polymer preparation

The synthesis of polymer **1** was adapted from Spivak *et al*.[10] To a solution of ethyl adenine-9-acetate (354 mg, 1.6 mmol) and AIBN (263 mg, 1.6 mmol) in acetonitrile (37.2 mL) was added methacrylic acid (1.6 mL, 19.2 mmol) and ethylene glycol dimethacrylate (25.7 mL, 136 mmol). The reaction mixture was sonicated under nitrogen for 15 min to remove unwanted gases. The polymerization was initiated photochemically by a standard laboratory UV light source [Hanovia (Newark, NJ, USA) medium pressure 450 W mercury arc lamp] at 0 °C and allowed to proceed for 24 h. The polymerization chamber was turned 180° after 20 min, 40 min and 10 h of polymerization. The polymers were crushed and Soxhlet extracted in acetonitrile–methanol (4 + 1) overnight, then dried under vacuum. The particles were ground with a Braun coffee grinder; particles in the 38–150 μm size range were used for batch rebinding studies.

Batch rebinding studies

A stock standard solution of 3 mM EA9A was prepared. Dilutions were made from 0.05 to 3.0 mM. An aliquot of 5 mL

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of each solution was added to 125 mg of polymer in screw-cap vials. Three samples were made for each solution. The vials were shaken for 2 h followed by centrifugation. UV measurements were taken at 255 nm on the supernatants. Dilutions were made to keep the absorbance in the 0.1–2.0 range, as necessary. From the absorbance values was subtracted the absorbance value from a sample where no EA9A had been added, in order to compensate for slow leakage of UV-active material from the polymer. This absorbance (A_F) corresponds to the absorbance of free (unbound) EA9A in solution. The free concentration was calculated as $F = A_F T/A_T$, where T and A_T are the concentration and absorbance of the stock standard solution, respectively. The concentration of bound EA9A was calculated as $B = T - F$.

Application of the AS method

Solutions to eqn. (2) (see later) were calculated using values for B interpolated from an experimental binding isotherm at F values of a/K, $1/aK$, a^2/K and $1/a^2K$. The shape of the distribution was found to be highly sensitive to the curvature in the binding isotherm. In particular, very slight changes in the slope of the B *vs*. log F graph result in the appearance of peaks in the distribution function $N(K)$. The concentration range of the guest in the batch rebinding studies limited the effective range of binding constants that could be accurately measured. Typically, binding parameters were calculated from $K_{min} = 1/F_{(max)}$ to $K_{max} = 1/F_{(min)}$. Increasing the concentration range and number of points in the experimental binding isotherm can diminish interpolation and extrapolation errors.

Results and discussion

A variety of methods have been developed for characterizing complex binding systems based on eqn. (1):

$$\frac{B}{R_0} = \int_{-\infty}^{\infty} \frac{N(K)KF}{1+KF} \mathrm{d}(\log K) \qquad (1)$$

where $N(K)$ is the fraction of sites having association constant K and B, F and R_0 are the molar concentrations of bound and free ligand and total receptors, respectively.[11] In this equation, $N(K)$ d(log K) is the probability of a binding site having an association constant between log K and log K + d(log K). The expression is general and does not assume a particular number or distribution of sites.

Various solutions can be found for eqn. (1) by approximating $N(K)$ to take the form of a specific function.[12] However, Hunston *et al.* showed that a general solution can be determined using a first order finite difference approximation:[11]

$$N(K) = \left| \frac{r}{2\log a} - \frac{a(s-2r)}{2(a-1)^2 \log a} \right|_{F=1/K} \qquad (2)$$

where a = constant > 1.0 (typically $a = 10^{0.2}$),

$r = B(a/K) - B(1/aK)$

$s = B(a^2/K) - B(1/a^2K)$

Eqn. (2) describes a continuous distribution of binding sites with respect to the association constant, known as an affinity spectrum (AS). The method is easily applied to MIPs, utilizing the same experimental procedures as existing batch rebinding studies. What is more, the AS approach produces a better picture of the binding parameters than current characterization methods for MIPs, most notably Scatchard plot analysis. This is because the underlying assumptions of these methods severely limit their ability to describe heterogeneous systems. By assuming a continuous distribution of sites, the AS approach has great flexibility in this regard. To demonstrate, a well-characterized MIP similar to that described by Spivak *et al.*[10] was made and tested. Polymer **1** was synthesized from methacrylic acid and ethylene glycol dimethacrylate and imprinted with ethyl adenine-9-acetate (Table 1).

A binding isotherm was experimentally determined by measuring the concentration of bound guest over a range of guest concentrations by UV spectrophotometry. The data were first displayed in a B *vs.* log F plot [Fig. 1(a)]. The corresponding N *vs*. log K graph was generated by calculating the number of binding sites from eqn. (2) at a set of association constants over the range $K = 1/F_{(max)}$ to $1/F_{(min)}$. Solutions to eqn. (2) necessitated interpolations for B when $F = a/K$, $1/aK$, a^2/K, and $1/a^2K$ from the experimental binding isotherm. The plot of B *vs.* log F was fitted with a smoothed spline from which the required B values were interpolated.[14] Smoothed spline gave an objective fit to the data set without having to impose any particular shape or function on the binding isotherm. The appropriateness of the fitted smoothed spline was best evaluated by plotting the B axis of the binding isotherm also in a log format, which better accentuates deviations between the experimental and fitted isotherms [Fig. 1(b)].

For polymer **1**, solutions to eqn. (2) revealed an asymptotic relationship between the number of binding sites and the association constant within the concentration range studied [Fig. 2(a)]. The observed distribution is qualitatively consistent with that reported in the literature for non-covalently imprinted polymers.[12a,15] Binding studies have shown that a relatively small population of sites is formed with high association constants whereas the majority of sites possesses relatively low association constants.[16]

A comparison was made between the binding parameters as determined by the AS method and those calculated from Scatchard plot analysis. Scatchard plots have been a common method for quantifying association constants in MIPs.[17,18] The advantage of the Scatchard method is that heterogeneity can be taken into account, to some degree, by fitting the continuum of sites to a bimodal distribution of 'high' and 'low' affinity sites by the limiting slopes method.[12a,15] The Scatchard plot (B/F *vs.* B) for polymer **1** displayed upward curvature, which is characteristic of the heterogeneity found in non-covalently imprinted polymers.[19] The curve can be fitted to two straight lines at low and high concentration limits, with the corresponding slopes and x-intercepts yielding estimates for the binding constants and the number of sites, respectively (Table 2, entry 1 for polymer **1**). A second set of binding parameters was determined by examining a narrower concentration range of B (3–20 mM) (Table 2, entry 2 for polymer **1**).

The Scatchard method finds a greater number of binding sites than the AS method at each association constant. The

Table 1 Preparation conditions for imprinted polymers

Polymer	Type of imprint	Composition	Template	Imprinting solvent	Ref.
1	Non-covalent	Methacrylic acid–ethylene glycol dimethacrylate (12 + 86 mol/mol)	Ethyl adenine-9-acetate	Acetonitrile	This work
2	Non-covalent	Methacrylic acid–ethylene glycol dimethacrylate (12 + 86 mol/mol)	9-Ethyl adenine	Chloroform	10a
3	Covalent	Ethylene glycol dimethacrylate	Cholesterol	Hexane–toluene (9 : 1 v/v)	13

underlying models for each analysis can explain this discrepancy. In an AS, *N* describes the number of binding sites within a *narrow* range of *K* values.[20] On the other hand, the number of sites found by Scatchard analysis represents a group of sites that have a relatively *broad* range of *K* values. Therefore, the Scatchard method should always find a higher number of sites. Additionally, the limiting slopes analysis of curved Scatchard plots intrinsically overestimates the number of binding sites.[21] Despite these differences, the two methodologies clearly yield similar binding parameters for polymer **1**. The correlation is best seen by a log–log plot of *N vs. K* [Fig. 2(b)] where both the AS and Scatchard binding parameters form relatively straight and parallel lines.

Based on the above analysis, the AS method appears to yield a more accurate measure of the number of binding sites. However, it should be emphasized that the AS represents a probability distribution of sites and not the actual number of sites at a particular *K*. This implies that the AS method cannot differentiate between binding sites of similar *K*, but instead measures the net complexation or observed energy for those sites.

The comparison of the two methodologies highlights some of the advantages of the AS method. (1) The analysis is easily applied, utilizing the same binding data as the common Scatchard analysis. (2) The AS method estimates the relative contribution of every possible association constant to the overall equilibrium for a given concentration range, whereas Scatchard analysis yields the contribution of only two association constants. (3) The continuous distribution makes comparison of imprinted polymers much easier. Comparisons by the Scatchard method are complicated because, typically, neither the number of sites nor the binding affinity remains constant from polymer to polymer. Furthermore, Scatchard analysis can yield different binding parameters for the same polymer depending on the concentration ranges considered (Table 2, entries 1 and 2 for polymer **1**).

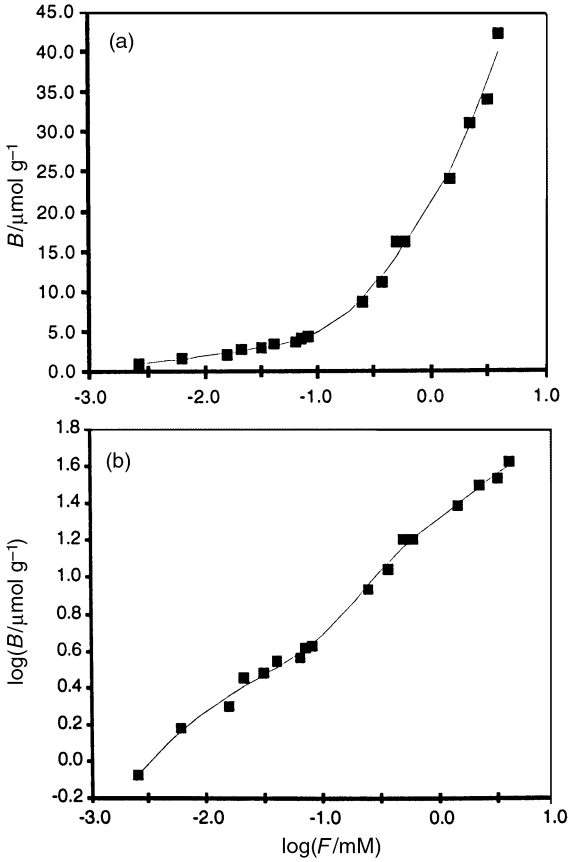

Fig. 1 (a) The binding isotherm (boxes) and interpolated smoothed spline (line, $S = 0.2$) for polymer **1** plotted in a *B vs.* log *F* format. (b) Binding isotherm for polymer **1** plotted in a log *B vs.* log *F* format.

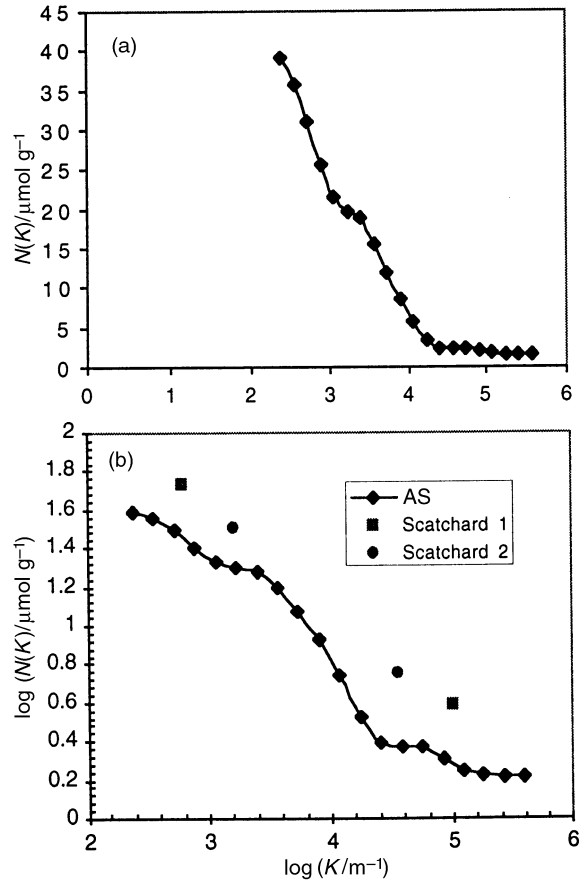

Fig. 2 (a) Distribution curve for polymer **1** found by the AS method. (b) Comparison of the binding parameters found by the AS and Scatchard methods for polymer **1**. Scatchard 1 is the binding parameters for the concentration range $F = 0.0025$–4.1 mM. Scatchard 2 is the binding parameters for the concentration range $F = 0.041$–0.61 mM.

Table 2 Theoretical and calculated binding parameters for imprinted polymers

Polymer	Theoretical number of sites[a]/μmol g^{-1}	Total number of sites (AS)[b]/ μmol g^{-1}	Total number of sites (Scatchard)/ μmol g^{-1}	Association constants/M^{-1}	Ref.
1	57	52 (±3)	58 (3.8 + 54)[c] 38 (5.6 + 32)[d]	101 000 and 610[c] 34 000 and 1600[d]	This work
2	57	50	75 (23 + 52)	70 000 and 7200	10a
3	183	103	114[e]	1 700[e]	13

[a] Calculated from moles of template extracted per gram of polymer. [b] Calculated by integration of curves in Fig. 3 in the *K* range 170–160 000 M^{-1}. [c] Calculated by limiting slopes Scatchard method with points 1–5 and 12–16. [d] Calculated by limiting slopes Scatchard method with points 5–10 and 9–14. [e] Recalculated from binding isotherm in ref. 10a.

Analysis of other MIPs

The distribution of binding sites in imprinted polymer **1** was found to decay asymptotically with increasing association constant within the analytical window. To determine whether this distribution is characteristic of all imprinted polymers or of only a particular class, distribution curves were calculated for representative MIPs from the literature. Polymers **2** and **3** are non-covalently and covalently imprinted polymers, respectively. Polymer **2** is the original adenine-binding polymer developed by Spivak *et al.* and is identical in composition with polymer **1** (Table 1, entry 2).[10] The primary difference is the imprinting solvent: chloroform for **2** and acetonitrile for **1**. On the other hand, MIP **3** is a covalently imprinted polymer synthesized and studied by Whitcombe *et al.*[13] The unique covalent imprinting approach developed by Whitcombe *et al.* leaves a single non-covalent binding group (a phenolic –OH) upon removal of the cholesterol template. Polymers **2** and **3** were selected because their reported binding isotherms contained enough data points (> 10) to resolve unique features in their corresponding distribution curves.

The distribution curves for **2** and **3** were calculated by the AS method and are shown overlaid on the distribution curve for polymer **1** (Fig. 3). Details of the calculations can be found in the Experimental section. The respective distribution curves are different in shape. The differences appear to be largest between polymers **1** and **2** *vs.* polymer **3**. Both **1** and **2** have a uniform decaying form, whereas **3** has a narrow distribution of binding sites with a maximum at $K = 1760\ M^{-1}$.

Comparison of the binding parameters found by the AS and Scatchard methods yields very similar values. For example, the total numbers of binding sites in polymers **1**, **2**, and **3** are in close agreement for both methodologies (Table 2) and were consistent with the theoretical number of binding sites. Calculations for the total number of sites were estimated as the sum of the 'high' and 'low' affinity sites for the Scatchard method and as the area under the curve of the plot of *N vs*. log *K* for the AS method. For polymer **3**, the total number of sites from the AS method was 103 μmol g^{-1} and that from the Scatchard method was 114 μmol g^{-1}. Likewise for polymer **1**, the Scatchard analysis estimates the total number of sites to be 58 or 38 μmol g^{-1}, depending on the concentration range. The AS analysis finds the intermediate value of 52 μmol g^{-1}.

Both methods also find a similar average association constant for polymer **3**. The binding constant ($K = 1700\ M^{-1}$) found from the slope of the Scatchard plot correlates with the maximum of the peak found in the distribution curve of the AS analysis (1760 M^{-1}). The correlation is most probably due to the appropriateness of both binding models to the homogeneous distribution of sites in polymer **3**. On the other hand, the AS and Scatchard methods find very different association constants for polymers **1** and **2**. This discrepancy is probably due to the inability of the Scatchard method to accommodate the more heterogeneous distribution found in polymers **1** and **2**.

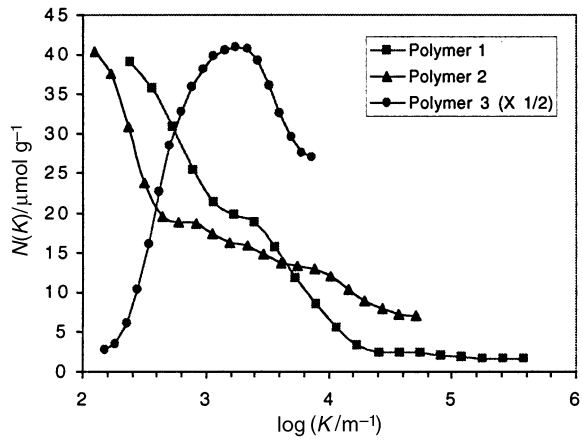

Fig. 3 Comparision of the distribution curves calculated by the AS method for polymers **1**, **2** and **3**.

Analysis of distribution curves

The polymers analyzed above represent a small subset of MIPs and therefore any definitive conclusions based on their distribution curves await the analysis of a greater number of polymers. Clearly, imprinted polymers can have very different distributions of binding sites as seen by polymers **1**, **2** and **3**. However, the differences in polymers **1**, **2** and **3** are highly suggestive of the operative imprinting processes in covalently and non-covalently imprinted polymers. In particular, the observed distribution curves appear to reflect the relative concentrations and diversity of the respective prepolymerization complexes.[22].

For covalently imprinted polymers, the template and functional monomers are irreversibly bound, ensuring stoichiometry and homogeneity of the prepolymerization complex. The resulting MIP is, therefore, expected to have a narrower distribution of binding sites which was observed in covalently imprinted polymer **3**. The distribution probably reflects both the benefits of the covalent imprinting process and the novel imprinting technique applied by Whitcombe *et al.* Support for the more homogeneous distributions in covalently imprinted polymers is found in the studies by Wulff *et al.* on a polymer covalently imprinted with a carbohydrate and having boronic acid functionalites.[9] The distribution curve was found to be a composite of the narrow distribution such as that in polymer **3** and an asymptotically decaying function that was attributed to swelling of the polymer.

In contrast, non-covalently imprinted polymers are expected to contain a more heterogeneous distribution of binding sites as seen in polymers **1** and **2**. An explanation may reside in the relatively weak cohesive forces that direct the imprinting process, leading to a greater diversity of structure and stoichiometry in the prepolymerization complexes. The imprinting mechanism can also explain the shape of the observed distribution curve in non-covalently imprinted MIPs. Calculations based on equilibrium equations predict that as the ratio of functional monomer to template increases, the relative concentration of the corresponding prepolymerization complexes will rapidly decrease.[23] This inverse relationship will also be reflected in the distribution of association constants because the affinity of a binding site has been correlated with the number of binding groups in that site.[24] Therefore, a rapidly decaying population of binding sites is predicted with increasing association constant, which is found for both polymers **1** and **2**.

The slight differences in the distribution curves for **1** and **2** give additional support for the above analysis. Polymers **1** and **2** are nearly identical in composition, template and stoichiometry and therefore the two distribution curves are similar in shape. The primary difference is that polymer **2** is imprinted in a less polar solvent (chloroform) which favors the non-covalent hydrogen bonding interactions that hold the prepolymerization complexes together. As a result, both the number and quality of imprints are expected to increase. This is seen by the greater number of high-affinity binding sites in polymer **2** and a corresponding smaller number of low-affinity sites.

Consequences for the imprinting process

While generalizations taken from polymers **1**, **2** and **3** are difficult because they represent only a small subset of MIPs, the respective shapes of the distribution curves suggest that

covalently and non-covalently imprinted polymers may be suited for different types of applications. For applications that require very high association constants and can tolerate low concentrations such as biosensors, the non-covalently imprinted polymers may be superior, whereas for applications that require a high percentage of good sites such as chromatography, the covalent imprinting process appears more appropriate.

The application of the AS method allows, for the first time, the facile characterization of the distribution of binding sites in molecularly imprinted polymers. The observed distributions in polymers **1**, **2** and **3** are suggestive of a more homogeneous distribution in covalently imprinted polymers and a more heterogeneous distribution in non-covalently imprinted polymers. However, definitive corroboration of this hypothesis awaits the study of a larger number of MIPs. Overall, the AS method is an improvement over Scatchard plots that apply what is largely a homogeneous model to a heterogeneous system.[25] The value lies not only in characterization and comparison of MIPs, but also in the ability of this method to be used as a tool for optimization and improvement of these polymers. The effects of changing different variables in the imprinting process can be more clearly evaluated not only by changes in the overall affinities but also in the distribution of binding sites.

Acknowledgements

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