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Interpretation of Ocular Melanin Drug Binding Assays. Alternatives to the Model of Multiple Classes of Independent Sites

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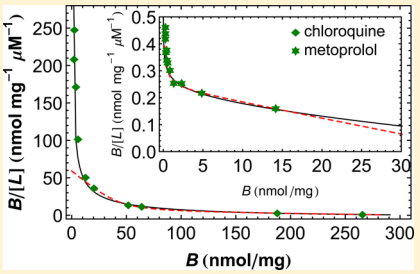
Supporting Information

ABSTRACT: Melanin has a high binding affinity for a wide range of drugs. The determination of the melanin binding capacity and its binding affinity are important, e.g., in the determination of the ocular drug distribution, the prediction of drug effects in the eye, and the trans-scleral drug delivery. The binding parameters estimated from a given data set vary significantly when using different isotherms or different nonlinear fitting methods. In this work, the commonly used bi-Langmuir isotherm, which assumes two classes of independent sites, is confronted with the Sips isotherm. Direct, log−log, and Scatchard plots are used, and the interpretation of the binding curves in the latter is critically analyzed. In addition to the goodness of fit, the emphasis is placed on the physical meaning of the binding parameters. The bi-Langmuir model imposes a bimodal distribution of binding energies for the sites on the melanin granules, but the actual distribution is most likely continuous and unimodal, as assumed by the Sips isotherm. Hence, the latter describes more accurately the distribution of binding energies and also the experimental results of melanin binding to drugs and metal ions. Simulations are used to show that the existence of two classes of sites cannot be confirmed on the sole basis of the shape of the binding curve in the Scatchard plot, and that serious doubts may appear on the meaning of the binding parameters of the bi-Langmuir model. Experimental results of melanin binding to chloroquine and metoprolol are used to illustrate the importance of the choice of the binding isotherm and of the method used to evaluate the binding parameters.



KEYWORDS: *ocular melanin, drug binding assays, multiple classes of independent sites, bi-Langmuir isotherm, Langmuir−Freundlich isotherm, Sips isotherm, site energy distribution function, Scatchard plot, chloroquine, metoprolol*

INTRODUCTION

Melanin has a high binding affinity for a wide range of basic drugs. Drug binding to melanin is important in fields such as ocular drug distribution,[1−4] onset and duration of drug effect in the eye,[5,6] trans-scleral drug delivery to the posterior segment of the eye,[7] melanoma imaging[8,9] and imaging-guided chemotherapy,[10] the toxicological analysis of some drugs,[11,12] the investigation of illicit drug use,[13] etc. Similarly, the binding of small ions to melanin has been extensively studied. The ability of melanin to sequester metals protects the cells from oxidative stress,[14] is implicated in Parkinson's disease,[9,15] and can be exploited in the purification of water contaminated by heavy metals.[16]

In the analysis of the drug binding assays, it is a general practice to choose the simplest model, within those of widespread use, that explains the data. The extension of the Langmuir isotherm to several classes of independent sites has become very popular because its versatility often leads to relatively high coefficients of determination when fitting the data.[17,18] There is a widespread idea[13,17−19] that a concave upward binding curve in the Scatchard plot is a clear indication of the existence of, at least, two classes of sites.

The Scatchard plot is also used in drug binding studies because of an attributed capacity to point out the existence of positive or negative cooperativity. However, the usual interpretations of the curvature of the binding curve in the Scatchard plot have been criticized as several mechanisms result in similar curvature.[20−22] For example, in the context of mutivalent antibody binding to B-cells,[23] it is known that the Sips isotherm is consistent with the same concave upward curvature that is often attributed to the existence of two classes of sites.

Other isotherms have also been considered for melanin binding, and it has been concluded that different drugs are best analyzed by different isotherms,[24] although the differences could not be very significant. Remarkably, several authors have found that the isotherms that generalize the Langmuir isotherm by adding a parameter related to the surface heterogeneity provided a better account of the experimental data.[24,25] The

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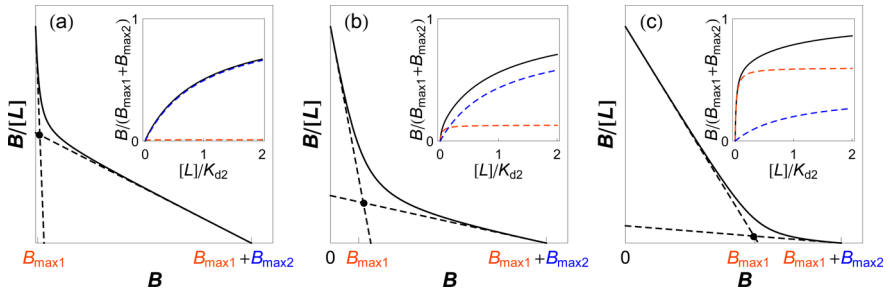

Figure 1. With four parameters, the bi-Langmuir model can reproduce different shapes in the Scatchard plot, which partly explains its utility. The binding isotherm is concave upward, and its intercept at the origin is $k \equiv (B_{max1}/K_{d1}) + (B_{max2}/K_{d2})$.

same conclusion was reached when studying the binding of metal ions to melanin. In particular, Bridelli et al.[26] found that the Sips isotherm was among the best in accounting for the binding of a number of metal ions to melanin.

The Sips isotherm is commonly used to describe adsorption on heterogeneous surfaces.[27] Natural melanin is packed in spherical or ellipsoidal granules whose surface has low porosity and is heterogeneous at the microscopic level (having different radii of curvature and potentially local surface irregularities).[18] Hence, the Sips isotherm is most relevant to analyze drug binding on melanin particles. Drug binding is considered a surface phenomenon, and a few recent studies have used information on the surface characteristics of the melanin particles for the interpretation of binding data.[12,13,18,26] Granules with a smaller size have a greater specific surface area and a higher binding capacity, but the size dispersity must also be considered. The size distribution depends on the melanin nature and origin, and so do the melanin binding parameters. Melanins isolated from different regions of the human body, as well as from other biological sources, have dissimilar particle size and shape. For instance, the sizes of synthetic melanin granules vary from 0.3 to 50 μm, those from human hair vary from 0.3 to 1 μm, bovine eye granules vary from 0.2 to 830 μm, and *Sepia* granules vary from 0.1 to 150 μm.[12,13,18]

The equilibrium binding constants are related to the binding energy released when a drug molecule binds to a site on the surface of a melanin granule. The binding of flexible drug molecules involves electrostatic and nonelectrostatic interactions, such as van der Waals and hydrophobic interactions, and, hence, a distribution of binding energies should be expected. In addition, the interactions between bound molecules might also contribute to this distribution. The Sips isotherm is theoretically derived from the assumption of a Gaussian-like distribution of binding energies.[27−29] On the contrary, the Langmuir model with two classes of sites considers only two binding energies.

With a focus on the binding assays of different drugs to eye melanin (located in, e.g., the RPE and uveal stroma),[30] in this work we critically analyze the selection of the binding isotherm and the method used to evaluate the binding parameters. Rather often, the drug binding data can be fitted to different models with similar values of the coefficient of determination. In any case, the model should be chosen from its physical relevance and that of its binding parameters. The quality of the fitting simultaneously in different representations (direct, log−log, and Scatchard plots) is an additional criterion. The values obtained for the binding capacity and the binding affinity depend on the method (e.g., best fit in direct, Scatchard, or log−log plot) used to obtain them. The models that are less sensitive to this method should be preferred. For instance, the binding parameters of the Sips model are more independent of the drug concentration range than those obtained from a Langmuir isotherm with two classes of sites,[31] which allows for a better comparison of different drug−melanin systems. The disparity of values of the maximum binding capacity of melanin is a significant drawback for the understanding of drug−melanin interactions, particularly in the context of ocular drug distribution. Therefore, the best choice of the binding isotherm and the best choice of the plot to evaluate its parameters are important, open questions in the analysis of drug binding to ocular melanin.

THEORY

The Langmuir, Bi-Langmuir, and Sips Models. The Langmuir model assumes localized drug binding on identical sites and predicts that the amount B of bound drug is

$$B = B_{max}\frac{[L]}{K_d + [L]} \quad (1)$$

where B_{max} is the maximum binding capacity. For a given free drug concentration [L], a lower dissociation constant K_d implies a higher affinity. Some drug−melanin interactions have been described using the Langmuir model,[32,33] especially when the drug concentration range is very small (e.g., only 1 order of magnitude in refs 25 and 34), but this model cannot account for most experimental observations.

The upward concavity of the binding curve in a Scatchard plot, $(B/[L])$ vs B, is considered as an indication of the existence of at least two classes of independent sites (Figure 1).[13,17−19] The bi-Langmuir model,[31]

$$B = \frac{B_{max1}[L]}{K_{d1} + [L]} + \frac{B_{max2}[L]}{K_{d2} + [L]} \quad (2)$$

where K_{d1} ($<K_{d2}$) corresponds to the high-affinity sites, can fit binding curves of different shapes in the Scatchard plot with high measures of the goodness of fit. However, the interpretation of the parameter values (B_{max1}, B_{max2}, K_{d1}, and K_{d2}) is often unclear, and the direct plot of B vs [L] seldom evidences confidently the existence of two classes of sites.

At low drug concentrations the binding data represented in a $\log_{10} B$ vs $\log_{10}$ [L] plot often shows a linear behavior with slope $n < 1$.[25,30,35] This observation cannot be explained by the bi-Langmuir model but agrees with the Sips or Langmuir−Freundlich isotherm,[24,26,30]

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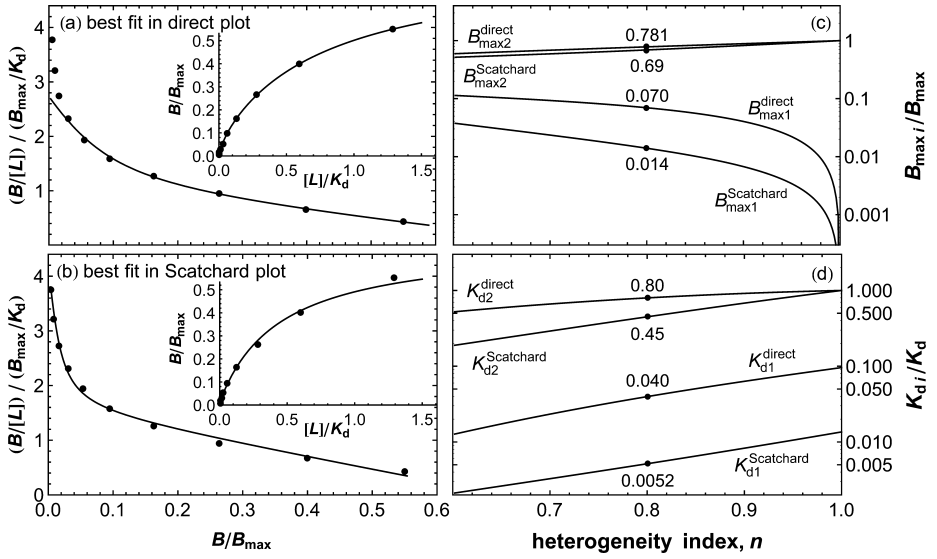

Figure 2. Drug binding data have been simulated using a Sips isotherm and the binding capacities and dissociation constants of the bi-Langmuir isotherm have been obtained from best fits in direct and Scatchard plots. Panels a and b correspond to $n = 0.8$, and their parameter values are shown at the points marked in panels c and d. The best fit from the direct plot (panel a, inset) produces a very poor agreement with the data in the Scatchard plot (a). The best fit in the Scatchard plot (panel b) produces a modest agreement with the data in the direct plot (b, inset). It is evidenced that (i) the binding parameters depend on the method used to evaluate them and (ii) the parameters of the high affinity sites (index 1) are very sensitive to n, that is, to minor variations of the binding data, what casts doubts on their physical meaning.

$$B = \frac{B_{max}[L]^n}{K_d{}^n + [L]^n} \quad (3)$$

where $n < 1$ is the heterogeneity index. (Its theoretical background is explained in Results, and its relation to the Hill isotherm with cooperativity index n can be found in the Supporting Information.) This isotherm can be approximated by $(B/[L]) \approx (k^{Sips})^{1/n} B^{(n-1)/n}$ when $B \to 0$, and hence the binding curve resembles very much that of Figure 1a, as the divergence at $B = 0$ cannot be observed when experimental data is analyzed.

The uncertainties of the estimates of the parameters (n, B_{max}, K_d) and (B_{max1}, B_{max2}, K_{d1}, K_{d2}) of the Sips and bi-Langmuir models can be rather high because the experimental data are usually restricted to the range $[L] < K_d$ and hardly ever reach the binding saturation. In the bi-Langmuir model, $B \approx k[L]$ at low [L], with $k \equiv (B_{max1}/K_{d1}) + (B_{max2}/K_{d2})$. Similarly, the Sips model reduces to $B \approx k^{Sips}[L]^n$ at low [L], with $k^{Sips} \equiv (B_{max}/K_d{}^n)$. The parameter k, introduced with somewhat different meanings in these two models, can be usually determined with low uncertainty, and, hence, it provides more accurate information in the low concentration range.

Lack of Reliability of the High Affinity Parameters of the Bi-Langmuir Model. The parameters of the bi-Langmuir model depend on the method used to evaluate them and on the concentration range of the experiments. To inquire about the physical meaning of the parameter values obtained from the bi-Langmuir model, ten data points corresponding to free drug concentrations $([L]/K_d) = 10^x$, with $x = 1/9, -2/9, -5/9, ..., -26/9$, have been simulated using a Sips isotherm with $n = 0.8$; these values of [L] closely resemble those used in actual experiments.[30] A best fit in the direct plot (Figure 2a, inset) gives large weight to the high [L] data and small weight to the low [L] data. Hence, the best fit from the direct plot often fails to describe the low concentration data in both the log−log plot and the Scatchard plot (Figure 2a); $1/y$ weighting could be used to partially solve this problem.[18] The best fit parameters to eq 2 obtained from the Scatchard plot (Figure 2b) produce a modest agreement with the data in the direct plot (Figure 2b, inset). The binding parameters of the high affinity sites (index 1) are very much dependent on the method used to evaluate them as, e.g., $(K_{d1}^{direct}/K_d) = 0.040 \pm 0.008$ is roughly eight times larger than $(K_{d1}^{Scatchard}/K_d) = 0.0052 \pm 0.0011$; standard errors are shown. More importantly, these parameters vary significantly with the concentration range considered. If we change only one data point, replacing the value $([L]/K_d) = 10^{1/9}$ by $10^{-29/9}$, the parameters of the high affinity sites are roughly halved, when estimated from both the direct and Scatchard plots. This result is in agreement with experimental observations.[21,35]

The analysis of simulated data (with $([L]/K_d) = 10^x$ and $x = 1/9, ..., -26/9$) for different values of the heterogeneity index n evidence that the best fit parameters of the high affinity sites are very much dependent on n (Figure 2c,d). As n tends to unity, B_{max1} tends to zero and the limiting value of K_{d1} depends on the method used to evaluate it; $\lim_{n\to1}(K_{d1}^{direct}/K_d) = 0.096$ and $\lim_{n\to1}(K_{d1}^{Scatchard}/K_d) = 0.014$. In conclusion, serious doubts are cast on the meaning of the values of B_{max1} and K_{d1}, which might be just artifacts arising from our choice of the bi-Langmuir model.

MATERIALS AND METHODS

Chloroquine diphosphate salt (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in DMSO for the highest concentration of stock solutions, which were then diluted with phosphate buffered saline (PBS, pH 7.4, Gibco, Invitrogen, Grand Island, NY, USA). The melanin was isolated from RPE and choroid of porcine eyes as described in ref 30. Melanin binding experiments with chloroquine were performed at pH 7.4 in the concentration range of 0.003−270 μmol/L. Melanin suspension and test compound solutions were prepared just before every experiment. Melanin was mixed with PBS buffer to form a 2 mg/mL suspension. The suspension was sonicated for 15 min before incubation with the test compounds (70 μL of

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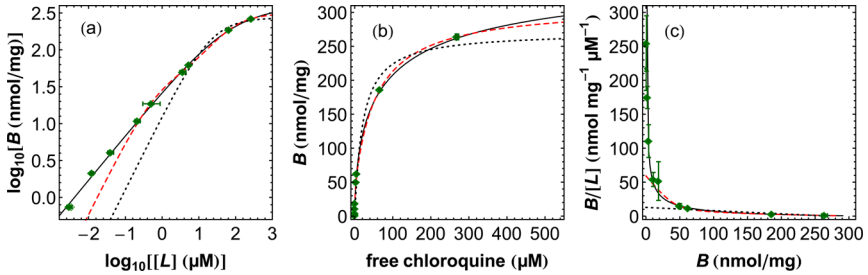

Figure 3. Experimental data of chloroquine binding to melanin at pH 7.4 in log−log, direct, and Scatchard plots. The solid, dashed, and dotted lines correspond to the Sips, bi-Langmuir, and Langmuir isotherms, respectively. The bi-Langmuir model predicts that the low concentration slope in the log−log plot is unity, while the data have slope $n < 1$, as predicted by the Sips model. A best fit in the direct plot, as used for the bi-Langmuir isotherm, often fails in the low concentration range when the log−log or the Scatchard plots are used.

melanin suspension + 70 μL of compound solution) at 37 °C in a shaker (220 rpm). Three replicates were made of each experiment. After incubation, the melanin suspension was centrifuged at 21000*g* for 15 min and the supernatant collected for analysis.

Samples with expected concentrations over 0.1 μmol/L were analyzed using UPLC with UV detection (Acquity UPLC with photodiode array detector, Waters, Milford, MA, USA). For samples with expected concentrations below 0.1 μmol/L, a mass spectrometric analysis with UPLC separation was used. Further details can be found in ref 30.

Data analysis for the determination of the binding parameters (based on nonlinear, ordinary least-squares) was performed in Wolfram Mathematica using the Levenberg−Marquardt algorithm and no weighting.

RESULTS

Analysis of Measurements of Chloroquine Binding to Melanin. The analysis of experimental data of chloroquine binding to melanin at pH 7.4 (Figure 3) shows that the Sips model is superior to the bi-Langmuir model. Conventionally, the parameters of the Langmuir and bi-Langmuir models are obtained from the best fit in the direct plot. The Langmuir isotherm fails dramatically when the best fit is analyzed in the log−log and Scatchard plots. The bi-Langmuir model yields parameters that vary significantly when estimated from different representations (Table 1), but no set of parameter values yields good agreement with the data simultaneously in the three plots of Figure 3. On the contrary, the best fit of the Sips model in the log−log plot also provides an excellent fit in the direct and Scatchard plots.

Table 1. Parameter Values (± Standard Error) of the Langmuir, Bi-Langmuir and Sips Isotherms for the Experimental Data in Figures 3 and 4[a]

chloroquine pH 7.4		metoprolol pH 5.0	
$B_{max}^{langmuir}$ = 271 ± 14	$K_d^{langmuir}$ = 21 ± 5		
B_{max1}^{direct} = 43 ± 3	K_{d1}^{direct} = 0.78 ± 0.15	B_{max1}^{direct} = 0.26 ± 0.11	K_{d1}^{direct} = 1.7 ± 1.2
B_{max2}^{direct} = 268 ± 4	K_{d2}^{direct} = 57 ± 3	B_{max2}^{direct} = 42.8 ± 2.0	K_{d2}^{direct} = 187 ± 15
$B_{max1}^{log-log}$ = 9.4 ± 2.2	$K_{d1}^{log-log}$ = 0.044 ± 0.014	$B_{max1}^{log-log}$ = 0.13 ± 0.04	$K_{d1}^{log-log}$ = 0.62 ± 0.19
$B_{max2}^{log-log}$ = 250 ± 30	$K_{d2}^{log-log}$ = 18 ± 5	$B_{max2}^{log-log}$ = 39 ± 7	$K_{d2}^{log-log}$ = 160 ± 30
B_{max}^{Sips} = 380 ± 40	K_d^{Sips} = 76 ± 23	B_{max}^{Sips} = 65 ± 22	K_d^{Sips} = 380 ± 160
n = 0.605 ± 0.012	k^{Sips} = 27.9 ± 1.1	n = 0.895 ± 0.009	k^{Sips} = 0.322 ± 0.006

[a]The units of B_{max} and K_d are (nmol/mg) and (μmol/L). Given the large uncertainties of B_{max} and K_d, it is convenient to report also the value of $k^{Sips} \equiv (B_{max}/K_d^n)$.

The parameter values of a model depend on the method used to evaluate them (Figure 2). The maximum binding capacity estimated from the bi-Langmuir model, $B_{max1}^{direct} + B_{max2}^{direct}$ = (311 ± 5) nmol/mg, is lower than that estimated from the Sips model, as expected from Figure 2c. However, the estimates of the binding capacity are poor when the ligand concentrations do not cover the binding saturation range. In relation to the reciprocal of the affinity constant, it is observed that $K_d^{langmuir}$, K_{d2}^{direct}, and K_d^{Sips} (Table 1) are of the same order. When the bi-Langmuir parameters are determined from the best fit in the log−log plot (values in Table 1; isotherm not shown in Figure 3), the maximum binding capacity $B_{max1}^{log-log} + B_{max2}^{log-log}$ = (260 ± 30) nmol/mg is smaller than $B_{max}^{langmuir}$ and B_{max}^{Sips}, and $K_{d2}^{log-log}$ is also smaller than K_{d2}^{direct} and K_d^{Sips}.

Remarkably, the meaning of the high-affinity constant K_{d1}^{direct} is doubtful. For the same isotherm and experimental data, the best fit in the log−log plot yields a much lower value, $K_{d1}^{log-log} \ll K_{d1}^{direct}$. Its value is very sensitive to small deviations of just one data point from the trend exhibited by the other ones.

Analysis of Measurements of Metoprolol Binding to Melanin. The experimental data of metoprolol binding to synthetic melanin from ref 18 have been analyzed as an additional case for the comparison of the Sips and bi-Langmuir models. From the shape of the binding curve in the Scatchard plot, two classes of binding sites were judged evident,[18] but the Sips isotherm also produces an excellent agreement with the experimental observations (Figure 4). In fact, the difference between the shapes on these two isotherms is negligible not only in the Scatchard plot but also in the direct and log−log plots.

The best fit parameters for two classes of independent sites found in ref 18 are reported in Table 1; note the large uncertainties of the parameters of the high affinity sites, B_{max1} and K_{d1}. The parameter values of the low affinity sites are closer to those obtained for the Sips isotherm in the log−log plot (Table 1). The binding parameters estimated for metoprolol using the Sips isotherm are also consistent with those reported in Table 1 of ref 30.

Distribution of Binding Energies. The equilibrium constants of the binding isotherms are related to the (free) energy $\varepsilon > 0$ that is released when a drug molecule binds to a site on the surface of a melanin granule; ε can also be interpreted as a binding enthalpy when a single temperature is considered. The dissociation constant in eq 1 is the reciprocal

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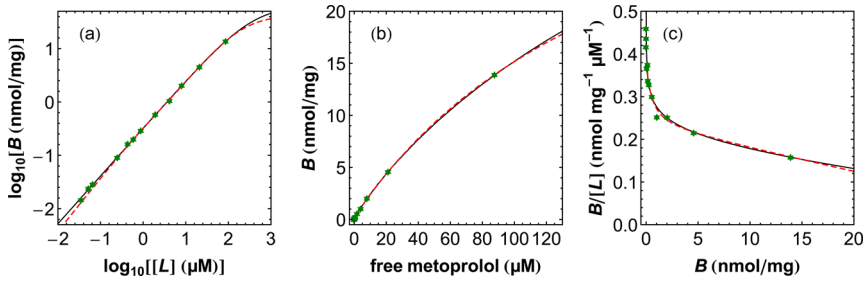

Figure 4. Experimental data of metoprolol binding to synthetic melanin from ref 18. The solid and dashed lines correspond to the Sips and bi-Langmuir isotherms, respectively.

of the affinity constant, $(1/K_d) = A\ \exp(\varepsilon/RT)$, where T is the absolute temperature, R is the gas constant, and A is a prefactor. The bi-Langmuir model imposes a bimodal distribution of binding energies; actually, two energies related to K_{d1} and K_{d2}.[31] However, the actual distribution is most likely continuous and unimodal.

The binding on a site is described by a Langmuir isotherm with a dissociation constant $(1/K_d(\varepsilon)) = A\ \exp(\varepsilon/RT)$. If $f(\varepsilon)\ d\varepsilon$ denotes the fraction of sites with binding energies between ε and $\varepsilon + d\varepsilon$, the overall binding isotherm is

$$\frac{B}{B_{max}} = \int \frac{[L]}{K(\varepsilon) + [L]} f(\varepsilon)\ d\varepsilon \tag{4}$$

The higher affinity (higher ε) sites are preferentially occupied at low [L], while the lower affinity ones can only be occupied at higher [L]. The Sips isotherm, eq 3, corresponds to the binding energy distribution[27]

$$f_{Sips}(\varepsilon) = \frac{1}{\pi RT} \frac{\sin(n\pi)}{e^{n(\varepsilon-\varepsilon_0)/RT} + 2\cos(n\pi) + e^{-n(\varepsilon-\varepsilon_0)/RT}} \tag{5}$$

which is Gaussian-like and centered at a value ε_0 determined by K_d^{Sips}. The heterogeneity index n determines the width of the distribution. The closer to unity, the narrower is the distribution. In the limit $n = 1$ only one binding energy is possible, that of the Langmuir isotherm, eq 1.

The distribution function is normalized so that $\int_{-\infty}^{\infty} f_{Sips}(\varepsilon)\ d\varepsilon = 1$. Negative values of the binding energy are meaningless,[29] but the distribution function restricted to $\varepsilon > 0$ should not differ significantly from that in eq 5 because both factors in the integrand of eq 4 are small in the range $\varepsilon < 0$. Indeed, $K(\varepsilon)$ is large for $\varepsilon < 0$ and $f_{Sips}(\varepsilon)$ has a peak at $\varepsilon_0 > 0$, the most likely binding energy, and takes small values for $\varepsilon < 0$. Thus, it seems reasonable to use eq 4 with $\varepsilon > 0$ and $f(\varepsilon) = (f_{Sips}(\varepsilon)/I)$, where $I = \int_0^{\infty} f_{Sips}(\varepsilon)\ d\varepsilon$. The most likely binding energy ε_0 can be obtained from the relation $(1/K_d^{Sips}) = A\ \exp(\varepsilon_0/RT)$ after a value is assigned to $1/A$. It is customary to assign a value close to the saturation limit of the drug,[29] and in Figure 5 we have taken $(1/A) = 1$ mmol/L. The binding energy distribution obtained with the (exact) method of the Stieljes transform, eq 5,[27] is similar to those obtained using the condensation approximation method;[29,31] see Supporting Information for further details.

DISCUSSION

The bi-Langmuir model, which assumes two classes of independent sites, is perhaps the most common approach to determine the drug binding parameters in ocular melanin studies because it is a simple model integrated in most

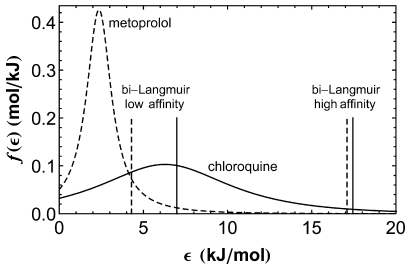

Figure 5. Binding energy distributions at 20 °C corresponding to the Sips parameters of the experimental data in Figures 3 and 4. Metoprolol has a narrower distribution ($n = 0.895$) peaked at lower energies ($K_d^{Sips} = 380\ \mu$mol/L), and chloroquine has a wider distribution ($n = 0.605$) peaked at higher energies ($K_d^{Sips} = 76\ \mu$mol/L), as expected for a cationic drug binding on an anionic surface. The two binding energies corresponding to the bi-Langmuir model are also shown as vertical lines for comparison.

equipment software and it has been widely used in the literature. With four fitting parameters, it often leads to good agreements between the fitted curve and the experimental data. However, the best fit parameter values should be critically analyzed and alternative isotherms should be considered if a sound explanation is not found for the binding parameter values. We have shown that the four parameters of this model are largely dependent on the nonlinear regression method used to obtain them, that is, on the type of plot used to represent the data, and on the concentration range considered in the experiments. The parameters, especially those of the high affinity sites, are also very sensitive to small deviations (from the general trend) of the data points resulting from experimental errors.

The affinity constant is related to the energy released when a drug molecule binds to a site on the melanin granule surface. The binding of flexible drug molecules involves electrostatic and nonelectrostatic interactions and, hence, it is not surprising that a distribution of binding energies is involved. The bi-Langmuir model imposes a discrete, bimodal distribution of binding energies for the sites on the melanin granules, but the actual distribution is most likely continuous and unimodal.[31] The Sips isotherm describes more accurately the distribution of binding energies[31] and, hence, also the experimental results of drug binding assays.[30]

Drug binding data often exhibit a linear behavior with slope $n < 1$ when represented in a $\log_{10} B$ vs $\log_{10}$ [L] plot.[25,30,35] The Sips isotherm accounts for this observation.[24,26,30] The parameter values of the Sips isotherm are routinely obtained from the best fit in the log−log plot, and it is remarkable that these parameter values also lead to good agreement between isotherm and experimental data when represented in the

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Scatchard plot and in the direct plot. On the contrary, the best fit parameters of the bi-Langmuir isotherm (calculated as usual from the direct plot) often lead to poor agreement when represented in the Scatchard and log−log plots. In addition, the parameter values of the Sips isotherm are not so sensitive to the method used to evaluate them.

Several mechanisms result in a similar upward concavity of the binding curve in the Scatchard plot (see Supporting Information).[20,21] The heterogeneity of the surface of the melanin granules, as described by the Sips isotherm, predicts the curvature often attributed[13,17−19] to multiple classes of binding sites. The existence of different classes of sites cannot be confirmed on the sole basis of the Scatchard plot. In any case, when proposing the existence of two classes of binding sites, some plausibility arguments should be given for the parameter values obtained, for their differences among drugs, as well as for their variability when determined using different methods (such as best fits in different plots or using different weight factors).

The bi-Langmuir model imposes a discrete, bimodal distribution of binding energies for the sites on the melanin granules, but this distribution is most likely continuous and unimodal. Because of this, the binding parameters estimated from the bi-Langmuir model depend upon the free drug concentration range that is studied.[31,35] Apparently, the Sips isotherm describes more accurately the distribution of binding energies and, hence, also the experimental results of binding assays.[24,26,30] As a result, the binding capacity and binding affinity estimated from the Sips isotherm are more concentration independent, which allow for an easier comparison of different drug−melanin systems.

The Sips isotherm adequately describes the melanin binding data of chloroquine and metoprolol considered in this work. This conclusion is confirmed in ref 30 for nadolol, timolol, methotrexate, and carboxydichlorofluorescein at two different pHs. Our data of metoprolol has been chosen to clearly illustrate that a slight deviation from $n = 1$ (Langmuir) to $n = 0.98$ yields the type of curve in the Scatchard plot that is often interpreted as an evidence for two classes of sites. Chloroquine, on the contrary, has been chosen because it is the drug with the lowest value of the heterogeneity index ($n = 0.6$), among those considered in ref 30. Since the other drug−melanin systems would likely have values of the heterogeneity index between 0.6 and 1.0, the examples used can be considered demonstrative.

The binding capacity estimated from a given data set varies significantly when using different models. Whenever possible, measurements over a wider concentration range should be used to find the most suitable binding model. Since melanin-containing tissues represent a depot that might be saturated at high continuous drug dosing, the widening of the drug concentration interval used in binding studies may reveal the saturation of melanin and provide valuable insight.

Finally, the evidence provided in this study in favor of the use of the Sips model for adsorption on heterogeneous surfaces supports the belief that drug binding to melanin is a surface phenomenon.[12,13,18,26] This implies that the binding capacity should be normalized to the surface area because it is related to the number and availability of binding sites. Unfortunately, there are few studies on the specific surface area of melanin granules, and these confirm the variability with the nature and origin of the melanins.[18] Therefore, further work is needed to provide reliable estimates of the maximum binding capacity per surface area of the melanin granules.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.5b00783.

Relation of the Sips isotherm with cooperativity, binding energy distribution from the condensation approximation method, and alternative binding isotherms (PDF)

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Notes

The authors declare no competing financial interest.

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